A study about the corpus luteum activity in high yielding dairy cows postpartum

Steven Cools

2014

UNIVERSITEIT GENT

FACULTEIT DIERGENEESKUNDE
approved by EAEVE

Dit onderzoek werd gesubsidieerd door het Bijzonder Onderzoeksfonds van de Universiteit Gent, Beursnummer 05B01906

Alle dierproeven uitgevoerd voor dit proefsschrift werden gunstig beoordeeld door de Ethische Commissie van de Faculteit Diergeneeskunde, Universiteit Gent

Het drukken van dit werk werd mede mogelijk gemaakt dankzij de financiële steun van de firma Zoetis.

A study about the corpus luteum activity in high yielding dairy cows postpartum

Steven Cools

Merelbeke, 2014

opdracht

Beautiful Holstein
whose milk helps give life to all;
a true dairy queen.
(a Haiku of Clover)

A study about the corpus luteum activity in high yielding dairy cows postpartum

Een studie handelend over de activiteit van het geel lichaam bij hoogproductieve melkkoeien gedurende de postpartum fase

(met een samenvatting in het Nederlands)

Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Diergeneeskundige Wetenschappen aan de Faculteit Diergeneeskunde, Universiteit Gent, 20 mei, 2014

door

Steven Cools

Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde
Faculteit Diergeneeskunde
Universiteit Gent

Department of Reproduction, Obstetrics and Herd Health
Faculty of Veterinary Medicine
Ghent University

Hoofdpromotor

Prof. dr. G. Opsomer

Faculty of Veterinary Medicine, Ghent University, Belgium

Promotor

Prof. dr. W. Van den Broeck

Faculty of Veterinary Medicine, Ghent University, Belgium

Additional Members of the Examination Committee

Prof. dr. H. Bollwein

Clinic of Reproduction, University of Zürich, Switzerland

Prof. dr. P. Bossaert

Department of Veterinary Sciences, Antwerp University, Belgium

Prof. dr. P. Cornillie

Faculty of Veterinary Medicine, Ghent University, Belgium

Prof. dr. S. De Vliegher

Faculty of Veterinary Medicine, Ghent University, Belgium

dr. M. Thys

Area Veterinary Manager Ruminants-Dairy Portfolio Specialist, Zoetis Belux, Belgium

Prof. dr. L. Vanhaecke

Faculty of Veterinary Medicine, Ghent University, Belgium

Table of Contents

List of Abbreviations

AHSG	alpha-2-HS-glycoprotein
ANPEP	alanyl (membrane) aminopeptidase
ASGR2	asialoglycoprotein receptor 2
ATP	adenosine triphophate
AUC	area under the curve
BAK1	B-cell lymphoma 2-antagonist/killer 1
BCL2L1	B-cell lymphoma 2-like protein 1
BCL2L12	B-cell lymphoma 2-like protein 12
bFGF	basic fibroblast growth factor
BM	basal lamina
BOHB	beta-hydroxybutyrate
BP	binding protein
bST	bovine somatotropin hormone
BW	body weight
cAMP	cyclic adenosine monophosphate
CASP3	caspase 3, apoptosis-related cysteine peptidase
CASP7	caspase 7, apoptosis-related cysteine peptidase
CDC2	cell division cycle protein 2 homolog
CIDR	controled internal drug release
C-LA	commencement of luteal activity
CI	calving interval
CL	corpus luteum
CLa	corpora lutea
CLDN4	claudin 4
COF	cystic ovarian follicles
COX	cyclo-oxygenase
CP	crude protein
CR	conception rate
CRYGS	gamma S crystallin
CTGF	connective tissue growth factor
$CYP450_{SCC}$	cholesterol side chain cleavage cytochrome P450

D	day
DCAD	dietary cation anion difference
DEG	differentially expressed genes
DG	deep glandular epithelium
DGAT2	diacylglycerol acyltransferase 2
DHD	dihydrodaidzein
DMI	dry matter intake
DNA	desoxyribonucleic acid
EC	endothelial cells
ECM	extracellular matrix
EED	early embryonic death
EIF4E	eukaryotic translation initiation factor 4E
EMT	epithelial-mesenchymal transition
ER	estradiol receptor
ES	extracellular space
ET	embryo transplantation
FABP	fatty acid binding protein
FADD	Fas (TNFRSF6)-associated via death domain
FASLG	Fas ligand (TNF superfamily, member 6)
FBS	fetal bovine serum
FGF	fibroblast growth factor
FIB	fibroblast
FR	fertilization rate
FSH	follicle stimulating hormone
G	gauge
GDF8	growth/differentiation factor 8
GE	glandular epithelium
GnRH	gonadotropin releasing hormone
GO	gene ontogony
hCG	human chorion gonadotropin
HDL	high density lipoprotein
HF	Holstein Friesian
HGF	hepatocyte growth factor
HMG-CoA	hydroxymethylglutaryl coenzyme A

IC_{50}	concentration of an inhibitor at which 50% inhibition of the response is seen
IDH1	isocitrate dehydrogenase 1
IFNτ	interferon tau
IFNAR	IFN alpha receptor 1 subunit
IGF	insulin like growth factor
IGFBP	IGF binding protein
IGF1-R	IGF1-receptor
IP_3	inositol triphosphate
IRE	IFN response element
IRF	IFN regulatory factor
ISG	interferon stimulated gene
JAK STAT	janus kinase signal transducer and activator of transcription
Ki67	antigen identified by monoclonal antibody Ki67
L	lactating
LBF	liver blood flow
LDL	low density lipoprotein
LE	luminal epithelium
LED	late embryonic death
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein
LGALS9	lectin, galactoside-binding, soluble, 9
LH	luteinizing hormone
LH-R	LH-receptor
LLC	large luteal cell
LOC286871	uterine milk protein
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LTC_4	leukotriene C_4
MAP	multiple angle probe
MAPK	mitogen-activated protein kinase
ME	metabolizable energy
MEP1B	zinc metallo-endopeptidase
MHC	major histocompatibility complex
MMI	microvessel maturation index

MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MSTN	myostatin
MY	milk yield
NA	noradrenaline
NDGA	nordihydroguaiaretic acid
NEB	negative energy balance
NEFA	non-esterified fatty acids
NID2	nidogen 2
NL	non lactating
NO	nitric oxide
NSC	non steroidogenic cell
O-DMA	O-desmethylangolensin
OPU	ovum pick-up
OT	oxytocin
OTH	other cell types
OTR	oxytocin receptor
P	significance level
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PG	prostaglandin
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin F2alpha
PGFM	prostaglandin F metabolite
P-gp	permeability glycoprotein
P_4R	progesterone receptor
pH	decimal logarithm of the reciprocal of the hydrogen ion activity in a solution
PI	post insemination
PIP	phosphatidyl inositol phosphate
PIP_2	phosphatidyl inositol biphosphate
PKA	protein kinase A
PKC	protein kinase C
PR	pregnancy rate

PRID	progesterone releasing intravaginal device
PRSS	protease serine
PTK	protein tyrosin kinase
P21	cyclin dependent kinase inhibitor 1
r	correlation coefficient
r^2	coefficient of determination
RBP4	retinol binding protein 4
RDP	rumen degradable protein
RNase	ribonuclease
RNAseq	RNA sequencing
SAMD9	sterile alpha motif domain containing 9
SCG5	secretogranin V
SCP2	sterol carrier protein 2
SD	standard deviation
SER	smooth endoplasmatic reticulum
SERMs	selective ER modulators
SERPING	serpin peptidase inhibitor clade G (C1 inhibitor), member 1
SF1	steroidogenic factor 1
SG	superficial glandular epithelium
SLC	small luteal cell
SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1
SLC5A1	solute carrier family 5 (sodium/glucose cotransporter) member 1
SLC5A6	solute carrier family 5 (sodium/multivitamin and iodide cotransporter) member 6
SLC25A10	solute carrier family 25 (mitochondrial carrier dicarboxylate transporter) member 10
SLC27A6	solute carrier family 27 (fatty acid transporter) member 6
SMC	smooth muscle cell
SR-B1	scavenger receptor type 1, class B
StAR	steroidogenic acute regulatory protein
TK	tyrosin kinase
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
US	ultrasound

V_d	distribution volume
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
VWP	voluntary waiting period
Wnt7a	wingless-type MMTV integration site family, member 7A
ZsF	zinc-salts fixative
3β HSD	3 beta-hydroxysteroid dehydrogenase

Chapter 1

General Introduction

After the harsh European famine during World War II, mankind concluded “never again” and created an agricultural policy with the intention to gradually increase food production to dispel starvation and to feed an increasing population (Frederico, 2009; UNPD, 2010). Also the dairy industry was growing. Ruminants, such as dairy cows, do have an added value for humans, as they are able to convert indigestible matter such as roughages into high-quality nutrients (milk and meat). But the other side of the coin is that dairy cows also have a polluting effect on the environment. Because of the ruminal fermentation process, they emit methane and because of the relatively low feed conversion ratio (0.2-0.3), their manure excretion is relatively high (70-80%) in comparison to their dry matter intake (DMI) (Garnsworthy, 2011). To cope with these disadvantages and to justify the environmental load to the society, cows should produce milk with maximal efficiency. Based on well thought selection programs and managerial optimization during the last decades, cows are created to produce the same amount of milk with significantly less animals, showing a significantly higher food conversion ratio and less manure production, leading to a reduced environmental impact (Capper et al., 2009). One should however not forget the most indispensable feature before a cow initiates milk production: she has to calve.

Consequently, reproductive efficiency has major impacts on profitability of livestock operations, including commercial dairy herds. Since several decades, reproductive performance of dairy cattle is decreasing (Royal et al., 2000a; Wiltbank et al., 2006): a longer period of anoestrus, a higher incidence of silent heats or suboestrus (1.6 versus 0.7 silent heats for 36 and 28 kg milk/day, respectively) (Harrison et al., 1990; Opsomer et al., 2000; Lopez et al., 2004), lowered conception rates (25-40% in lactating dairy cows versus 60-75% in dairy heifers), and an impaired embryonic quality (Butler, 2000; Wiltbank et al., 2006; Dobson et al., 2007; 2008; Friggens et al., 2010) have been reported. Leroy et al. (2005) classified only 13% of day-6-embryos flushed out of high-yielding dairy cows as excellent in comparison to 62.5% that were harvested out of non-lactating heifers of the same breed. These reduced fertility parameters result in an increase of the calving interval (CI) (Lucy, 2001; Inskeep and Dailey, 2005; Leroy and de Kruif, 2006). The mean Dutch-Flemish CI has increased with 35 days (from 394 days in 1993 to 422 days in 2011) (CRV, Jaarstatistieken, 2011), which costs the dairy industry a lot of money. An increase in milk production seems to be an important risk factor for an extension of the CI, as this interval is rising more obviously for cows in the higher milk production classes. It even rises to 431 days in the milk-production-class above 10,000 kg (CRV, Jaarstatistieken, 2011). When the CI increases from 395 days to 432 days on average 2.08 Euros will be lost daily per cow present in the herd

(Hogeveen, 2008; Cools et al., 2008). This economic loss could be mitigated by optimizing the cows' profits per day, i.e. by increasing the milk production peak or selecting for a higher persistency (Dekkers et al., 1998; Haile-Mariam et al., 2003; Muir et al., 2004). But even when the farmer should succeed this goal, in most of the herds, the increasing CI still entails an economic loss (Swalve, 2000; Hogeveen, 2008).

Beside other causes, such as the increased herd size leading to a less intensive individual care, the increased negative energy balance (NEB) associated with the intensive genetic selection for milk yield (MY) plays a key role in this problem (Wiltbank et al., 2006). Genetic selection during the last decennia, mainly oriented to milk production capacity, has led to Holstein Friesian cows with an increased milk production (more than 10,000 kg/year is common) (Diskin and Morris, 2008; Opsomer et al., 2000) which is only partly compensated by an increased DMI (Miglior et al., 2005; Dobson et al., 2007). Veerkamp et al. (2003) found a genetic correlation between MY and DMI of 0.44 to 0.65, indicating that only half of the extra energy requirements for the increase in MY is covered by a correlated response in DMI. The other half is covered by fat mobilization (i.e. the cow is dealing with a 'metabolic load') and energy partitioning (i.e. the cow is coping with 'metabolic stress'), as there is no indication of more efficient digestion or utilization of metabolizable energy (ME) (Ingvartsen et al., 2003). So, while creating a dairy cow gaining in genetic merit for MY, man has also shaped the conditions for a more pronounced NEB during the first 10-12 weeks post partum (Butler, 2003; Pryce et al., 2003). Indeed, several studies found a negative genetic correlation between MY or NEB and multiple fertility parameters in Holstein cows (Oltenacu et al., 1991, Dematawewa and Berger, 1998, Veerkamp et al., 2000).

This NEB is threatening cows' fertility but increases also cows' susceptibility to typical peripartal diseases including lameness, harming farmers' profit, as these disorders increase medical expenses and can further decrease cows' fertility (Royal et al., 2000a; Butler, 2003. Wiltbank et al., 2006), particularly in the Holstein breed.

Two main factors are responsible for the currently mentioned prolonged CI, namely an increased (voluntary) waiting period (Opsomer et al., 2000) and an increased incidence of early conceptus loss (Mann and Lamming, 2001; Diskin and Morris, 2008).

1.1 Increased (voluntary) waiting period (VWP)

Some farmers simply decide to delay the first insemination of cows with a higher yield (Royal et al., 2000b), but since this measure seldom returns into profit (Hogeveen, 2008), the extended period between partus and first insemination is mostly involuntary. There are

several underlying causes inducing a delay in the moment of first insemination: anoestrus (Opsomer et al., 2000; Royal et al., 2000a; Lopez-Gatius et al., 2003; Garnsworthy, 2006), suboestrus (Dobson et al., 2008), cystic ovarian follicles (Lamming and Darwash, 1998) and an extended luteal phase (Opsomer et al., 2000; Royal et al., 2000a). Based on data of Crowe (2008), the fate of the first postpartum dominant follicle in high yielding dairy cows is as follows: 30 to 80% will ovulate and will be transformed into a functional corpus luteum (CL); 15 to 60% will become atretic and 1 to 5% evolves to a cyst.

So, the majority of animals has already a follicular growth cycle in the first two weeks post partum, even resulting into a dominant follicle (Crowe, 2008). In other words: the problem seems not to be situated in a modulation of follicle stimulating hormone (FSH), but rather in luteinizing hormone (LH) (Vanholder et al., 2006). As LH is also necessary for luteogenesis, this could also impair consequent luteal development (Schams and Berisha, 2004). Indeed, in several studies a negative impact of NEB or MY-level on commencement of luteal activity (C-LA) has been demonstrated (Senatore et al., 1996; De Vries and Veerkamp, 2000; Kawashima et al., 2007).

1.2 Increased incidence of early embryonic death

When the cow ovulates and is served, one obstacle has been taken. But serving the cow will not always result in calving.

During the last decades, the conception rate (CR) after first insemination has decreased from 60% to 40% while the fertilization rate (i.e. the relative number of oocytes that are fertilized; FR) does not show such a dramatic decrease (Royal et al., 2000a; Sartori et al., 2002a). Depending on the study and the age of the animals included in the study, the FR varies between 76 and 100% (Mann and Lamming, 2001; Santos et al., 2004) and it appears that this rate is independent of MY-level (Diskin and Morris, 2008). On the other hand, MY-level does impair early embryonic development (during the first week), as significantly more embryos show an abnormal or retarded development in lactating heifers and cows versus non lactating animals (47 to 67% versus 17 to 28%; Sartori et al., 2002b). The latter is also true for higher (11-67%) versus lower yielding (17-28%) dairy heifers and cows (Cerri et al., 2009 a; b). So, despite comparable FR, the major component of embryo loss occurs before day 16 after breeding, with emerging evidence of greater losses before day 8 in present-day high yielding dairy cows compared to their lower producing counterparts (Diskin et al., 2012).

What are the reasons of such a high wastage of bovine life?

Compared to the reproductive outcome of the British Friesian (of 1980), the Holstein Friesian cows (of 2006) are coping with an increased loss in offspring caused by a significantly higher incidence of the early embryonic death (EED), while fertilization failure and late embryonic death (LED) were stable (Diskin and Morris, 2008).

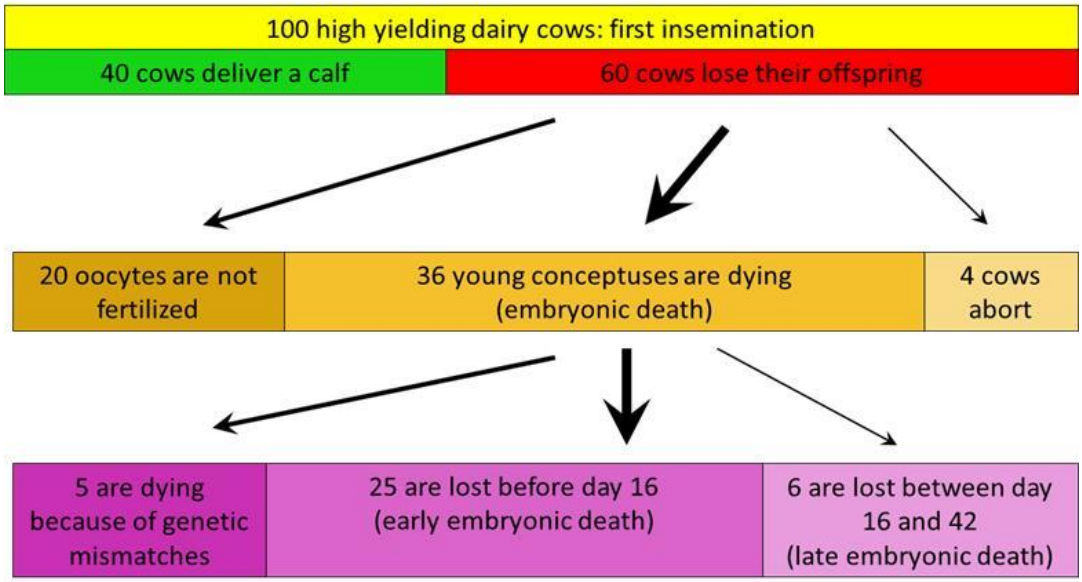


Figure 1: *distribution and importance of the loss of offspring during the gestation period*

Figure 1 gives an overview of the distribution of this 'loss of life' in the present-day high yielding dairy cows. Sixty percent of these cows do not calve after first insemination (Diskin et al., 2012). Within this subgroup, in 33% (percentage on total base: 20) the oocyte was not fertilized, 60% (36%) experienced embryonic death and 7% (4%) of those cows aborted. Within the group dealing with embryonic death, 14% (5%) of the embryos are confronted with genetic mismatches, 69% (25%) shows EED (loss before day 21 of pregnancy) and 17% (6%) did conceive but the embryo died between 21 and 42 days (LED). So, the majority of the loss of potential offspring in modern dairy cattle is concentrated in the first 3 weeks of pregnancy (Sreenan et al., 2001; Inskeep and Dailey, 2005). During this period the size and protein content of the embryo is increasing exponentially; there is a peak in embryonic synthetic activity; the embryonic morphology is changing dramatically as a result of compaction, blastocyst formation and elongation. At this time, the embryo is completely dependent on the salpingeal and uterine environment and is not yet attached to the endometrium. In practice, this means that animals dealing with EED are repeat breeders with normal cyclicity. As the variation in embryonic mortality between different herds is

substantial and has large impacts on the economic benefits of a dairy herd, it makes sense to analyze the possible causes to minimize their effects (Inchaisri et al., 2010).

The causes of EED are visualized in figure 2 and listed below; they can be categorized in management-linked factors and cow/embryo-linked factors.

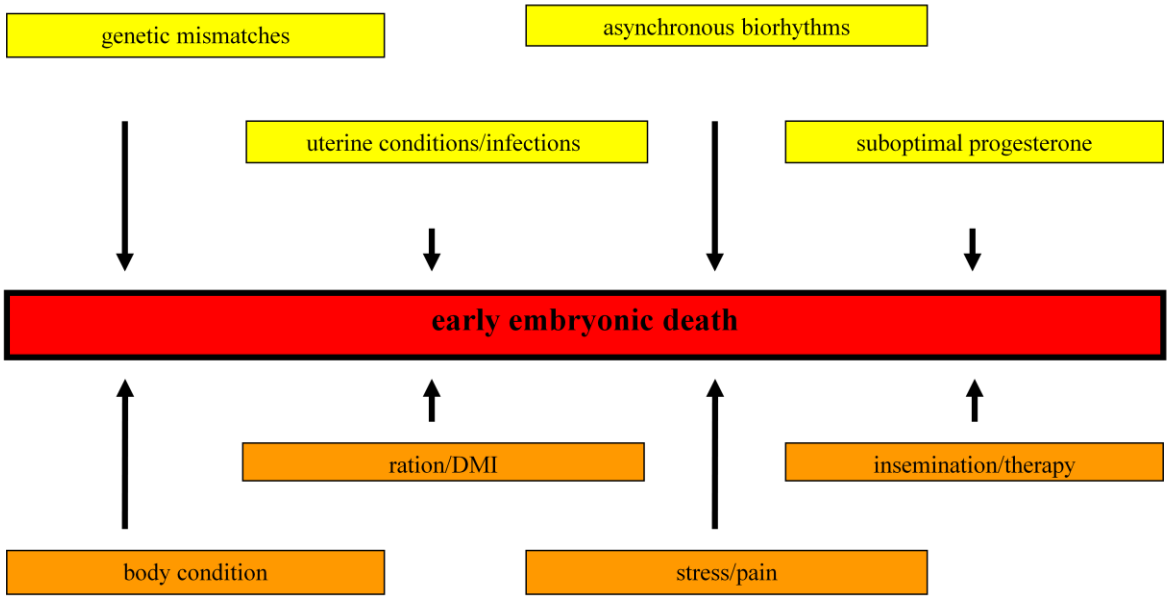


Figure 2: *overview of the multitude of factors influencing specifically early embryonic death. Factors are separated in cow/embryo-linked (yellow) and management-linked factors (orange)*

1.2.1 Management-linked factors

1.2.1.1 Insemination and therapy

The bovine oocyte has a fertilizable life of about six hours after ovulation. As the interval between ovulation and fertilization increases, so does the incidence of embryonic death after conception (Sreenan and Diskin, 1983).

Erroneously inseminated pregnant cows expressing heat symptoms and rectal palpation for pregnancy diagnosis can also cause embryo mortality (Donald, 1943; Abbitt et al., 1978 Kummerfeld et al.,1978; Cavestony and Foote, 1985; Sheldon, 1997; Sturman et al., 2000). Accidental injection of prostaglandin (PG) $F_{2\alpha}$ or oxytocin (OT) injection to induce milk letdown can induce partial to total luteolysis possibly leading to embryonic mortality (Roberts et al., 1975; Sheldon, 1997).

1.2.1.2 Stress and pain

Sickness, poor cow handling, poor housing design or maintenance (slippery floors, poorly bedded cubicles,…), overcrowding and other management shortcomings can induce stress and pain (Sheldon, 1997) disrupting the LH-secretion pattern (Dobson et al., 2008) and inducing the secretion of cytokines, which can have harmful effects on the uterine environment or directly on the embryo (Sheldon et al., 2002).

In case of heat stress, the increment of the rectal temperature with 1°C induces a decrease in pregnancy rate (PR) with 16% by decreasing the FR of defective oocytes and by increasing the EED (Dobson et al., 2007).

1.2.1.3 Ration and energy status

Negative effects on fertility may be exerted via a lower energy status, increased blood urea concentrations, or via an increased feed intake level per se (Leroy et al., 2008a and b).

A multitude of studies demonstrate the negative impact of NEB on conception (Butler, 2001; Gillund et al., 2001; Butler, 2003). High yielding dairy cows are confronted with a NEB when they are also expected to resume ovarian cyclicity (Opsomer et al., 2000; Royal et al., 2000b). Increasing the energy density of the ration to moderate the NEB is only a partial solution as high genetic merit cows translate the extra energy in an increase in MY rather than in decreasing the energy gap (Veerkamp et al., 2003). A NEB leads to a decreased GnRH and FSH/LH pulse frequency (Villa-Godoy et al., 1988; Butler, 2000), possibly affecting subsequent luteogenesis. Additionally, Britt (1994) and Leroy et al. (2005) hypothesized that follicles exposed to NEB during their initial growth result in inferior quality oocytes and dysfunctional corpora lutea (CLa), leading to a higher incidence of EED. The high crude protein (CP) content (17 to 19%) in most rations of high yielding dairy cows (Kung and Huber, 1983; Roffler and Thacker, 1983; Butler, 1998) can induce a decrease in fertility, such as a declie of the CR (Canfield et al., 1990; Larson et al., 1997; Butler, 1998; Westwood et al., 1998). Reason is the increased concentrations of urea in the blood and the uterine fluid, which is toxic for the embryo (Ferguson et al., 1993; Butler et al., 1996; Wittwer et al., 1999; Hojman et al., 2004). The gradual increase in DMI as noticed in postpartum dairy cows is associated with an acute and chronic increase in portal liver blood flow (Sangsritavong et al., 2002; Rhinehart et al., 2009), leading to an increase in the hepatic progesterone catabolism and consequently to a reduction of the peripheral progesterone concentration.

1.2.2 Cow or embryo-linked factors

1.2.2.1 Genetic mismatches

As meiosis is a game of chance in which the chromatin is divided partly by translocations, 5% of the ovum-sperm combinations leads to a lethal combination. Such an unfit genotype can be caused by chromosomal defects, individual genes and genetic interactions (Peters, 1996; VanRaden and Miller, 2006). Most of those embryos die before day 8 (Sheldon, 1997). Embryos, originating from cows that were genetically high milk producers showed a lower cleavage rate than those from genetically lower producing cows (Snijders et al., 2000).

1.2.2.2 Asynchrony between biorhythm of mother and embryo

A perfect synchrony between uterus, ovary and embryo is an absolute necessity for conception in cattle (Rowson et al., 1972; Wright, 1981). The mean time necessary for pregnant cows to initiate luteal function (3.27 days), approximates the time of transport of the embryo from the fallopian tube to the uterus, which is linked to an early influence of progesterone on the musculature of the isthmus (Chang, 1966). The uterus will not wait for embryos to become synchronous (Pope, 1988), but the embryonic development can be decelerated or accelerated to match uterine synchrony. Nevertheless, the performance of bovine embryos to adapt for asynchrony is rather limited compared to other species (Pope, 1988). An aberrant oestrogen/progesterone ratio can induce uterine (maternal) asynchrony (Testart, 1987) and can be created by individual variation in luteogenesis, heat stress, age or undernourishment (Quirke et al., 1979; Parr et al., 1982; Putney et al., 1988). Causes of embryonic asynchrony are gender, moment of ovulation, variation in timing of first and second meiotic division and embryonic cleavage rate (Pope et al., 1988; Sheldon, 1997). Those factors could lead to an aberrant interferon tau (IFNτ) production, leading to a lower chance of maternal recognition.

1.2.2.3 Infections and uterine conditions

Infections may affect fertility by interfering with ovarian cyclicity as well as with conception (Dhaliwal et al., 1996; Sheldon, 1997; Fray et al., 2002). *Escherichia coli* is the most common isolate from the uterus during endometritis, and lipopolysaccharide (LPS), the main pathogenic component from *E. coli*, is found in the plasma of cows with postpartum uterine infection (Sheldon et al., 2002). Lipopolysaccharide induces not only uterine damage but also disruption of the ovarian cycle control mechanisms (Sheldon et al., 2002; Morris et al., 2009; Wathes et al., 2009). . The endometrium produces an increased amount of $PGF_{2\alpha}$, which is

embryotoxic, and can induce a premature luteolysis (Manns et al., 1985). Additionally, cytokines and hyperthermia associated with intra- and extra-uterine infections may disrupt fertility via interactions with the hypothalamo-pituitary axis, the ovaries, the fallopian tubes, the uterus and the embryo itself (Gilbert, 2012).

1.2.2.4 Suboptimal progesterone concentration

Luteal progesterone is necessary for the establishment and maintenance of pregnancy (Sreenan and Diskin, 1983). Embryo transplantation results are better when transplantation is done in the horn ipsilateral to the CL, because of the higher local progesterone concentration (Newcomb and Rowson, 1978). Repeat breeders are often dealing with a delayed progesterone increase or a lowered progesterone concentration (Shelton et al., 1990). Plasma progesterone concentrations post partum in cows with a high genetic merit for MY were 25-50% lower during the second and third luteal phase than in control line cows (Lucy and Crooker, 2001).

Progesterone concentrations during the cycle preceding service as well as during the cycle of insemination itself seem to influence PR (Mann and Lamming, 2001; Diskin et al., 2012; Wiltbank et al., 2012). A positive association between the progesterone concentration during the luteal phase preceding conception and the embryonic survival rate in the subsequent cycle was found (Diskin et al., 2004; Inskeep, 2004). The relation between the progesterone concentration post insemination and EED is equivocal. Some studies described a positive relationship between the luteal phase progesterone level and PR (Fahey et al., 2002), while others do not (Sreenan and Diskin, 1983; Wiltbank et al., 2012). Proposed reasons for the delayed progesterone rise were impaired luteinisation and inadequate LH-support of progesterone secretion (Lamming et al., 1989; Mann and Lamming, 2001). The degree of embryonic development is positively correlated with maternal progesterone levels (Mann and Lamming, 2001). Sixty to 80% of the high yielding dairy cows had a suboptimal blood progesterone concentration during the first week after insemination (Stronge et al., 2005).

Progesterone decreases the endometrial oxytocin receptor (OTR) expression, leading to inhibition of luteolysis (Wathes and Lamming, 1995). So, a low concentration of progesterone during the luteal phase induces a stronger luteolytic signal (Mann and Lamming, 1995). Consequently, the future pre-ovulatory follicle escapes too early to the inhibiting progesterone influence. The latter leads to a follicle which enters the phase of dominance prematurely finally resulting in persistency of larger, but older dominant follicles. The result is the senescence of the oocyte which is still conceivable but shows a delayed

development (Savio et al., 1993; Armstrong et al., 2001). The musculature tone of the isthmus and utero-tubal junction is also influenced by progesterone. When the postovulatory progesterone rise is delayed, the conceptus stays too long in the alkaline fallopian tubal milieu, leading to an impaired embryo development (Baltz et al., 1995).

So, it is out of question that the majority of our high yielding Holstein cows are suffering from a NEB during 10 to 12 weeks post partum, which has an important negative impact on the dynamics of the blood progesterone concentration. As farmers want their cows to be fertile again in this period, they are often confronted with a reduced fertility in their livestock. As explained above, the etiology of this impaired reproduction is complex, but one of the main factors is the increased incidence of EED. Again, although the underlying reasons of EED are diverse and situated on different levels (management, cow, embryo), suboptimal LH-secretion, luteogenesis, CL-function and progesterone concentration represent a large part of the causal factors leading to the increased incidence of EED in high yielding dairy cattle. This implicates that if we could optimize this progesterone concentration, a significant amelioration in cows' postpartum fertility could be obtained. From now on, we will only focus on the problem of the suboptimal progesterone concentration in high yielding dairy cows.

Reference List

Abbitt B, Ball L, Kitto GP, Sitzman CG, Wilenburg B, Raim LW, Seidel GE, 1978: Effect of three methods of palpation for pregnancy diagnosis per rectum on embryonic and fetal attrition in cows. J Am Vet Med Assoc **173**, 973-977.

Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Wood KJ, Webb R, Sinclair KD, 2001: Effect of dietary energy and protein on bovine follicular dynamics and embryo production : associations with the ovarian insulin-like growth factor system. Biol Reprod **64**, 1624-1632.

Baltz JM, Zhao Y, Phillips KP, 1995: Intracellular pH and its regulation during fertilization and early embryogenesis. Theriogenology **44**, 1133-1144.

Britt JH, 1994: Follicular development and fertility, potential impacts of negative energy balance. In: Proceedings of National Reproductive Symposium, Pittsburgh, PA, pp. 103-112.

Butler WR, 1998: Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. J Dairy Sci **81**, 2533-2539.

Butler WR, 2001: Nutritional effects on resumption of ovarian cyclicity and conception rate in postpartum dairy cows. Anim Sci Occas Publ **26**, 133-145.

Butler WR, 2003: Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest Prod Sci **83**, 211-218.

Butler WR, Calaman JJ, Beam SW, 1996: Plasma and milk urea nitrogen in relation to pregnancy rate in lactating dairy cattle. J Anim Sci **74**, 858.

Butler WR, 2000: Nutritional interactions with reproductive performance in dairy cattle. Anim Reprod Sci **60-61**, 449-457.

Canfield RW, Sniffen CJ, Butler WR, 1990: Effects of excess degradable protein on postpartum reproduction and energy balance in dairy cattle. J Dairy Sci **73**, 2342-2349.

Capper JL, Cady RA, Bauman DE, 2009: The environmental impact of dairy production: 1944 compared with 2007. J Anim Sci **87**, 2160-2167.

Cavestony D, Foote RH, 1985: Prostaglandin F_2 alpha-induced estrus in open cows and presumed abortion in pregnant cows in the unobserved estrus in a herd monitored by milk progesterone assay. Cornell Vet **75**, 393-397.

Cerri RLA, Rutigliano HM, Chebel RC, Santos JEP, 2009a: Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reprod **137**, 813-823.

Cerri RLA, Juchem SO, Chebel RC, Rutigliano HM, Bruno RGS, Galvao KN, Thatcher WW, Santos JEP, 2009b: Effect of fat source differing in fatty acid profile on metabolic parameters, fertilization, and embryo quality in high-producing dairy cows. J Dairy Sci **92**, 1520-1531.

Chang MC, 1966: Effects of oral administration of medroxyprogesterone acetate and ethinyl estradiol on the transportation and development of rabbit eggs. Endocrinology **79**, 939.

Cools S, Bossaert P, Caluwaerts T, Hostens M, Opsomer G, de Kruif A, 2008: The economic consequences of extending the calving interval in high producing dairy cows. VDT **77**, 406-413.

Crowe M, 2008: Resumption of ovarian cyclicity in postpartum beef and dairy cows. Reprod Domestic Anim **43** (suppl 5), 20-28.

CRV, 2012: Jaarstatistieken 2011, CRV, Arnhem, march, 2012.

Dekkers JCM., Ten Hag JH, Weersink A, 1998. Economic aspects of persistency of lactation in dairy cattle. Livest Prod Sci 53, 237-252.

Dematawewa CMB, Berger PJ, 1998. Genetic and phenotypic parameters for 305-day yield, fertility and survival in Holsteins. J Dairy Sci **81**, 2700-2709.

De Vries MJ, Veerkamp RF, 2000. Energy balance of dairy cattle in relation to milk production variables and fertility. J Dairy Sci **83**, 62-69.

Dhaliwal GS, Murray RD, Dobson H, Montgomery J, Ellis WA, 1996: Reduced conception rates in dairy cattle associated with serological evidence of *Leptospira interrogans serovar hardjo* infection. Vet Rec **139**, 110-114.

Diskin MG, Morris DG, 2008: Embryonic and early fetal losses in cattle and other ruminants. Reprod Domest Anim **43** (suppl 2), 260-267.

Diskin MG, Parr MH, Morris DG, 2012: Embryo death in cattle: an update. Reprod Fertil Dev **24**, 244-251.

Diskin MG, Stronge AJH, Morris DG, Kenny DA, Sreenan JM, 2004: The association between early luteal phase concentrations of progesterone and embryo survival in heifers and dairy cows. J Anim Sci **82**, 101.

Dobson H, Smith RF, Royal MD, Knight CH, Sheldon IM, 2007: The high producing dairy cow and its reproductive performance. Reprod Domest Anim **42**, 17-23.

Dobson H, Walker SL, Morris MJ, Routly JE, Smith RF, 2008: Why is it getting more difficult to successfully artificially inseminate dairy cows? Animal **2**, 1104-1111.

Donald HF, 1943: Heat during pregnancy in dairy cows. Vet Rec **55**, 297-298.

Fahey J, McNamara S, Murphy JJ, Mee JF, Diskin MG, O'Callaghan D, 2002: The relationship between plasma progesterone concentrations post insemination and conception success in Holstein Friesian dairy cows. Ir Vet J **55**, 284.

Ferguson JD, Galligan DT, Blanchard T, Reeves M, 1993. Serum urea nitrogen and conception rate: the usefulness of test information. J Dairy Sci **76**, 37412.

Fray MD, Mann GE, Bleach ECL, Knight PG, Clarke MC, Charleston B, 2002: Modulation of sex hormone secretion in cows by acute infection with bovine viral diarrhea virus. Reprod **123**, 281-289.

Frederico G, 2009: Was the CAP the worst agricultural policy of the 20th century? In: Kiran Patel (ed.). Fertile ground for Europe? The history of European integration and the common agricultural policy since 1945. Baden-Baden Nomas, pp. 257-271.

Friggens NC, Disenhaus C, Petit HV, 2010. Nutritional sub-fertility in the dairy cow: towards improved reproductive management through a better biological understanding. Animal **4**,1197-1213.

Garnsworthy PC, 2006: Body condition score in dairy cows: targets for production and fertility. In: Garnsworthy PC, Wiseman J (eds.). Recent advances in animal nutrition, 2006. Nottingham University Press; Nottingham, UK, pp. 150-200.

Garnsworthy, PC, 2011: Sustainable intensive farming systems. in: Animal farming and environmental interactions in the mediterranean region. Casasús I., Rogosić J., Rosati A., Stoković I., Gabiña D. (eds.). Wageningen Academic Publishers, Amsterdam, The Netherlands, pp. 139-143.

Gilbert RO, 2012: The effects of endometritis on the establishment of pregnancy in cattle. Reprod Fertil Dev **24**, 252–257.

Gillund P, Reksen O, Gröhn YT, Karlberg K, 2001: Body condition related to ketosis and reproductive performance in Norwegian dairy cows. J Dairy Sci **84**, 1390-1396.

Haile-Mariam M, Bowman PJ, Goddard ME, 2003: Genetic and environmental relationship among calving interval, survival, persistency of milk yield and somatic cell count in dairy cattle. Livest Prod Sci **80**, 189-200.

Harrison RO, Ford SP, Young JW, Conely AJ, Freeman AE, 1990: Increased milk production versus reproductive and energy status in high-producing dairy cows. J Dairy Sci **73**, 2749-2758.

Hogeveen H, 2008: Is it economically feasible to improve health and fertility in dairy cows? WIAS Seminar, Strategies to improve health and fertility in dairy cows, Wageningen, 2008.

Hojman D, Kroll O, Adin G, Gips M, Hanochi B, Ezra E, 2004: Relationships between milk urea and production, nutrition, and fertility traits in Israeli dairy herds. J Dairy Sci **87**, 1001-1011.

Inchaisri C, Jorritsma R, Vos PLAM, van der Weijden GC, Hogeveen H, 2010: Economic consequences of reproductive performance in dairy cattle. Theriogenology **74**, 835-846.

Ingvartsen KL, Dewhurst RJ, Friggens NC, 2003: On the relationship between lactational performance and health: is it yield or metabolic imbalance that causes diseases in dairy cattle? A position paper. Livest Prod Sci **83**, 277-308.

Inskeep EK, 2004: Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. J Anim Sci **82** (E Suppl), E24-E39.

Inskeep EK, Dailey RA, 2005: Embryonic death in cattle. Vet Clin North Am Food Anim Pract **21**, 437-461.

Kawashima C, Amaya Montoya C, Masuda Y, Kaneko E, Matsui M, Shimizu T, Matsunaga N, Kida K, Miyake YI, Suzuki M, Miyamoto A, 2007: A positive relationship between the first ovulation postpartum and the increasing ratio of milk yield in the first part of lactation in dairy cows. J Dairy Sci **90**, 2279-2282.

Kummerfeld HL, Oltenacu EAB, Foot RH, 1978: Embryonic mortality in dairy cows estimated by nonreturns to service, estrus, and cyclic milk progesterone patterns. J Dairy Sci **61**, 1773-1777.

Kung Jr L, Huber JT, 1983: Performance of high producing cows in early lactation fed protein of varying amounts, sources and degradability. J Dairy Sci **66**, 227-234.

Lamming GE, Darwash AO, 1998: The use of milk progesterone profiles to characterise components of subfertility in milked dairy cows. Anim Reprod Sci **52**, 175-190.

Lamming GE, Darwash AO, Back HL, 1989: Corpus luteum function in dairy cows and embryo mortality. J Reprod Fert Suppl **37**, 245-252.

Larson SF, Butler WR, Currie WB, 1997: Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. J Dairy Sci **80**, 1288-1295.

Leroy JLMR, de Kruif A, 2006: Reduced reproductive performance in high producing dairy cows: is there actually a problem? VDT **75**, 55-60.

Leroy JLMR, Vanholder T, Opsomer G, Van Soom A, de Kruif A, 2005: The *in vitro* development of bovine oocytes after maturation in glucose and β-hydroxybutyrate concentrations associated with NEB in dairy cows. Reprod Dom Anim **41**, 119-123.

Leroy JL, Opsomer G, Van Soom A, Goovaerts IG, Bols PE, 2008a: Reduced fertility in high-yielding dairy cows: Are the oocyte and embryo in danger? Part I - The importance of negative energy balance and altered corpus luteum function to the reduction of oocyte and embryo quality in high-yielding dairy cows. Reprod Domest Anim **43**, 612-622.

Leroy JL, Van Soom A, Opsomer G, Goovaerts IG, Bols PE,. 2008b: Reduced fertility in high-yielding dairy cows: Are the oocyte and embryo in danger? Part II - Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows. Reprod Domest Anim **43**, 623-632.

Lopez H, Satter LD, Wiltbank MC, 2004: Relationship between level of milk production and estrous behavior of lactating dairy cows. Anim Reprod Sci **81**, 209-223.

Lopez-Gatius F, Yanis J, Madriles-Helm D, 2003: Effects of body condition score and score change on the reproductive performance of dairy cows: a meta-analysis. Theriogenology **59**, 801-812.

Lucy MC, 2001: Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci **84**,1277-1293.

Mann GE, Lamming GE, 1995: Progesterone inhibition of the development of the luteolytic signal in the cow. J Reprod Fertil **104**, 1-5.

Mann GE, Lamming GE, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reprod **121**, 175-180.

Manns JG, Nkuuhe JR, Bristol F, 1985: Prostaglandin concentrations in uterine fluid of cows with pyometra. Can J Comp Med **49**, 436-438.

Miglior F, Muir BL, van Doormaal BJ, 2005: Selection indices in Holstein cattle of various countries. J Dairy Sci **88**, 1255-1263.

Morris DG, Waters S, McCarthy S, Patton J, Earley B, Fitzpatrick R, Murphy JJ, Diskin MG, Kenny D, Brass A, Wathes DA, 2009: Pleiotropic effects of negative energy balance in the post partum dairy cow on splenic gene expression: repercussions for innate and adaptive immunity. Physiol Genomics **39**, 28-37.

Muir BL, Fatch J, Schaeffer LR, 2004: Genetic relationships between persistency and reproductive performance in first-lactation Canadian Holsteins. J Dairy Sci **87**, 3029-3037.

Oltenacu PA, Frick A, Lindhe O, 1991: Relationship of fertility to milk yield in Swedish cattle. J Dairy Sci **74**, 264-268.

Opsomer G, Gröhn Y, Hertl J, Coryn M, Deluyker H, de Kruif A, 2000: Risk factors for post partum ovarian dysfunction in high producing dairy cows in Belgium: a field study. Theriogenology **53**, 841-857.

Parr RA, Cumming IA, Clark IJ, 1982: Effects of maternal nutrition and plasma progesterone concentrations on survival and growth of the sheep embryo in early gestation. J Agric Sci **98**, 39-46.

Peters AR, 1996: Embryo mortality in the cow. Anim Breed Abst **64**, 587-598. Pope WF, 1988: Uterine asynchrony: a cause of embryonic loss. Biol Reprod **39**, 999-1003.

Pope WF, Wilde MH, Xie S, 1988: Effect of electrocautery of non-ovulated day 1 follicles on subsequent morphological variation among day 11 porcine embryos. Biol Reprod **39**, 882-887.

Pryce JE, Royal MD, Garnsworthy PC, Mao IL, 2003: Fertility in the high-producing dairy cow. Livest Prod Sci **86**, 125-135.

Putney DJ, Drost M, Thatcher WW, 1988: Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between days 1 to 7 post insemination. Theriogenology **30**, 195-209.

Quirke JF, Hanrahan JP, Gosling JP, 1979: Plasma progesterone levels throughout the oestrous cycle and release of LH at oestrus in sheep with different ovulation rates. J Reprod Fertil **55**, 37-44.

Rhinehart JD, Starbuck-Clemmer MJ, Flores JA, Milvae RA, Yao J, Poole DH, Inskeep EK, 2009: Low peripheral progesterone and late embryonic/early fetal loss in suckled beef and lactating dairy cows. Theriogenology **71**, 480-490.

Roberts JS, Barcikowski B, Wilson L Jr, Skarnes RC, McCracken JA, 1975: Hormonal and related factors affecting the release of prostaglandin F2α from the uterus. J Steroid Biochem **6**, 1091-1097.

Roffler RE, Thacker DL, 1983: Influence of reducing dietary crude protein form 17 to 13.5% on early lactation. J Dairy Sci **66**, 51-58.

Rowson LEA, Lawson RAS, Moor RM, Baker AA, 1972: Egg transfer in the cow: synchronization requirements. J Reprod Fertil **28**, 427-431.

Royal MD, Darwash AO, Flint APF, Webb R, Woolliams JA, Lamming GE, 2000a: Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. Anim Sci **70**, 487-501.

Royal M, Mann GE, Flint AP, 2000b: Strategies for reversing the trend towards subfertility in dairy cattle. Vet J **160**, 53-60.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC, 2002: High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle J Dairy Sci **85**, 2831-2842.

Santos JE, Thatcher WW, Chebel RC, Cerri RL, Galvao KN, 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Anim Reprod Sci **82**-83, 513-535.

Sartori R, Rosa GJM, Wiltbank MC, 2002: Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci **85**, 2813-2822.

Sartori R, Sartori-Bergfelt R, Mertens SA, Guenther JN, Parish JJ, Wiltbank MC, 2002: Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci **85**, 2803-2812.

Savio JD, Thatcher WW, Morris GR, Entwistle K, Drost M, Mattiacci MR, 1993: Effect of induction of low plasma progesterone concentration with a progesterone-releasing intravaginal device on follicular turnover and fertility in cattle. J Reprod Fertil **98**, 77-84.

Schams D, Berisha B, 2004: Regulation of corpus luteum function in cattle - an overview. Reprod Domest Anim **39**, 241-251.

Senatore EM, Butler WR, Oltenacu PA, 1996: Relationships between energy balance and postpartum ovarian activity and fertility in first lactation dairy cows. J Anim Sci **62**, 17-23.

Sheldon M, 1997: Bovine fertility - practical implications of the maternal recognition of pregnancy. In practice, November/December.

Sheldon IM, Noakes EE, Rycroft AN, Pfeiffer DU, Dobson H, 2002: Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reprod **123**, 837-845.

Snijders SEM, Dillon P, Callaghan DO, Boland MP, 2000: Effect of genetic merit, milk yield, body condition and lactation number on *in vitro* oocyte development in dairy cows. Theriogenology **53**, 981-990.

Sreenan JM, Diskin MG, 1983: Early embryonic mortality in the cow: its relationship with progesterone concentration. Vet Rec **112**, 517-521.

Sreenan JM, Diskin MG, Morris DG, 2001: Embryo survival rate in cattle: a major limitation to the achievement of high fertility. In: Diskin M.G. (ed.). Fertility in the high-producing dairy cow. Occasional Publication 26, British Society of Animal Science. Galway, pp. 93-104.

Stronge AJH, Sreenan JM, Diskin MG, Mee JF, Kenny DA, Morris DG, 2005: Post-insemination milk progesterone concentration and embryo survival in dairy cows. Theriogenology **64**, 1212-1224.

Sturman H, Oltenacu EAB, Foote RH, 2000: Importance of inseminating only cows in estrus. Theriogenology **53**, 1657-1667.

Swalve HH, 2000: Theoretical basis and computational methods for different test-day genetic evaluation methods. J Dairy Sci **83**, 1115-1124.

Testart J, 1987: Evidence of uterine inadequacy to egg implantation in stimulated in vitro fertilization cycles. Fertil Steril **47**, 855-857.

UNPD, 2010. World population prospects, the 2010 revision. United Nations Population Division. New York, New York.

Vanholder T, Opsomer G, de Kruif A, 2006: Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. Reprod Nutr Dev **46**,105-119.

VanRaden PM, Miller RH, 2006: Effects of nonadditive genetic interactions, inbreeding and recessive defects on embryo and fetal loss by seventy days. J Dairy Sci **89**, 2716-2721.

Veerkamp RF, Oldenbroeck JK, Van Der Gaast HJ, Van der Werf JH, 2000: Genetic correlation between days until start of luteal activity and milk yield, energy balance, and live weight. J Dairy Sci **83**, 577-583.

Veerkamp RF, Beerda B, van der Lende T, 2003: Effects of genetic selection for milk yield on energy balance, levels of hormones and metabolites in lactating cattle and possible links to reduced fertility. Livest Prod Sci **83**, 257-265.

Villa-Godoy A, Hughes TL, Emery RS, Chapin LT, Fogwell RL, 1988: Association between energy balance and luteal function in lactating dairy cows. J Dairy Sci **71**, 1063-1072.

Wathes DC, Cheng Z, Chowdhury W, Fenwick MA, Fitzpatrick R, Morris DG, Patton J, Murphy JJ, 2009: Negative energy balance alters global gene expression and immune responses in the uterus of postpartum dairy cows. Physiol Genomics **39**, 1-13.

Wathes DC, Lamming GE, 1995: The oxytocin receptor, luteolysis and the maintenance of pregnancy. J Reprod Fertil **49**, 53-67.

Westwood CT, Lean IJ, Kellaway RC, 1998: Indications and implications for testing of milk urea in dairy cattle: a quantitative review. Part 2. Effect of dietary protein on reproductive performance. New Zeal Vet J **46**, 123-130.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A, 2006: Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology **65**, 17-29.

Wiltbank MC, Souza AH, Carvalho PD, Bender RW, Nascimento AB, 2012: Improving fertility to timed artificial insemination by manipulation of circulating progesterone concentrations in lactating dairy cattle. Reprod Fertil Devel **24**, 238-243.

Wittwer FG, Gallardo P, Reyes J, Optiz H, 1999: Bulk milk urea concentrations and their relationship with cow fertility in grazing dairy herds in southern Chile. Prev Vet Med **38**, 159-166.

Wright J, 1981: Non-surgical embryo transfer in cattle embryo-recipient interactions. Theriogenology **15**, 43-56.

Chapter 2

Literature Review:

Why are high yielding dairy cows struggling with a lowered peripheral progesterone concentration and why does this lead to an increased incidence of early embryonic death?

Chapter 2.1

Progesterone in dairy cows, a key stone in fertility?

Progesterone is a prerequisite in sustaining pregnancy (McDonald et al., 1953; Sreenan and Diskin, 1983). Nevertheless, the relation between the peripheral progesterone concentration (blood, milk) during the first 2 weeks post insemination (PI) and the PR stays equivocal. Some found already a significant difference between pregnant and non-pregnant cows in the first week, others do not (Wiltbank et al., 2012). Table one gives an overview of those studies.

Table 1. *An overview of studies dealing with the evolution of the progesterone concentration (in milk or blood) during the early pregnancy.*

author (year of publication)	**sample size**	**matrix**	**cycle stage**	**effect[3]**
Erb et al. (1976)	30 (HF[1]; L[2])	blood	D 6	+
Bulman and Lamming (1978)	535 (HF; L)	milk	D 13	-
Lukasewska and Hansel (1980)	47 (HF; NL)	blood	D 10	-
Roche et al. (1985)	29 (HF; NL)	blood	D 16	-
Lamming et al. (1989)	2093 (HF; L)	milk	D 9	+
Shelton et al. (1990)	55 (HF; NL, L)	blood	D 6	-
Butler et al. (1996)	315 (HF; L)	blood	D 4	+
Larson et al. (1997)	228(HF;L)	milk	D 4,5	+
Lamming and Darwash (1998)	1682 (HF; L)	milk	D 4	+
Chagas e Silva et al. (2002)	407 (HF; L)	blood	D 7	-
Do Carmo Feliciano et al. (2003)	60 (HF; NL; L)	blood	D 17	+
Hommeida et al. (2004)	19 (HF; L)	milk	D 9	-
Green et al. (2005)	20 (HF; L)	blood	D 5	-

[1]HF: Holstein Friesian

[2]L: Lactating; NL: Non lactating

[3]significant difference between pregnant and non-pregnant animals (+); no difference detected (-)

In more recent studies the importance of progesterone in the establishment and maintenance of pregnancy is more and more refined. Shelton et al. (1990) found that the interval between the LH-peak and the initiation of the progesterone increase was significantly elongated in

subfertile cows versus fertile heifers. Also the progesterone increase rate was lower and the peripheral blood progesterone concentration was lower in subfertile cows, while the luteal mass was even larger. These phenomena are merged under the definition "luteal inadequacy". Larson et al. (1997) found in Holstein Friesian (HF) cows (MY ≥ 11,000 kg), in which conception succeeded, that the luteal function initiates at 3.27 days PI (i.e. more or less the duration of the transport through the salpinx), while this interval was extended (3.71 to 3.99 days) in case of failure of conception. A delayed increase of progesterone (even with less than one day) impairs the embryonic development, resulting in a decreased PR (Mann and Lamming, 2001; Hommeida et al., 2004; Robinson et al., 2005). Besides a delay in the progesterone increase, a subnormal progesterone concentration can lead to a stronger uterine luteolytic signal (Mann and Lamming, 1995) and a reduced vitality of the embryo (McNeill et al., 2006a).

Lamming and Darwash (1998) postulated a very nuanced statement:

"As there is an overlap of the progesterone concentration interval of pregnant versus non-pregnant animals, the maternal peripheral progesterone concentration must not be seen as an absolute cut-off determinant whether the animal will retain pregnancy or not, but it is rather a factor influencing the chance of success."

This positive relationship between the post ovulatory progesterone rise and the early embryonic development was also confirmed by Kerbler et al. (1997), Mann and Lamming (2001) and Mann et al. (2006). Green et al. (2005) stated that the plasma progesterone concentration was not significantly different between pregnant and non-pregnant animals, but the animals bearing an embryo in the morula stage showed a higher progesterone concentration than those animals with an embryo in the earlier, more immature 16-cell stage.

The story about the importance of progesterone has become already more refined, but it is still not complete. As the embryonic developmental rate is a measure of the viability of the conceptus, a higher progesterone concentration creates an embryo with a higher vitality already on day 5 (Dominko and First, 1997). Starbuck et al. (2001) found that cows with a milk progesterone concentration of 7-8 ng/ml on day 5 PI showed a maximal embryonic survival rate. Stronge et al. (2005) found a positive linear but also a quadratic relationship between milk progesterone concentration and embryonic survival rate on day 5, 6 and 7 PI. This quadratic relation indicates the existence of an optimal progesterone concentration (with a maximal embryonic survival rate) at every time point: 7.4 ng/ml (day 5 PI); 13.2 ng/ml (day 6 PI) and 16.8 ng/ml (day 7 PI). They even found an optimal progesterone increase rate (from day 4 to day 7) corresponding with a minimal EED (4.7 ng/(ml*d)). But, they also found that

the progesterone concentration on day 4 was negatively correlated with the embryonic survival rate, indicating that a progesterone concentration increasing too early or too fast could also induce an increase in EED. This linear and quadratic relation was also found between milk progesterone on cycle day 4 to 6 PI and the embryonic survival rate (McNeill et al., 2006b).

Progesterone seems to play a nuanced but essential role during the embryonic development. Surprisingly, the embryo itself does not contain progesterone receptors (P_4R) and progesterone seems to exert her effects indirectly on the conceptus. As systemic progesterone concentrations are influencing the volume of the uterine secretions and the expression of the endometrial genes, progesterone seems to use the uterine site to influence conceptus growth and survival. Namely, a slower rate of postovulatory progesterone rise through cycle day 4-5 has been associated with decreased embryonic growth by day 16 (Mann et al., 1996) and progesterone supplementation during the first 5 days altered the synthesis and release of polypeptides of the endometrium, stimulating the embryonic growth rate (Garrett et al., 1988). An extra argument of the uterine effect of progesterone is the presence of P_4R in the uterine endometrium, of which the expression is modified by progesterone itself (Stronge et al., 2005; McNeill et al., 2006b).

Elevated peripheral progesterone concentrations in the immediate post-conception period were associated with an advancement of conceptus elongation (Garrett et al., 1988; Geisert et al., 1992; Kerbler et al., 1997; Carter et al., 2008), and an increase in IFN τ production, a new protagonist with an essential role in the maternal recognition (Kerbler et al., 1997; Mann and Lamming, 2001). On the contrary, progesterone supplementation in a later stage (from day 20 to 39 post insemination) did not affect maintenance of pregnancy after its removal (Pilz et al., 2012).

This leads us to a final conclusion:

Maternal progesterone is influencing embryonic growth rate indirectly via endometrial actions. Sub- and supra-optimal progesterone concentrations on day 4 to 7 PI or a suboptimal progesterone increasing rate are all associated with a decrease in embryonic survival rate, as both extreme situations can induce uterine asynchrony not compensative anymore by the embryo. The missing link of the interaction between both, mother and embryo, is interferon tau (IFN τ).

2.1.1 the role of progesterone till maternal recognition

Now we will focus on the importance of progesterone during the first phase ofpregnancy, the period until implantation. In cattle, the implantation process is characterized by a protracted period of elongation of the hatched blastocyst followed by apposition, attachment and adhesion of the trophectoderm to the uterine luminal epithelium (Gharib-Hamrouche et al., 1993; Chavatte-Palmer and Guillomot, 2007). During the first 20 days of the embryogenesis, three phases can be recognised: 1) the evolution from zygote to morula (day 1 to 5); 2) the formation of a blastocyst (day 5 to 12); 3) the blastocyst elongation (day 12 to 20).

2.1.1.1 the first week of pregnancy

After fertilization, the embryo resides approximately 3 days in the salpinx. On the fourth day of pregnancy, the embryo arrives in the uterus. During this period the zygote evolves to a morula and three essential physiological events are needed to be established. First of all, the cow needs to create an appropriate salpingeal climate for the evolving conceptus, which means the provision of sufficient nutrients (ions, amino acids, glucose) and growth factors, such as insulin like growth factor (IGF)1 and 2. A second crucial event is the coordination of the action of the salpingeal cilia and smooth muscle contractions to make sure that the morula stage enters the uterus at the correct moment (Robinson et al., 2008). This process is influenced by the progesterone increasing rate (Chang, 1966). This brings us seamlessly to the third essential event in this first week: the increase in progesterone (i.e. the luteogenic process). This process needs an increase in translation or activity of the progesterone generating components (such as 3 β -hydroxysteroiddehydrogenase (3β-HSD), cytochrome $P450_{SCC}$ ($CYP450_{SCC}$), steroidogenic acute regulatory protein (StAR), peripheral type benzodiazepine receptors (PBDP-R)) and a decrease of the estrogen producing molecules (such as aromatase) (Niswender et al., 2000). The peripheral or systemic blood progesterone concentration starts to increase at cycle day 4 PI and evolves to a plateau at pregnancy day 10 to 14. This increasing progesterone concentration, together with the fast decreasing estradiol concentration creates and controls the salpingeal and endometrial milieu (Robinson et al., 2008), in which the embryo evolves relatively autonomously.

The answer to the question whether the presence of the embryo itself induces luteotrophic effects seems not that unambiguous. Chagas e Silva and Lopes da Costa (2005) found no evidence of any peripherally detectable luteotrophic influence of the embryo during the first 5 to 7 days, while several studies indicate a significant increase of the peripheral progesterone concentration in the early luteal phase (day 5-7) in pregnant females versus non-pregnant

cows (Mann et al., 1999; Kenny et al., 2001). Cows undergoing embryo transplantation (ET), showed no higher progesterone profiles until the moment of transfer (day 6-8), suggesting again a possible luteotrophic influence of the embryo which can even be delayed by postponing the ET (Spell et al., 2001; Chagas e Silva et al., 2002). Moreover, young bovine embryos exert *in vitro* luteotrophic stimuli upon co-cultured luteal cells (Thibodeaux et al., 1994). As the peripheral progesterone concentration could be confounded by several factors (such as metabolic status, milk yield) and as early embryonic death before day 16 has no effect on luteal lifespan (O'Callaghan and Boland, 1999; Humblot, 2001; Hommeida et al., 2004), the local (central) progesterone concentration could be a more sensitive parameter versus the peripheral blood progesterone concentration during this phase to monitor the supposed luteotrophic effects of the embryo.

2.1.1.2 the second week of pregnancy

This period is the pre-attachment period, and is the first part of the implantation phase. Mann and Lamming (2001) found that the rate of the blastocyst development was positively correlated with the increase in blood progesterone. It is the period of histotroph production. Histotroph, also called 'uterine milk', is composed of molecules secreted by the uterine epithelium or transported into the uterine lumen by the uterine epithelium (Bazer et al., 2011). Histotroph is essential for the conceptus development during the post-hatching period and the secretion is controlled by progesterone via the endometrial P_4R (Gray et al., 2001). In this period, the expression levels of the P_4R are maximal in the epithelial cells of the endometrial glands and in the subepithelial stroma from day 4 to 10 post ovulation (Robinson et al., 2001). This upregulation is initiated by estradiol, produced during the oestrus and the first dominant follicle on cycle day 4-5. Progesterone, on its turn, induces a down regulation of its own receptor (Spencer and Bazer, 1995) and creates an increase in the endometrial glandular density, stimulating histotroph production (Wang et al., 2007). During the luteal phase, several genes coding for transport proteins are up-regulated in the endometrium. These are dealing with the transport of glutamate, metal-ions, selenium, thyroid hormone, and are important as they are likely to control histotroph composition (Bauersachs et al., 2005; Mitko et al., 2008).

This means that luteal inadequacy could impair embryonic development, caused by a decreased up-regulation of these transporters and by retarded structural modification of the bovine endometrium (Robinson et al., 2008).

A blastocyst can produce *in vitro* progesterone by itself (Shemesh et al., 1979; O' Callaghan et al., 1999). This could eventually explain the pregnancy maintenance in cows with low peripheral plasma progesterone concentration (Chagas e Silva et al., 2002).

2.1.1.3 the third week of pregnancy

During this pre-attachment period, there are already interactions between endometrium and embryo. The main events are the strong elongation of the embryo and the inhibition of the luteolytic mechanism, which is initiated by an up-regulation of the uterine oxytocin receptors (OTR) (Mann et al., 1999). Progesterone initially decreases the OTR expression and the estradiol receptor (ER) α during the first 10 days. The expression of OTR was spontaneously up-regulated in the endometrial luminal epithelial cells even in the absence of oestradiol, but oestradiol seems to speed up the OTR expression *in vitro* (Leung and Wathes, 2000). Meanwhile, the conceptus is elongated from less than 1 cm (day 12) to more than 10 cm (day 16) and it produces IFN τ (Spencer et al., 2007). This IFN τ is detectable in uterine flushings from day 12 to 25 (Farin et al., 1990; Roberts et al., 2003), but shows a rapid increase from day 14 to 18, because of an increased elongation of the trophoblast (Robinson et al., 2006). Interferon τ interacts with a common receptor complex consisting of two subunit polypeptides (IFNAR 1 and 2) (Pestka et al., 2004) mainly present on the surface epithelium of the uterine lumen, but there is also evidence that it can reach stromal and even myometrial cells (Johnson et al., 1999; Hicks et al., 2003). Interferon τ inhibits the expression of the OTR (Telgmann et al., 2003). This IFN τ stimulates several endometrial genes, which are important for embryonic development and attachment (Demmers et al., 2001; Spencer et al., 2007a; b; 2008).

Also IGF1 and 2 stimulate the IFN τ production (Ko et al., 1991) and IGFBP can sequester IGF in the uterine lumen to promote IGF-action in this period. In the luminal epithelium, IGFBP-1 is increased in the presence of an embryo, coinciding with the blastocyst elongation (Robinson et al., 2000). This could increase the IGF1 availability for the embryo by regulating the transport of IGF1 from the endometrium to the uterine lumen. Transcription of this BP is switched on once the progesterone block is lost. So, a delayed progesterone increase, which can be seen under 'luteal inadequacy' could induce a delay in increase of IGFBP1 (Robinson et al., 2008). The expression of IGFBP 2 and 3 is decreased during pregnancy (Robinson et al., 2000). This could be suppressed by the embryo to increase IGF1 and 2-availability (Robinson et al., 2008).

For the maternal recognition of pregnancy (around day 16), a two way communication is necessary (Roberts et al., 2008): on the one hand the conceptus needs to block maternal ovarian cyclicity and ensure a sustained luteal progesterone production; on the other hand the mother needs to assess the fitness of the conceptus as early as possible (preferably before implantation is completed). The phase before hatching is not of importance for the maternal recognition. Rapidly after the blastocyst formation, the conceptus hatches and the outer surface of the trophectoderm can make instantly contact to the uterine environment (Roberts et al., 2008). On the one hand, the uterine milieu of a cyclic cow is still receptive at the end of the second week for an embryo (Betteridge et al., 1978), but on the other hand a robust luteoprotective intervention by the conceptus is absolutely necessary around cycle day 17, the moment of onset of luteolysis to maintain the pregnancy (Chagas e Silva and Lopes da Costa, 2005). Interferon tau is one of the signaling molecules, allowing firstly the embryo to declare its presence to its mother, and allowing secondly the mother to assess the conceptus' viability.

This means that the amount of interferon tau synthesized by the conceptus is influenced by the development status and the quality of the embryo, and may be used as an objective indicator of embryo quality (Hernandez-Ledezma et al., 1993).

Nevertheless the maternal system seems to have a coordinating role in the IFN τ production, the production of IFN τ begins at the blastocyst stage and rises as the conceptus begins to elongate (Farin et al., 1990; Ealy et al., 2001). The moment of the onset of elongation and maximal IFN τ production is variable from cow to cow but there is a correlation between the maternal plasma progesterone concentration and IFN τ of 0.593 (Kerbler et al., 1997). The most important regulating hormone is progesterone (Mann et al., 1999). Increased progesterone concentrations during metestrus and early diestrus (day 0 to 7) enhances the growth of embryos and the production of IFN τ (Geisert et al., 1992) and the progesterone concentration around maternal recognition and placentation plays also a role in the pregnancy establishment and retention (Starbuck et al., 2004; Scanavez et al., 2011). When the postovulatory progesterone increase was delayed with one day (in ewes), the IFN τ production decreased with a factor 3 and the embryos were smaller (Nephew et al., 1991). Mann and Lamming (2001) analyzed this progesterone effect on bovine embryos of day 16. In a first group of pregnant cows, which expressed a delayed increase in progesterone and a lowered progesterone plateau concentration between day 12 and 16 PI, IFN τ was not detectable and the recovered embryos were spherical to tubular (< 1 cm). The second group

of cows with a normal progesterone profile was IFN τ positive and the embryos were tubular or filamentous (>0.5cm). They also proved that IFN τ inhibits the endometrial $PGF_{2\alpha}$ secretion after an OT-challenge. So, a lower circulating progesterone concentration allows a stronger effect of the uterine luteolytic mechanism (Lamming and Mann, 1995a; b). Besides the inhibiting effect on the $PGF_{2\alpha}$ synthesis, Asselin et al. (1997), Xiao et al. (1999), and Chen et al. (2007) found in cattle or ewes that IFN τ stimulates the production of PGE. Prostaglandin E supports luteal maintenance and progesterone production (Magness et al., 1981; Bowolaksono et al., 2008). On the contrary, to make it more complicated, one study (Gray et al., 2006) found that IFN τ stimulated the endometrial production of $PGF_{2\alpha}$ by up-regulating cyclooxygenase (COX)-2.

2.1.1.4 the effect of progesterone supplementation on embryonic survival during the different periods

In cows, an increase in progesterone from day 2 to 5 (i.e. the first week of pregnancy) leads to embryos which had 10 times the normal length on day 14. Kerbler et al. (1997) studied the effect of progesterone supplementation from day 8 (i.e. the second week of pregnancy). This induces only a small increase in IFN tau on day 18. Mann et al. (1999) tested the effect of both periods: progesterone supplementation on day 5 to 9 induces IFN τ increase, while progesterone supplementation on day 12 to 16 had no effect.

So, it seems that the progesterone increase is more important than the progesterone level as such.

Can progesterone act directly on the embryo? On the one hand, Clemente et al. (2009) found mRNA of the nuclear P_4R and of the P_4R membrane component, but the progesterone concentration had *in vitro* no effect on the blastocyst yield, blastocyst cell number or relative abundance of certain selected transcripts in the blastocyst in the presence or absence of salpinx epithelial cells (Carter et al., 2010; Larson et al., 2011). So, probably progesterone does not have a direct effect on the embryo (Clemente et al., 2009). Moreover, an elevated progesterone concentration only during the period before ET (day 3 to 6) resulted in longer embryos on day 14, but when embryos were exposed *in vitro* to increased progesterone concentrations before ET there was no effect on elongation on day 14 (Clemente et al., 2009). So, progesterone does not affect directly the development to blastocyst stage or post-hatching elongation, but progesterone has an effect on elongation before maternal recognition and IFN τ production by regulation of uterine histotroph secretion (Carter et al., 2008; Rings et al., 2008). A minimal progesterone concentration is required for embryo survival after embryo

transfer in lactating Holstein cows (Kenyon et al., 2013): when a cow had a peripheral progesterone concentration lower than 5 ng/ml on cycle day 14, she had significantly more chance to lose the embryo in the period from 28 days to 42 days post insemination (P=0.01), and she tended to lose it in the period from day 28 to 63 (P=0.07). A faster rise in progesterone during the metestrus and early diestrus (day 0 to 7) are associated with a higher chance for pregnancy establishment following embryo transfer, which suggests that early rise in progesterone has an indirect effect on embryo development through modulation of uterine environment and secretion of histotroph (Lonergan, 2011). An early rise in progesterone resulted in a greater uterine capacity for histotroph production (increased mRNA for lipoprotein lipase, connective tissue growth factor) and an increased IFN τ production leading to an increase in the transcription of genes related to glucose and fructose transport (Forde et al., 2010; 2011a; b). So, an early increase in progesterone leads to an increased availability of nutrients for embryonic growth (elongation) and development (Kenyon et al., 2013). Furthermore, the positive effects of early rise in progesterone appear to go beyond the phase of maternal recognition of pregnancy through adhesion and placentation stages. The progesterone concentration from day 7 to 14 was associated with the establishment of the pregnancy. When the progesterone concentration was above 5 ng/ml, the pregnancy loss significantly reduces (Kenyon et al., 2013). But, apparantly contradictory with the important role of progesterone during the early pregnancy, the P_4R in the luminal and superficial glandular endometrial epithelium are already decreasing. Nevertheless, there are still P_4R present in the endometrial stromal cells and myometrium (Bazer et al., 2009; Forde et al., 2011a). Progesterone can still regulate the secretion of prostamedins (FGF7 and 10; HGF, IGF1 and 2) with paracrine effects on endometrial luminal and glandular epithelium and the trophectoderm (Bazer et al., 2009). The profile of these prostamedins is identical for cyclic and pregnant cows before maternal recognition.

Hugentobler et al. (2010) studied the progesterone effect on the composition of salpingeal and uterine fluids from day 3 to 6: 1) progesterone had no effect on the salpingeal fluid secretion rate, while uterine fluid secretion rate was decreasing; 2) progesterone decreased salpingeal sulphate and sodium, while uterine glucose was increasing, and no other effects were detected on uterine ions; 3) the salpingeal amino-acid content was increasing, the glucose content was increasing with a factor 2, while in the uterus only valine was increasing. This could explain partly the early differences in recovery rate and growth rate on day 7 (Forde et al., 2011a). So, progesterone level modifies the composition of the salpingeal fluid in which the embryo develops for the first 3-4 days. Besides that, the developmental capacity

of an embryo exposed to a maternal environment in vivo is higher than embryos produced in vitro (Enright et al., 2000; Rizos et al., 2002).

So, progesterone has an indirect effect on the embryonic growth by modulation of 1) the histotroph secretion, 2) the endometrial structure, 3) the adhesion and placentation.

2.1.2 The effect of progesterone on the genomic level

Progesterone affects the conceptus development partly by altering the temporal and spatial expression of key regulatory genes required as part of the biochemical cascade that controls early embryonic development by preparing the endometrium for histotroph production and implantation (Spencer et al., 2008). So, a considerable proportion of the early embryonic loss may be attributable to inadequate circulating progesterone concentrations and the downstream consequences on endometrial gene expression and histotroph secretion into the uterine lumen (Bazer et al., 1986; Forde et al., 2009a). The preparation of the uterine endometrium for embryo attachment and implantation involves extensively coordinated spatiotemporal alterations in the endometrial transcriptomic profiles. But in cyclic as well as in pregnant cows the endometrial gene expression is similar up to the initiation of conceptus elongation, and from day 16 (maternal recognition) on significant changes in the transcriptomic profile are detectable between cyclic and pregnant endometria (Forde et al., 2009b; Forde et al., 2011a; b), the moment when the filamentous conceptus produces increasing amounts of IFN τ. Forde et al. (2009a) analyzed the effect of elevated progesterone concentration on the endometrial gene expression advancing the conceptus elongation. Significant temporal changes in the transcriptional profile of the endometrium coincide with different developmental stages of the conceptus in early pregnancy. These changes are affected by an increased progesterone concentration and both contribute to and are a consequence of advanced development of the conceptus. Progesterone supplementation during the first week induces modifications in the endometrial gene expression at critical stages of peri-implantation embryo development. Genes with the largest fold change increase in translation activity (such as DGAT2 (diacylglycerol acyltransferase 2) and MSTN (myostatin) (= GDF8; growth/differentiation factor 8)) were associated with triglyceride synthesis and glucose transport, which can be used as an energy source for the developing blastocyst. Progesterone supplementation advances endometrial gene expression by altering the time (FABP (fatty acid binding protein), DGAT2, and MSTN) or the duration (CRYGS; gamma-S crystallin) of the expression pattern of genes that contribute to the composition of

histotroph. Diacylglycerol acyltransferase 2 catalyses the final step in the catabolism of triglycerides to acylcoenzyme A (Ferguson and Leese, 2006). Myostatin may increase glucose secretion into histotroph, promoting the conceptus development after hatching (McPherron and Lee, 2002). The differentially expressed genes (DEGs) between day 5 and 7 are mainly involved in the gene ontogeny (GO) terms: 1) triglyceride synthesis, cholesterol biosynthesis, retinoic acid metabolism and acylcoenzyme A synthesis (Suzuki et al., 1990; Faergeman and Knudsen, 1997; Su et al., 2008; Lutz et al., 2009); 2) protein transport/localization: intracellular transport of proteins possibly involved in trafficking of potential components of histotroph and endocytosis of serum glycoproteins, modifying histotroph composition (Johannes and Popoff, 2008); 3) members of glucose/fructose transporters; 4) transcription activity. These GOs can, on their turn, statistically be assembled to biochemical pathways and networks. But for more details, the reader is referred to the studies of Forde et al. (2011a; b; 2012). Progesterone could induce some of these genes, which could be further stimulated by IFN τ, such as the facilitative and sodium-dependent glucose-transporters in the ovine endometrium (Gao et al., 2009a). But progesterone did not induce an advancement in the expression of these genes (Forde et al., 2009a).

From day 7 to 13, a major shift in uterine gene expression occurs together with the loss of the P_4R in the uterine luminal epithelia. This loss of P_4R was associated with the induction of some genes associated with cell adhesion (Spencer et al., 2008). The DEGs between day 7 and 13 were mainly involved in the GO-terms of 1) mitochondrial electron transport and ATP-production; 2) cytoskeletal remodeling and cell cycle progression; 3) transport and synthesis of nucleotides, proteins and energy sources. Again these GOs can be collected into certain biochemical pathways and networks (Forde et al., 2011a; b). From day 16 on several genes were up-regulated or down-regulated (764 DEGs) in the endometrium of pregnant heifers versus cyclic counterparts, which can be attributed to 25 biological process terms (GOs), 10 canonical pathways and several networks (OT-R signaling pathway; apoptotic pathways; etc.) (Forde et al., 2011b).

Progesterone supplementation in heifers induces an earlier increase of the transport of the protein products into the uterine lumen, required to advance conceptus development and also of the genes associated with ATP-production (Forde et al., 2009a). A progressive increase of Interferon stimulated genes (ISGs) was seen comparing pregnancy day 13 versus 7. The endometrium shows an increased responsiveness on day 13 versus 7 but also on day 16 versus 13 to IFN τ, when progesterone was supplemented (Forde et al., 2011a). On the other hand, low circulating progesterone concentrations impair the ability of the salpinx/uterus to

support embryo development compared with that of dairy heifers with normal progesterone (Rizos et al., 2010) and induced a delay in the down-regulation of the P_4R in the luminal and glandular uterine epithelium till day 13 (Forde et al., 2011a; b). Forde et al. (2012) identified endometrial genes that are regulated by circulating concentrations of progesterone *in vivo* and likely impact the conceptus elongation and consequently embryonic IFN τ secretieon. The expression pattern of DGAT2, ASGR2, and FABP3 are delayed when progesterone levels are low. The temporal regulation of the expression of some genes is disrupted because of the delayed down-regulation of P_4R when progesterone is too low: ANPEP (a peptidase cleaving neutral amino acids from peptides), LPL (delivers triglycerides to tissues) were too low on day 7 and too high on day 13 in heifers with low progesterone levels; CTGF (role in proliferation of trophectoderm: cellular proliferation, migration, adhesion) was too low on day 13 in low progesterone heifers (Forde et al., 2011a; b). In general, genes which are more associated with the expression profiles during the early diestrus (day 7) persisted to day 13, when progesterone level was low, and genes with a likely biological role in elongation were lower when progesterone level was reduced (Forde et al., 2012).*This leads to a suboptimal uterine environment, independent of the presence of an embryo. It seems acceptable to believe that the modulation of endometrial genes by the progesterone level can influence the developmental capacity of the embryo, even before the blastocyst stage.*

Several genes could be influenced by progesterone level, namely genes encoding for growth factors, cytokines; genes involved in the progression of the estrous cycle (Forde and Lonergan, 2012). So, a change in progesterone level can induce changes in the endometrial transcriptome profile with a modification in histotroph and elongation rate. Besides that, there can also be an effect on IFN τ expression and maternal pregnancy recognition.
The progesterone level has an impact on elongation, even when the progesterone change was only before the moment of ET (day 7). This indicates that the effect of progesterone is rather indirect on the endometrial transcriptome, rather than direct effects on the embryo itself.

In general, we may conclude that progesterone is indeed a key stone in fertility. Progesterone regulates the most important key events during pregnancy establishment in bovines, namely blastocyst elongation and IFN τ production, essential for maternal recognition of the conceptus.

Reference List

Asselin E, Bazer FW, Fortier MA, 1997: Recombinant ovine and bovine interferons tau regulate prostaglandin production and oxytocin response in cultured bovine endometrial cells. Biol Reprod **56**, 402-408.

Bauersachs S, Ulbrich SE, Gross K, Schmidt SE, Meyer HH, Einspanier R, Wenigerkind H, Vermehren M, Blum H, Sinowatz F, Wolf E, 2005: Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. J Mol Endocrinol **34**, 889-908.

Bazer FW, Spencer TE, Johnson GA, Burghardt RC, Wu G, 2009: Comparative aspects of implantation. Reproduction **138**, 195-209.

Bazer FW, Vallet JL, Roberts RM, Sharp DC, Thatcher WW, 1986: Role of conceptus secretory products in establishment of pregnancy. J Reprod Fertil **76**, 841-850.

Bazer FW, Wu G, Johnson GA, Kim J, Song G, 2011: Uterine histotroph and conceptus development: select nutrients and secreted phosphoprotein 1 affect mechanistic target of rapamycin cell signaling in ewes. Biol Reprod **85**, 1094-1107.

Betteridge K, Eaglesome N, Randall G, Mitchell D, Lugden E, 1978: Maternal progesterone levels as evidence of luteotrophic or antiluteolytic effects on embryos transferred to heifers 12-17 days after estrus. Theriogenology **9**, 86-93.

Bowolaksono A, Nishimura R, Hojo T, Sakumoto R, Acosta TJ, Okuda K, 2008: Anti-apoptotic roles of prostaglandin E2 and F2alpha in bovine luteal steroidogenic cells. Biol Reprod **79**, 310-317.

Bulman DC, Lamming GE, 1978: Milk progesterone levels in relation to conception, repeat breeding and factors influencing acyclicity in dairy cows. J Reprod Fertil **54**, 447-458.

Butler WR, Calaman JJ, Beam SW, 1996: Plasma and milk urea nitrogen in relation to pregnancy rate in lactating dairy cattle. J Anim Sci **74**, 858-865.

Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans AC, Kenny DA, Roche JF, Lonergan P, 2008: Effect of increasing progesterone concentration from day 3 of pregnancy on subsequent embryo survival and development in beef heifers Reprod Fertil Dev **20**, 368-375.

Carter F, Rings F, Mamo S, Holker M, Kuzmany A, Besenfelder U, Havlicek V, Mehta JP, Tesfaye D, Schellander K, Lonergan P, 2010: Effect of elevated circulating progesterone concentration on bovine blastocyst development and global trancriptome following endoscopic transfer of *in vitro* produced embryos to the bovine oviduct. Biol Reprod **83**, 707-719.

Chagas e Silva J, Lopes da Costa L, 2005: Luteotrophic influence of early bovine embryos and the relationship between plasma progesterone concentrations and embryo survival. Theriogenology **64**, 49-60.

Chagas e Silva J, Lopes da Costa L, Silva J, 2002: Plasma progesterone profiles and factors affecting embryo-fetal mortality following embryo transfer in dairy cattle. Theriogenology **58**, 51-59.

Chang MC, 1966: Effects of oral administration of medroxyprogesterone acetate and ethinyl estradiol on the transportation and development of rabbit eggs. Endocrinology **79**, 939.

Chavatte-Palmer P, Guillomot M, 2007: Comparative implantation and placentation. Gynecol Obstet Invest **64**, 166-174.

Chen F, Wang B, Chen LZ, 2007: Comparison of safety and sensitivity in diagnosis of liver lesion between ultrasound-guided 16 gauges and 18 gauges core needle biopsy. Zhonghua Yi Xue Za Zhi **87**, 823-825.

Clemente M, de la Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, Rizos D, Lonergan P, 2009: Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? Reproduction **138**, 507-517.

Demmers KJ, Derecka K, Flint A, 2001: Trophoblast interferon and pregnancy. Reproduction **121**, 41-49.

do Carmo Feliciano M, Mateus L, da Costa LL, 2003: Luteal function and metabolic parameters in relation to conception in inseminated dairy cattle. RPCV **98**, 25-31.

Dominko T, First NL, 1997: Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Mol Reprod Dev **47**, 456-467.

Ealy AD, Larson SF, Liu L, Alexenko AP, Winkelman GL, Kubish HM, Bixby JA, Roberts RM, 2001: Polymorphic forms of expressed bovine interferon-tau genes: relative transcript abundance during early placental development, promoter sequences of genes and biological activity of protein products. Endocrinology **142**, 2906-2915.

Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X, Boland MP, 2000: Culture of *in vitro* produced bovine zygotes *in vitro* vs *in vivo*: implications for early embryo development and quality. Theriogenology **54**, 659-673.

Erb RE, Garverick HA, Randel RD, Brown BL, Callahan CJ, 1976: Profiles of reproductive hormones associated with fertile and nonfertile inseminations of dairy cows. Theriogenology **5**, 227-242.

Faergeman NJ, Knudsen J, 1997: Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and cell signaling. Biochem J **323** (part 1), 1-12.

Farin CE, Imakawa K, Hansen TR, McDonnell JJ, Murphy CN, Farin PW, Roberts RM, 1990: Expression of trophoblastic interferon genes in sheep and cattle. Biol Reprod **43**, 210-218.

Ferguson EM, Leese HJ, 2006: A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol Reprod Dev **73**, 1195-1201.

Forde N, Beltman ME, Duffy GB, Duffy P, Mehta JP, O'Gaora P, Roche JF, Lonergan P, Crowe MA, 2011a: Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. Biol Reprod **84**, 266-278.

Forde N, Carter F, Fair T, Crowe MA, Evans ACO, Spencer TE, Bazer FW, McBride R, Bolandt MP, O'Gaora P, Lonergan P, Roche JF, 2009a: Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. Biol Reprod **81**, 784-794.

Forde N, Carter F, Fair T, Crowe MA, Evans ACO, Spencer TE, Bazer FW, O'Gaora P, McBride R, Bolandt MP, Lonergan P, Roche JF, 2009b: Effects of pregnancy and progesterone on gene expression in the uterine endometrium of cattle. In: Abstracts of the 42nd annual meeting of the society for the study of reproduction, july 18-22, 2009, Pittsburgh, Pennsylvania. Biol. Reprod., Special Issue: Abstract 37.

Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, Okumu LA, McGettigan PA, Mehta JP, McBride R, O'Gaora P, Roche JF, Lonergan P, 2011b: Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? Biol Reprod **85**, 144-156.

Forde N, Duffy PA, McGettingen PA, Browne JA, Mehta JP, Kelly AK, Mansouri-Attia N, Sandra O, Loftus BJ, Crowe MA, Fair T, Roche JF, Lonergan P, Evans ACO, 2012: Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. Physiol. Genomics **44**, 799-810.

Forde N, Lonergan P, 2012: Transcriptomic analysis of the bovine endometrium: what is required to establish uterine receptivity to implantation in cattle? J Reprod Dev **58**, 189-195.

Forde N, Spencer TE, Bazer FW, Song G, Roche JF, Lonergan P, 2010: Effect of pregnancy and progesterone concentration on expression of genes encoding for transporters or secreted proteins in the bovine endometrium. Physiol Genomics **41**, 53-62.

Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW, 2009a: Select nutrients in the ovine uterine lumen, II: glucose transporters in the uterus and periimplantation conceptuses. Biol Reprod **80**, 94-104.

Garrett JE, Geisert RD, Zavy MT, Morgan GL, 1988: Evidence for maternal regulation of early conceptus growth and development in beef cattle. J Reprod Fertil **84**, 437-446.

Geisert RD, Morgan GL, Short EC Jr, Zavy MT, 1992: Endocrine events associated with endometrial function and conceptus development in cattle. Reprod Fertil Dev **4**, 301-305.

Gharib-Hamrouche N, Chene N, Guillomot M, Martal J, 1993: Localization and characterization of EGF/TGF-alpha receptors on peri-implantation trophoblast in sheep. J Reprod Fertil **98**, 385-392.

Gray CA, Abbey CA, Beremand PD, Choi Y, Farmer JL, Adelson DL, Thomas TL, Bazer FW, Spencer TE, 2006: Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. Biol Reprod **74**, 383-394.

Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, Spencer TE, 2001: Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod **64**, 1608-1613.

Green MP, Hunter MG, Mann GE, 2005: Relationships between maternal hormone secretion and embryo development on day 5 of pregnancy in dairy cows. Anim Reprod Sci **88**, 179-189.

Hernandez-Ledezma JJ, Mathialagan M, Villanueva C, Sikes JD, Roberts RM, 1993: Expression of bovine trophoblast interferons by in vitro-derived blastocysts is correlated with their morphological quality and stage of development. Mol Reprod Dev **36**, 1-6.

Hicks BA, Etter SJ, Carnahan KG, Joyce MM, Assiri AA, Carling SJ, Kodali K, Johnson GA, Hansen TR, Mirando MA, Woods GL, Vanderwall DK, Ott TL, 2003: Expression of the uterine Mx protein in cyclic and pregnant cows, gilts, and mares. J Anim Sci **81**, 1552-1561.

Hommeida A, Nakao T, Kubota H, 2004: Luteal function and conception in lactating cows and some factors influencing luteal function after first insemination. Theriogenology **62**, 217-225.

Hugentobler SA, Sreenan JM, Humpherson PG, Leese HJ, Diskin MG, Morris DG, 2010: Effects of changes in the concentration of systemic progesterone on ions, amino acids and energy substrates in cattle oviduct and uterine fluid and blood. Reprod Fertil Dev **22**, 684-694.

Humblot P, 2001: Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology **56**, 1417-1433.

Johannes L, Popoff V, 2008: Tracing the retrograde route in protein trafficking. Cell **135**, 1175-1187.

Johnson G, Austin K, Collins A, Murdoch W, Hansen T, 1999: Endometrial ICG17 mRNA and a related mRNA are induced by IFN τ and localized to glandular epithelial and stromal cells from pregnant cows. Endocrine **10**, 243-252.

Kenny DA, Boland MP, Diskin MG, Sreenan JM, 2001: Effect of pasture crude protein and fermentable energy supplementation on blood metabolite and progesterone concentrations and on embryo survival in heifers. Anim Sci **73**, 501-511.

Kenyon AG, Mendonca LGD, Lopes G Jr, Lima JR, Santos JEP, Chebel RC, 2013: Minimal progesterone concentration required for embryo survival after embryo transfer in lactating Holstein cows. Anim Reprod Sci **136**, 223-230.

Kerbler TL, Buhr MM, Jordan LT, Leslie KE, Walton JS, 1997: Relationship between maternal plasma progesterone concentration and interferon-tau synthesis by the conceptus in cattle. Theriogenology **47**, 703-714.

Ko Y, Lee CJ, Ott TL, Davis MA, Simmen RCM, Bazer FW, Simmen FA, 1991: Insulin-like growth factors in sheep uterine fluids: concentrations and relationship to ovine trophoblastic protein-1 production during early pregnancy. Biol Reprod **45**, 135-142.

Lamming GE, Darwash AO, 1998: The use of milk progesterone profiles to characterise components of subfertility in milked dairy cows. Anim Reprod Sci **52**, 175-190.

Lamming GE, Darwash AO, Back HL, 1989: Corpus luteum function in dairy cows and embryo mortality. J Reprod Fert Suppl **37**, 245-252.

Lamming GE, Mann GE, 1995a: A dual role for progesterone in the control of cyclicity in ruminants. J Reprod Fertil Suppl **49**, 561-566.

Lamming GE, Mann GE, 1995b: Control of endometrial oxytocin receptors and prostaglandin F2 alpha production in cows by progesterone and oestradiol. J Reprod Fertil **103**, 69-73.

Larson SF, Butler WR, Currie WB, 1997: Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cow. J Dairy Sci **80**, 1288-1295.

Larson JE, Krisher RL, Lamb GC, 2011: Effects of supplemental progesterone on the development, metabolism and blastocyst cell number of bovine embryos produced *in vitro*. Reprod Fertil Dev **23**, 311-318.

Leung ST, Wathes DC, 2000: Oestradiol regulation of oxytocin receptor expression in cyclic bovine endometrium. Reprod Fertil **119**, 287-292.

Lonergan P, 2011: Influence of progesterone on oocyte quality and embryo development in cows. Theriogenology **76**, 1594-1601.

Lukaszewska J, Hansel W, 1980: Corpus luteum maintenance during early pregnancy in the cow. J Reprod Fertil **59**, 485-493.

Lutz JD, Dixit V, Yeung CK, Dickmann LJ, Zelter A, Thatcher JE, Nelson WL, Isoherranen N, 2009: Expression and functional characterization of cytochrome P450 26A1, a retinoic acid hydroxylase. Biochem Pharmacol **77**, 258-268.

Magness RR, Huie JM, Hoyer GL, Hueckstead TP, Reynolds LP, Seperich GJ, Whysong G, Weems CW, 1981: Effect of chronic ipsilateral or contralateral intrauterine infusion of prostaglandin E2 (PGE2) on luteal function of unilatera0lyl ovariectomized ewes. Prostaglandins Med **6**, 389-401.

Mann GE, Lamming GE, 1995: Progesterone inhibition of the development of the luteolytic signal in the cow. J Reprod Fertil **104**, 1-5.

Mann GE, Lamming GE, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction **121**, 175-180.

Mann GE, Lamming GE, Robinson RS, Wathes DC, 1999: The regulation of interferon-tau production and uterine hormone receptors during early pregnancy. J Reprod Fertil Suppl **54**, 317-328.

Mann GE, Mann SJ, Lamming GE, 1996: The interrelationship between the maternal hormone environment and the embryo during the early stages of pregnancy in the cow. J Reprod Fertil Abstr Seer **17**, 21.

McDonald LE, Nichols RE, McNutt SH, 1953: Replacement therapy with a slowly absorbed progesterone product. Am J Vet Res **14**, 385-387.

McNeill RE, Diskin MG, Sreenan JM, Morris DG, 2006a: Associations between milk progesterone concentration on different days and with embryo survival during the early luteal phase in dairy cows. Theriogenology **65**, 1435-1441.

McNeill RE, Sreenan JM, Diskin MG, Cairns MT, Fitzpatrick R, Smith TJ, Morris DG, 2006b: Effect of systemic progesterone concentration on the expression of progesterone-responsive genes in the bovine endometrium during the early luteal phase. Reprod Fertil Dev **18**, 573-583.

McPherron AC, Lee SJ, 2002: Suppression of body fat accumulation in myostatin-deficient mice. J Clin Invest **109**, 595-601.

Mitko K, Ulbrich SE, Wenigerkind H, Sinowatz F, Blum H, 2008: Dynamic changes in messenger RNA profiles of bovine endometrium during the oestous cycle. Reproduction **135**, 225-240.

Nephew KP, McClure KE, Ott TL, Dubois DH, Bazer FW, Pope WF, 1991: Relationship between variation in conceptus development and differences in estrous cycle duration in ewes. Biol Reprod **44**, 536-539.

Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW, 2000: Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev **80**, 1-29.

O'Callaghan D, Boland MP, 1999: Nutritional effects on ovulation, embryo development and the estrablishment of pregnancy in ruminants. Anim Sci **68**, 299-314.

O'Callaghan D, Lozano JM, Fahey J, Gath V, Snijders S, Boland MP, 1999: Relationships between nutrition and fertility in dairy cattle. In: Diskin M.G., editor. Fertility in the high-producing dairy cow, vol. I, no. 26. British Society of Animal Science Occasional Publication, 147-159.

Pestka S, Krause CD, Walter MR, 2004: Interferons, interferon-like cytokines, and their receptors. Immunological Reviews **202**, 8-32.

Pilz M, Fischer-Tenhagen C, von Krueger X, Thiele G, Heuwieser W, 2012: Efficacy of progesterone inserts (CIDR) to reduce early embryonic losses in dairy cows. Tieraerztliche Praxis Ausgabe Grosstiere Nutztiere **40**, 7-13.

Rings F, Carter F, Holker M, Kuzmany A, Besenfelder U, Havlicek V, Tefaye D, Schellander K, Lonergan P, 2008: Effect of elevated circulating progesterone concentration on development of *in vitro* produced bovine zygotes *in vivo*. Reprod Fertil Dev **20**, 147-148.

Rizos D, Carter F, Besenfelder U, Havlicek V, Lonergan P, 2010: Contribution of the female reproductive tract to low fertility in postpartum lactating dairy cows. J Dairy Sci **93**, 1022-1029.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P, 2002: Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev **61**, 234-248.

Roberts RM, Chen Y, Ezashi T, Walker AM, 2008: Interferons and the maternal-conceptus dialog in mammals. Seminars in Cell & Developmental Biology **19**, 170-177.

Roberts RM, Ezashi T, Rosenfeld CS, Ealy AD, Kubisch HM, 2003: Evolution of the interferon tau genes and their promoters, and maternal-trophoblast interactions in control of their expression. Reprod Suppl **61**, 239-251.

Robinson RS, Fray MD, Wathes DC, Lamming GE, Mann GE, 2006: *In vivo* expression of interferon tau mRNA by the embryonic trophoblast and uterine concentrations of interferon tau protein during early pregnancy in the cow. Mol Reprod Dev **73**, 470-474.

Robinson RS, Hammond AJ, Hunter MG, Mann GE, 2005: The induction of a delayed post-ovulatory progesterone rise in dairy cows: a novel model. Domest Anim Endocrinol **28**, 285-295.

Robinson RS, Hammond AJ, Wathes DC, Hunter MG, Mann GE, 2008: Corpus luteum-endometrium-embryo interactions in the dairy cow: underlying mechanisms and clinical relevance. Reprod Dom Anim **43** (Suppl. 2), 104-112.

Robinson RS, Mann GE, Gadd TS, Lamming GE, Wathes DC, 2000: The expression of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy. J Endocrinol **165**, 231-243.

Robinson RS, Mann GE, Lamming GE, Wathes DC, 2001: Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. Reproduction **122**, 965-979.

Roche JF, Ireland JJ, Boland MP, McGeady TM, 1985: Concentrations of luteinising hormone and progesterone in pregnant and non-pregnant heifers. Vet Rec **116**, 153-155.

Scanavez ALA, Moraes JGN, Bruno RGS, Lager KJ, Hernandez-Rivera JA, Silva PRB, Mendonca LGD, Bilby TR, Chebel RC, 2011: Effects of sequential injections of GnRH at 17 and 24 d after AI on progesterone concentration and pregnancy losses. J Dairy Sci **94** (E-suppl. 1), 348.

Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE, 1990: Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil **90**, 1-10.

Shemesh M, Milaguir F, Ayalon N, Hansel W, 1979: Steroidogenesis and prostaglandin synthesis by cultured bovine blastocysts. J Reprod Fertil **56**, 181-185.

Spell AR, Beal WE, Corah LR, Lamb GC, 2001: Evaluating recipient and embryo factors that affect pregnancy rates of embryo transfer in beef cattle. Theriogenology **56**, 287-297.

Spencer TE, Bazer FW, 1995: Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. Biol Reprod **53**, 1527-1543.

Spencer TE, Johnson GA, Bazer FW, Burghardt RC, 2007a: Fetal-maternal interactions during the establishment of pregnancy in ruminants. Soc Reprod Fertil Suppl **64**, 379-396.

Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M, 2007b: Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev **19**, 65-78.

Spencer TE, Sandra O, Wolf E, 2008: Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction **135**, 165-179.

Sreenan JM, Diskin MG, 1983: Early embryonic mortality in the cow: its relationship with progesterone concentration. Vet Rec **112**, 517-521.

Starbuck MJ, Dailey RA, Inskeep EK, 2004: Factors affecting retention of early pregnancy in dairy cattle. Anim Reprod Sci **84**, 27-39.

Starbuck GR, Darwash AO, Mann GE, Lamming GE, 2001: The detection and treatment of post-insemination progesterone insufficiency in dairy cows. Fertility in the high-producing dairy cow. Brit Soc Anim Sci Occasional Publication **26**, 447-450.

Stronge AJH, Sreenan JM, Diskin MG, Mee JF, Kenny DA, Morris DG, 2005: Post-insemination milk progesterone concentration and embryo survival in dairy cows. Theriogenology **64**, 1212-1224.

Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA, Matzuk MM, Eppig JJ, 2008: Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. Development **135**, 111-121.

Suzuki H, Kawarabayashi Y, Kondo J, Abe T, Nishikawa K, Kimura S, Hashimoto T, Yamamoto T, 1990: Structure and regulation of rat long-chain acyl-coA synthetase. J Biol Chem **265**, 8681-8685.

Telgmann R, Bathgate RA, Jaeger S, Tillmann G, Ivell R, 2003: Transcriptional regulation of the bovine oxytocin receptor gene. Biol Reprod **68**, 1015-1026.

Thibodeaux JK, Broussard JR, Godke RA, Hansel W, 1994: Stimulation of progesterone production in bovine luteal cells by coincubation with bovine blastocyst-stage embryos or trophoblastic vesicles. J Reprod Fertil **101**, 657-662.

Wang CK, Robinson RS, Flint AP, Mann GE, 2007: Quantitative analysis of changes in endometrial gland morphology during the bovine estrous cycle and their association with progesterone levels. Reproduction **134**, 365-371.

Wiltbank MC, Souza AH, Carvalho PD, Bender RW, Nascimento AB, 2012: Improving fertility to timed artificial insemination by manipulation of circulating progesterone concentrations in lactating dairy cattle. Reprod Fertil Devel **24**, 238-243.

Xiao CW, Murphy BD, Sirois J, Goff AK, 1999: Down-regulation of oxytocin-induced cyclooxygenase-2 and prostaglandin F synthase expression by interferon-tau in bovine endometrial cells. Biol Reprod **60**, 656-663.

Chapter 2.2

The peripheral blood progesterone concentration, a resultant of several factors

The peripheral plasma progesterone concentration is a dynamic equilibrium, set between production rate and clearance rate. Progesterone is produced by the luteal gland. That is the one and only significant input factor. On the other side, progesterone is drained towards the milk and this hormone is also metabolized mainly by the liver, besides other less important clearing sites (brain, kidneys, adrenal gland mammary gland, body fat, …). Below, we will discuss these factors that may determine the peripheral blood progesterone concentration in dairy cows.

2.2.1 Progesterone production

Although it has been suggested as a significant contributing factor in the increased early embryonic death rate (Shelton et al., 1990), the role of inadequate progesterone production by the CL, described as 'luteal inadequacy', in suboptimal peripheral progesterone levels in lactating dairy cows has not yet been completely cleared. Progesterone production is mainly depending on two physiologic processes: luteal steroidogenesis and angiogenesis (Bollwein et al., 2013). Luteal steroidogenesis refers to the progesterone production by the steroidogenic cells of the CL, and depends mainly on the total number of these cells and the individual activity of each of these 'production units' (Robinson et al., 2006). Luteal angiogenesis on the other hand, refers to the physiological process through which new blood vessels form out of pre-existing ones. Luteal angiogenesis is a prerequisite to obtain an intense luteal steroidogenesis as it is responsible for the supply of nutrients and compounds towards the luteal gland and the conveyance of progesterone towards the peripheral circulation (Acosta et al., 2002). The importance of angiogenesis in the process of luteal progesterone production and release in lactating dairy cows was recently demonstrated by Herzog et al. (2010) who found a correlation of 0.71 ($P < 0.001$) between the luteal blood flow measured by Doppler ultrasound and plasma progesterone levels. Both processes, however, are very complicated and strictly regulated and intertwined (Augustin et al., 1995; Diaz et al., 2002; Shirasuna et al., 2012). Indeed, Yamashita et al. (2008) found that the regulation of angiogenesis and steroidogenesis are interconnected by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2). One of the purposes of our research is the selection of histological traits suitable to analyse based on a subsample. So, in the next paragraph we will analyse the morphometric structure of an intact luteal gland, but as she is the final result of the reorganization of the follicular remnant after ovulation, we will firstly describe the origin of the luteal gland, i.e. the ovulatory Graafian follicle.

2.2.1.1 Ovulation and luteogenesis: a morphometric point of view

As the corpus luteum originates from the follicular remnant of the Graafian follicle (figure a), the luteal morphology depends on the histology of the ovulatory follicle. Consequently, the starting point of our story is the Graafian or pre-ovulatory follicle (figure b). The inner avascular membrana granulosa is separated from the outer theca layer by a basal lamina (or lamina basalis). The richly vascularized theca layer consists of a more central, cell rich layer, the theca interna (containing theca cells), and a peripheral more collagen rich layer, the theca externa (Banks, 1992). The blood supplementation towards the follicle is provided by one artery, dividing into 3-4 arterioles in the theca externa, forming a dense network of capillaries in the theca interna (König et al., 1988). Already 6 hours before the initiation of oestrus there are signs of luteinization in the granulosa and theca interna cells (Donaldson and Hansel, 1965). The granulosa cells are already transforming towards progesterone-producing cells (Bao et al., 1997), in coordination with a decrease in the FSH-receptor expression and an increase in the LH-receptor expression (Xu et al., 1995; Bao et al., 1997; Soumano et al., 1998). In this period, mitotic activity in the membrana granulosa is still high (Donaldson et Hansel, 1965). When ovulation approaches, the follicular basal lamina is degraded by the matrix metalloproteinases (MMP) (Donaldson and Hansel, 1965; Smith et al., 1999; Curry and Osteen, 2001). From this moment on, cell types of the theca interna (theca cells, pericytes, endothelial cells, …) infiltrate through this degrading basal lamina into the membrana granulosa (O'Shea et al., 1980; O'Shea et al., 1987; Meidan et al., 1990; Smith et al., 1994; Amselgruber et al., 1999). Granulosa cells undergo hypertrophia and evolve from an epithelial polar to a mesenchymal apolar cell type. This process is defined as luteinization and is a type of epithelial-mesenchymal transition (EMT; Rodgers et al., 2001; Rodgers and Irving-Rodgers, 2002). This loss of polarity or loss of cell-adhesion-molecules (Hay, 1995) is caused by the degeneration of the follicular basement membrane. During the ovulation the follicular fluid, the oocyte and a part of the granulosa cells are driven out. The follicular wall collapses into a folded structure, the starting point of luteogenesis (figure c). A peak of LH is necessary for the initiation of luteinization in bovines, only a follicle rupture is not sufficient (Hayashi et al., 2006).

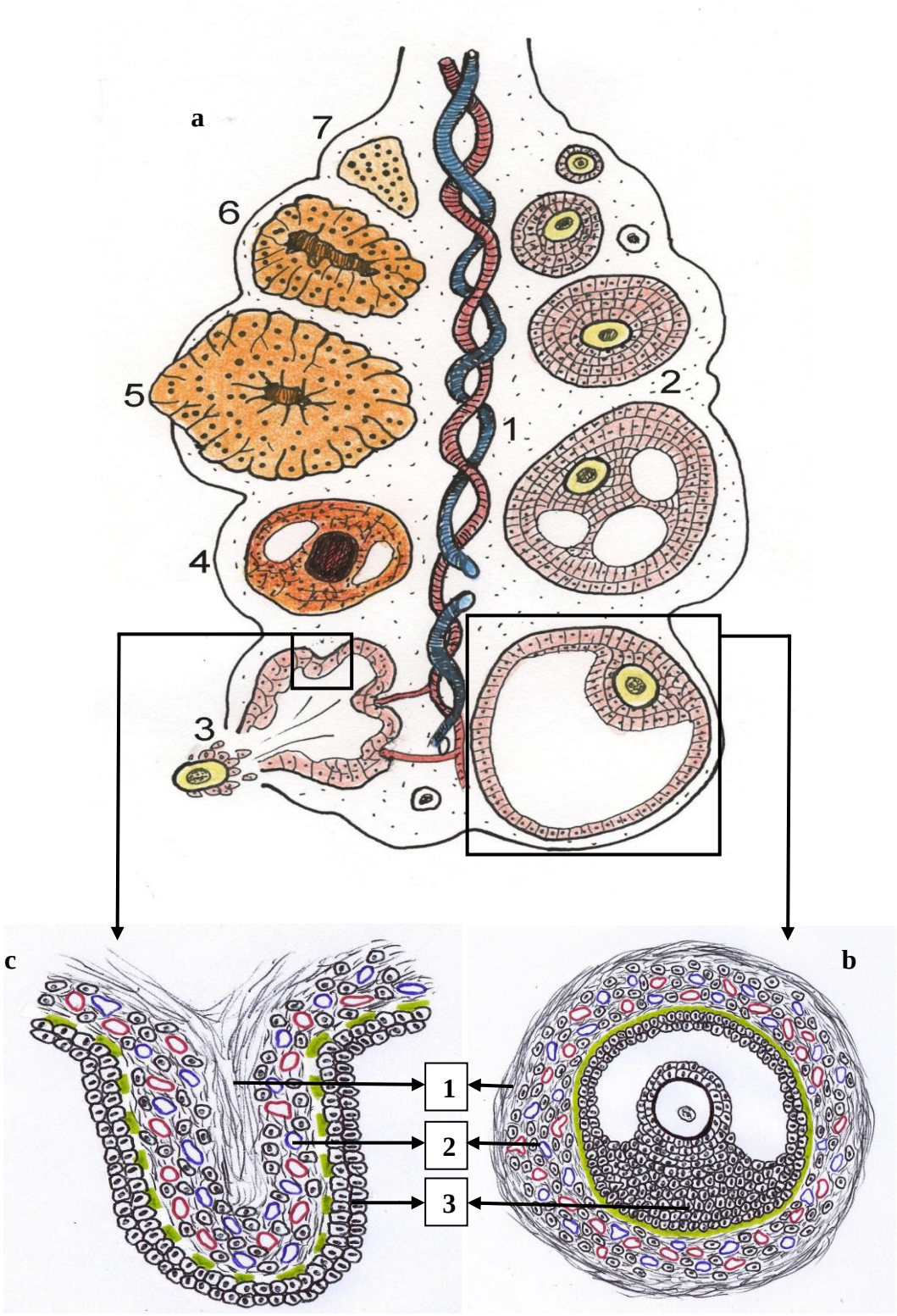


Figure 3 a: *schematic drawing of ovarian structures: 1) zona vasculosa ovarii; 2) zona parenchymatosa ovarii containing ovarian follicles; 3) ovulating follicle and oocyte; 4) corpus hemorrhagicum; 5) corpus luteum; 6) corpus luteum regressum; 7) corpus albicans (source: professor Paul Simoens)*

***Figure 3 b and c**: schematic drawing of respectively a Graafian follicle and a detail of the collapsed follicular remnant after ovulation: 1) theca externa; 2) theca interna; 3) membrana granulosa; lamina basalis (green line; which is degenerating in figure c)*

The final goal of luteogenesis is the creation of a luteal gland, composed of three functional compartments: 1) the steroidogenic production units, composed of large luteal cells (LLC) and small luteal cells (SLC) (production of progesterone), 2) the circulatory system, containing endothelial cells (EC), pericytes and smooth muscle cells (SMC) (provision of constituents for steroidogenesis and removal of progesterone and residuals) and 3) the extracellular matrix (ECM) with fibroblasts and collagen fibers (supporting function).

We go back to the starting point, the follicular remnant (figure c). The folds of the collapsed follicular remnant consist of a central connective tissue axis with blood vessels (the theca externa). Each axis is surrounded by a theca interna layer with capillaries. This layer is delineated by the disintegrating basement membrane. The most peripheral layer is the originally avascular granulosa layer, lining the antral cavity filled with blood. This results in an inner zone of the collapsed follicle containing initially relatively more granulosa cells, while the theca interna cells were more abundant in the outer zone. In all cell types mitotic activity is seen (Donaldson and Hansel, 1965). The degenerated basal lamina allows the invasion of vessel sprouts and other cell types into the membrana granulosa (O'Shea et al., 1980; Smith et al., 1994; Irving-Rodgers et al., 2006). A corpus hemorrhagicum is the result. From cycle day 4 on, the antral cavity is gradually filled up with cellular mass and extracellular matrix (ECM), the folds make contact with each other but the central connective tissue axes are still obvious. The corpus hemorrhagicum evolves to a corpus rubrum. This corpus rubrum has a red colour because of the high angiogenetic activity (Nickel et al., 1995). During the second week of luteogenesis, the corpus rubrum acquires a yellow colour caused by the accumulation of lipochrome, leading to the name corpus luteum or luteal gland (O'Shaughnessy, 1989).

The luteal gland is a result of the cooperation of different cell types, steroidogenic and non-steroidogenic ones. The non-steroidogenic cells are endothelial cells, pericytes, smooth muscle cells, immune cells (macrophages, granulocytes, and other leukocytes). The two cell types LLC and SLC are very important, as they are responsible for the progesterone production. Now, we will zoom into each luteal cell type.

2.2.1.1.1 Large luteal cells

The hypertrophia in the LLC leads to polyhedrical cells with a large amount of clear cytoplasm with a spotted view and a centrally localized, round vesicular nucleus with an obvious nucleolus on cycle day 3-4 (Priedkalns et al., 1968; Lei et al., 1991; Milvae et al., 1991). The LLC is a mastodon compared to the SLC. The mean LLC cell volume was 29.6 ± 6.3 x 10^3 µm^3 (diameter 38.4 µm) compared to 2.7 ± 0.4 x 10^3 µm³ (diameter 17.2 µm) for the SLC. The cytoplasm/nucleus ratio of LLC was 4.5 times the ratio of SLC (O'Shea et al., 1989).

The LLC develop numerous 'enlarged' mitochondria and the cellular volume occupied by mitochondria is increasing from 14±1% on day 4 towards 20 ±1% on day 17 (Fields et al., 1985). These mitochondria are essential for steroidogenesis. These cells contain 3 types of granules: microperoxisomes, primary lysosomes and secretory granules. The number of the latter ones is correlated with the progesterone secretion pattern (Priedkalns and Weber, 1968a; b; Gemmell and Stacy, 1979; Quirke et al., 1979; Fields et al., 1985). These secretory granules are rather clustered within the LLC (Fields et al., 1985). Initially, they start to appear around the nucleus and then spread out in cluster formation towards the cell membrane, the site of exocytosis (Chegini and Rao, 1987; Fields et al., 1985; 1992). In the cyclic CLa, 80-85% of these granules contain oxytocin (OT) and neurofysin (Fields et al., 1992).

2.2.1.1.2 Small luteal cells

The ultrastructural characteristics of the SLC indicate steroidogenic activity. Compared to the LLC, they had less, but more dense cytomplasm; an oval eccentric nucleus with dense chromatine, tubular and vesicular distended SER, mitochondria with variable forms and tubular cristae, lipid droplets (Priedkalns and Weber, 1968a; b; Lei et al., 1991; Milvae et al., 1991). Like the LLC, the cellular volume occupied by mitochondria is increasing from 8±1% on cycle day 4 towards 12 ±1% on cycle day 17 in SLC (Priedkalns and Weber, 1968a; b).

2.2.1.1.3 Non-steroidogenic cells

The non-steroidogenic cells are small, have no round shape, and have a strongly condensed nucleus (Lei et al., 1991). The relative amount of non-steroidogenic cells (NSC%) is depending on the luteal stage, e.g. stage 2: 74%, stage 3: 60% (Lei et al., 1991). Based on the study of O'Shea et al. (1989), the volume density of the NSC takes up 21.4% in stage III CLa.

2.2.1.1.4 All cells together, creating a luteal gland

Lei et al. (1991) analyzed the quantitative cell composition by light microscopy of bovine CLa from various cycle stages. The number of non-steroidogenic cells is higher than steroidogenic cells, and the number of SLC is higher than LLC except during the late luteal stage (Lei et al., 1991). Not surprisingly, the ratio of cell types in the CL changes as the CL evolves. The number of steroidogenic cells was increasing from the early to the mid luteal phase, while the number of non-steroidogenic cells was decreasing. From the mid to the late luteal stage, the evolution was the opposite (Lei et al., 1991).

Every 60 to 70 hours the mass and the number of luteal cells double during the first 8 to 10 days. The growth of the CL is a result of hypertrophy (SLC, LLC) and hyperplasia (SLC, endothelial cells, fibroblasts). The effect of the hypertrophy is the most important, but also the effect of hyperplasia is not negligible (Smith et al., 1994, Zheng et al., 1994). Depending on the study and cycle stage, 50 to 85% of the cellular proliferation was accounted for the microvascular compartment, making endothelial cells the cell type with the highest proliferation rate (Reynolds et al., 1994; Zheng et al., 1994; Christenson and Stouffer, 1996; Redmer et al., 2001; Al-Zi'abi et al., 2003).

Maximal angiogenesis was seen during cycle day 2 to 3. From day 1 to 4 the number of organized parenchymal capillaries was scarce (Zheng et al., 1993). They were more prevalent in the connective tissue trabeculae. From cycle day 5 to 17 (mid-luteal) the capillary network was very dense in the parenchyma, occupying 8 to 12% of the surface area from cycle day 3 to 8. The result is that almost every steroidogenic cell is neighboring at least one capillary (Azmi and O'Shea, 1984; Grazul-Bilska et al., 1992; Reynolds et al., 1992; Reynolds et al., 2000; Redmer et al., 2001). Forty to 70% of the total cell population are pericytes and endothelial cells till cycle day 17 (Hünigen et al., 2005). On cycle day 12, endothelial cells and pericytes take account of 52.3% of the total number of luteal cells (393.4 ± 52.0 x $10^3/mm^3$) (O'Shea et al., 1989). The surface area occupied by endothelial cells and pericytes is increasing from early to mid-luteal phase and decreasing during late luteal phase (Lei et al., 1991).

The vascular bed has a lobular organization, consisting of capsular arteries, dividing into septal and further into parenchymal arteries. The number of arterioles is rather low. Consequently, the blood flow is largely depending on the systemic blood flow (Stouffer et al., 2001). These arterioles branch into fenestrated capillaries with heterogeneous distribution. Capillary blood is collected towards centrally located venules and venes (Findlay, 1986; König and Amselgrüber, 1987; Zheng et al., 1993; Stouffer et al., 2001). Already on day 5,

the proliferation rate is decreasing in bovine CL (Zheng et al., 1994), while regression is appearing during the last quartile of the luteal phase.

Several studies analyzed the morphometrics of the bovine CL. The number of SLC is increasing in the early luteal phase (i.e. hyperplasia), while the number of LLC is constant, but when the luteal phase evolves, LLC diameter is increasing (i.e. hypertrophia) (Parkinson et al., 1994). Together, LLC and SLC take up 60 to 70% of the luteal volume during the mid-luteal phase in Shorthorns (O'Shea et al., 1989; Wiltbank et al., 1994; Sawyer, 1995). The fewer but larger LLC occupy 40-52% of the volume against 20-28% by the smaller, but numerous SLC (O'Shea et al., 1989; Diaz et al., 2002; Neves and Marques, 2006). The large variation in these results between the different studies can be explained by the application of different techniques to obtain luteal tissue samples (dispersion of the cells versus intact tissue) (Rodgers et al., 1984; O'Shea et al., 1986; Farin et al., 1986) and cut off values for the identification of LLC versus SLC (Ursely and Leymarie, 1979; Chegini et al., 1984; Fields et al., 1985; Weber et al., 1987; Hansel et al., 1987; Villa-Godoy et al., 1990). In figure 3 the number and volume occupation of the steroidogenic and non-steroidogenic cells in a mature (mid luteal) bovine CL are presented, analyzed by Fields and Fields (1996).

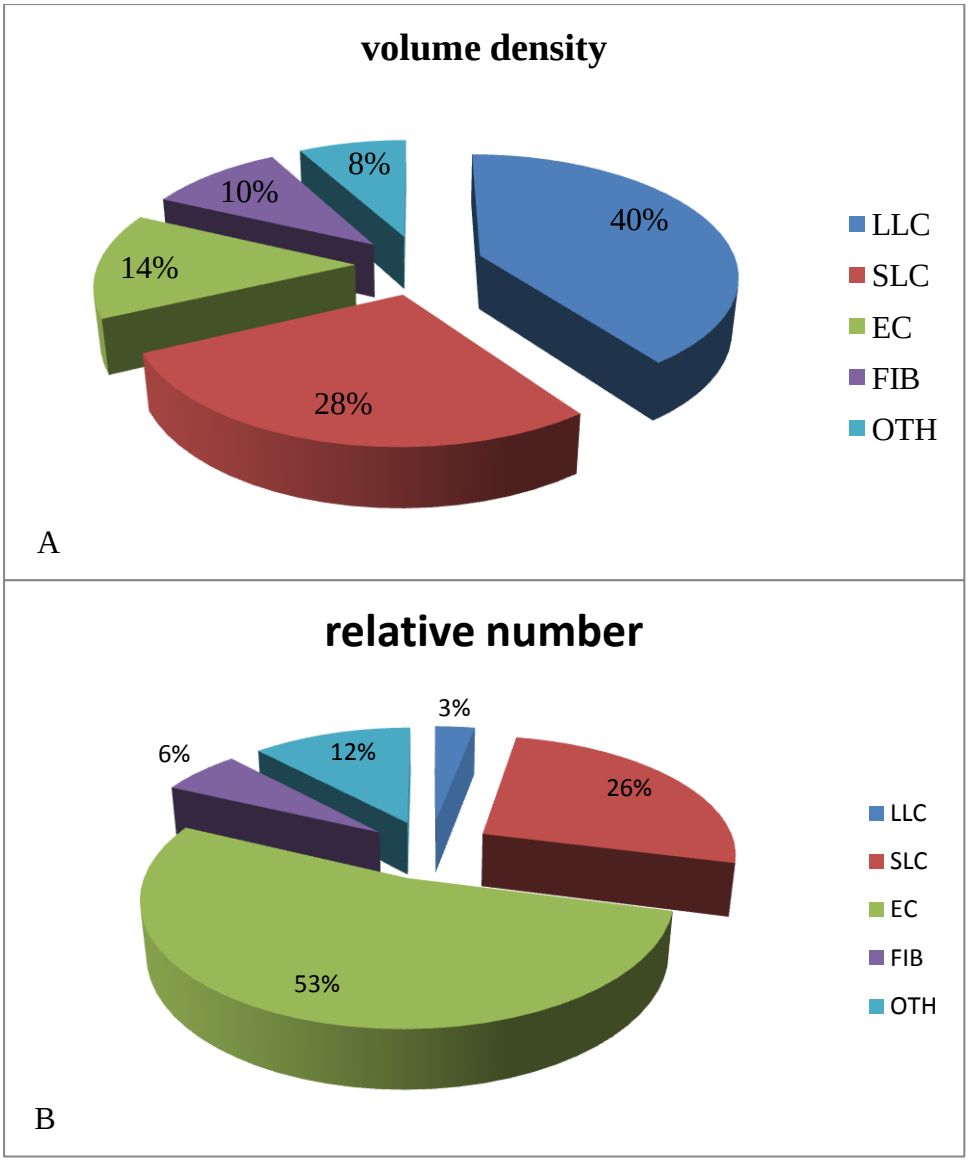


Figure 4: *A: The volume density of the different cell types within the total volume occupied by the cells in a mature (mid luteal) bovine CL; B: the relative number of the different cell types within the total number of cells. (LLC: large luteal cells; SLC: small luteal cells; EC: endothelial cells; FIB: fibroblasts; OTH: other cell types). Wiltbank (1994) reviewed the different morphological characteristics of the cells in the mid-cycle CL of a cow.*

2.2.1.1.5 The extracellular space

Parry et al. (1980) stated that the extracellular space ('ES'; i.e. ECM and collagen fibers) comprised 30% of the cross-sectional area on cycle day 6, while O'Shea et al. (1989) calculated an ES of 10.7% in CLa of cycle day 12. The relative collagen fiber content found in the study of Parkinson et al. (1994) was 1.5-2% on cycle day 7 or 8. From cycle day 7, the

connective trabeculae become less obvious. Within a luteal lobe, the amount of the fibrillar collagen type I increased in the inner parenchymal tissue, while there was no significant increase detected in the outer region. The latter means that as well between the parenchymal lobes as within one lobe, the chance of attendance of collagen type 1 is higher in the inner zone when compared to the outer (Luck and Zhao, 1993; Silvester and Luck, 1999). In figure 4 an overview of the dynamics of the amount of collagen type I in the different topographic zones is visualized.

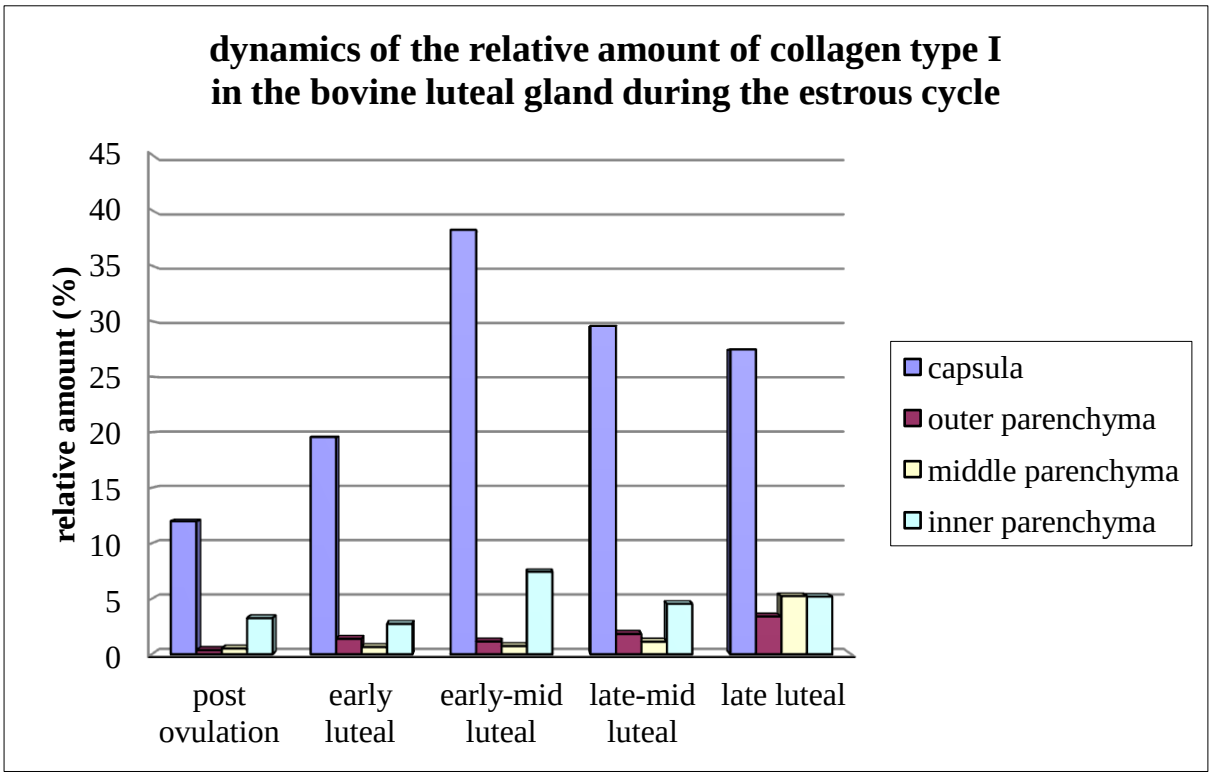


Figure 5: *distribution of the collagen type I over the different luteal topographic regions throughout the different luteal phases*

So, we can state that the luteal histology is constructed around two key processes, angiogenesis and steroidogenesis, united as luteinisation, to obtain an optimal progesterone secretion. Angio- and steroidogenesis rely on several common regulators, such as the gonadotrophins.

In the next section, both key processes are analyzed more in detail as both processes will be analysed immunohistochemically in our study in high yielding dairy cows.

2.2.1.2 Angiogenesis in the bovine CL

The luteal gland is one of the few adult tissues exhibiting regular periods of growth, functionality and lysis. It is a heterogeneous tissue where various cell types interact to establish a functional blood supply (Schams and Berisha, 2004).

Angiogenesis means the construction of new blood vessels, starting from existing ones after degradation of the capillary basement membrane (Schams and Berisha, 2004). Starting point is the Graafian or pre-ovulatory follicle. The blood supplementation is realized by one artery, dividing into 3-4 arterioles in the theca externa, forming a dense network of capillaries in the theca interna (König et al., 1988) infiltrating into the membrana granulosa (Redmer et al., 1991). After the LH-peak, blood vessels dilate and an increase in permeability and fenestration leads to edema, ischemia and formation of the ovulation stigma, the predilection place for follicular rupture. After the ovulation, the luteal gland is developing from the collapsed follicular remnant (Augustin et al., 1995). The bovine luteal gland is confronted with only a short period of intense angiogenesis (until day 5) (Skarzynski et al., 2013).

2.2.1.2.1 Chronological dynamics of luteal angiogenesis

Phase 1: corpus hemorrhagicum

The follicle remnant collapses. Erythrocytes and granulocytes accumulate in the edematous interstitial tissue of the membrana granulosa. Hemorrhage is filling the follicular cavity. The capillaries are growing out of the central axis (preceded by pericytes) after the degeneration of the BM and they create a vascular tuft in the corpus hemorrhagicum (Amselgruber et al., 1999). Histologically, one can see the invasion of individual sprouts into the parenchyma 1 to 2 days after ovulation creating an intense vascular sprouting during the initial third of the oestrous cycle (Augustin et al., 1995).

Phase 2: corpus rubrum

The luteal gland grows to the maximal diameter (Augustin et al., 1995). At the end of the first week of the estrous cycle, the growth of the vessel sprouts continues, forming a dense network of capillaries, creating the red colour. Histologically, EC are seen with large, oval nuclei, delineating cavernae (with strong leakage), while there are also maturing arterioles and venules visible. Fifty percent of the cells in the corpus rubrum are EC (Augustin et al., 1995).

Phase 3: corpus luteum

This is a plateau phase concerning the macroscopic growth (Augustin et al., 1995). There is still a strong angiogenic activity, but there is no increasing trend anymore (plateau phase). Histologically, the typically elongated EC delineate a well differentiated dense vascular network (relatively immature). The structure of the luteal vascular bed is as follows: capsular arterioles form septal arterioles and parenchymatous arterioles, leading to fenestrated capillaries lobulary (and heterogeneously) distributed. As mentioned before, every steroidogenic cell is neighboring at least one capillary (Azmi and O'Shea, 1984; Grazul-Bilska et al., 1992; Reynolds et al., 1992; Reynolds et al., 2000; Redmer et al., 2001), but Goede et al. (1998) found that for every luteal cell, even 2 to 4 capillaries were seen.

Eberhard et al. (2000) compared the angiogenesis in the CL with this in some malignant human tumors. The proliferation index of the EC (i.e. the ratio between the number of microvessels with proliferating EC and the total number of microvessels) in an early luteal bovine CL was 40.6 ± 11%, while this was for the tumors 2-10%. The microvessel maturation index (MMI; i.e. the fraction of EC coupled to pericytes) of a young CL was 60%, of a mature CL it was 62 to 64%, in the tumors it was 12-67% (Goede et al., 1998). This means that the vascular network stays relatively immature, giving the opportunity for remodeling. The difference with the tumors is that the angiogenesis in the CL is tightly regulated.

Acosta et al. (2003) studied the hemodynamics of the follicle wall and CL in bovines. Before the LH-peak takes place, the blood supply of the follicle was mostly situated in the follicular basis. After the LH-peak, the follicular blood supply is increasing until ovulation. Then it is decreasing until day 2. From day two to five, the luteal blood flow is gradually increasing (surface and intensity) in parallel with the increase in CL-volume and plasma progesterone concentration (Acosta et al., 2003).

2.2.1.2.2 Angiogenesis in detail

The angiogenic stimulus is frequently induced by cells, having a role in inflammation processes (macrophages, mast cells) (Amselgruber et al., 1999) combined with growth factors (secreted by luteal cells, EC) and ECM-components (fibrin, type IV collagen, non-collagen molecules such as laminin, fibronectin) (Amselgruber et al., 1999). The angiogenetic activity is the result of a balance between positive and negative regulatory molecules (Hanahan and Folkman, 1996).

The initial morphological step in the angiogenic process is the migration phase followed by mitoses (Furcht, 1986). A pericyte is forming pseudopods through the BM, which becomes fragmented. This process is only happening at the level of the capillaries and postcapillary venules (Amselgrüber et al., 1999). Two or more endothelial cells are following the pericyte, forming a small lumen. So, they maintain their polarity (Amselgrüber et al., 1999). During this process, the pericyte has an intense, but non-structural contact with the granulosa cells.
The leading role of the pericytes during the initiation of the angiogenesis is controversial (Nehls et al., 1992; Goede et al., 1998; Amselgrüber et al., 1999; Liekens et al., 2001; Arfuso et al., 2005). Moreover, the pericytes seems to have a very nuanced role: initiation of the angiogenesis, but also stabilizing the vessel sprouts afterwards (Sato and Rifkin, 1989).
The peripheral progesterone concentration is closely related to the luteal blood flow (Acosta et al., 2003; Herzog et al., 2010). Inhibition of the angiogenesis decreases the postovulatory progesterone rise (Yamashita et al., 2008), while Robinson et al. (2006) found no significant difference in angiogenic activity between high and low progesterone cows on day 5 and 8. Pericyte staining on day 8 was positively correlated with the peripheral progesterone concentration (Robinson et al., 2008). So, degree of vasculature maturation might influence progesterone production. And pericytes could be the key drivers of angiogenesis and vascular function (Robinson et al., 2008). So, the results concerning the correlation between the vasculare density and progesterone secreting activity seems to be inconsistent. Therefore, we analyzed again in our study the density of the luteal vascular bed within a group of cows kept under identical experimental conditions, but we followed them up longitudinally during three oestrous cycles, attempting to reduce interindividual variation influencing the luteal angiogenic and secretory activity.

2.2.1.3 Steroidogenesis

2.2.1.3.1 Progesterone production: physiology

When ovulation is approaching, the granulosa cells are transforming towards progesterone-producing cells (Bao et al., 1997), in coordination with a decrease in FSH-receptor expression and an increase in LH-receptor expression (Xu et al., 1995; Bao et al., 1997; Soumano et al., 1998). Progesterone synthesis is already initiated before ovulation (Rouillier et al., 1996; Bao et al., 1997). The activity and diameter of the follicle during the pre-ovulatory phase is positively correlated with the progesterone increase during the post-ovulatory luteogenesis (Mann et al., 2001; Robinson et al., 2005; Starbuck et al., 2006). But this correlation

disappears from cycle day 8 (Vasconcelos et al., 2001; Wathes et al., 2003; Robinson et al., 2005).

The pre-ovulatory LH-peak is necessary for luteinization (i.e. the morphological adaptations and the shift in steroidogenesis from estrogens towards progesterone; Smith et al., 1994), while follicle rupture is not essential for a normal luteinization (Smith et al., 1994). The luteal sensitivity for LH increases from day 5 to 8 (Robinson et al., 2006). The regulation of the steroid production is largely dependent on LH (Niswender et al., 2000). LH has direct effects on the luteal cells by LH-R, but also by indirect modulation of the luteal steroidogenesis via growth factors, cytokines and others. Also gonadotrophin releasing hormone (GnRH) can induce luteinization *in vitro* (analogous to the LH-effects). The luteal gland consists of LLC (granulosa origin, as stated above) and SLC (theca origin). The LLC has the greatest progesterone production (Niswender et al., 1985). Despite similar numbers of LH-R on LLC and SLC, LLC are less or not responsive to LH. In contrast, SLC bind to a high degree LH and respond with an increase in progesterone (Murphy, 2000; Diaz et al., 2002). Consequently, progesterone production under LH-stimulation increases with a factor 40 in the SLC (Fitz et al., 1982; Lemon and Loir, 1977).

2.1.3.2 Progesterone production: biochemistry

The 'core business' of the CL is progesterone production. The progesterone concentrations continuously increase until cycle day 14, while the CL-volume does not significantly increase anymore after day 7 (Sangsritavong et al., 2002). In figure 6, a schematic overview is given of the progesterone production process (Diaz et al., 2002).

The luteal steroidogenic cells can obtain their cholesterol by three different ways, in order of importance: 1) uptake of cholesterol from circulatory HDL and LDL and storage of cholesterol esters in cytoplasmic fat droplets; 2) from the cell membrane; 3) by *de novo* synthesis (Diaz et al., 2002). In the next paragraph, we will explain these different options more in detail.

1) Cholesterol esters, in ruminants mostly supplied by HDL resorption out of the blood, are accumulated in the cytoplasm of the LLC and SLC. This selective uptake of cholesterol esters out of the HDL is carried out by the scavenger-receptor type 1, class B (SR-B1) (Acton et al., 1996; Li et al., 1998). This molecule regulates both, the transport of cholesterol from the HDL towards the cytoplasmic lipid droplets and the efflux out of the cell. Sterol carrier protein (SCP)-2 transports the hydrophobic free cholesterol towards the mitochondria (Chanderbhan et al., 1983; Lavoie et al., 1997). Niemann-Pick C disease-1 transports the

LDL-derived cholesterol out of the lysosomes towards the cytosol (Storch and Cheruku, 2005).

2) Cholesterol, residing in the plasma membrane can be transported towards the endoplasmatic reticulum. The permeability glycoprotein (P-gp) should be involved in this transport mechanism as a flippase, facilitating the translocation of phospholipids from the outer towards the inner membrane (Liscum and Munn, 1999).

3) The *de novo* synthesis of cholesterol by hydroxymethylglutaryl coenzyme A (HMG-CoA)-reductase in the endoplasmatic reticulum has a minor role in the luteal steroidogenesis (Gwynne and Strauss, 1982). After synthesis, the cholesterol is transported towards the plasma membrane (Liscum and Munn, 1999).

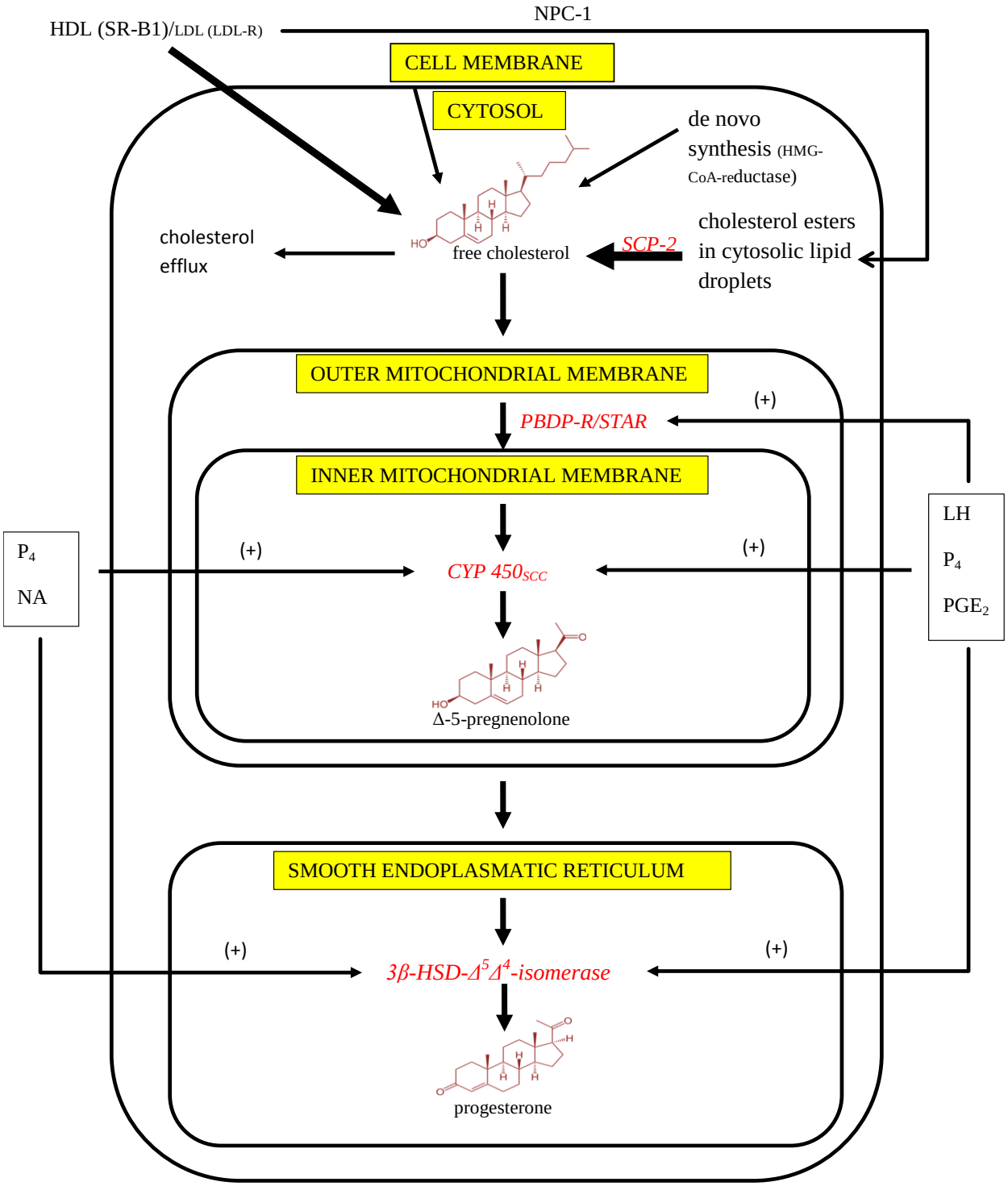


Figure 6: *a schematic overview of the progesterone production process: HDL and LDL: high and low density lipoproteins; SR-B1: scavenger-receptor type 1, class B; LDL-R: LDL-receptor; HMG-CoA-reductase: hydroxymethylglutaryl coenzyme A reductase; NPC-1: Niemann-Pick C disease: SCP-2: sterol carrier protein-2; StAR: Steroidogenic acute*

regulatory protein; PBDP-R: peripheral benzodiazepine receptor ; $CYP450_{SCC}$: cholesterol side-chain-cleavage cytochrome P450; 3β-HSD-Δ^5 Δ^4-isomerase: 3 β hydroxysteroiddehydrogenase-Δ^5 Δ^4-isomerase; P_4: progesterone; NA: noradrenaline; LH: luteinizing hormone; PGE_2: prostaglandin E_2; (+): stimulating effect on transcription of the mRNA or the activity of the protein

Nevertheless, the main goal is steroidogenesis. For this purpose, cholesterol needs to enter the mitochondria. Cholesterol is stored as cholesterol esters (i.e. cholesterol esterified through the 3β-hydroxyl group to poly-unsaturated fatty acids or sulfate). These cholesterol esters are accumulated in lysosomes. Free cholesterol comes into the outer mitochondrial membrane and has to get inside the inner mitochondrial membrane. This transport from the outer to the inner mitochondrial membrane is carried out by the steroidogenic acute regulatory protein (StAR) and the peripheral benzodiazepine receptor (PBDP-R) and is the rate limiting step (Stocco, 2000). Steroidogenic acute regulatory protein transfers cholesterol across the aqueous space separating the cholesterol-rich outer mitochondrial membrane and the cholesterol-poor inner mitochondrial membrane (Christenson and Strauss, 2001). Prior to the LH-surge, StAR is virtually absent in granulosa cells, while it is already present in high levels in the periovulatory theca cells (Pescador et al., 1996; Chaffin et al., 2000). StAR mRNA and protein are maximal during the early and mid-luteal phase and decrease during the late luteal phase (Juengel et al., 1995). Its transcription is stimulated by LH (Pon and Orme-Johnson, 1988) and by insulin (in humans) (Devoto et al., 1999) via Steroidogenic factor-1 (SF-1) (Sugawara et al., 1997). The promotor sequence of the bovine StAR-gene contains also SF-1 binding sites (Rust et al., 1998). Besides the major hormonal regulation of the steroidogenic cell function, also the para- and autocrine factors influence StAR-gene expression, such as IGF's (stimulatory effect on StAR-gene expression) (Chaffin et al., 2000; Christenson and Strauss, 2000). Even progesterone seems to stimulate StAR-gene expression in a mouse Leydig cell line devoid of progesterone receptors (Schwarzenbach et al., 2003). Altogether, these findings illustrate that StAR transcription is tightly regulated during the luteal cycle and plays a key role in steroidogenesis. After cholesterol transport to the inner mitochondrial membrane, the biosynthesis of progesterone consists only of two enzymatic steps (Christenson and Devoto, 2003). The cholesterol side-chain-cleavage cytochrome P450 ($CYP450_{SCC}$), situated on the matrix side of the inner mitochondrial membrane, modifies the cholesterol towards Δ5-pregnenolon (Christenson and Strauss, 2001; Niswender, 2002). Cytochrome $P450_{SCC}$ needs to cooperate with electron transfer partners

(adrenodoxin/adrenodoxin reductase) (Chaffin et al., 2000). Only the luteal cells stained (obtained by dispersion): LLC stained more intense than SLC because of a higher number of mitochondria (Rodgers et al., 1986). The staining is more intense in a mature CL compared to young or regressed CL (Rodgers et al., 1986). This molecule is less stable but migrates out of the mitochondria. The progesterone is finished in the SER by 3β-hydroxysteroiddehydrogenase (HSD)-Δ^5 Δ^4-isomerase (Niswender, 2002). Before ovulation, the mRNA of this enzyme is only situated in the theca interna cells, while it is also found in the granulosa-derived cells after the LH-peak (Couet et al., 1990). It increases after the LH-peak and is maximal at day 8 to 11, but decreases strongly by $PGF_{2\alpha}$. Lobel and Levy (1968) detected a decrease in steroiddehydrogenase activity from day 14 on. Both mentioned enzymes are no rate limiting steps (Wiltbank et al., 1993; Belfiore et al., 1994). Both steps are responsible for the ultrastructural modifications and increase in quantity or number of SER and mitochondria. The initial increase in progesterone is rather due to the steroidogenesis at the level of the theca internal zone, than at the level of the remnant of the membrana granulosa, as in the very early luteal phase the enzymatic machinery and vascular network is not yet completely established in the zone originating from the membrana granulosa (Conley et al., 1995; Reynolds and Redmer, 1999).

Figure 6 illustrates also the molecular regulation of the progesterone synthesis in the bovine luteal cell. Luteinizing hormone, progesterone, and PGE_2 increase the gene expression of StAR, $CYP450_{SCC}$, and 3β-HSD, which are crucial enzymes of steroidogenesis. Progesterone influences its own synthesis by increasing of $CYP450_{SCC}$ and 3β-HSD activity. Even though noradrenaline (NA) stimulates progesterone secretion and increases $CYP450_{SCC}$ and 3β-HSD activity, but it does not affect gene expression of these enzymes.

The bovine luteal gland consists of a relatively high autonomy, thanks to local factors regulating the progesterone production and inhibiting premature luteolysis (Kotwica, 2004) ***and with changing capacity for steroidogenesis as progesterone does not only stimulate its own synthesis, but also directly influences luteolysis*** (Rothchild, 1996).

2.2.2 Drainage towards milk

In high yielding dairy cows, the type of cows we were dealing with in our phd-research, the progesterone loss via the milk is increasing with the increasing milk production. In literature this way of progesterone 'spoiling' is discussed extensively. In the next paragraph, we will summarize the contribution of this factor in the progesterone loss.

Because of its liposolubility, 80% of the progesterone in the milk is accumulated in the milk fat, 19% in the casein and only 1% in the milk serum or whey (Laing and Heap, 1971, Heap et al., 1975). So, progesterone loss via the milk is mainly depending on the milk fat production. Logically, several studies found a positive correlation between milk fat content and progesterone content of the milk (r=0.8) (Heap et al., 1974; Ball and Pope, 1976). Ginther et al. (1976) even found a correlation of 0.98. Hoffman and Hamburger (1973) found that the milk progesterone content was increasing with 3 ng/ml when milk fat was increasing with one percent. This milk fat production is influenced by the breed (Pennington et al., 1981), the individual animal, the lactation stage (Macfarlane et al., 1977) and even the cycle stage (King, 1963). Nevertheless, the dynamics of milk fat content did not completely elucidate the variation in milk progesterone dynamics. Progesterone concentration in the alveolar milk was higher compared to cysternal milk, which could not be explained by the difference in milk fat content (Waldmann et al., 1999). The reason of this topographic difference is still unknown.

Depending on the sampling protocol, the correlation between the progesterone concentrations in milk and blood plasma was 0.78 to 0.91 (Dobson et al., 1975; Pope et al., 1976; Mather et al., 1978) and this correlation increased to 0.92-0.985 by correcting the milk progesterone concentration for milk fat content (Hoffmann and Hamburger, 1973; Pope et al., 1976). During the luteal phase, milk progesterone concentrations (0.75-31.7 ng/ml) were 2-3 times higher than the blood plasma concentrations (0.1-9.7 ng/ml) (Hoffmann and Hamburger, 1973; Pope et al., 1976), while during the oestrous period milk progesterone concentration was 3 (± 0.7) ng/ml versus 0.3 (± 0.07) ng/ml in the blood (Dobson et al., 1975). Also, Schiavo et al. (1975), Heap et al. (1973; 1975) and Hoffmann et al. (1976) found that parallelism between milk and blood, with systematically higher concentrations in the milk versus the blood.

The milk progesterone concentration is a resultant of passive diffusion. And as the dilution effect of an increase in milk production level on the milk progesterone concentration is very small, the flux of progesterone into the milk could be a contributing factor to the lowered blood progesterone concentration in high yielding dairy cows. On the contrary, Heap et al. (1975) stated that the progesterone loss by the milk (3.1 µg/min) should be only 3% of the progesterone production rate (173 ± 23.3 µg/min) in Jersey cows. Whether the milk progesterone concentration is the result of passive diffusion only is not clear. Heap et al. (1986) postulated that the bovine's mammary gland also actively extracts progesterone out of the blood based on facilitated diffusion. Belvedere et al. (1996) even showed that the bovine

lactating mammary gland has limited steroidogenic capabilities for terminal activation of circulating steroids coming from steroidogenic endocrines.

The milk progesterone concentration in high yielding (27-29 L/day) dairy cows was the same as in the low yielding (17-22 L/day) cows (Rabiee et al., 2001), leading to a higher progesterone loss in the high yielding dairy cows.Surprisingly, the blood concentration was not lowered. But cows were inserted with a CIDR, giving them the opportunity to extract progesterone out of the device into the blood circulation. The CIDRs in the high yielding dairy cows had lost more progesterone versus the lower yielding ones. Hommeida et al. (2004) found that the area under the curve (AUC) of milk progesterone during the first 15 days PI was negatively correlated with the milk yield (r=-0.83).

As the progesterone losses via the milk are relatively small (only 3% of the progesterone production rate), we can conclude that the total progesterone loss via the milk is rather limited. On the other side, we see that cows with a higher milk production extract significantly more progesterone out of an external progesterone source when they have the opportunity, indicating they are dealing with a lowered peripheral blood progesterone concentration compared to cows with a lower milk production. But, till now we did not take into account that cows with a higher milk production, have also a higher dry matter intake (DMI), leading to an increased liver blood flow (LBF). This aspect will be discussed in the next paragraph.

2.2.3 Hepatic clearance

The metabolic activity of an organ is depending on the mass of that organ and the metabolic activity per tissue unit. The liver mass of the cow was increasing with one kilogram during the first 8 weeks post partum (Gibb et al., 1992). During that period the circulatory volume of the cow is increasing (Reynolds et al., 2003). As the authors did not analyze the hepatic microscopic characteristics, the mechanism behind this mass increase could not be defined. It could be caused by hyperplasia and hypertrophia, but as the liver has a large compliance, it could also be caused by an increase in blood content (Lautt, 1996; Reynolds et al., 2004). Bell (1995) found that the bovine hepatic protein synthesis was increased 10 days pre partum, indicating an increase in tissue mass. Moreover, Johnson et al. (1990) found a positive relation between energy uptake and hepatic tissue mass.

The liver is the most vascularized large organ of the body; 25% of its mass is blood (Lautt, 1996). The liver receives venous portal blood and arterial blood. This arterial contribution varies from 2.3% to 21% (Brockman and Bergman, 1975; Barnes et al., 1983). This arterial

supply is regulated by the sympathetic nervous system (Lautt, 1996) and by the liver itself. The venous portal supply regulates the arterial blood supply ('hepatic arterial buffer response'), obtaining a constant blood flow towards the liver and a constant clearance rate of certain hormones. The portal blood supply is the resultant of the outflow from the extrahepatic splanchnic organs. Several studies monitored the effect of feed intake on portal blood flow. A higher DMI (i.e. a higher level of digestible energy) induced an increase in portal blood flow in lactating and non-lactating dairy cows (Wieghart et al., 1986). Lactation (and the accompanied increase in DMI) increases significantly the portal blood flow in HF cows (Lomax and Baird, 1983; Reynolds et al., 1988; Sangsritavong, 2002). No significant effect was found on arterial hepatic supply. On the contrary, starvation during two days induces a decreased portal and arterial blood supply, as well in lactating as in non-lactating cows (Sangsritavong et al., 2002). Reynolds et al. (1989) found also an increase in bovine hepatic blood flow on 8 weeks post partum versus 4 weeks post partum. So, the transition period induces a significant increase in LBF, mainly attributed to the venous portal blood flow, as the arterial blood supply stays below 20%.

Huntington (1990) found a high correlation between energy-uptake and the venous portal blood supply in bovines (r^2=0.98). This statement needs to be finetuned as feeding level has an acute and chronic effect. The act of feed uptake induces an acute increase in hepatic blood flow (Sangsritavong et al., 2002). Baseline LBF in lactating cows is more than 2 times the LBF of non-lactating cows and lactating cows had lower progesterone levels (Sangsritavong et al., 2002). The acute increase in LBF induces an increase in metabolic clearance rate of progesterone, both are positively correlated with each other (r=0.92). But feed level induces also a chronic increase in the hepatic blood supply (Parr et al., 1993). The efficiency of the hepatic progesterone clearance is depending on the portal blood flow. The lower the portal blood flow, the more efficient the progesterone clearance. Bedford et al. (1972) found that 57% of the progesterone was cleared by the liver in lactating cows and 42% in non-lactating cows. The DMI-level was negatively correlated with plasma progesterone concentration in non-lactating cows (Rabiee et al., 2001). DMI level influences progesterone clearance rate, leading to a negative correlation between the progesterone concentration in the peripheral blood or milk and DMI (Cumming et al., 1971; Hommeida et al., 2004).

To monitor the hepatic clearance rate of progesterone, we need to take into account the enterohepatic recycling of progesterone. This means that progesterone, excreted by the liver into the bile fluid, will be partly reabsorbed in the intestine after bile ejection as a native, conjugated or metabolized form passing again the liver via the portal blood flow. Besides the

hepatic progesterone clearance, Watson (1986) found that 4 to 14% of the progesterone was cleared by the heart, lungs, body fat of the cow. Bedford et al. (1974) found that even 43 to 58% of the progesterone was cleared by kidneys, brain, ovaries and adrenal glands. The goat's mammary gland also metabolizes progesterone (Heap et al., 1986).

So, as the hepatic metabolic clearance rate of progesterone (MCR; l/(min*kg $BW^{0.75}$)) depends on the liver blood flow (Sangsritavong et al., 2002; Rhinehart et al., 2009), **which on its turn is mainly dependent on the dry matter intake (DMI)** (Cumming et al., 1971; Sangsritavong et al., 2002)**, there is a general agreement nowadays that lactating high yielding dairy cows are dealing with a higher progesterone loss mainly via an increased metabolization rate in the liver and via some extra loss via the milk. These processes contribute undoubtedly to the problemacy of the lowered blood progesterone levels in high yielding dairy cows, but, on the other side, data about the contribution of the luteal dysfunction in this issue are rather scarce in literature. This lack of information leads to the hypothesis of our study: 'Also the luteal inadequacy contributes significantly to the reduced blood progesterone concentration in high yielding dairy cows.'**

Reference List

Acosta TJ, Hayashi KG, Ohtani M, Miyamoto A, 2003: Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. Reproduction **125**, 759-767.

Acosta TJ, Yoshizawa N, Ohtani M, Miyamoto A, 2002: Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F(2 alpha) injection in the cow. Biol Reprod **66**, 651-658.

Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M, 1996: Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science **271**, 518-520.

Al-Ziabi MO, Watson ED, Fraser HM, 2003: Angiogenesis and vascular endothelial growth factor expression in the equine corpus luteum. Reproduction **125**, 259-270.

Amselgruber WM, Schafer M, Sinowatz F, 1999: Angiogenesis in the bovine corpus luteum: an immunocytochemical and ultrastructural study. Anat Histol Embryol **28**, 157-166.

Arfuso F, McGeachie JK, Meyer GT, 2005: A quantitative study of blood capillary formation (angiogenesis) concomitant with parenchymal tissue differentiation. Endothelium **12**, 171-177.

Augustin HG, Braun K, Telemenakis I, Modlich U, Kuhn W, 1995: Ovarian angiogenesis. Phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. Am J Pathol **147**, 339-351.

Azmi TI, O'Shea JD, 1984: Mechanisms of deletion of endothelial cells during regression of the corpus luteum. Lab Invest **51**, 206-217.

Ball PJ, Pope GS, 1976: Proceedings: Measurement of concentrations of progesterone in fat-free cows' milk: its potential value in studies of reproduction. J Endocrinol **69**, 40P-41P.

Banks WJ, 1992: Applied veterinary histology, 3rd edn. Williams & Wilkins, Baltimore.

Bao B, Thomas MG, Williams GL, 1997: Regulatory roles of high-density and low-density lipoproteins in cellular proliferation and secretion of progesterone and insulin-like growth factor I by enriched cultures of bovine small and large luteal cells. J Anim Sci **75**, 3235-3245.

Barnes RJ, Comline RS, Dobson A, 1983: Changes in the Blood-Flow to the Digestive Organs of Sheep Induced by Feeding. Quarterly J Exp Physiol CMS **68**, 77-88.

Bedford CA, Harrison FA, Heap RB, 1974: Splanchic, Uterine, Ovarian and Adrenal Uptake of Progesterone and 20-Alpha-Dihydroprogesterone in Pregnant and Nonpregnant Sheep. J Endocrinol **62**, 277-290.

Belfiore CJ, Hawkins DE, Wiltbank MC, Niswender GD, 1999: Regulation of Cytochrome P450(Scc) Synthesis and Activity in the Ovine Corpus-Luteum. J Steroid Biochem **51**, 283-290.

Bell AW, 1995: Regulation of Organic Nutrient Metabolism During Transition Form Late Pregnancy to Early Lactation. J Anim Sci **73**, 2804-2819.

Belvedere P, Gabai G, Dalla Valle L, Accorsi P, Trivoletti M, Colombo L, Bono G, 1996: Occurrence of steroidogenic enzymes in the bovine mammary gland at different functional stages. J Steroid Biochem Mol Biol **59**, 339-347.

Bollwein H, Lüttgenau J, Herzog K, 2013: Bovine luteal blood flow: basic mechanism and clinical relevance. Reprod Fertil Dev **25**, 71-79.

Brockman RP, Bergman EN, 1975: Effect of Glucagon on Plasma Alanine and Glutamine Metabolism and Hepatic Gluconeogenesis in Sheep. Am J Physiol **228**, 1627-1633.

Chaffin CL, Dissen GA, Stouffer RL, 2000: Hormonal regulation of steroidogenic enzyme expression in granulosa cells during the peri-ovulatory interval in monkeys. Mol Hum Reprod **6**, 11-18.

Chanderbhan R, Tanaka T, Strauss JF, Irwin D, Noland BJ, Scallen TJ, Vahouny GV, 1983: Evidence for sterol carrier protein2-like activity in hepatic, adrenal and ovarian cytosol. Biochem Biophys Res Commun **117**, 702-709.

Chegini N, Ramani N, Rao CV, 1984: Morphological and biochemical characterization of small and large bovine luteal cells during pregnancy. Mol Cell Endocrinol **37**, 89-102.

Chegini N, Rao CV, 1987: Dynamics of Nuclear Associated Granules in Bovine Luteal Cells After Treatment Invitro with Prostaglandin-F2-Alpha. Endocrinology **121**, 1870-1878.

Christenson LK, Devoto L, 2003: Cholesterol transport and steroidogenesis by the corpus luteum. Reprod Biol Endocrinol **1**, 90-99.

Christenson LK, Stouffer RL, 1996: Proliferation of microvascular endothelial cells in the primate corpus luteum during the menstrual cycle and simulated early pregnancy. Endocrinology **137**, 367-374.

Christenson LK, Strauss JF, 2000: Steroidogenic acute regulatory protein (StAR) and the intramitochondrial translocation of cholesterol. BBA-Mol Cell Biol L **1529**, 175-187.

Christenson LK, Strauss JF, 2001: Steroidogenic acute regulatory protein: An update on its regulation and mechanism of action. Arch Med Res **32**, 576-586.

Conley AJ, Kaminski MA, Dubowsky SA, Jablonka-Shariff A, Redmer DA, Reynolds LP, 1995: Immunohistochemical localization of 3 beta-hydroxysteroid dehydrogenase and P450 17 alpha-hydroxylase during follicular and luteal development in pigs, sheep, and cows. Biol Reprod **52**, 1081-1094.

Couet J, Martel C, Dupont E, Luu-The V, Sirard MA, Zhao HF, Pelletier G, Labrie F, 1990: Changes in 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase messenger ribonucleic acid, activity and protein levels during the estrous cycle in the bovine ovary. Endocrinology **127**, 2141-2148.

Cumming IA, Mole BJ, Obst J, Blockey MA, Winfield CG, Goding JR, 1971: Increase in plasma progesterone caused by undernutrition during early pregnancy in the ewe. J Reprod Fertil **1**, 146-147.

Curry TE Jr, Osteen KG, 2001: Cyclic changes in the matrix metalloproteinase system in the ovary and uterus. Biol Reprod **64**, 1285-1296.

Devoto L, Christenson LK, McAllister JM, Makrigiannakis A, Strauss JF, 1999: Insulin and insulin-like growth factor-I and -II modulate human granulosa-lutein cell steroidogenesis: enhancement of steroidogenic acute regulatory protein (StAR) expression. Mol Hum Reprod **5**, 1003-1010.

Diaz FJ, Anderson LE, Wu YL, Rabot A, Tsai SJ, Wiltbank MC, 2002: Regulation of progesterone and prostaglandin F2alpha production in the CL. Mol Cell Endocrinol **191**, 65-80.

Dobson H, Midmer SE, Fitzpatrick RJ, 1975: Relationship between progesterone concentrations in milk and plasma during bovine estrous cycle. Vet Rec **96**, 222-223.

Donaldson L, Hansel W, 1965: Histological study of bovine corpora lutea. J Dairy Sci **48**, 905-909.

Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG, 2000: Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res **60**, 1388-1393.

Farin CE, Moeller CL, Sawyer HR, Gamboni F, Niswender GD, 1986: Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. Biol Reprod **35**, 1299-1308.

Fields MJ, Barros CM, Watkins WB, Fields PA,1992: Characterization of large luteal cells and their secretory granules during the estrous cycle of the cow. Biol Reprod **46**, 535-545.

Fields MJ, Dubois W, Fields PA, 1985: Dynamic Features of Luteal Secretory Granules - Ultrastructural-Changes During the Course of Pregnancy in the Cow. Endocrinology **117**, 1675-1682.

Fields MJ, Fields PA, 1996: Morphological characteristics of the bovine corpus luteum during the estrous cycle and pregnancy. Theriogenology **45**, 1295-1325.

Findlay JX, 1986: Angiogenesis in reproductive tissues. J Endocrinol **111**, 357-366.

Fitz TA, Mayan MH, Sawyer HR, Niswender GD, 1982: Characterization of 2 Steroidogenic Cell-Types in the Ovine Corpus-Luteum. Biol Reprod **27**, 703-711.

Furcht LT, 1986: Critical factors controlling angiogenesis: cell products, cell matrix, and growth factors. Lab Invest **55**, 505-509.

Gemmell R, Stacy BD, 1979: Ultrastructural-Study of Granules in the Corpora-Lutea of Several Mammalian-Species. Am J Anat **155**, 1-13.

Gibb MJ, Ivings WE, Dhanoa MS, Sutton JD, 1992: Changes in Body Components of Autumn-Calving Holstein-Friesian Cows Over the 1St 29 Weeks of Lactation. Anim Prod **55**, 339-360.

Ginther OJ, Nuti LC, Garcia MC, Wentworth BC, Tyler WJ, 1976: Factors affecting progesterone concentration in cow's milk and dairy products. J Anim Sci **42**, 155-159.

Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG, 1998: Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest **78**, 1385-1394.

Grazul-Bilska AT, Reynolds LP, Slanger WD, Redmer DA, 1992: Production of heparin-binding angiogenic factor(s) by bovine corpora lutea during pregnancy. J Anim Sci **70**, 254-262.

Gwynne JT, Strauss JF, 1982: The role of lipoprotein in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocr Rev **3**, 299.

Hanahan D, Folkman J, 1996: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell **86**, 353-364.

Hansel W, Alila HW, Dowd JP, Yang XZ, 1987: Control of Steroidogenesis in Small and Large Bovine Luteal Cells. Aust J Biol Sci **40**, 331-347.

Hay ED, 1995: An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) **154**, 8-20.

Hayashi KG, Matsui M, Acosta TJ, Kida K, Miyamoto A, 2006: Effect of the dominant follicle aspiration before or after luteinizing hormone surge on the corpus luteum formation in the cow. J Reprod Dev **52**, 129-135.

Heap RB, Fleet IR, Hamon M, Brown KD, Stanley CJ, Webb AE, 1986: Mechanisms of transfer of steroid hormones and growth factors into milk. Endocrinol Exp **20**, 101-118.

Heap RB, Gwyn M, Laing JA, Walters DE, 1973: Pregnancy Diagnosis in Cows - Changes in Milk Progesterone Concentration During Estrous-Cycle and Pregnancy Measured by A Rapid Radioimmunoassay. Pregnancy Diagnosis in Cows - Changes in Milk Progesterone Concentration During Estrous-Cycle and Pregnancy Measured by A Rapid Radioimmunoassay. J Agr Sci **81**, 151-157.

Heap RB, Henville A, Linzell JL, 1975: Metabolic clearance rate, production rate, and mammary uptake and metabolism of progesterone in cows. J Endocrinol **66**, 239-247.

Heap RB, Linzell JL, Laing JA, 1974: Proceedings: Pregnancy diagnosis in cows: use of progestagen concentration in milk. Vet Rec **94**, 160.

Herzog K, Brockhan-Ludemann M, Kaske M, Beindorff N, Paul V, Niemann H, Bollwein H, 2010: Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. Theriogenology **73**, 691-697.

Hoffmann B, Gunzler O, Hamburger R, Schmidt W, 1976: Milk progesterone as a parameter for fertility control in cattle; methodological approaches and present status of application in Germany. Br Vet J **132**, 469-476.

Hommeida A, Nakao T, Kubota H, 2004: Luteal function and conception in lactating cows and some factors influencing luteal function after first insemination. Theriogenology **62**, 217-225.

Hünigen H, Bisplinghoff P, Plendl J, 2005: Angiogenesis and vascular regression in the corpus luteum periodicum. Anatomia, Histologia, Embryologia **34**, 22.

Huntington GB, Eisemann JH, Whitt JM, 1990: Portal Blood-Flow in Beef Steers - Comparison of Techniques and Relation to Hepatic Blood-Flow, Cardiac-Output and Oxygen-Uptake. J Anim Sci **68**, 1666-1673.

Irving-Rodgers HF, Catanzariti KD, Aspden WJ, D'Occhio MJ, Rodgers RJ, 2006: Remodeling of extracellular matrix at ovulation of the bovine ovarian follicle. Mol Reprod Dev **73**, 1292-1302.

Johnson DE, Johnson KA, Baldwin RL, 1990: Changes in Liver and Gastrointestinal-Tract Energy Demands in Response to Physiological Workload in Ruminants. J Nutr **120**, 649-655.

Juengel JL, Meberg BM, Turzillo AM, Nett TM, Niswender GD, 1995: Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea. Endocrinology **136**, 5423-5429.

King RL, 1963: Effect of Washing and Homogenization on Oxidation of Milk Fat Globules. J Dairy Sci **46**, 605.

König HE, Amselgrüber W, 1986: Vaskularisation des Corpus luteum des Rindes. Fertilität **2**, 205-208.

König HE, Amselgrüber W, Rüsse I, 1988: Zur Mikrozirkulation in Follikeln und Corpora lutea des Rinderovars-eine korrosionsanatomische Studie. Tierärztl Prax **16**, 25-31.

Kotwica J, Rekawiecki R, Duras M, 2004: Stimulatory influence of progesterone on its own synthesis in bovine corpus luteum. Bull Vet Inst Pulawy **48**, 139-145.

Laing JA, Heap RB, 1971: The concentration of progesterone in the milk of cows during the reproductive cycle. Br Vet J **127**, 19-22.

Lautt WW, 1996: The 1995 Ciba-Geigy Award Lecture. Intrinsic regulation of hepatic blood flow. Can J Physiol Pharm **74**, 223-233.

Lavoie HA, Benoit AM, Garmey JC, Dailey RA, Wright DJ, Veldhuis,J D, 1997: Coordinate developmental expression of genes regulating sterol economy and cholesterol side-chain cleavage in the porcine ovary. Biol Reprod **57**, 402-407.

Lei ZM, Chegini N, Rao CV, 1991: Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol Reprod **44**, 1148-1156.

Lemon M, Loir M, 1977: Steroid Release Invitro by 2 Luteal Cell-Types in Corpus-Luteum of Pregnant Sow. J Endocrinol **72**, 351-359.

Li X, Peegel H, Menon KM, 1998: In situ hybridization of high density lipoprotein (scavenger, type 1) receptor messenger ribonucleic acid (mRNA) during folliculogenesis and luteinization: evidence for mRNA expression and induction by human chorionic gonadotropin specifically in cell types that use cholesterol for steroidogenesis. Endocrinology **139**, 3043-3049.

Liekens S, De Clercq E, Neyts J, 2001: Angiogenesis: regulators and clinical applications. Biochem Pharmacol **61**, 253-270.

Liscum L, Munn NJ, 1999: Intracellular cholesterol transport. Biochim Biophys Acta **1438**, 19-37.

Lobel BL, Levy E, 1968: Enzymic Correlates of Development Secretory Function and Regression of Follicles and Corpora Lutea in Bovine Ovary. Acta Endocrinol-Cop **S132**, 7.

Lomax,M.A.; Baird,G.D., 1983: Blood-Flow and Nutrient Exchange Across the Liver and Gut of the Dairy-Cow - Effects of Lactation and Fasting. Br J Nutr **49**, 481-496.

Luck MR, Zhao Y, 1993: Identification and Measurement of Collagen in the Bovine Corpus-Luteum and Its Relationship with Ascorbic-Acid and Tissue-Development. J Reprod Fertil **99**, 647-652.

Macfarlane JS, Booth JM, Deas DW, Lowman BG, 1977: Pregnancy test and evaluation of embryonic and fetal mortality based on progesterone concentrations fore-milk. Vet Rec **100**, 565-566.

Mann GE, Merson P, Fray MD, Lamming GE, 2001: Conception rate following progesterone supplementation after second insemination in dairy cows. Vet J **162**, 161-162.

Mather EC, Camper PM, Vahdat F, Whitmore HL, Gustafsson BG, 1978: Assessment of Ovarian Activity in Postpartum Dairy Cow by Use of A Milk Progesterone Assay. Theriogenology **10**, 119-129.

Meidan R, Girsh E, Blum O, Aberdam E, 1990: In vitro differentiation of bovine theca and granulosa cells into small and large luteal-like cells: morphological and functional characteristics. Biol Reprod **43**, 913-921.

Milvae RA, Alila HW, Bushmich SL, Hansel W, 1991: Bovine corpus luteum function after removal of granulosa cells from the preovulatory follicle. Domest Anim Endocrin **8**, 439-443.

Murphy BD, 2000: Models of luteinization. Biol Reprod **63**, 2-11.

Nehls V, Denzer JA, Drenckhahn D, 1992, Pericyte involvement in capillary sprouting during angiogenesis in situ. Cell Tissue Res **270**, 469-474.

Neves MM, Marques Jr, AP, 2006: Volumetric proportions of the corpus luteum constituents of Nelore. Arq Bras Med Vet Zootec **58**, 944-946.

Nickel R, Schummer A, Seiferle D, 1995: Lehrbuch der Anatomie der Haustiere, II, Eingeweide. Paul Parey, Berlin.

Niswender GD, 2002: Molecular control of luteal secretion of progesterone. Reproduction **123**, 333-339.

Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW, 2000: Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev **80**, 1-29.

Niswender GD, Roess DA, Sawyer HR, Silvia WJ, Barisas BG, 1985: Differences in the Lateral Mobility of Receptors for Luteinizing-Hormone (Lh) in the Luteal Cell Plasma-Membrane When Occupied by Ovine Lh Versus Human Chorionic-Gonadotropin. Endocrinology **116**, 164-169.

O'Shaughnessy PJ, 1989: Die Kontrolle der Entwicklung des Corpus luteum und seine Aufrechterhaltung beim Rind. Tierärztl Prax Suppl **4**, 7-11.

O'Shea JD, 1987: Heterogeneous cell types in the corpus luteum of sheep, goats and cattle. J Reprod Fertil Suppl **34**, 71-85.

O'Shea JD, Cran DG, Hay MF, 1980: Fate of the theca interna following ovulation in the ewe. Cell Tissue Res **210**, 305-319.

O'Shea JD, Rodgers RJ, D'Occhio MJ, 1989: Cellular composition of the cyclic corpus luteum of the cow. J Reprod Fertil **85**, 483-487.

O'Shea JD, Rodgers RJ, Wright PJ, 1986: Cellular composition of the sheep corpus luteum in the mid- and late luteal phases of the oestrous cycle. J Reprod Fertil **76**, 685-691.

Parkinson TJ, Turvey A, Jenner LJ, 1994: A morphometric analysis of the corpus luteum of the cow during the estrous cycle and early pregnancy. Theriogenology **41**, 1115-1126.

Parr RA, Davis IF, Miles MA, Squires TJ, 1993: Liver blood flow and metabolic clearance rate of progesterone in sheep. Res Vet Sci **55**, 311-316.

Parry DM, Willcox DL, Thorburn GD, 1980: Ultrastructural and Cytochemical Study of the Bovine Corpus-Luteum. J Reprod Fertil **60**, 349-357

Pennington JA, Spahr SL, Lodge JR, 1981: Influences on progesterone concentration in bovine milk. J Dairy Sci **64**, 259-266.

Pescador,N.; Soumano,K.; Stocco,D.M.; Price,C.A.; Murphy,B.D., 1996: Steroidogenic acute regulatory protein in bovine corpora lutea. Biol Reprod **55**, 485-491.

Pon LA, Orme-Johnson NR, 1988: Acute stimulation of corpus luteum cells by gonadotrophin or adenosine 3',5'-monophosphate causes accumulation of a phosphoprotein concurrent with acceleration of steroid synthesis. Endocrinology **123**, 1942-1948.

Pope GS, Majzlik I, Ball PJH, Leaver JD, 1976: Use of Progesterone Concentrations in Plasma and Milk in Diagnosis of Pregnancy in Domestic Cattle Br Vet J **132**, 497-506.

Priedkalns J, Weber AF, 1968b: Ultrastructural Studies of Bovine Graafian Follicle and Corpus Luteum. Z Zellforsch Mikrosk Anat **91**, 554-573.

Priedkalns J, Weber AF, 1968a: Quantitative Ultrastructural Analysis of Follicular and Luteal Cells of Bovine Ovary. Z Zellforsch Mikrosk Anat **91**, 574-585.

Priedkalns J, Weber AF, Zemjanis R, 1968: Qualitative and Quantitative Morphological Studies of Cells of Membrana Granulosa Theca Interna and Corpus Luteum of Bovine Ovary. Z Zellforsch Mikrosk Anat **85**, 501-520.

Quirke JF, Hanrahan JP, Gosling JP, 1979: Plasma progesterone levels throughout the oestrous cycle and release of LH at oestrus in sheep with different ovulation rates. J Reprod Fertil **55**, 37-44.

Rabiee AR, MacMillan KL, Schwarzenberger F, 2001: Excretion rate of progesterone in milk and faeces in lactating dairy cows with two levels of milk yield. Reprod Nutr Dev **41**, 309-319.

Redmer DA, Doraiswamy V, Bortnem BJ, Fisher K, Jablonka-Shariff A, Grazul-Bilska AT, Reynolds LP, 2001: Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. Biol Reprod **65**, 879-889.

Redmer DA, Kirsch JD, Reynolds LP, 1991: Production of mitogenic factors by cell types of estrogen-active preovulatory bovine follicles. J Anim Sci **69**, 237-245.

Reynolds CK, Aikman PC, Lupoli B, Humphries DJ, Beever DE, 2003: Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. J Dairy Sci **86**, 1201-1217.

Reynolds CK, Durst B, Lupoli B, Humphries DJ, Beever DE, 2004: Visceral tissue mass and rumen volume in dairy cows during the transition from late gestation to early lactation. J Dairy Sci **87**, 961-971.

Reynolds LP, Grazul-Bilska AT, Redmer DA, 2000: Angiogenesis in the corpus luteum. Endocrine **12**, 1-9.

Reynolds CK, Huntington GB, Elsasser TH, Tyrrell HF, Reynolds PJ, 1989: Net metabolism of hormones by portal-drained viscera and liver of lactating holstein cows. J Dairy Sci **72**, 1459-1468.

Reynolds CK, Huntington GB, Tyrrell HF, Reynolds PJ, 1988: Net Metabolism of Volatile Fatty-Acids, D-Beta-Hydroxybutyrate, Nonesterified Fatty-Acids, and Blood Gasses by Portal-Drained Viscera and Liver of Lactating Holstein Cows. J Dairy Sci **71**, 2395-2405.

Reynolds LP, Killilea SD, Grazul-Bilska AT, Redmer DA, 1994: Mitogenic factors of copora lutea. Progr Growth Factor Res **5**, 159-175.

Reynolds LP, Killilea SD, Redmer DA, 1992: Angiogenesis in the female reproductive system. Faseb J **6**, 886-892.

Reynolds LP, Redmer DA, 1999: Growth and development of the corpus luteum. J Reprod Fertil Suppl **54**, 181-191.

Rhinehart JD, Starbuck-Clemmer MJ, Flores JA, Milvae RA, Yao J, Poole DH, Inskeep EK, 2009: Low peripheral progesterone and late embryonic/early fetal loss in suckled beef and lactating dairy cows. Theriogenology **71**, 480-490.

Robinson RS, Hammond AJ, Hunter MG, Mann GE, 2005: The induction of a delayed post-ovulatory progesterone rise in dairy cows: a novel model. Domest Anim Endocrinol **28**, 285-295.

Robinson RS, Hammond AJ, Nicklin LT, Schams D, Mann GE, Hunter MG, 2006: Endocrine and cellular characteristics of corpora lutea from cows with a delayed post-ovulatory progesterone rise. Domestic Animal Endocrinol **31**, 154-172.

Robinson RS, Hammond AJ, Wathes DC, Hunter MG, Mann GE, 2008: Corpus luteum-endometrium-embryo interactions in the dairy cow: underlying mechanisms and clinical relevance. Reprod Dom Anim **43** (Suppl. 2), 104-112.

Rodgers RJ, Irving-Rodgers HF, 2002: Extracellular matrix of the bovine ovarian membrana granulose. Moll Cell Endocrinol **191**, 57-64.

Rodgers RJ, Irving-Rodgers HF, van Wezel IL, Krupa M, Lavranos TC, 2001: Dynamics of the membrana granulosa during expansion of the ovarian follicular antrum. Moll Cell Endocrinol **171**, 41-48.

Rodgers RJ, O'Shea JD, Bruce NW, 1984: Morphometric analysis of the cellular composition of the ovine corpus luteum. J Anat **138**, 757-770.

Rodgers RJ, Rodgers HF, Waterman MR, Simpson ER, 1986: Immunolocalization of Cholesterol Side-Chain-Cleavage Cytochrome-P-450 and Ultrastructural Studies of Bovine Corpora-Lutea. J Reprod Fertil **78**, 639-652.

Rothchild I, 1996: The corpus luteum revisited: are the paradoxical effects of RU486 a clue to how progesterone stimulates its own secretion? Biol Reprod **55**, 1-4.

Rouillier P, Matton P, Sirard MA, Guilbault LA, 1996: Follicle-stimulating hormone-induced estradiol and progesterone production by bovine antral and mural granulosa cells cultured in vitro in a completely defined medium. J Anim ScI **93**, 3012-3019.

Rust W, Stedronsky K, Tillmann G, Morley S, Walther N, Ivell R, 1998: The role of SF-1/Ad4BP in the control of the bovine gene for the steroidogenic acute regulatory (StAR) protein. J Mol Endocrinol **21**, 189-200.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC, 2002: High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle. J Dairy Sci **85**, 2831-2842.

Sato Y, Rifkin W, 1989: Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. J Cell Biol **109**, 309-315.

Sawyer HR, 1995: Structural and functional properties of the corpus luteum of pregnancy. J Reprod Fertil Suppl **49**, 97-110.

Schams D, Berisha B, 2004: Regulation of corpus luteum function in cattle - an overview. Reprod Domest Anim **39**, 241-251.

Schiavo JJ, Matuszczak RL, Oltenacu EB, Foote RH, 1975: Milk Progesterone in Postpartum and Pregnant Cows As A Monitor of Reproductive Status. J Dairy Sci **58**, 1713-1716.

Schwarzenbach H, Manna PR, Stocco DM, Chakrabarti G, Mukhopadhyay AK, 2003: Stimulatory effect of progesterone on the expression of steroidogenic acute regulatory protein in MA-10 Leydig cells. Biol Reprod **68**, 1054-1063.

Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE, 1990: Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil **90**, 1-10.

Shirasuna K, Nitta A, Sineenard J, Shimizu T, Bollwein H, Miyamoto A, 2012: Vascular and immune regulation of corpus luteum development, maintenance, and regression in the cow. Domestic Animal Endocrinol. **43**, 198-211.

Silvester LM, Luck MR, 1999: Distribution of extracellular matrix components in the developing ruminant corpus luteum: a wound repair hypothesis for luteinization. J Reprod Fertil **116**, 187-198.

Skarzynski DJ, Piotrowska-Tomala KK, Lukasik K, Galvao A, Farerov S, Zalman Y, Meidan R, 2013: Growth and regression in bovine corpora lutea: regulation by local survival and death pathways. Reprod Dom Anim **48**, 25-37.

Smith MF, McIntush EW, Ricke WA, Kojima FN, Smith GW, 1999: Regulation of ovarian matrix remodeling by metalloproteinases and their tissue inhibitors: Effects on follicular development, ovulation and luteal function. J Reprod Fertil Suppl **54**, 367-381.

Smith MF, McIntush EW, Smith GW, 1994: Mechanisms associated with corpus luteum development. J Anim Sci **72**, 1857-1872.

Soumano K, Lussier JG, Price CA, 1998: Levels of messenger RNA encoding ovarian receptors for FSH and LH in cattle during superovulation with equine chorionic gonadotrophin versus FSH. J Endocrinol **156**, 373-378.

Starbuck MJ, Inskeep EK, Dailey RA, 2006: Effect of a single growth hormone (rbST) treatment at breeding on conception rates and pregnancy retention in dairy and beef cattle. Anim Reprod Sci **93**, 349-359.

Stocco DM, 2000: Intramitochondrial cholesterol transfer. Biochim Biophys Acta **1486**, 184-197.

Storch J, Cheruku SR, 2005: Cholesterol transport in lysosomes. In: Saftig P (ed.), Lysosomes. Eurekah.com and Springer Science.

Stouffer RL, Martinez-Chequer JC, Molskness TA, Xu FH, Hazzard TM, 2001: Regulation and action of angiogenic factors in the primate ovary. Arch Med Res **32**, 567-575.

Sugawara T, Kiriakidou M, McAllister JM, Holt JA, Arakane F, Strauss JF, 1997: Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: a central role for steroidogenic factor 1. Steroids **62**, 5-9.

Ursely J, Leymarie P, 1979: Varying Response to Luteinizing-Hormone of 2 Luteal Cell-Types Isolated from Bovine Corpus-Luteum. J Endocrinol **83**, 303-310.

Vasconcelos JLM, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC, 2001: Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology **56**, 307-314.

Villa-Godoy A, Hughes TL, Emery RS, Enright WJ, Ealy AD, Zinn SA, Fogwell RL, 1990: Energy balance and body condition influence luteal function in Holstein heifers. Domest Anim Endocrinol **7**, 135-148.

Waldmann A, Ropstad E, Landsverk K, Sorensen K, Solverod L, Dahl E, 1999: Level and distribution of progesterone in bovine milk in relation to storage in the mammary gland. Anim Reprod Sci **56**, 79-91.

Wathes DC, Taylor VJ, Cheng Z, Mann GE, 2003: Follicle growth, corpus luteum function and their effects on embryo development in postpartum dairy cows. Reprod.Suppl **61**, 219-237.

Watson ED, 1986: A Note on the Effect of Postparturient Fatty Liver on Clearance Rate of Exogenous Progesterone from Blood. Anim Prod **42**, 425-426.

Weber DM, Fields PA, Romrell LJ, Tumwasorn S, Ball BA, Drost M, Fields MJ, 1987: Biol Reprod **37**, 685-697.

Wieghart M, Slepetis R, Elliot JM, Smith DF, 1986: Glucose-Absorption and Hepatic Gluconeogenesis in Dairy-Cows Fed Diets Varying in Forage Content. J Nutr **116**, 839-850.

Wiltbank MC 1994: Cell types and hormonal mechanisms associated with mid-cycle corpus luteum function. J Anim Sci **72**, 1873-1883.

Wiltbank MC, Belfiore CJ, Niswender GD, 1993: Steroidogenic enzyme activity after acute activation of protein kinase (PK) A and PKC in ovine small and large luteal cells. Mol Cell Endocrinol **97**, 1-7.

Xu Z., Garverick HA, Smith GW, Smith MF, Hamilton SA, Younquist RS, 1995: Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol Reprod **53**, 951-957.

Yamashita H, Kamada D, Shirasuna K, Matsui M, Shimizu T, Kida K, Berisha B, Schams D, Miyamoto A, 2008: Effect of local neutralization of basic fibroblast growth factor or vascular endothelial growth factor by a specific antibody on the development of the corpus luteum in the cow. Mol Reprod Dev **75**, 1449-1456.

Zheng J, Fricke PM, Reynolds LP, Redmer DA, 1994: Evaluation of growth, cell proliferation, and cell death in bovine copora lutea throughout the estrous cycle. Biol Reprod **51**, 623-632.

Zheng J, Redmer DA, Reynolds LP, 1993: Vascular development and heparin-binding growth factors in the bovine corpus luteum at several stages of the estrous cycle. Biol Reprod **49**, 1177-1189.

Chapter 2.3

Ration induced effects on luteal and endometrial function, with emphasis on NEB and isoflavones

2.3.1 The impact of a negative energy balance (NEB)

The 'Resources Allocation Theory' of Goddard and Beilharz (1977) declared that animal resources are limited. This means that if the output by one biological process is increased (such as milk production), the results of other systems are affected (such as reproduction). The availability of resources can be increased or optimized by a higher DMI, but this increase is limited, leading to a NEB. The NEB elicits a lowered plasma level of glucose, insulin, and IGF1 (Butler, 2003), inducing a more intense lipolysis in the body fat reserves. Plasma NEFA levels increase and lead to hepatic storage of triglycerides. When the liver becomes overcrowded by the NEFAs, ketone bodies (such as BOHB) are produced and released into the circulation, leading to ketonemia. Besides those players, β-endorphin (an endogenous opioid) induces an increase of DMI together with hypoglycemia, hypo-insulinemia and lipolysis. The common effect of those modifications (hypoglycemia, ketonemia, the production of β- endorphins) is a depression of the LH-pulse frequency (Butler and Smith, 1989; Butler, 2003).

Additionally, StAR may be a linking molecule between energy balance and luteal function in dairy cows. Indeed, the level of the mentioned regulators of the StAR-transcription (LH, insulin, and IGF) decreases during NEB, possibly leading to a reduction in the StAR transcription and progesterone production. Also progesterone is decreased in cows dealing with NEB, possibly leading to a reduced positive feedback of the StAR transcription.

As there is a strong interaction between the metabolic status of the cow and her reproductive status, IGF could be the link between cow's metabolism and fertility (Wathes et al., 2003; Velazquez et al., 2008). Indications are: 1) when the systemic concentration of IGF1 is decreased during the first two weeks post partum, also the ovarian and uterine function is impaired (Beam and Butler, 1998; Wathes et al., 2007); 2) in the endometrium IGF1 and 2 mRNA are increasing in the presence of a growing embryo during early pregnancy, leading to the possibility that, or the embryo up-regulates the expression of these genes, or the embryonic development was enhanced in cows with increased IGF1 and 2 concentrations (Robinson et al., 2000; Bilby et al., 2006). Moreover, maternal IGF1 and 2 stimulate the embryonic IFN τ production (Ko et al., 1991); 3) IGFBP can sequester IGF or promote IGF-action. A cow in NEB undergoes during day 1 to 5 postpartum a modification in the salpingeal expression pattern of IGFBP 2 and 6, both having a higher affinity for IGF2. This possibly leads to a modified IGF signaling pathway within the salpingeal environment (Fenwick et al., 2008), which could also be present later on in the postpartum period.

Transcription of this BP is switched on once the progesterone block is lost. So, a delayed progesterone increase in cows, which is described in cows with 'luteal inadequacy', could induce a delay in increase of IGFBP1, as progesterone regulates the down regulation of its P_4R (Robinson et al., 2008). In the luminal epithelium, endometrial IGFBP-1 is increased in the presence of an embryo, coinciding with the blastocyst elongation (Robinson et al., 2000). This increased IGFBP-1 could increase the IGF1 availability of the embryo by regulating the transport of IGF1 from the endometrium to the uterine lumen. So, 'luteal inadequacy' could influence embryonic development during the early pregnancy.The expression of IGFBP 2 and 3 is decreased during pregnancy (Robinson et al., 2000), probably by the embryo to increase IGF1 and 2-availability in the uterine lumen (Robinson et al., 2008).

When we look more in detail to the bovine CL itself, the angiogenic as well as the luteogenic process are partly regulated by insulin, IGF, growth hormone and gonadotrophines (FSH, LH), all hormones of which the secretion is influenced by the energy status of the cow (Toutges et al., 1999; Stouffer et al., 2001). The NEB suppresses the GnRH, FSH and LH pulse frequency and amplitude, possibly affecting subsequent luteogenesis (Rutter and Manns, 1987; Villa-Godoy et al., 1988; Butler, 2000).

The NEB has a carryover effect on blood progesterone concentrations. Progesterone in the peripheral circulation increases during the first two or three postpartum ovulatory cycles and the rate of the increase in progesterone levels is reduced or moderated by a NEB early postpartum (Villa-Godoy et al., 1988; Spicer et al., 1990; Staples et al., 1990). Cows with the severest NEB during the first 9 days postpartum still show decreased serum progesterone levels during their third estrous cycles (Villa-Godoy et al., 1988). In the study of Villa-Godoy et al. (1988) it was stated that the NEB had no effect on the luteal progesterone production during the first cycle, but in the second and third cycle the effect was obvious. The luteal lifespan was normal, but the progesterone production was reduced. Possible reasons could be reduced luteal development or decreased secretory activity per luteal cell or both. This reduced luteal steroidogenesis is what we will analyze in our study.

Britt (1994) and Mann and Lamming (2001) postulated that follicles exposed to a period of NEB led to smaller and even dysfunctional CLa. Shelton et al. (1990) found a delayed progesterone increase in repeat breeding cows. He proposed, together with Mann and Lamming (2001), that an impaired luteinisation or an inadequate LH-support of the steroidogenesis could be the reason. Researchers describe a phenomenon named ‘luteal inadequacy’ in Holstein Friesian cows. 'Luteal inadequacy' means a lowered progesterone secretion or a delayed progesterone secretion increase during luteogenesis, leading to an

increased incidence of early embryonic death. As we discussed already, the energy balance of these animals influences the luteal functionality. Robinson et al. (2006) analyzed this hypothesis, but found no impairing effects on LH-response *in vitro*, on steroidogenesis nor on angiogenesis. The latter study indicated that the LH-support, the degree of vascularisation and the steroidogenic capacity are probably not the major factors explaining luteal inadequacy.

On the contrary, Ikeda et al. (1995) and Mukhopadhyay et al. (1995) described that the regulation of the expression of the most important regulator of luteal angiogenesis, vascular endothelial growth factor (VEGF) was modified by metabolic parameters such as hypoglycemia. Fasting gilts and ewes had to cope with an increase in blood NEFA-concentrations, leading to a decreased mRNA level of VEGF and VEGF-receptor (VEGFR)-2 in newly formed corpora lutea or placenta (Galeati et al., 2005; McMullen et al., 2005).

Also certain steps in the luteal steroidogenesis are influenced by the energy balance of the cows. Hormone sensitive lipase (i.e. neutral cholesterol ester hydrolase) is regulated by FSH, LH and hCG of which the expression is depending on the energy balance (Devoto et al., 2002; Christenson and Devoto, 2003). Insulin like growth factor (IGF) stimulates StAR-gene expression. Luteinizing hormone, progesterone and PGE_2 also stimulate the gene expression of StAR, CYP_{450SCC} and 3β-HSD. Noradrenaline increases the activity of CYP_{450SCC} and 3β-HSD, but has no influence on the gene expression (Christenson and Strauss, 2000). Vanholder et al. (2005) found that NEFAs, which are liberated in the cow's blood during a period of NEB induce an impairment of the steroidogenesis and cell proliferation in granulosa cell cultures, the precursors of luteal steroidogenic cells.

Based on these data, it can be stated that the NEB, present during the early postpartum period in high yielding dairy cows, can deteriorate the luteal angiogenesis and steroidogenesis exerting direct or indirect impairing effects on the salpingeal and uterine environment and the early embryonic evolution. But besides the negative contribution of the NEB, there is a second important factor, the high yielding cows are dealing with, namely the presence of isoflavones in the most important protein source of their ration. This will be discussed in the next paragraph.

2.3.2 The impact of nutritional isoflavones

In human society, an interesting observation has been done at the end of the last century: the incidence of hormone-sensitive breast- and prostate cancer was the highest in the United States of America and the lowest in China (Dhom, 1991). Surprisingly, when Chinese human beings migrated to North-America, the incidence of these cancer types was increasing to half

the local incidence (Shimizu et al., 1991). Moreover, the incidence in American vegetarians was also lower as in local omnivorous people (Mills et al., 1989). The common aspects between the foreign Chinese and the local American vegetarian people was the increased consumption of plant-derived oestrogenic components, named phytooestrogens, which should have a cancer protective effect (Adlercreutz, 1990). Several epidemiological studies found a relationship between soy food consumption in humans and the lower cancer risk (Mills et al., 1989; Dhom, 1991; Shimizu et al., 1991; Kurzer and Xu, 1997; Setchell and Cassidy, 1999).

Soy is known to be rich in isoflavones, a subgroup of the phytooestrogens, (Coward et al., 1993; Mazur and Adlercreutz, 1998), making soybeans and their dietary derivates a major nutritional source of phytooestrogens for humans (Bingham et al., 1998; Hollman, 2001). The cancer preventive properties were credited to several of these isoflavonic molecules and metabolites present in soy products (Adlercreutz, 1990). Isoflavones and their metabolites are generally recognized as phytooestrogens with a high oestrogenic potency (COT, 2003). The mechanism suggested to be behind the anti-cancer effect of these molecules, is the significant impairment of angiogenesis (Cassidy, 1996; Chapin et al., 1996; Fotsis et al., 1993; Kurzer and Xu, 1997; Tham et al., 1998; Ying et al., 2001; Zhou et al., 1999) through inhibition of cell proliferation and interruption of the breakdown of extracellular matrix surrounding growing vessels and tumours (Ibarreta et al., 2001; Su et al., 2005).

Also in dairy industry, high yielding dairy cows are frequently supplemented with soybeans or soybean meal, supplementary to their basic ration to meet their requirements in terms of protein intake (Chouinard et al., 1997; Steinfeld et al., 2006), exposing these animals also to the mentioned phytooestrogens.

2.3.2.1 What are these molecules and where do they come from?

Phytooestrogens are agonists or antagonists of the oestrogen receptor (ER) (Murkies et al., 1998). They are also defined as ‘SERMs’ (selective ER modulators) (Brzezinski and Debi, 1999). These SERMs are non-steroidal molecules with a structure comparable to oestradiol and an affinity for the ER (Riggs and Hartmann, 2003). The agonistic or antagonistic character is depending on the tissue type, the ER-subtype locally present in the tissue and the concentration of endogenous oestradiol (Gruber et al., 2002). Besides the ER-modulation, they also exert effects independent of the ER (Wang et al., 1996).

Benassayag et al. (2002) described 6 classes of phytooestrogens with oestrogenic as well as anti-oestrogenic effects: 1) flavonoids (such as kaempferol, quercitin), 2) isoflavonoids (such as genisteine, daidzeine, glycitein, formonetin, equol), 3) coumestans (such as coumestrol),

4) lignans (such as enterolacton, enterodiol, nordihydroguaiaretic acid), 5) mycotoxins (such as resorcylic acid lactones, zearalenol) and 6) stilbenes (such as resveratrol). Nevertheless, the structural classification and identification of phytooestrogenic members is still a story with open ending. Under the name 'dietary phytooestrogens', the flavonoids in combination with the related, metabolic generated components, including isoflavones, coumestans and mammalian lignans are collected (Chapin et al., 1996).

Out of this large group of phytooestrogens, one subgroup will be highlighted, namely the isoflavones, as these are important representatives in soy. The common structure of the isoflavones is visualized in figure 7. The backbone structure is the molecular structure of flavone (2-phenyl-1-benzopyran-4-one). But isoflavones differ from flavones in the location of the phenyl group. Their phenyl ring (B-ring) is carried rather on the C3 instead of the C2 of the heterocyclic ring (Mazur en Adlercreutz, 1998).

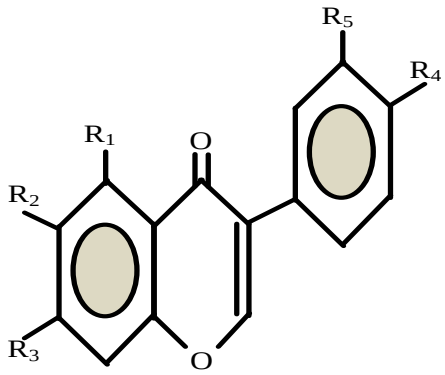
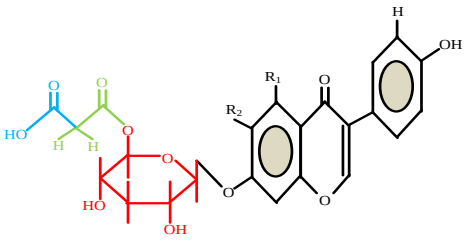


Figure 7: *the backbone structure of isoflavones. R_1 till R_5 represent specific residual groups.*

Within the class of isoflavones, the members genistein, daidzein, and glycitein and their conjugates are important. In table 2 an overview is given of the different chemical structures of the aglycones genistein, daidzein and glycitein, together with their conjugates. Also the methyl ether derivatives of genistein and daidzein, respectively biochanin A and formononetin, are incorporated in the table. The black structure represents the aglycone; when combined with the red structure, the β-glycoside form is made; when the green and blue structures are added, the acetyl-, respectively malonylglucosides are born.

Table 2. *structure of the most important members of the class of isoflavones*

isoflavone	**formula**	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**
biochanin A	$C_{16}H_{12}O_5$	OH	H	OH	OCH_3	H
genistein	$C_{15}H_{10}O_5$	OH	H	OH	OH	H
genistin	$C_{21}H_{20}O_{10}$	OH	H	O-$C_6H_{10}O_5$	OH	H
acetylgenistin	$C_{23}H_{22}O_{11}$	OH	H	O-$C_6H_9O_5$-C_2H_3O	OH	H
malonylgenistin	$C_{24}H_{22}O_{13}$	OH	H	O-$C_6H_9O_5$- $C_3H_3O_3$	OH	H
formononetin	$C_{16}H_{12}O_4$	H	H	OH	OCH_3	H
daidzein	$C_{15}H_{10}O_4$	H	H	OH	OH	H
daidzin	$C_{21}H_{20}O_9$	H	H	O-$C_6H_{10}O_5$	OH	H
acetyldaidzin	$C_{23}H_{22}O_{10}$	H	H	O-$C_6H_9O_5$-C_2H_3O	OH	H
malonyldaidzin	$C_{24}H_{22}O_{12}$	H	H	O-$C_6H_9O_5$-$C_3H_3O_3$	OH	H
glycitein	$C_{16}H_{12}O_5$	H	OCH_3	OH	OH	H
glycitin	$C_{22}H_{22}O_{10}$	H	OCH_3	O-$C_6H_{10}O_5$	OH	H
acetylgenistin	$C_{24}H_{24}O_{11}$	H	OCH_3	O-$C_6H_9O_5$-C_2H_3O	OH	H
malonylgenistin	$C_{25}H_{24}O_{13}$	H	OCH_3	O-$C_6H_9O_5$- $C_3H_3O_3$	OH	H



R_1 till R_5 represent the different residual groups, specific for each molecule.

Several factors such as growth rate of the plants, stress (caused by acute changes in temperature, humidity and insects), plant variety (influences the topographic distribution of isoflavones within the plant), harvest- and conservation method, have been mentioned to affect the isoflavone concentration in soy compounds (Ibarreta et al., 2001; Liggins et al., 2000; Price and Fenwick, 1985; Tsukamoto et al., 1995). This leads to a substantial variation in the isoflavone concentrations in soybeans. One can find that soybeans contain 2.88 to 3 mg isoflavones per g fresh meal (Seo and Morr, 1984; Zdunczyk et al., 2006) and 1.2 to 4.2 mg isoflavones per g dry matter (Bingham et al., 1998; Eldridge and Kwolek, 1983; Hollman, 2001; Mazur and Adlercreutz, 1998; Wang and Murphy, 1994), with mostly genistein,

daidzein and their conjugates, and in lesser amounts glycitein present (Kurzer and Xu, 1997; Tham et al., 1998). The isoflavones are polar, hydrophilic molecules, leading to the fact that soy oil is devoid of these molecules, while the protein fraction (such as soy flakes) is very rich (Mazur, 1998; Setchell and Cassidy, 1999). The latter may result in an enrichment of the concentration of isoflavones present in soybean meal versus the oil fraction or in whole soybeans as such. Notwithstanding this enrichment, only moderate concentrations of 2.2 till 3.0 mg/g fresh product have been reported in those soybean meal products frequently used as very important dietary protein source for dairy cows (Coward et al., 1993; Farmakalidis and Murphy, 1985; Seo and Morr, 1984). Moreover, heat treatment stimulates the decarboxylation of malonyl esters towards acetyl-glucosides (Barnes et al., 1994) and further towards β-glycosides (by ester hydrolysis) and aglycones (also by hydrolysis) (see figure 8). The β-glycosides and aglycones seem to be rather stable to high temperatures when present in feed or food, leading to a higher bioavailability of these bio-active compounds (Barnes et al., 1994). Also fermentation induces hydrolysis of glucosides towards aglycones (Coward et al., 1993; Matsuura en Obata, 1993; Setchell, 1998). According to Adlercreutz (1998), the aglycones are biphenolic structures with exceptional stability. Their structures and molecular weights make them suitable for passing cell membranes and interaction with enzymes and other proteins such as the ER.

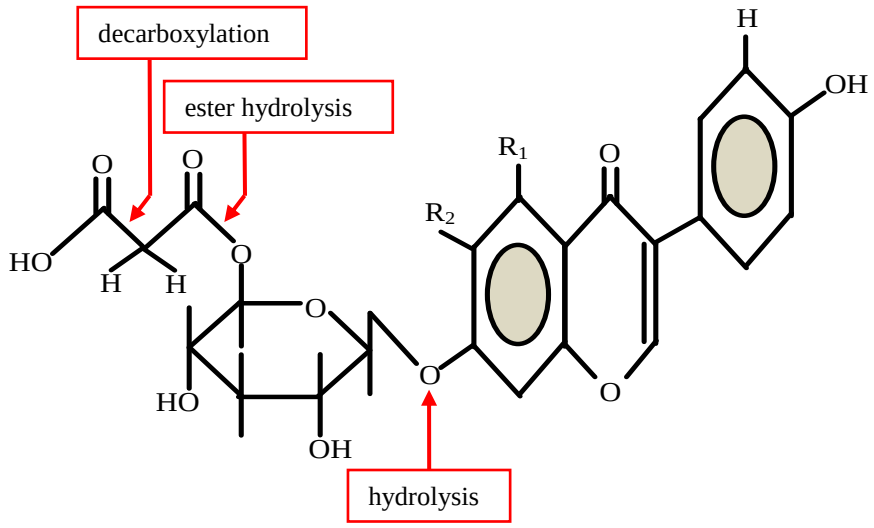


Figure 8: *overview of the different chemical degradation pathways of isoflavonic molecules*

2.3.2.2 Metabolism of isoflavones

2.3.2.2.1 Bioavailability within ruminants

The bioavailability, which is the effectivity of a chemical component to evoke a response in a target tissue, is different for each molecule and is influenced by several phenomena within the organism: absorption, metabolisation, distribution, and excretion (Rowland et al., 2003).

In ruminants (bovines, sheep) the metabolisation and resorption mainly take place in the rumen (figure 9) (Lundh, 1990). Formononetin and biochanin A are demethylated respectively to daidzein and genistein (Braden et al., 1971). Other conjugates (β-glycosides, acetyl- and malonylglucosides) are first hydrolyzed to aglycones (Lundh et al., 1990). The aglycone daidzein can be further degraded to equol or O-desmethyl-angolensin (O-DMA) (Setchell et al., 2002) by hydrogenation and ring cleavage, while the aglycone genistein can be degraded to p-ethyl phenol or 4-hydroxyphenyl-2-propionic acid by ring cleavage (Dickinson et al., 1988; Saloniemi et al., 1995; Setchell et al., 1984). Genistein is more sensitive to microbial degradation than daidzein (Bowey et al., 2003), as genistein carries an OH-group on the C5 of ring A (Griffiths and Smith, 1972), making the molecule more sensitive for bacterial cleavage of ring C. This leads to lowered plasma genistein concentrations, despite the fact that genistein is the most prominent molecule in soy.

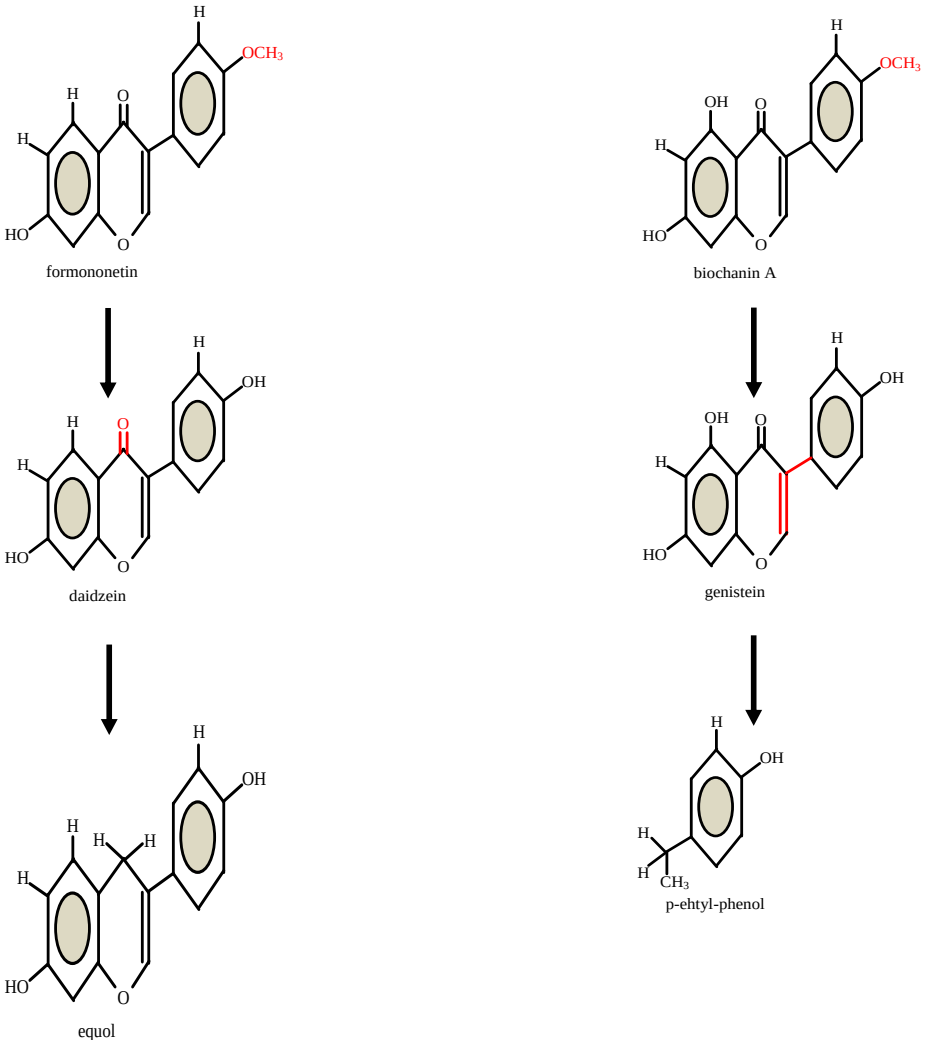


Figure 9: *ruminal biodegradation of formononetin and biochanin A*

The ruminal flora needs 6 to 10 days to fully adapt to these substrates (Adams, 1995; Duquesnoy, 2005). So, it is mainly during these first days after the introduction to new fodder that isoflavones can induce obvious clinical oestrogenic effects (Adams, 1995).

The structure or molecular weight itself is not a limiting factor for the resorption of isoflavones, but diffusion of the hydrophylic isoflavonoid-glycosides through biological membranes seems unlikely. So, resorption is possible when the β-glycoside would be hydrolyzed towards the more lipophylic aglycones or when a specific active transport mechanism should exist. For the hydrolysis of β-glycosides, β-glycosidases are necessary, expressed by the ruminal microbiota. Consequently, most of the plant-oestrogens, especially isoflavones, are absorbed at the ruminal level (Dickinson et al., 1988; Lundh et al., 1990).

The conjugative activity is substrate specific (Lundh, 1990). The highest conjugative activity was seen for equol: 3 to 10 times higher compared to daidzein and formononetin.

The consumption of (higher amounts of) soybeans results in bovine into higher blood isoflavone concentrations (Woclawek-Potocka et al., 2005; Piotrowska et al., 2006), lasting for at least 10 weeks notwithstanding the ruminal adaptation (Zdunczyk et al., 2006). The most prominent isoflavone molecules detectable in the peripheral circulation were daidzein, genistein, equol, and p-ethyl phenol (Table 3; Zdunczyk et al., 2006). Blood concentrations of the native molecules (daidzein and genistein) were relatively low, while the concentrations of the ruminal born metabolites (equol and p-ethyl phenol) were obviously higher (Nilsson et al., 1967).

Table 3. *the mean concentration (µmol/L ± SD) of daidzein, genistein (aglycon and glycosides), equol and p-ethyl phenol (Zdunczyk et al., 2006)*

group	daidzein	genistein	equol	p-ethyl phenol	total
1 (n=20)	0.3 ± 0.2	<0.1	1.3 ± 0.3	3.9 ± 1.7	5.5 ± 1.9
2 (n=20)	<0.1	<0.1	0.1 ± 0.1	1.8 ± 0.6	1.9 ± 0.6

An alternative for the daidzein and genistein rich soybeans, as protein source could be rapeseed, in which both molecules are more scarce. Analogous to our field study, Trinacty et al. (2009) examined the effect of supplementation of extruded soybeans versus extruded rapeseed on the blood isoflavone concentration (table 4).

Table 4. *the difference of the mean blood concentration (ng/ml), including standard error (SD) between soybean and rapeseed supplemented cows*

blood concentration (ng/ml)	rape	soy	SD	P
daidzein	13.5	49.3	7.58	<0.001
genistein	42.9	78.7	10.75	<0.001
equol	18.3	218.8	11.13	<0.001

The achievable blood concentrations of isoflavones are much higher than the concentrations of endogenous oestrogens, but their activity is lower (factor 0.01-0.001) (Benassayag et al., 2002).

Soy supplementation even leads to a significant increase of p-ethyl phenol and equol in the luteal tissue (Piotrowska et al., 2006) and an accumulation in the body fat reserves. The

lipophylic aglycones could be 20 times higher than plasma concentrations, leading to an increase in isoflavone blood concentrations during postpartal fat mobilisation. In addition, genistein and daidzein induce an inhibition of lipogenesis and an activation of lipolysis (Nowicka et al., 2006).

But the story becomes more complicated. The blood profiles of isoflavones depend on the sampling time after consumption. Reasons are the molecular specific ruminal absorption rates (faster for daidzein and genistein versus equol and p-ethyl phenol) and half-lives (Woclawek-Potocka et al., 2005; Piotrowska et al., 2006; Trinacty et al., 2009). Moreover, the half-lives of isoflavones depend also on the physiological state (pregnancy; sickness) of the animal (Woclawek-Potocka et al., 2008). This could lead to the fact that, when sampling time is different, also the detected molecules could be different. Differences in digestive conditions, hormonal status and immunological status modulate isoflavone absorption, biotransformation, metabolisation and bioavailability (Kelemen et al., 1998; Shimoi, 2001; Woclaweck-Potocka et al., 2006; Majewska et al., 2007).

2.3.2.2.2 Modification after absorption

Genistein, daidzein and glycitein are inactive glucosides, which after consumption are partially metabolized to heterocyclic phenols, known to have oestrogen-like activities. Soybeans themselves do not contain equol, nor do they contain O-DMA and dihydrodaidzein (DHD). The latter three are known as isoflavone metabolites detectable in blood, among which equol is known to be more oestrogenic than daidzein, while there is a lot of debate concerning the oestrogenic activity of O-DMA and DHD. The metabolite p-ethyl phenol is not supposed to be oestrogenically active (Lundh, 1995; Setchell et al., 2002), nevertheless Woclawek-Potocka et al. (2008) stated the opposite.

After absorption of isoflavones in ruminants, only a small quantity of aglycones and equol appears in the blood circulation as aglycone; more than 95% appears as conjugated form in circulation, mostly as glucuronides, and in lesser amount as sulphates (Duquesnoy, 2005; Lundh, 1990).

The hepatic metabolisation of isoflavones seems to be less important than the metabolisation in the gastro-intestinal tract in bovines (Lundh, 1990). Isoflavones are conjugated with glucuronic acid or sulphates in the liver by hepatic phase II enzymes (such as UDP-glucuronosyltransferase) (figure 10).

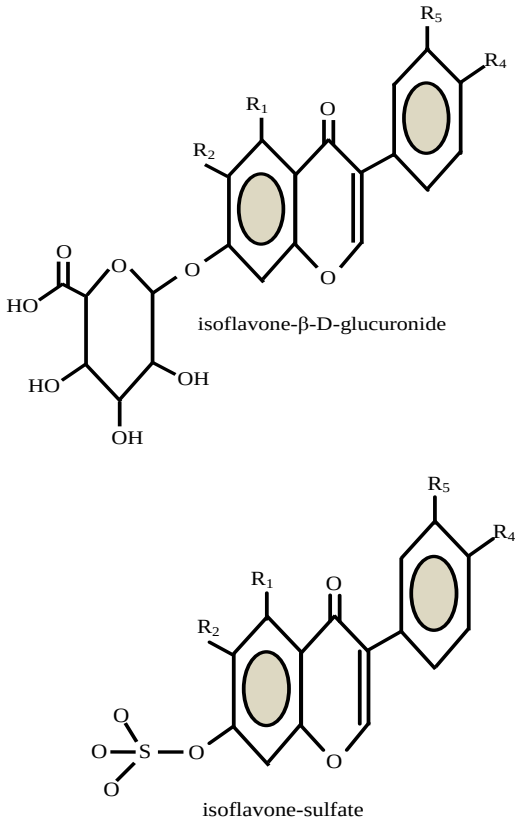


Figure 10: *general structure of isoflavones after glucuronic acid conjugation or sulphatation*

The majority of the molecules will be conjugated (Thomas et al., 2001), making it difficult to assess blood concentrations of free isoflavones. The metabolisation seems to be stimulated during chronical supplementation, leading to a gradual decrease of the plasma concentration of daidzein and genistein over 14 days (Barnes, 1996). Atherton et al. (2006) found that cytochrome P_{450} 1A2 (CYP1A2) delivers the most important contribution to the hepatic metabolism of daidzein and genistein. Moreover, significant enterohepatic circulation has been reported for these molecules (Setchell et al., 1982; Lee et al. 1995; Supko and Malspeis, 1995), leading to molecules that can again be subjected to deconjugation with reabsorption or further metabolisation, degradation by the colonic flora in the large intestine. In ruminants the majority is mainly excreted via the urine and partly via the milk (Adlercreutz et al., 1985; Trinacty et al., 2009). The water solubility is important (daidzein > glycitein > genistein). The higher the water solubility, the higher the urinary excretion (Zhang et al., 1999b), the shorter the half-life of these molecules.

2.3.2.2.3 Tissue distribution

Concentrations were tissue specific (Urpi-Sarda et al., 2008). Renal concentrations were remarkably high by 'ion trapping'. High hepatic concentrations can be explained by the high hepatic uptake capacity of xenobiotics, such as isoflavones, for metabolisation. Concentrations in ovaries, uterus and mammary gland were situated within the levels of biological activity and these tissues are indeed estrogenic sensitive (Doerge and Chang, 2002).

2.3.2.3 The clinical relevance of isoflavones

Whitehead et al. (2002) stated that biochanin A was the most potent inhibitor (IC_{50}=0.5 µM), followed by genistein (1.5 µM) and finally daidzein and formononetin (3.7 µM), while genistein is known to be the isoflavone molecule most abundantly present in soybeans and (Shutt and Cox, 1972).

The physiological concentration of phytooestrogens in biological fluids is much higher compared to these of the endogenous oestrogens. But their biological activity is also only 0.01 to 0.001 of these of the oestrogens (Benassayag et al., 2002). Nevertheless, because of the higher concentrations they still can modulate the effects of oestrogens. But, we have to take into account that phytooestrogens can circulate as conjugated or free molecules. Only 0 to 5% of the molecules is free and consequently biologically active (Lundh, 1995; Lundh et al., 1988; 1990).The effects of phytooestrogens seem to be agonistic as well as antagonistic depending on the level of endogenous oestrogens (Adlercreutz 1990, Whitten and Naftolin, 1998; Chapin et al., 1996; Kurzer and Xu, 1997), as this concentration influences

a) the induction of a down-regulation of the ER-expression with suppression of oestradiol-mediated responses (genistein); and

b) the competition of the isoflavones with endogenous oestradiol at the level of the ER: when the concentration is 100 to 1000 times higher than that of oestradiol (which is achievable by frequent consumption of phytooestrogens), this will influence the feedback regulation mechanism to the hypothalamus (Zhang et al., 1999a).

2.3.2.3.1 Effects on fertility (i.e.steroidogenesis)

The most obvious clinical signs induced by isoflavone consumption are infertility and clinical oestrogenism (Adams, 1995). The clinical signs of estrogenism were mammogenesis, vulvar swelling, prolapsus vaginae/recti, abnormal cervical mucus production and enlarged uterus, COF (irregular cycles, nymphomania, anoestrous), leading to bad pregnancy results.

Other studies such as Kallela et al. (1984), Zdunczyk et al. (2005; 2006) described more discrete disadvantages, such as an increased incidence of irregular oestrous cycles and silent heats, a delay in C-LA, a reduction of the conception rates and an increase of embryonic losses in cattle. Moreover, not only high, but also long-lasting low concentrations of phytooestrogens have substantial deleterious effects on reproductive processes (Hughes et al., 1991; McGarvey et al., 2001; Rosselli et al., 2000). Feeding cows soy induces an increase in the insemination rate and a decrease in successful pregnancies (Adams, 1995; Woclawek-Potocka et al., 2005).

Nwannenna et al. (1994) and McGarvey et al. (2001) stated that these phenomena could be declared by a reduction in the hypothalamic GnRH-pulse frequency and also by a reduction of the intensity and duration of the hypophyseal response for GnRH, possibly leading to an impaired luteogenic process. Besides this central effect, Kaplanski et al. (1981) described a dose dependent and biphasic suppressing effect of the isoflavones biochanin A and genistein on the *in vitro* progesterone synthesis of bovine granulosa cells. This means that below a certain concentration, progesterone synthesis was stimulated, and above a certain threshold progesterone synthesis was suppressed. This threshold concentration was shown to be dependent on the LH-concentration. The more LH a cow secreted, the higher the isoflavone concentration should be in order to efficiently suppress progesterone synthesis. Nichols and Morimoto (2000) found that genistein can inhibit the degradation of cAMP by inhibition of certain phosphodiesterase isozymes, leading to an increase in PKA-activity and even steroid synthesis. On the contrary, Wong and Keung (1999) showed that genistein, daidzein, biochanin A and formononetin inhibit the 3-beta-HSD/isomerase-complex, inhibiting the conversion from pregnenolone to progesterone. Whitehead et al. (2002) reported a dose and time dependent effect of genistein on the progesterone secretion of human luteinized granulosa cells *in vitro*. Genistein directly and dose dependently inhibited 3-β-HSD synthesis (acutely at 4 h and chronicly at 24-48 h). The inhibiting effect during the acute phase was stronger compared to the inhibition during the chronic phase. Analogous to the positive effect of LH in the cow, hCG clearly showed a stimulatory effect on progesterone secretion of

human cells. Also biochanin A and daidzein inhibited progesterone production or had no effect (Dusza et al., 2006).

Such a direct effect of isoflavones on the luteal cells should be possible, as Van den Broeck et al. (2002) showed the presence of ERα in the bovine CL. The expression of ERα in the luteal cells depended on the progesterone concentration in blood and the pregnancy state: cows with a blood progesterone concentration beneath 0.5 ng/ml had no ERα in their CL; cows with 0.5 to 3.0 ng/ml and pregnant animals showed intermediate ERα-levels; and finally non-pregnant cows with a blood progesterone concentration higher than 3 ng/ml expressed the highest luteal ERα levels.

Piotrowska et al. (2006) found that cows daily fed 2.5 kg bruised soybeans showed increased concentrations of p-ethyl phenol and equol in the plasma together with a decreased progesterone concentraton in blood and even in the luteal tissue itself. On the contrary, no effect was found on the *in vitro* basic progesterone secretion of luteal steroidogenic cells coming from the same cows. The *in vitro* progesterone production of the luteal tissue (coming from day 8 CL) was stimulated by PGE_2, $PGF_{2\alpha}$, and LH when the luteal tissue originates from the control group (no soy supplementation) while these stimulatory effects were not seen in luteal tissue of the soy group. Nevertheless, on the basic progesterone secretion, there was no effect of equol and p-ethyl phenol.

A possible explanation for the contradiction *in vivo* versus *in vitro* could be that phytooestrogens exert their effects indirectly via luteolysis, increasing several cytokines and endometrial $PGF_{2\alpha}$, causing a decrease in LH- and PGE_2-stimulated luteal progesterone secretion. On the one hand, phytooestrogens block the nuclear ER of luteal cells leading to an inhibition of the translation and secretion of luteal luteotrophic $PGF_{2\alpha}$ and as such render luteal cells more sensitive to luteolysis. On the other hand, isoflavones fortify the stimulatory effect of cytokines on endometrial luteolytic $PGF_{2\alpha}$, leukotriene C_4 (LTC_4) and NO secretion and even make that cytokines acquire a cytotoxic effect, stimulating luteolysis. Progesterone is the auto-/paracrine factor preventing lysis (apoptosis) of luteal cells by suppression of the Fas and Casp-3-mRNA expression, while phytooestrogens (such as equol and p-ethyl phenol) do not induce a decrease of progesterone production, but rather induce a decrease in the sensitivity of the CL for luteotropic factors (LH, PGE_2) and induce an increase in the local mediators of lysis (endometrial $PGF_{2\alpha}$, NO, LTC_4) (Woclawek-Potocka et al., 2006). They also found that both molecules (equol and p-ethyl phenol) modulate the endometrial secretion of $PGF_{2\alpha}$ in a dose dependent way. In another study, Woclawek-Potocka et al. (2005) found

that feeding soy (daily 2.5 kg of soy (1900 µg/g of isoflavones) versus no soy (300 µg/g)), indeed induces a significant increase of PGFM (the most important metabolite of $PGF_{2\alpha}$ metabolisation) concentrations in serum of these animals with an increase in the number of inseminations and a decrease of PR. Reason could be a disruption of the $PGF_{2\alpha}/PGE_2$ ratio, disturbing the initiation of the pregnancy or the maternal recognition, which is depending on this ratio. A lowered ratio is in favour of maternal recognition as it induces vasodilation and an increased blood supply to the uterus (Kennedy, 1983; McCracken et al., 1999; Okuda et al., 2002) and a stimulation of the luteal progesterone production (Weems et al., 1998). A disruption of this ratio, can induce a local luteolytic signal (Woclawek-Potocka et al., 2005; 2006). Prostaglandin $F_{2\alpha}$ exerts also a direct and negative effect on the *in vitro* bovine embryonic development. So, an increased secretion of $PGF_{2\alpha}$ caused by a disruption of the ratio could be a reason for the increased incidence of embryonic death.

As a conclusion, we can state that it is important to differentiate the effect of phytooestrogens on the luteal steroidogenesis into an indirect (or central) and a direct (or peripheral) effect. On the one hand, genistein inhibits the progesterone synthesis in a dose-dependent manner, but it mainly inhibits the steroidogenic response of the gonadotropins LH and FSH on the luteal cells (i.e. the indirect effect; Nejaty et al., 2001; Whitehead en Lacey, 2000; McGarvey et al., 2001), leading indirectly to a decrease in progesterone-synthesis and an increase in embryonic death, on the other hand phytooestrogens directly inhibit several enzymes with a role in steroidogenesis in human follicles and CLa (Whitehead et al., 2002).

2.3.2.3.2 Effects on angiogenesis

Apart from luteal steroidogenesis, blood progesterone concentrations also depend on the blood supply and removal in the CL (Arfuso et al., 2005). Pathologic angiogenesis (i.e. in aggressive tumors) was decreased following soy supplementation (Fotsis et al., 1993, 1997). Zhou et al. (1999) found a reduction in microvessel density in a human prostate tumor transplanted in mice, when soy was consumed. They found a possible explanation: feeding soy proteins decreases the angiogenetic IGF1 in blood. Chapin et al. (1996), Kurzer and Xu (1997), and Tham et al. (1998) describe an anti-angiogenic effect of genistein. Fotsis et al. (1993; 1997) demonstrated that genistein inhibits the basic fibroblast growth factor (bFGF)-induced invasion into gels and the formation of capillary like structures. Besides that, they saw that genistein was cytostatic (between 10µM and 25 µM) and even cytotoxic (above 25 µM) in a non-confluent monolayer of endothelial cells (Cassidy, 1996). Li and Sarkar (2002) stated that genistein down regulates the transcription and translation of certain genes

controlling angiogenesis in human prostate tumours. This effect was dose- and time dependent. Genistein inhibits the endothelial cell proliferation and the *in vitro* angiogenesis (half maximal concentration of 5 and 10 µM, respectively). It seems to selectively inhibit fast dividing cells (Fotsis et al., 1995) and it reduces the degradation of ECM by lowering the bFGF-stimulated and basic levels of urokinase plasminogen activator and plasminogen activator inhibitor in the endothelial cells. It induces for example a down-regulation of matrix metalloproteinase (MMP)-2.

Nevertheless, we cannot neglect the studies reporting a stimulating effect of soy supplementation on tumour growth (Makela et al., 1994; Wang en Kurzer, 1998, Hsu et al., 1999). Also Makarevich et al. (1997) found a biphasic effect of genistein on cell growth: a lower concentration induced cell growth, a higher concentration inhibited cell growth.

Also biochanin A inhibits angiogenesis (of human endothelial cells) at physiologically achievable concentrations (Ying et al., 2001). The *in vitro* effect of isoflavones on angiogenesis is depending on: 1) the dose, 2) the percentage of fetal bovine serum (FBS) in the medium modifying the level of inhibition of cell proliferation, 3) the cell density (a lower cell density results in a stronger inhibition, caused by the more active growth phase).

As a conclusion, we can state that several studies indicate that nutritional isoflavones modify or impair steroidogenic and angiogenic processes. The pending question is, whether the supplementation of soy, the most important protein concentrate in Belgium for high yielding dairy cows, in practice induces the same negative effects on luteal steroidogenesis and angiogenesis. That is one of the research topics of this thesis.

Reference List

Adams NR, 1995: Detection of the effects of phytoestrogens on sheep and cattle. J Animal Sci **73**, 1509-1515.

Adlercreutz H, 1990: Diet, breast cancer, and sex hormone metabolism. Ann NY Acad Sci **595**, 281-290.

Adlercreutz H, 1998: Epidemiology of phytoestrogens. Baillieres Clin Endocrinol Metab **12**, 605-623.

Adlercreutz H, Fotsis T, Banwart C, Hamalainen E, Bloigu S, Ollus A, 1985: Urinary estrogen profile, including catechol estrogens, in young Finnish lactovegetarian and omnivorous women. Acta Endocrinologica **109**, 92.

Arfuso F, McGeachie JK, Meyer GT, 2005: A quantitative study of blood capillary formation (angiogenesis) concomitant with parenchymal tissue differentiation. Endothelium **12**, 171-177.

Atherton KM, Mutch E, Ford D, 2006: Metabolism of the soyabean isoflavone daidzein by CYP1A2 and the extra-hepatic CYPs 1A1 and 1B1 affects biological activity. Biochem Pharmacol **72**, 624-631.

Barnes S, 1996: The soy isoflavonoid genistein and cancer risk. Abstr Pap Am Chem S **212**, 113.

Barnes S, Kirk M, Coward L, 1994: Isoflavones and their conjugates in soy foods - extraction conditions and analysis by HPLC Mass-Spectrometry. J Agric Food Chem **42**, 2466-2474.

Beam SW, Butler WR, 1998: Energy balance, metabolic hormones, and early postpartum follicular , development in dairy cows fed prilled lipid. J Dairy Sci **81**, 121-131.

Benassayag C, Perrot-Applanat M, Ferre F, 2002: Phytoestrogens as modulators of steroid action in target cells. J Chromatogr B Analyt Technol Biomed Life Sci **777**, 233-248.

Bilby TR, Sozzi A, Lopez MM, Silvestre FT, Ealy AD, Staples CR, Thatcher WW, 2006: Pregnancy, bovine somatotropin, and dietary n-3 fatty acids in lactating dairy cows: I. Ovarian, conceptus, and growth hormone-insulin-like growth factor system responses. J Dairy Sci **89**, 3360-3374.

Bingham SA, Atkinson C, Liggins J, Bluck L, Coward A, 1998: Phyto-oestrogens: where are we now? Br J Nutr **79**, 393-406.

Bowey E, Adlercreutz H, Rowland I, 2003: Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. Food Chem Toxicol **41**, 631-636.

Braden AWH, Thain RI, Shutt DA, 1971: Comparison of plasma phyto-oestrogen levels in sheep and cattle after feeding on fresh clover. Aust J Agr Res **22**, 663.

Britt JH, 1994: Follicular development and fertility, potential impacts of negative energy balance. In: Proceedings of National Reproductive Symposium, Pittsburgh, PA, pp. 103-112.

Brzezinski A, Debi A, 1999: Phytoestrogens: the 'natural' selective estrogen receptor modulators? Eur J Obstet Gyn R B **85**, 47-51.

Butler WR, 2000: Nutritional interactions with reproductive performance in dairy cattle. Anim Reprod Sci **60-61**, 449-457.

Butler WR, 2003: Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest Prod Sci **83**, 211-218.

Butler WR, Smith G.E. 1989: Interrelationships between energy balance and postpartum reproductive function in dairy cows. J Dairy Sci **72**, 767-783.

Cassidy A, 1996: Physiological effexcts of phyto-oestrogens in relation to cancer and other human health risks. P Nutr Soc **55**, 399-417.

Chapin RE, Stevens JT, Hughes CL, Kelce WR, Hess RA, Daston GP, 1996: Endocrine modulation of reproduction. Fundam Appl Toxicol **29**, 1-17.

Chouinard PY, Girard V, Brisson GJ, 1997: Performance and profiles of milk fatty acids of cows fed full fat, heat-treated soybeans using various processing methods. J Dairy Sci **80**, 334-342.

Christenson LK, Devoto L, 2003: Cholesterol transport and steroidogenesis by the corpus luteum. Reprod Biol Endocrinol **1**, 90-99.

Christenson LK, Strauss JF, 2000: Steroidogenic acute regulatory protein (StAR) and the intramitochondrial translocation of cholesterol. BBA-Mol Cell Biol L 1529, 175-187.

Committee on toxicity of chemicals in food, consumer products and the environment (COT), 2003: COT-report, Phytoestrogens and health. Food standards agency.

Coward L, Barnes NC, Setchell KDR, Barnes S, 1993: Genistein, daidzein, and their beta-glycoside conjugates - antitumor isoflavones in soybean foods from American and Asian diets. J Agr Food Chem **41**, 1961-196.

Devoto L, Kohen P, Vega M, Castro O, Gonzalez RR, Retamales I, Carvallo P, Christenson LK, Strauss JF, 2002: Control of human luteal steroidogenesis. Mol Cell Endocrinol **186**, 137-141.

Dhom G, 1991: Epidemiologic and morphogenetic aspects of prostate cancer. Urologe A **30**, 3-4.

Dickinson JM, Smith GR, Randel RE, Pemberton IJ, 1988: *In vitro* metabolism of formononetin and biochanin A in bovine rumen fluid. J Anim Sci **66**, 1969-1973.

Doerge DR, Chang HC, 2002: Inactivation of thyroid peroxidase by soy isoflavones, in vitro and in vivo. J Chromatogr B **777**, 269-279.

Duquesnoy N, 2005: Estrogen like substances in ruminant diets. Ann Med Vet **149**, 202-212.

Dusza L, Ciereszko R, Skarzynski DJ, Nogowski L, Opalka M, Kaminska B, Nynca A, Kraszewska O, Stomczynska M, Woclawek-Potocka I, Korzekwa A, Pruszynska-Oszmalek E, Szkudelska K, 2006: Mechanism of phytoestrogen action in reproductive processes of mammals and birds. Reprod Biol **6** (suppl 1), 151-174.

Eldridge AC, Kwolek WF, 1983: Soybean Isoflavones - Effect of Environment and Variety on Composition. J Agric Food Chem **31**, 394-396.

Farmakalidis E, Murphy PA, 1985: Isolation of 6"-O-Acetylgenistin and 6"-O-Acetyldaidzin from toasted defatted soyflakes. J Agric Food Chem **33**, 385-389.

Fenwick MA, Llewellyn S, Fitzpatrick R, Kenny DA, Murphy JJ, Patton J, Wathes DC, 2008: Negative energy balance in dairy cows is associated with specific changes in IGF-binding protein expression in the oviduct. Reproduction **135**, 63-75.

Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L, 1993: Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis. Proc Natl Acad Sci USA **90**, 2690-2694.

Fotsis T, Pepper M, Adlercreutz H, Hase T, Montesano R, Schweigerer L, 1995: Genistein, a dietary ingested isoflavonoid, inhibits cell-proliferation and in-vitro angiogenesis. J Nutr **125**, S790-S797.

Fotsis T, Pepper MS, Aktas E, Breit S, Rasku S, Adlercreutz H, Wahala K, Montesano R, Schweigerer L, 1997: Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. Cancer Res **57**, 2916-2921.

Galeati G, Forni M, Spinaci M, Zannoni A, Govoni N, Ribeiro LA, Seren E, Tamanini C, 2005: Fasting influences steroidogenesis, vascular endothelial growth factor (VEGF) levels and mRNAs expression for VEGF, VEGF-receptor type 2 (VEGFR-2), Endothelin 1 (ET-1), Endothelin receptor type A (ETA) and endothelin converting enzyme 1 (ECE-1) in newly formed pig corpora lutea. Domest Anim Endocrinol **28**, 272-284.

Goddard ME, Beilharz RG, 1977: Natural selection and animal breeding. Proc 3rd Int Congr, S.A.B.R.A.O., Animal Breeding Papers **4**.19-4.21.

Griffith LA, Smith GE, 1972: Metabolism of apigenin and related compounds in rat metabolite formation in vivo and by intestinal microflora in vitro. Biochem J **128**, 901.

Gruber CJ, Tschugguel W, Schneeberger C, Huber JC, 2002: Mechanisms of disease - Production and actions of estrogens. New Engl J Med **346**, 340-352.

Hollman PCH, 2001: Evidence for health benefits of plant phenols: local or systemic effects? J Sci Food Agricult **81**, 842-852.

Hsu JT, Hung HC, Chen CJ, Hsu WL, Ying CW, 1999: Effects of the dietary phytoestrogen biochanin A on cell growth in the mammary carcinoma cell line MCF-7. J Nutr Biochem **10**, 510-517.

Hughes CL Jr, Kaldas RS, Weisinger AS, McCants CE, Basham KB, 1991: Acute and subacute effects of naturally occurring oestrogens on luteinizing hormone secretion in the ovariectomized rat. Reprod Toxicol **5**, 127-132.

Ibarreta D, Daxenberger A, Meyer HHD, 2001: Possible health impact of phytoestrogens and xenoestrogens in food. Apmis **109**, S402-S425.

Ikeda E, Achen MG, Breier G, Riseau W, 1995: Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. J Biol Chem **270**, 19761-19766.

Kallela K, Heinonen K, Saloniemi H, 1984: Plant oestrogens; the cause of decreased fertility in cows. A case report. Nord Vet Med **36**, 124-129.

Kaplanski O, Shemesh M, Berman A, 1981: Effects of phyto-oestrogens on progesterone synthesis by isolated bovine granulosa cells. J Endocrinol **89**, 343-348.

Kelemen K, Paldi A, Tinneberg H, Torok A, Szekeres-Bartho J, 1998: Early recognition of pregnancy by the maternal immune system. Am J Reprod Immunol **39**, 351-355.

Kennedy TG, 1983: Prostaglandin-E2, adenosine 3'-5'-cyclic monophophate and changes in endometrial vascular permeability in rat uteri sensitized for the decidual cell reaction. Biol Reprod **29**, 1069-1076.

Ko Y, Lee CJ, Ott TL, Davis MA, Simmen RCM, Bazer FW, Simmen FA, 1991: Insulin-like growth factors in sheep uterine fluids: concentrations and relationship to ovine trophoblastic protein-1 production during early pregnancy. Biol Reprod **45**, 135-142.

Kurzer MS, Xu X, 1997: Dietary phytoestrogens. Annu Rev Nutr **17**, 353-381.

Lee KW, Wang HJ, Murphy PA, Hendrich S, 1995: Soybean isoflavone extract suppresses early but not later promotion of hepatocarcinogenesis by phenobarbital in female rat liver. Nutr Cancer **24**, 267-278.

Li YW, Sarkar FH, 2002: Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. Cancer Lett **186**, 157-164.

Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA, 2000: Daidzein and genistein contents of vegetables. Br J Nutr **84**, 717-725.

Lundh TJO, 1990: Conjugation of the plant estrogens formononetin and daidzein and their metabolite equol by gastrointestinal epithelium from cattle and sheep. J Agr Food Chem **38**, 1012-1016.

Lundh T, 1995: Metabolism of estrogenic isoflavones in domestic-animals. Proc Soc Exp Biol Med **208**, 33-39.

Lundh TJO, Petterson H, Kiessling KH, 1988: Liquid chromatographic determination of the estrogens daidzein, formononetin, coumestrol, and equol in bovine blood plasma and urine. J Assoc Off Ana Chem **71**, 938-941.

Lundh TJO, Pettersson HI, Martinsson KA, 1990: Comparative levels of free and conjugated plant estrogens in blood-plasma of sheep and cattle fed estrogenic silage. J Agr Food Chem **38**, 1530-1534.

Majewska M, Chelmonska-Soyta A, Bah MM, Woclawek-Potocka I, Skarzynski DJ, 2007: Lymphocyte subsets distribution in peripheral blood and endometrium during early pregnancy in cows. In: The 11th annual conference of Europian society for domestic animal reproduction, Cell, Germany.

Makarevich A, Sirotkin A, Taradajnik T, Chrenek P, 1997: Effects of genistein and lavendustin on reproductive processes in domestic animals *in vitro.* J Steroid Biochem **63**, 329-337.

Makela S, Davis VL, Tally WC, Korkman J, Salo L, Vihko R, Santti R, Korach KS, 1994: Dietary estrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environ Health Persp **102**, 572-578.

Mann GE, Lamming GE, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction. **121**, 175-180.

Matsuura M, Obata A, 1993: Beta-glucosidases from soybeans hydrolyze daidzein and genistein. J Food Sci **58**, 144-147.

Mazur W, 1998: Phytoestrogen content in foods. Bailliere Clin Endoc **12**, 729-742.

Mazur W, Adlercreutz H, 1998: Naturally occurring oestrogens in food. Pure Appl Chem **70**, 1759-1776.

McCracken JA, Custer EE, Lamsa JC, 1999: Luteolysis: a neuroendocrine-mediated event. Physiol Rev **79**, 263-323.

McGarvey C, Cates PS, Brooks N, Swanson IA, Milligan SR, Coen CW, O'Byrne KT, 2001: Phytoestrogens and gonadotropin-releasing hormone pulse generator activity and pituitary luteinizing hormone release in the rat. Endocrin **124**, 1202-1208.

McMullen S, Osgerby JC, Milne JS, Wallace JM, Wathes DC, 2005: The effect of acute nutrient restriction in the mid-gestational ewe on maternal and fetal nutrient status, the expression of placental growth factors and fetal growth. Placenta **26**, 25-33.

Mills PK, Beeson WL, Phillips RL, Fraser GE, 1989: Cohort study of diet, lifestyle, and prostate cancer in Adventist men. Cancer **64**, 598-604.

Mukhopadhyay D, Tsiokas L, Zhou XM, Foster D, Brugge JS, Sukhatme VP, 1995: Hypoxic induction of human vascular endothelial growth factor expression through c-SrC activation. Nature **375**, 577-581.

Murkies AL, Wilcox G, Davis SR, 1998: Clinical review 92: Phytoestrogens. J Clin Endocrinol Metab **83**, 297-303.

Nejaty H, Lacey M, Whitehead SA, 2001: Differing effects of endocrine-disruptinng chemicals on basal and FSH-stimulated progesterone production in rat granulosa-luteal cells. Exp Biol **226**, 570-576.

Nichols MR, Morimoto BH, 2000: Differential inhibition of multiple cAMP phophodiesterase isozymes by isoflavones and tyrphostins. Mol Pharmacol **57**, 738-745.

Nilsson A, Hill JL, Davies HL, 1967: An in vitro study of formononetin and biochanin A metabolism in rumen fluid from sheep. Biochim Biophys Acta **148**, 92.

Nowicka E, Szkudelski R, Nogowski L, 2006: Influence of phytoestrogens on humans and animals. Med Weter **62**, 736-738.

Nwannenna AI, Madej A, Lundh TJ, Fredriksson G, 1994: Effects of oestrogenic silage on some clinical and endocrinological parameters in ovariectomized heifers. Acta Vet Scand **35**, 173-183.

Okuda K, Miyamoto Y, Skarzynski DJ, 2002: Regulation of endometrial prostaglandin F-2 alpha synthesis during luteolysis and early pregnancy in cattle. Domest Anim Endocrin **23**, 255-264.

Piotrowska K, Woclawek-Potocka I, Bah MM, Piskula M, Pilawski W, Bober A, Skarzynski DJ, 2006: Phytoestrogens and their metabolites inhibit the sensitivity of the bovine corpus luteum on luteotrophic factors. J Reprod Dev **52**, 33-41.

Price KR, Fenwick GR, 1985: Naturally-occurring estrogens in foods - A Review. Food Addit Contam **2**, 73-106.

Riggs BL, Hartmann LC, 2003: Selective estrogen-receptor modulators-Reply. New Engl J Med **348**, 2259.

Robinson RS, Fray MD, Wathes DC, Lamming GE, Mann GE, 2006: *In vivo* expression of interferon tau mRNA by the embryonic trophoblast and uterine concentrations of interferon tau protein during early pregnancy in the cow. Mol Reprod Dev **73**, 470-474.

Robinson RS, Hammond AJ, Wathes DC, Hunter MG, Mann GE, 2008: Corpus luteum-endometrium-embryo interactions in the dairy cow: underlying mechanisms and clinical relevance. Reprod Dom Anim **43** (Suppl. 2), 104-112.

Robinson RS, Mann GE, Gadd TS, Lamming GE, Wathes DC, 2000: The expression of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy. J Endocrinol **165**, 231-243.

Rosselli M, Reinhard K, Imthurn B, Keller PJ, Dubey RK, 2000: Cellular and biochemical mechanisms by which environmental estrogens influence reproductive function. Hum Reprod Update **6**, 332-350.

Rowland I, Faughnan M, Hoey L, Wahala K, Williamson G, Cassidy A, 2003: Bioavailability of phyto-estrogens. Br J Nutr **89**, S45-S58.

Rutter LM, Manns JG,1987: Hypoglycemia alters pulsatile luteinizing hormone secretion in the postpartum beef cow. J Anim Sci **64**, 479-488.

Saloniemi H, Wahala K, Nykanenkurki P, Kallela K, Saastamoinen I, 1995: Phytoestrogen content and estrogenic effect of legume fodder. Proceedings of the Society for Experimental Biology and Medicine **208**, 13-17.

Seo A, Morr CV, 1984: Improved high-performance liquid-chromatographic analysis of phenolic-acids and isoflavonoids from soybean protein products. J Agric Food Chem **32**, 530-533.

Setchell KDR, 1998: Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr **68**, 1333S-1336S.

Setchell KDR, Borriello SP, Hulme P, Kirk DN, Axelson M, 1984: Nonsteroidal estrogens of dietary origin - possible roles in hormone-dependent disease. Am J Clin Nutr **40**, 569-578.

Setchell KDR, Brown NM, Lydeking-Olsen E, 2002: The clinical importance of the metabolite equol - A clue to the effectiveness of soy and its isoflavones. J Nutr **132**, 3577-3584.

Setchell KD, Cassidy A, 1999: Dietary isoflavones: biological effects and relevance to human health. J Nutr **129**, 758-767.

Setchell KDR, Lawson AM, Blackstock EJ, Murphy GM, 1982: Diurnal changes in serum unconjugated bile-acids in normal man. Gut **23**, 637-642.

Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE, 1990: Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil **90**, 1-10.

Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM, 1991: Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. Br J Cancer **63**, 963-966.

Shimoi K, 2001: Deglucuronidation of flavonoid, luteolin monoglucuronide, during inflammation. Drug Metabol Disposit **12**, 20-24.

Shutt DA, Cox RI, 1972: Steroid and phytoestrogen binding to sheep uterine receptors *in vitro*. J Endocrinol **52**, 299-310.

Spicer LJ, Tucker WB, Adams GD, 1990: Insulin-like growth factor-I in dairy cows: relationships among energy balance, body condition, ovarian activity, and estrous behavior. J Dairy Sci **73**, 929-937.

Staples CR, Thatcher WW, Clark JH, 1990: Relationship between ovarian activity and energy status during the early postpartum period of high producing dairy cows. J Dairy Sci **73**, 938-947.

Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M, de Haan C, 2006: Livestock's long shadow, FAD, Rome 2006.

Stouffer RL, Martinez-Chequer JC, Molskness TA, Xu FH, Hazzard TM, 2001: Regulation and action of angiogenic factors in the primate ovary. Arch Med Res **32**, 567-575.

Su SJ, Yeh TM, Chuang WJ, Ho CL, Chang KL, Cheng HL, Liu HS, Cheng HL, Hsu PY, Chow NH, 2005: The novel targets for anti-angiogenesis of genistein on human cancer cells. Biochem Pharmacol **69**, 307-318.

Supko JG, Malspeis L, 1995: Plasma pharmacokinetics of genistein in mice. Int J Oncol **7**, 847-854.

Tham DM, Gardner CD, Haskell WL, 1998: Clinical review 97 - Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. J Clin Endocr Metab **83**, 2223-2235.

Thomas BF, Zeisel SH, Busby MG, Hill JM, Mitchell RA, Scheffler NM, Brown SS, Bloeden LT, Dix KJ, Jeffcoat AA, 2001: Quantitative analysis of the principle soy isoflavones genistein, daidzein and glycitein, and their primary conjugated metabolites in human plasma and urine using reversed-phase high-performance liquid chromatography with ultraviolet detection. J Chromatogr B **760**, 191-205

Toutges MJ, Grazul-Bilska AT, Kirsch JD, Reynolds LP, Redmer DA, 1999: Effects of luteinizing hormone (LH) and oxygen (O_2) levels on vascular endothelial growth factor (VEGF) mRNA expression by ovine luteal cells in culture. Biol Reprod **60** (suppl. 1), abstract 601.

Trinacty J, Krizova L, Schulzova V, Hajslova J, Hanus O, 2009: The effect of feeding soybean-derived phytoestogens on their concentration in plasma and milk of lactating dairy cows. Arch Tierernahr **63**, 219-229.

Tsukamoto C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K, 1995: Factors affecting isoflavone content in soybean seeds - changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J Agric Food Chem **43**, 1184-1192.

Urpi-Sarda M, Morand C, Besson C, Kraft G, Viala D, Scalbert A, Besle JM, Manach C, 2008: Tissue distribution of isoflavones in ewes after consumption of red clover silage. Arch Biochem Biophys **476**, 205-210.

Van den Broeck W, Coryn M, Simoens P, Lauwers H, 2002: Cell-specific distribution of oestrogen receptor-alpha in the bovine ovary. Reprod Domest Anim **37**, 291-293.

Vanholder T, Leroy JL, Soom AV, Opsomer G, Maes D, Coryn M, de Kruif A, 2005: Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. Anim Reprod Sci **87**, 33-44.

Velazquez MA, Spicer LJ, Wathes DC, 2008: The role of endocrine insulin-like growth factor-I (IGF-1) in female bovine reproduction. Domest Anim Endocrinol **35**, 324-342.

Villa-Godoy A, Hughes TL, Emery RS, Chapin LT, Fogwell RL 1988: Association between energy balance and luteal function in lactating dairy cows. J Dairy Sci **71**, 1063-1072.

Wang CF, Kurzer MS, 1998: Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. Nutr Cancer **31**, 90-100.

Wang HJ, Murphy PA, 1994: Isoflavone composition of American and Japanese soybeans in Iowa - Effects of variety, crop year, and location. J Agr Food Chem **42**, 1674-1677.

Wang TT, Sathyamoorthy N, Phang JM, 1996: Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis **17**, 271-275.

Wathes DC, Fenwick M, Cheng Z, Bourne N, Llewellyn S, Morris DG, Kenny D, Murphy J, Fitzpatrick R, 2007: Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. Theriogenology **68S**, S232-S241.

Wathes DC, Taylor VJ, Cheng Z, Mann GE, 2003: Follicle growth, corpus luteum function and their effects on embryo development in postpartum dairy cows. Reprod Suppl **61**, 219-237.

Weems YS, Lammoglia MA, Vera-Avila HR, Randel RD, King C, Sasser RG, Weems CW, 1998: Effect of luteinizing hormone (LH), PGE(2), 8-EPI-PGE(1), 8-EPI-PGE(2), trichosanthin, and pregnancy specific protein B (PSPB) on secretion of progesterone in vitro by corpora lutea (CL) from nonpregnant and pregnant cows. Prostag Oth Lipid M **55**, 27-42.

Whitehead SA, Cross JW, Burden C, Lacey M, 2002: Acute and chronic effects of genistein, tyrphostin and lavendustin A on steroid synthesis in luteinized human granulosa cells. Hum Reprod **17**, 589-594.

Whitehead SA, Lacey M, 2000: Protein tyrosine kinase activity on lavendustin A and the phytoestrogen genistein on progesterone synthesis in cultured rat ovarian cells. Fertil Steril **73**, 613-619.

Whitten PL, Naftolin F, 1998: Reproductive actions of phytoestrogens. Baillieres Clin Endocrinol Metab **12**, 667-690.

Woclawek-Potocka I, Bah MM, Korzekwa A, Piskula M, Wiczkowski W, Depta A, Skarzynski DJ,2005, Soybean-derived phytoestrogens regulate prostaglandin secretion in endometrium during the estrous cycle and early pregnancy in cattle. Exp Biol Med **230**, 189-199.

Woclawek-Potocka I, Bober A, Korzekwa A, Okuda K, Skarzynski DJ, 2006: Equol and para-ethyl-phenol stimulate prostaglandin F2alfa secretion in bovine corpus luteum: intracellular mechanisms of action. Prostag Oth Lipid M **79**, 287-297.

Woclawek-Potocka I, Piskula MK, Bah MM, Siemieniuch MJ, Korzekwa A, Brzezicka E, Skarzynski DJ, 2008: Concentrations of isoflavones and their metabolites in the blood of pregnant and non-pregnant heifers fed soyu bean. J Reprod Dev **54**, 358-363.

Wong CK, Keung WM, 1999: Bovine adrenal 3 beta-hydroxysteroid dehydrogenase (EC 1.1.1.145)/5-ene-4-ene isomerase (EC 5.3.3.1): characterization and its inhibition by isoflavones. J Steroid Biochem Mol Biol **71**, 191-202.

Ying CW, Hsu JT, Shieh SC, 2001: Growth inhibition of human endothelial cells by the phyto-oestrogen biochanin A, a metabolite of genistein. Br J Nutr **85**, 615-620.

Zdunczyk S, Janowski T, Baranski W, Ras M, 2006: Studies on the effect of isoflavones on postpartum ovarian activity in cows. Tieraerztl Umschau **61**, 127-132.

Zdunczyk S, Piskula M, Janowski T, Baranski W, Ras M, 2005: Concentrations of isoflavones in blood plasma of dairy cows with different incidence of silent heat. B Vet I Pulawy **49**, 189-191.

Zhang Y, Song TT, Cunnick JE, Murphy PA, Hendrich S, 1999a: Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations. J Nutr **129**, 399-405.

Zhang Y, Wang GJ, Song TT, Murphy PA, Hendrich S, 1999b: Urinary disposition of the soybean isoflavones daidzein, genistein and glycitein differs among humans with moderate fecal isoflavone degradation activity. J Nutr **129**, 957-962.

Zhou JR, Gugger ET, Tanaka T, Guo YP, Blackburn GL, Clinton SK, 1999: Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. J Nutr **129**, 1628-1635.

Chapter 2.4

In vivo sampling technique of the luteal gland

2.4.1 Practical implications when sampling a bovine luteal gland *in vivo*

The principle of using ultrasound (US) imaging to guide a biopsy instrument for obtaining specimens of abdominal organs has been used in both humans (Chan et al., 2000) and animals (Modransky, 1986). Two types of biopsy instruments can be used: fine needles (20-22 G) for cell aspirations and large tissue cutting needles (14-18 G) with an automated spring-loaded feature (Hoppe et al., 1986). Several studies described this ultrasound-guided biopsy technique with a cutting needle as a simple and safe way to obtain specimens for accurate histological diagnosis (Jennings et al., 1989; Yeow et al., 2001; Pratap et al., 2009). Concerning the luteal gland, in the past luteal tissue specimens were obtained by dissection from bovine ovaries after slaughter (Wiltbank et al., 1995), colpotomy (Watson and Sertich, 1990) or laparatomy (Elecko et al., 1990).

Kot et al. (1999) designed an ultrasound-guided transvaginal biopsy technique to sample *in vivo* luteal glands in cattle. He used a 5.0 mHz convex array transducer (Aloka, UST-9111-5), mounted in a plastic handle equipped with a needle guidance system of 45 cm. The biopsy needle itself was 48 cm long and was constructed on the one hand by an inner needle (outer diameter 1.0 mm, 48 cm of length), containing a specimen notch (dimensions 20x1x0.5 mm) and on the other hand an outer needle (18 G, 47.5 cm of length) with a cutting edge. This biopsy needle was equipped with an automated spring-loaded handle with a trigger. The monitor showed a dashed line, which was the path of the biopsy needle.

The sampling protocol was as follows. The transducer was inserted intravaginally and was placed in the vaginal fornix against the vaginal wall ipsilateral to the luteal gland bearing ovary. The biopsy needle is guided via a needle guidance system through the handle till against the vaginal wall. By means of rectal manipulation, the CL-bearing ovary was immobilized against the vaginal wall and the transducer face. The luteal gland was positioned correctly when the ultrasound image of the CL was transected by the built-in dashed line on the US-monitor. Then, the inner needle of the biopsy system was punctured through the vaginal wall into the CL under ultrasound guidance. Next, the cutting needle was fired, cutting and trapping the luteal tissue in the specimen notch. The biopsy needle was withdrawn immediately. By retracting the plunger and pushing forward the inner needle, the tissue core within the biopsy notch was exposed. The advantages of this described protocol were that it was tolerated by the animals by only an epidural anesthesia and the required sampling time was only one minute.

Kot et al. (1999) prelevated 6 biopsies at cycle day 10. Its success rate (i.e. luteal tissue present in the specimen notch) was 92%. The problems encountered during a failure were

inadequate needle penetration when a large central cavity was present in the luteal gland or inadequate maintenance of the needle in a solid gland. The failures were followed by a second attempt, always giving a success. The average size of the obtained biopsy cores was 1 mm in diameter and 11.7 (11-12) mm in length. The average weight was 4.6 (1.8-7.5) mg. The color of the samples was uniform yellowish, eventually spotted with hemorrhagic points. All specimens contained large and small luteal cells on histological analysis. The effect of this sampling technique on the luteal function was followed up based on three end points: 1) maximal luteal area on ultrasound, 2) peripheral blood progesterone concentration and 3) the interval between moment of biopsy and ovulation. No significant differences within these 3 end points were found between biopted heifers and control counterparts. Nevertheless, they found in 4 of 6 heifers 1 to 3 detectable echoic lines starting 4 hours to 1 day after the biopsy. They lasted for 1 to 3 days and thereafter, the continuous lines disrupted and disappeared in 1 to 2 days. These echoic lines were probably local hemorrhages. Macroscopic blood cloths were never seen by the daily ultrasound sessions.

So, Kot et al. (1999) concluded that they created a practical procedure for repeated transvaginal ultrasound-guided biopsy sampling of the bovine luteal gland. This method should be useful for experimental and diagnostic purposes.

Tsai et al. (2001) used the same technique as Kot et al. (1999), but made the sampling protocol more intensive. They took at cycle day 9 to 11, 3 luteal biopsies at each of the three different time points (0 hours, 0.5 hours and 4 hours) obtaining 9 biopsies in total. They concluded that this technique allowed analysis of serial biopsies, taken at multiple time points, without altering the subsequent luteal function, as there was no significant effect on the serum progesterone and diameter of the luteal gland afterwards.

On the contrary, Beg et al. (2005) found a negative effect of the transvaginal ultrasound-guided luteal biopsy technique on blood progesterone concentration 12 hours after taking biopsies in mares. They used a biopsy needle of 12 G instead of 18 G, with a specimen notch of 15 mm in length. They did 6 to 10 attempts per session, with 2 to 8 times the prelevation of luteal tissue, leading to a success rate of 68%. So, this protocol seems to be too invasive.

Bols et al. (2004) compared two different types of transducers, a linear array and a mechanical multiple angle sector (MAP) transducer, for ultrasound-guided transvaginal oocyte retrieval (ovum pick-up, OPU) in the cow. The principles of the OPU-technique are comparable with those of the biopsy technique desribed by Kot et al. (1999). No significant difference was found in the success rate of oocyte retrieval between both transducer types,

but the MAP-transducer gives images with a higher resolution, leading to the identification of a higher number of small follicles.

Kot et al. (1999) compared 2 needle types, concerning the sustainability. The difference was situated in the depth of the specimen notch. When the depth was 0.6 mm, the needle bended to easily and makes repeated use not possible; while a needle with a notch depth of 0.4 mm could be used during on average 42 (39-45) attempts. Chen et al. (2007) analyzed whether the use of a 16 G needle leads to more complications compared to an 18 G needle in sampling the liver. Concerning the vascularization, the liver is comparable to the luteal gland. They found that the ultrasound-guided 16 G core needle biopsy is safe for the diagnosis of hepatic disease and more sensitive than the 18 G biopsy. Kim et al. (2008) even used a 14 G biopsy needle for the biopsy of axilla lymph nodes and evaluated the technique as safe.

2.4.2 Some histological points of attention

As the luteal histology is dynamic during luteogenesis, accurate estimation of the age is necessary for the objective comparison between different CLa. Initially, corpora lutea can be classified as corpora hemorrhagica, corpora rubra and corpora lutea, which regress towards corpora albicantia, but Augustin et al. (1995) stated that luteal stage can be estimated more precisely with intervals of 2-3 days based on morphological criteria. Ireland et al. (1980) divided the bovine cycle into four stages: I (cycle day 1 to 4), II (day 5-10), III (day 11-17), IV (day 18-20). Other divisions of CL age were suggested by other authors taking into account luteal, follicular and uterine characteristics (Miyamoto et al., 2000. D'haeseleer et al., 2006). Our study is based on the Ireland nomenclature.

Bovine luteal histology has been described by numerous studies (Donaldson and Hansel 1965; Fields and Fields 1996; Lei et al. 1991; Neves and Marques 2006; O'Shea 1987; O'Shea et al. 1989; Parkinson et al. 1994). Most studies assumed homogeneity of the topographic distribution of the different components. This means that these studies assumed that the topographic distribution of the different components composing a luteal gland is equal throughout the luteal gland. This is reflected in the sampling protocols used in these studies. The only condition is the fact that the locations of observation has to be equally spread to obtain a representative 'total image' of the luteal gland. Nevertheless, Estergreen et al. (1968) and Niswender et al. (1976) described functional heterogeneity within a bovine or ovine CL. Estergreen et al. (1968) found significantly different progesterone concentrations between different sections of bovine corpora lutea. Niswender et al. (1976) found that the *in*

vivo hCG binding capacity of the ovine luteal tissue showed significant regional differences. As the binding mechanism is receptor mediated, one can expect also morphological variations. But Niswender et al. (1976) did not find apparent morphological differences between these areas. What he did found was a positive correlation between the hCG binding capacity and the local blood flow. Altogether this indicates rather functional than structural regional differences. On his turn, Rodgers et al. (1984) reported even some small structural differences in cellular composition (or more specifically the volume density of the different cell types) between the inner and outer region of the ovine CL. Consequently, equal pooling of data from the larger outer region with that from the smaller inner region may bias the findings with respect to the inner region. This nuancing implicates that the possibility of regional variation according to the depth within the luteal tissue needs to be considered in creating sampling procedures. Silvester and Luck (1999) described an increase of collagen type I content going from the outer over mid to the inner parenchyma in the bovine luteal gland. The amount of collagen type I in the inner parenchyma was between four- and tenfold greater than in the mid-parenchymal region.

Neves and Marques (2002) analyzed how many observations one has to do, to obtain a representative estimation of the volume percentage of the steroidogenic cells, fibroblast, endothelial cells, and pericytes within a bovine CL. The estimation was representative when the standard deviation stabilizes and minimizes. They stated that it is necessary to analyze 35 images with each 25 cross-points (i.e. 750 hits; at a magnification of 400x), made in 3 parallel slices of different cell layers.

As a conclusion, we can state that it is possible to sample luteal glands probably without significantly hampering the luteal function. Whether such a sample is a representative witness of the intact luteal gland is much more complicated to answer. It seems to depend on the number of samples, the sampling site and the analyzed histological trait.

Reference List

Augustin HG, Braun K, Telemenakis I, Modlich U, Kuhn W, 1995: Ovarian angiogenesis. Phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. Am J Pathol **147**, 339-351.

Beg MA, Gastal EL, Gastal MO, Ji S, Wiltbank MC, Ginther OJ, 2005: Changes in steady-state concentration of messenger ribonucleic acids in luteal tissue during prostaglandin F2alpha induced luteolysis in mares. Anim Reprod Sci **90**, 273-285.

Bols PEJ, Leroy JLMR, Vanholder T, Van Soom A, 2004: A comparison of a mechanical sector and a linear array transducer for ultrasound-guided transvaginal oocyte retrieval (OPU) in the cow. Theriogenology **62**, 906-914.

Chan R, Common AA, Marcuzzi D, 2000: Ultrasound-guided renal biopsy experience using an automated core biopsy system. Can Assoc Radiol J **51**, 107-113.

Chen F, Wang B, Chen LZ, 2007: Comparison of safety and sensitivity in diagnosis of liver lesion between ultrasound-guided 16 gauges and 18 gauges core needle biopsy. Zhonghua Yi Xue Za Zhi **87**, 823-825.

D'haeseleer M, Cocquyt G, Van Cruchten S, Simoens P, Van den Broeck W, 2006: Cell-specific localization of apoptosis in the bovine ovary at different stages of the estrous cycle. Theriogenology **65**, 757-772.

Donaldson L, Hansel W, 1965: Histological study of bovine corpora lutea. J Dairy Sci **48**, 905-909.

Elecko J, Sevcika I, Bekeova E, Choma J, Kacmarik J, 1990: Changes in progesterone, triiodotyronine and tyroxin concentrations in the blood and the results of the quantitative histological analysis of the placenta and corpus luteum in cows after protracted parturition. Vet Med (Praha) **35**, 529-535.

Estergreen VL Jr, Holtan DW, Smith SN, 1968: Heterogeneity of progestin distribution in the bovine corpus luteum. J Dairy Sci **51**, 948.

Fields MJ, Fields PA, 1996: Morphological characteristics of the bovine corpus luteum during the estrous cycle and pregnancy. Theriogenology **45**, 1295-1325.

Hoppe FE, Hager DA, Poulos PW, Ekman S, Lindgren PG, 1986: A comparison of manual and automatic utrasound-guided biopsy techniques. Vet Radiol **27**, 99-101.

Ireland JJ, Murphee RL, Coulson PB, 1980: Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J Dairy Sci **63**, 155-160.

Jennings PE, Coral A, Donald JJ, Rode J, Lees WR, 1989: Ultrasound-guided core biopsy. Lancet **1**, 1369-1371.

Kim KH, Son EJ, Kim EK, Ko KH, Kang H, Oh YK, 2008: The safety and efficiency of the ultrasound-guided large needle core biopsy of axilla lymph nodes. Yonsei Med J **49**, 249-254.

Kot K, Anderson LE, Tsai SJ, Wiltbank MC, Ginther OJ, 1999: Transvaginal, ultrasound-guided biopsy of the corpus luteum in cattle. Theriogenology **52**, 987-993.

Lei ZM, Chegini N, Rao CV, 1991: Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol Reprod **44**, 1148-1156.

Miyamoto Y, Skarzynski DJ, Okuda K, 2000: Is tumor necrosis factor alpha a trigger for the initiation of endometrial prostaglandin F(2alpha) release at luteolysis in cattle? Biol Reprod **62**, 1109-1115.

Modransky PD, 1986: Ultrasound-guided renal and hepatic biopsy techniques. Vet Clin North Am Equine Pract **1**, 115-126.

Neves MM, Marques Jr AP, 2002: Tamanho da amostra para estudo da proporção volumétrica dos constituintes do corpo lúteo bovino. Arq Bras Med Vet Zootec **7**, 81-85.

Neves MM, Marques Jr AP, 2006: Volumetric proportions of the corpus luteum constituents of Nelore. Arq Bras Med Vet Zootec **58**, 944-946.

Niswender GD, Reimers TJ, Diekman MA, Nett TM, 1976: Blood flow: a mediator of ovarian function. Biol Reprod **14**, 64-81.

O'Shea JD, 1987: Heterogeneous cell types in the corpus luteum of sheep, goats and cattle. J Reprod Fertil Suppl **34**, 71-85.

O'Shea JD, Rodgers RJ, D'Occhio MJ, 1989: Cellular composition of the cyclic corpus luteum of the cow. J Reprod Fertil **85**, 483-487.

Parkinson TJ, Turvey A, Jenner LJ, 1994: A morphometric analysis of the corpus luteum of the cow during the estrous cycle and early pregnancy. Theriogenology **41**, 1115-1126.

Penninck DG, Cristal MA, Matz ME, Pearson SH, 1993: The technique of percutaneous ultrasound guided fine-needle aspiration biopsy and automated microcore biopsy in small animal gastrointestinal diseases. Vet Radiol Ultrasound **34**, 433-436.

Pratap R, Qayyum A, Ahmad N, Jani P, Berman LH, 2009: Ultrasound-guided core needle biopsy of parotid gland swellings. J Lar Otol **123**, 449-452.

Rodgers RJ, O'Shea JD, Bruce NW, 1984: Morphometric analysis of the cellular composition of the ovine corpus luteum. J Anat **138**, 757-770.

Silvester LM, Luck MR, 1999: Distribution of extracellular matrix components in the developing ruminant corpus luteum: a wound repair hypothesis for luteinization. J Reprod Fertil **116**, 187-198.

Tsai SJ, Kot K, Ginther OJ, Wiltbank MC, 2001: Temporal gene expression in bovine corpora lutea after treatment with PGF2alpha based on serial biopsies *in vivo*. Reprod **121**, 905-913.

Watson ED, Sertich PL, 1990: Secretion of prostaglandins and progesterone by cells from corpora lutea of mares. J Reprod Fertil **88**, 223-229.

Wiltbank MC, Shiao TF, Bergfelt DR, Ginther OJ, 1995: Prostaglandin $F_{2\alpha}$ receptors in the early bovine corpus luteum. Biol Reprod **52**, 74-78.

Yeow KM, Lo YF, Wang CS, Chang HK, Tsai CS, Hsueh C, 2001: Ultrasound-guided core needle biopsy as an initial diagnostic test for palpable breast masses. J Vasc Interv Radiol **12**, 1313-1317.

Chapter 3

Aims of the thesis

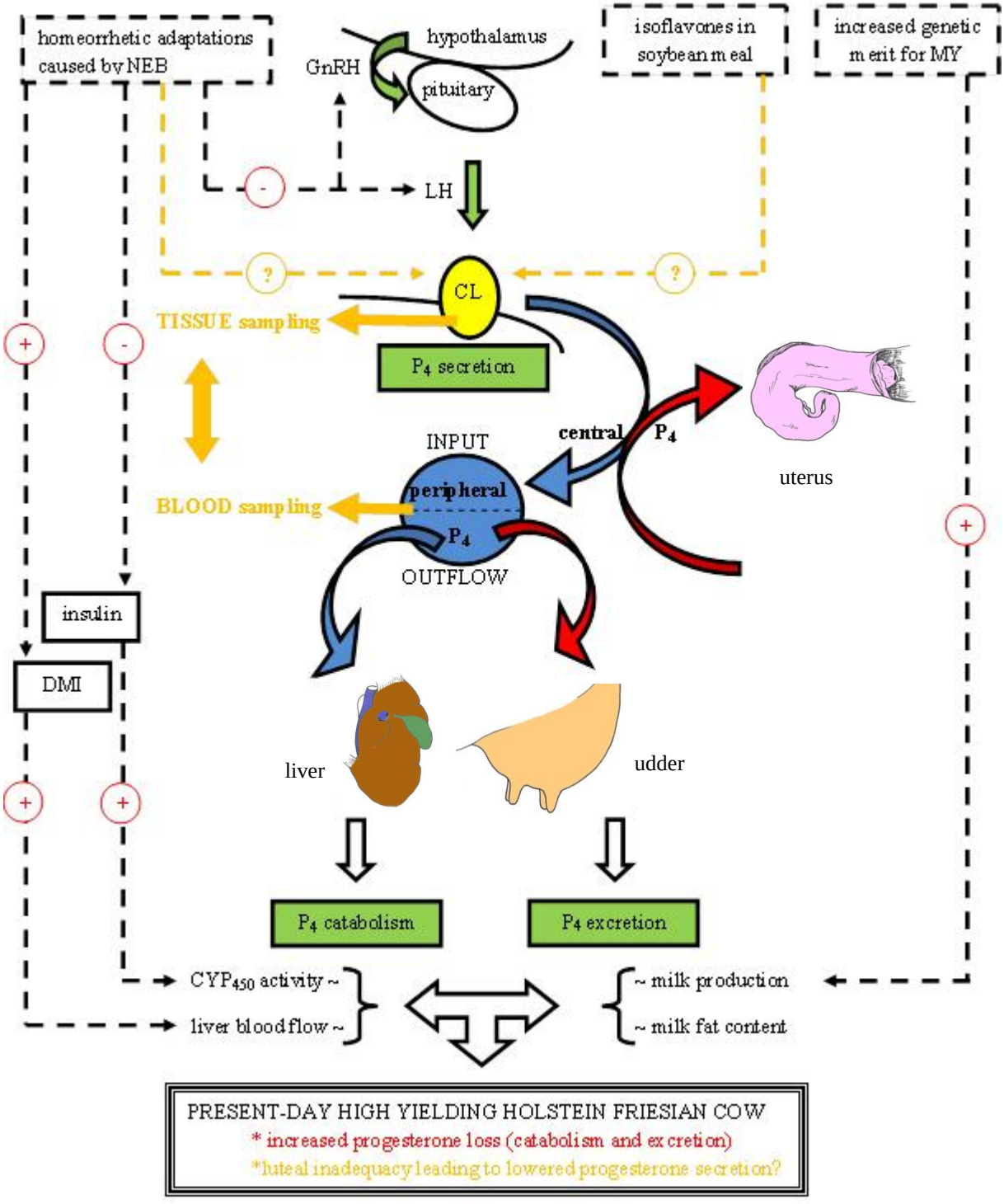


Figure 11: *schematic overview of the current knowledge concerning the establishment of a peripheral blood progesterone level and visualizing the pending research questions (blue and red arrows represent respectively venous and arterial blood)*

In figure 11 the current knowledge and the pending questions concerning the establishment of the peripheral blood progesterone concentration in present-day high yielding HF cows are visualized. The peripheral blood progesterone concentration is a dynamic equilibrium between progesterone secretion (i.e. the INPUT, depending on the hypothalamus, pituitary and CL) and progesterone loss (i.e. the OUTPUT, depending on the mammary excretion and the hepatic catabolism).

The only significant progesterone source is the CL. The most important regulators of the luteal progesterone production are GnRH and LH.

The impact of the mammary progesterone excretion is extensively described in literature and is depending on MY and milk fat content. Genetic selection created dairy cows with an increased genetic merit for MY, leading to a higher progesterone loss via the milk. Also the hepatic catabolism is tediously analyzed in earlier studies. The hepatic clearance of progesterone is depending on CYP_{450} and the hepatic blood flow, which are influenced by respectively the blood insulin concentration and the DMI.

Present-day dairy cows are dealing with a NEB during the transition period. To cope with this NEB, the cow induces homeorrhetic adaptations, which are extensively described in literature: 1) a depression of insulin secretion, leading to a stimulation of the CYP_{450} activity; 2) an increase of the DMI with an increase in LBF. The NEB induces also a decrease in GnRH and LH pulse frequency, with inhibiting effects on ovarian activity. Altogether these homeorrhetic adaptations and side effects of the NEB contribute to an increased progesterone loss in high yielding dairy cows. This can partially explain the decreased peripheral blood progesterone concentration in these cows.

At the moment there however is a serious gap in the knowledge about luteal progesterone secretion in modern dairy cows. Some studies suggested that modern cows experience a 'luteal inadequacy'. Sound data confirming this hypothesis are however scarce. One of the major problems is that most of these studies based the assessment of luteal function on the analysis of the peripheral progesterone concentration, which is significantly confounded by the above mentioned factors decreasing the peripheral progesterone concentration. This problem could be countered by directly sampling the CL itself. Further research is certainly necessary on this topic.

When such a technique should be available, it should also be possible to monitor whether the NEB exerts directly effects on luteal activity.

To compensate for the increased protein requirements due to the currently high milk yield level, soybean meal is generally used as supplementary protein source in many dairy herds.

An interesting topic for further research should be the effect of soybean meal supplementation on the luteal function. Soybean meal is known to contain relatively high levels of isoflavones of which in human medicine is known that they have tumor preventive characteristics. In literature, the fast CL-growth following ovulation is frequently compared with the growth of an aggressive tumor. Making this topic also an interesting subject for further research.

These considerations leads to the **main hypothesis** of this thesis: "The luteal activity in present-day high yielding Holstein Friesian cows is compromised during the post-partum period leading to a suboptimal peripheral blood progesterone concentration."

The **specific aims** of this thesis are

1) to analyze the topographic distribution of several luteal histological traits in order to identify parameters suitable for further analysis of the luteal histophysiology based on just a sample of the complete luteal gland **(chapter 4.1 and 4.2)**;
2) to optimize and validate the biopsy technique *in vitro* for monitoring the selected histological characteristics *in vivo* **(chapter 4.3)**;
3) to assess the effect of the biopsy technique on luteal function (**chapter 5.1**);
4) to analyze the effect of soybean meal supplementation (under representative Belgian conditions) on peripheral blood isoflavone concentration, on the luteal angio- and steroidogenesis, and peripheral progesterone concentration (**chapter 5.1**);
5) to longitudinally monitor luteal angio- and steroidogenesis during the post-partum period and to analyze whether these processes are affected by homeorrhetic adaptations associated with negative energy balance (**chapter 5.2**).

Chapter 4

Ex vivo research:

Optimization of the biopsy sampling technique

Chapter 4.1

The bovine luteal histological composition: a topographic point of view

Cools S[a], Van den Broeck W[b], De Vliegher S[a], Piepers S[a], Opsomer G[a]

[a]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

[b]Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Reproduction in Domestic Animals 2013, **48**, e29-32

Abstract

High yielding dairy cows are struggling with a high incidence of embryonic loss, among other reproductive disorders, caused by an insufficient peripheral progesterone concentration which for its part might be associated with an impaired luteal progesterone production. This impaired capacity to produce progesterone might be reflected in the histology of the gland. The aim of the present pilot study was the assessment of the variation in cell density within a bovine luteal gland, in order to examine whether it is possible to analyze histologically the activity of the gland based on one single tissue sample. Six luteal glands (stage II or III) were harvested out of just as many healthy cows at the slaughterhouse. The luteal cell density was assessed by calculating the nuclear density of the different luteal cell types on Haematoxylin-Eosin-stained histological sections from a number of topographic regions evenly spread throughout the glands, in order to give an overview of the pattern of cellular distribution within the whole gland. Cells were differentiated into 'large luteal cells', 'small luteal cells' and 'non-steroidogenic cells'. Results show that the cellular density, within a tissue sample is not significantly influenced by its location in relation to the gland's equatorial plane. However, the position with respect to the polar axis of the gland has a decisive effect, as the nuclear density is significantly higher ($P < 0.05$) in the peripheral regions (outer zone) when compared with the central regions (inner zone) of the gland, and this counts for all three cell types.

Keywords: nuclear density; histology; topography; luteal gland; bovine

Introduction

An insufficient peripheral blood progesterone concentration is associated with a higher incidence of (early) embryonic death in high yielding dairy cows (Mann and Lamming 2001; Diskin and Morris 2008). One hypothesis to explain this inadequate progesterone concentration might be the inability of the corpus luteum (LG, luteal gland) to produce sufficient progesterone. As the glandular progesterone output is a result of the collaboration between several luteal cells, the deficiency in steroidogenesis could be indicative for a modified luteal histological composition. Several studies have dealt with the histological composition of the bovine LG (O'Shea et al. 1989; Parkinson et al. 1994; Fields and Fields 1996), but none scrutinized the topographic variation in the cellular composition within the gland itself. Furthermore, profound knowledge of the variation in histological composition of the LG is needed in case one wants to examine the effect of environmental factors on the histology of the LG in living animals over time by means of a biopsy technique. Or in other words, if one wants to examine longitudinally the effect of different environmental factors on the histological composition of the bovine LG, it is necessary to know how many tissue samples should be taken and how the localization of the sampling in relation to the topography of the gland should be performed.

In the present study we aimed to examine whether the nuclear density within a tissue sample from a bovine LG depends on the location within the gland.

Materials and methods

In a slaughterhouse, six LGs of stage II (i.e. cycle day 5 to 10) and III (i.e. cycle day 11 to 17) (Miyamoto et al. 2000; D'haeseleer et al. 2006) were separated from normal non-gravid genital tracts at most 20 minutes after stunning and bleeding of the cows (4 Holstein Friesian and 2 Belgian Blue cows). As luteogenesis is a dynamic process, standardization of the gland's age was necessary. To do so, we applied the methodology described by Miyamoto et al. (2000) and D'Haeseleer et al. (2006), and decided to only take into account stage II and III LGs. We opted for these two stages as they are the stages in which the LG consists of an organized tissue structure from which it is possible to harvest biopsies, and because these stages are of most interest with regard to the hypothesis of insufficient progesterone production in relation to embryonic death. Further processing of the glands is visualized in Figure 1.

After excision, each LG was partitioned into 6 tissue disks (sections parallel to the equatorial plane). Four of these disks (1st, 3rd, 4th and 6th) were selected for further analysis. Fixation of the tissue disks was realized by immersion into neutral buffered 3.5% formaldehyde during 48 hours at RT (room temperature), followed by dehydration during 22 hours and embedding in paraffin following a standardized protocol (STP 420 D, Microm, Prosan, Merelbeke, Belgium). Out of each tissue disk, a series of histological slices (n=32; 5 µm thickness) was made, starting from the plane of the disk giving origin to the arrow (HM 360, Microm) (Fig. 1). The sections were mounted on gelatin-coated slides and dried overnight at RT. Based on a systematic-random-sampling procedure (with a sampling interval of eight slices), three slices were selected from each of the four tissue disks for further research, resulting in twelve histological slices to be examined within each LG (Fig. 1). Selected slices were HE (Haematoxylin-Eosin) stained, as described by Stevens (1977) by means of the Linear Stainer II (Sakura Finetek Belgium, Berchem, Belgium). The area of interest, i.e. the luteal parenchyma, was defined and separated into an inner and outer zone (Fig. 2).

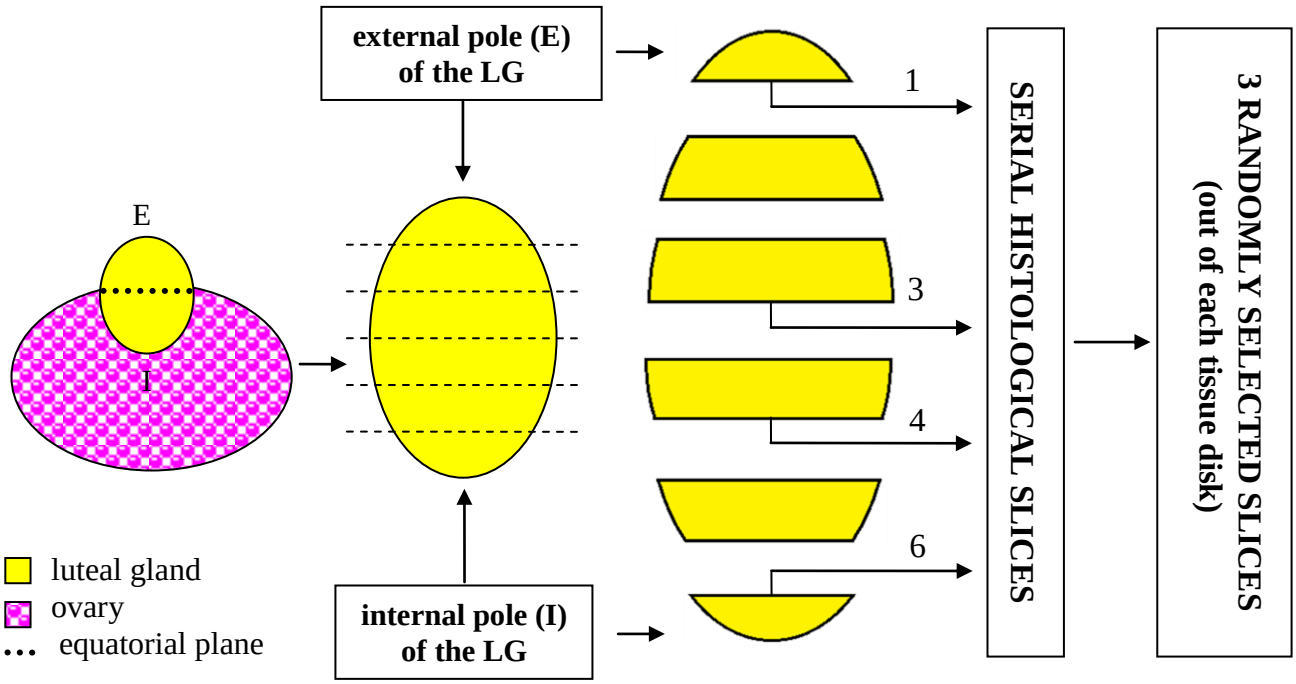


Figure 1: *sampling protocol for the luteal gland (LG). After excision, the LG is partitioned into 6 tissue disks (sections parallel to the equatorial plane). Four tissue disks (1st, 3rd, 4th and 6th) were selected for further study. Out of each tissue disk, a series of histological slices was made, starting from the plane giving origin to the arrow. Based on a systematic-random-sampling procedure (with a sampling interval of eight histological slices), three histological slices from each of the four tissue disks were chosen.*

The central border of the inner zone was situated in the region closest to the center of the tissue still containing steroidogenic cells, while the peripheral border of the outer zone was situated in the region as close as possible to the luteal capsule, but still presenting progesterone producing cells. The peripheral boundary of the inner zone and the central border of the outer zone were determined geometrically, aiming to compare similar surfaces in both zones, preventing overlap of both zones. This means that the distance of the border of the inner zone to the center (inner radius) is approximately 71% of the radius of the entire corpus luteum. In each zone, five micrographs (magnification x 1000) were made randomly spread over each entire zone (inner and outer) by means of an Olympus DP 50 digital camera mounted on a motorized Olympus BX61 light microscope (Olympus Belgium N.V., Aartselaar, Belgium), resulting in a total of 30 micrographs of each tissue disk, and consequently 120 micrographs per LG.

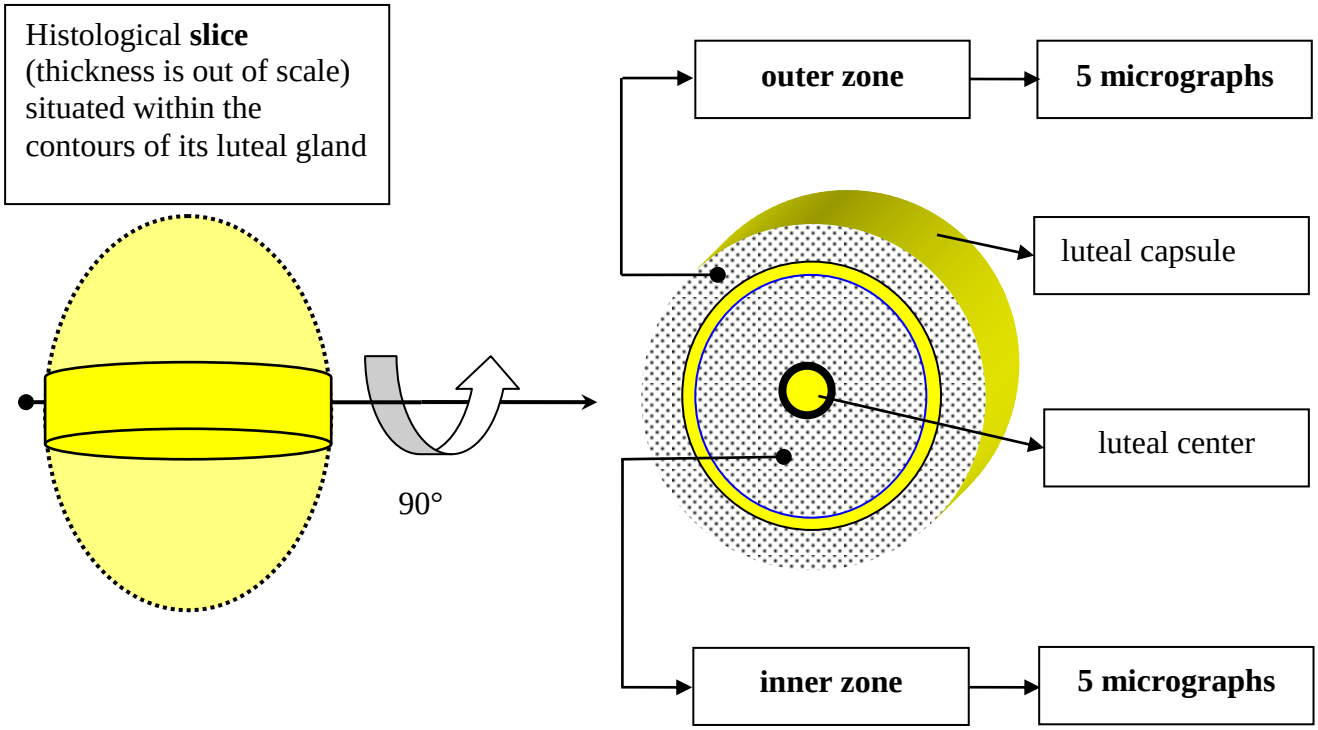


Figure 2: *schematic view of the delineation of the outer and inner zone relative to the luteal capsule and luteal center in the histological slices*

As we were mainly interested in the variation within one and the same gland, we analyzed a higher number of micrographs within a relatively low number of glands instead of the opposite. We also opted to make the micrographs with a high magnification (x 1000) to

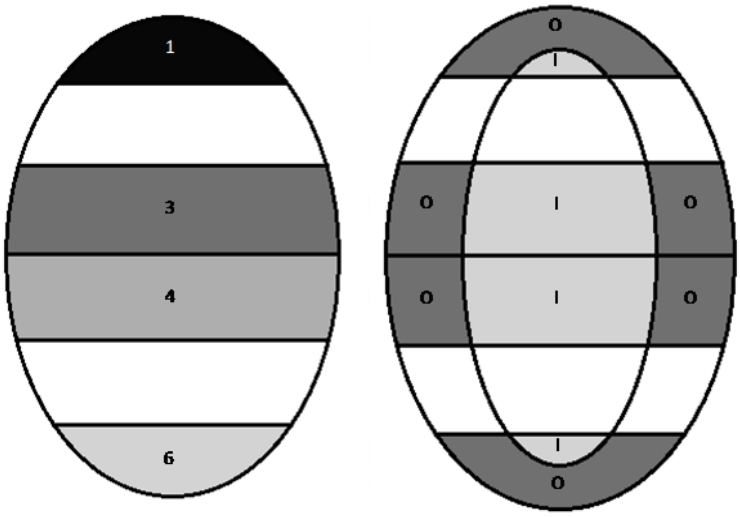
obtain the highest resolution for optimal identification of the three cell types. Cell type differentiation was based on light microscopic characteristics described in literature (O'Shea et al. 1989; Wiltbank 1994; Fields and Fields 1996).

'Total ND' (Total nuclear density), i.e. the total number of nuclei within a micrograph (with a fixed area of 11 875 µm²), was analyzed using Cell F (Soft Imaging System, Olympus Belgium N.V.). The luteal cells were further differentiated into 'LLC' ('large luteal cells'), 'SLC' ('small luteal cells'), and 'NSC' ('non steroidogenic cells') based on nuclear and cytoplasmic characteristics as described by O'Shea et al. (1989), Wiltbank (1994), Fields and Fields (1996).

The results were combined within each LG in two different ways: 1) the results of the micrographs made in the histological slices of each tissue disk were combined to determine the topographic effect of the location of the tissue disk in view of the equatorial plane within the LG on nuclear density (DEP, effect of the distance to the equatorial plane), leading to sixteen results within each LG (four results per outcome variable: 'total ND', 'LLC', 'SLC', 'NSC'); 2) the results of the micrographs made in the inner zone were grouped and compared with the results of the micrographs made in the outer zone (independent of the tissue disk) to determine the topographic effect of the distance to the axis through both poles on nuclear density (DPA, effect of the distance to the polar axis), leading to eight results within each LG (two results per outcome variable) (Fig. 3).

The data were 'sqrt' (square root) transformed to obtain normality. The association between the four outcome variables ('sqrtTotal ND', 'sqrtLLC', 'sqrtSLC', and 'sqrtNSC', respectively) and the two different topographic effects (DEP (4 categories) and DPA (2 categories)) was tested using linear mixed regression models including 'LG' as random effect (accounting for multiple observations within a gland) and 'topographic combinations' (DEP and DPA, respectively) as fixed effects using MLwiN 2.16 (Centre for Multilevel Modeling, Bristol, UK).

A power analysis was done by means of Win Episcope 2.0 to assess whether we were able to detect the observed difference in nuclear density with sufficient power based on the sample size we used. We also determined the minimal number of micrographs one has to analyze to estimate the observed topographic difference in nuclear density in a confident way (P-value < 0.05 and power > 80%).



a b

Figure 3: *in this figure the two different topographic combinations are presented: a) the results of the micrographs made in the histological slices within the four tissue disks (number 1, 3, 4, 6) were compared to each other to determine the topographic effect of the location of the tissue disk in view of the equatorial plane within the LG on nuclear density (effect of the distance to the equatorial plane, DEP); b) the results of the micrographs made in the outer zones (o) of the histological slices are compared with the results of the micrographs made in the inner zones (i) of the histological slices (independent of the tissue disk), to assess the effect of the distance to the polar axis (DPA) on the nuclear density. The white zones are the tissue disks which were not analyzed*

Results

The DEP had no significant influence on the 'sqrtTotal ND', 'sqrtLLC', 'sqrtSLC' and 'sqrtNSC' ($P > 0.05$), indicating that the mean and cell type specific nuclear density did not differ between the diverse tissue disks within a bovine LG. On the other hand, the DPA influenced the 'sqrtTotal ND' significantly ($0.001 < P < 0.01$) (Fig. 4) with 'sqrtTotal ND' always being lower in the inner zone. The same was seen for the different cell types (i.e. sqrtLLC, sqrtSLC and sqrtNSC) ($P < 0.001$).

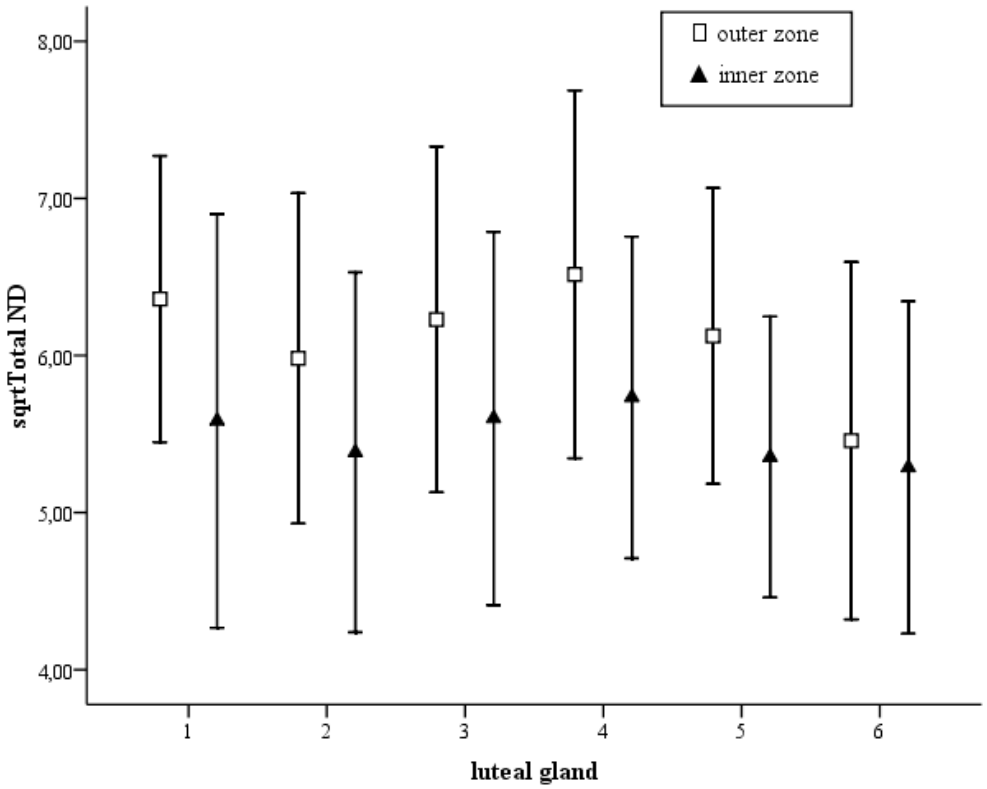


Figure 4: *error bar chart visualizing the square root transformed values of the total nuclear densities ('sqrtTotal ND') averaged for the outer zone (μ_o; open squares) and for the inner zone (μ_i; plane triangles), together with the standard deviations multiplied by 2 (bars) within each luteal gland (1 to 6)*

The power of our study (analyzing per LG 60 micrographs in the inner zone and 60 in the outer) turned out to be higher than 95%, indicating that in fact we could have come to the same conclusions based on a smaller sample size. Minimal sample size estimation indicated that one needs at least 33 randomized observations in each zone to assess the observed difference in the 'Total ND' between the inner and the outer zone with a power of 80% and 95% confidence.

Discussion

The purpose of the present study was to assess the regional variation in cell density within a stage II/III bovine LG. We opted to use the nuclear density as histological parameter based on several reasons. The nuclear density can be linked with the cellular density. Furthermore nuclear density is a parameter which can be analyzed in a relatively easy and economical way by light microscopy using HE-overview stainings, which makes it applicable on a larger

scale. An even more important reason is the fact that several studies indicate that the nuclear density could be influenced by several environmental factors, with or without effects on the luteal progesterone content or plasma progesterone concentration. Donaldson and Hansel (1965) stated that the number and size of the luteal cells within the gland reflect its progesterone concentration and content. Their results were more or less confirmed by Robinson et al. (2006), who described that in the bovine there is a tendency towards a positive correlation (r^2=0.41; P=0.09; n=8) between the luteal cell volume and the plasma progesterone concentration on day 5 of the estrous cycle.

Milvae et al. (1991) showed that when the number of granulosa cells is reduced in the bovine pre-ovulatory follicle, the luteal progesterone synthesis will subsequently also be reduced. Mee et al. (1993) described a stimulating effect of GnRH therapy on the proportion of LLC and SLC, and consequently on their nuclear density in bovine LGs. In mice, Hsieh et al. (2005) described a mutation inducing LGs with a higher nuclear density. Rutter et al. (1985) found in the LGs of spontaneously cycling cows a higher number of SLC and LLC per gram of tissue when compared to LGs from GnRH-induced ovulations.

Data concerning the topographic variation in cell distribution within a LG is currently lacking. And the minimal number of samples that has to be taken in case one wants to examine longitudinally the effect of environmental factors on the histophysiology of the LG by using an *in vivo* sampling technique is not yet elucidated.

The fact that there is no significant effect of DEP, suggests that the distance of the sampling site in relation to the equatorial plane of the luteal gland, does truly not affect the total cell number nor the distribution of the different cell types as substantiated by the fact that the power of our study was higher than 95%.

The fact that the nuclear density is significantly higher in the peripheral part of the LG (outer zone) versus the central part (inner zone) makes sense biologically. During ovulation, the follicle (with an inner granulosa layer and an outer theca interna layer) collapses and forms folds pointing centrally to a cavity filled with blood. So, one can expect that, on that moment the zone which will later become the inner zone, contains more extracellular matrix and relatively more granulosa versus theca interna cells per surface unit. As hypertrophia is more important in luteinizing granulosa cells (versus hyperplasia in theca interna cells) (Robinson et al., 2006), and as luteal tissue is growing by invading the central hematoma, one can expect that the inner zone of a LG will have a lower number of cells per surface unit in comparison with the outer zone.

Sample size estimation using the standard deviation from the present study, revealed that the examination of at least 33 randomized micrographs in both the inner and outer zone (making a total of 66 micrographs per LG) is needed to obtain results concerning the ND within the luteal gland with sufficient power (80%) and confidence (95%). This number is situated in the same range as presented in the study of Neves and Marques (2002).

Based on the results of the present pilot study, we can conclude that, in bovine stage II and III LGs, the distance from the sampling site to the axis between both poles has a significant effect on the total cell number and the distribution of the different cell types, with a higher cell density in the outer zone compared with the inner zone. To assess the nuclear density within a LG with sufficient power (> 80%) and confidence (95%), at least 33 randomized observations in both the inner and the outer zone are necessary.

Acknowledgements

The BOF is thanked for their financial support (05B01906). Also Lobke De Bels and Liliana Standaert of the laboratory of the Department of Morphology are thanked for their technical support and advice.

Reference List

D'haeseleer M, Cocquyt G, Van Cruchten S, Simoens P, Van den Broeck W, 2006: Cell-specific localization of apoptosis in the bovine ovary at different stages of the estrous cycle. Theriogenology **65**, 757-772.

Diskin MG, Morris DG, 2008: Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim **43** (suppl 2), 260-267.

Donaldson L, Hansel W, 1965: Histological study of bovine corpora lutea. J Dairy Sci **48**, 905-909.

Fields MJ, Fields PA, 1996: Morphological characteristics of the bovine corpus luteum during the estrous cycle and pregnancy. Theriogenology **45**, 1295-1325.

Hsieh M, Boerboom D, Shimada M, Lo Y, Parlow AF, Luhmann UFO., Berger W, Richards JS, 2005: Mice Null for Frizzled 4 ($Fzd4^{-/-}$) are infertile and exhibit impaired corpora lutea formation and function. Biol Reprod **73**, 1135-1146.

Mann GE, Lamming GE, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction **121**, 175-180.

Mee MO, Stevenson JS, Alexander BM, Sasser RG, 1993: Administration of GnRH at estrus influences pregnancy rates, serum concentrations of LH, FSH, estradiol-17 beta, pregnancy-specific protein B, and progesterone, proportion of luteal cell types, and in vitro production of progesterone in dairy cows. J Anim Sci **71**, 185-198.

Milvae RA, Alila HW, Bushmich SL, Hansel W, 1991: Bovine corpus luteum function after removal of granulosa cells from the preovulatory follicle. Domest Anim Endocrinol **8**, 439-443.

Miyamoto Y, Skarzynski DJ, Okuda K, 2000: Is tumor necrosis factor alpha a trigger for the initiation of endometrial prostaglandin F(2alpha) release at luteolysis in cattle? Biol Reprod **62**, 1109-1115.

Neves MM, Marques Jr AP, 2002: Tamanho da amostra para estudo da proporção volumétrica dos constituintes do corpo lúteo bovino. Arq Bras Med Vet Zootec **7**, 81-85.

O'Shea JD, Rodgers RJ, D'Occhio MJ, 1989: Cellular composition of the cyclic corpus luteum of the cow. J Reprod Fertil **85**, 483-487.

Parkinson TJ, Turvey A, Jenner LJ, 1994: A morphometric analysis of the corpus luteum of the cow during the estrous cycle and early pregnancy. Theriogenology **41**, 1115-1126.

Robinson RS, Hammond AJ, Nicklin LT, Schams D, Mann GE, Hunter MG, 2006: Endocrine and cellular characteristics of corpora lutea from cows with a delayed post-ovulatory progesterone rise. Domest Anim Endocrinol **31**, 154-172.

Rutter LM, Carruthers TD, Manns JG, 1985: The postpartum induced corpus luteum: functional differences from that of cycling cows and the effects of progesterone pretreatment. Biol Reprod **33**, 560-568.

Stevens A, 1977: The haematoxylins. In: Bancroft JD, Stevens A (Eds.), Theory and practice of histological techniques. Churchill Livingstone New York, p. 89.

Wiltbank MC 1994: Cell types and hormonal mechanisms associated with mid-cycle corpus luteum function. J Anim Sci **72**, 1873-1883.

Chapter 4.2

Topographic distribution of the different cell types, connective tissue and vascular tissue/lumina within a functional bovine corpus luteum and its association with breed, type of fixation protocol and stage during the cycle

Cools S[a], Van den Broeck W[b], De Vliegher S[a], Piepers S[a], Hostens M[a], Opsomer G[a]

[a]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

[b]Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Reproduction in Domestic Animals, 2013, **48**, 627-635

Abstract

In the present study, we analyzed the effect of fixative, breed, luteal stage and location on the nuclear density, volume density of connective tissue and vascular tissue/lumina within a bovine luteal gland in view of the development of an *in vivo* sampling technique to longitudinally monitor luteal histophysiology. The inner zone defined as the zone geometrically closest to the center of the gland, shows a significantly lower nuclear density (for all cell types) and a higher volume density of collagen fibers and vessels when compared with the outer zone ($P < 0.001$). The nuclear density in luteal glands from Holstein Friesian cows is not significantly different from that in Belgian Blue cows, nor is it in stage II versus stage III glands. The collagen fiber content was significantly lower in glands of Belgian Blue cows ($P=0.01$) and in younger glands ($P=0.003$). Hence, it seems that the lower nuclear density in the inner zone was compensated by a higher amount of collagen fibers. As the type of fixative applied has a significant effect on the nuclear density of the different cell types, the present study warrants future research to further optimize the fixation protocol. As a conclusion, we can state that the topographic difference in nuclear distribution for the different cell types in a bovine luteal is only significant when comparing the inner versus the outer zone. This implies that, if a sample representative for the whole gland has to be taken, e.g. when taking an *in vivo* sample, it is necessary that the biopsy goes through the inner zone and contains the total diameter of the gland.

Key words: cow; luteal gland; nuclear density; collagen; vessels

Introduction

There currently is a lot of debate concerning the reasons of the reduced peripheral progesterone concentration in high yielding dairy cows. This reduction in progesterone concentration is associated with the increased incidence of embryonic death (Diskin and Morris 2008; Mann and Lamming 2001; Shelton et al. 1990; Sreenan and Diskin 1983). So, a lowered blood progesterone concentration has been identified as a major contributor to the reduction in reproductive capacity of modern dairy cows. Whether the luteal progesterone production is impaired and can hence be seen as an underlying reason for the unsatisfactory progesterone levels, has not yet been fully elucidated. Measuring the peripheral progesterone level to monitor luteal function is inadequate since it has been demonstrated in several species including the bovine, that the progesterone concentration in serum depends on a wide number of interfering factors (Abecia et al. 1997; Parr et al. 1993; Rabiee et al. 2001; Sangsritavong et al. 2002; Virolainen et al. 2004; 2005a; 2005b; Wiltbank et al. 2006).

An innovative approach to more accurately assess the *in vivo* histophysiology of the luteal gland (CL) in terms of its capacity to produce and secrete progesterone, could be the analysis of biopsies (Kot et al. 1999). To correctly analyze the physiology of a bovine corpus luteum (CL) based on its histology, it is necessary to identify parameters which are measurable in a straightforward way and of which the results, obtained via one single tissue sample can be extrapolated to the whole gland. As progesterone production is a cell-associated parameter, it is recommended to assess the nuclear density. This trait can be analyzed in a straightforward and relatively economic way by means of light microscopy, making this parameter suitable for application in studies with larger sample sizes. However, the peripheral progesterone concentration is not only the result of progesterone production, but also depends on the supply of constituents and on the evacuation of the hormone. The latter is largely influenced by the blood circulation within the gland. Consequently, also the distribution of vascular tissue/lumina and fibrous collagen needs to be assessed if one wants to scrutinize the histophysiology of the gland.

Bovine luteal histology has already been described earlier (Donaldson and Hansel 1965; Fields and Fields 1996; Lei et al. 1991; Neves and Marques 2006; O'Shea 1987; O'Shea et al. 1989; Parkinson et al. 1994). Except for one study describing functional heterogeneity within an ovine CL (Niswender et al. 1976) and one study reporting some small structural differences in cellular composition between the inner and outer region of the ovine CL (Rodgers et al. 1984), there are to the best of our knowledge no papers reporting regional

differences in histology within the bovine CL. This hypothetical topographic cellular variation will probably not influence the peripheral blood progesterone concentration as this is the result of the progesterone production of the whole gland, but the knowledge about this variation is strictly necessary when one wants to take a biopsy that is representative for the whole gland.

Hence, the hypotheses we aimed to test in the present study were:

1) There is a significant difference between the inner and outer part of the CL concerning the distribution of the different cell types, collagen fibers and vascular tissue/lumina within the gland, making the location and direction when taking a biopsy of critical importance.

2) As the CL is a transient, evolving gland, its age has a significant influence on the density of the different cell types, collagen fibers and vascular tissue/lumina.

3) There is a significant difference in the density of the different cell types, collagen fibers and vascular tissue/lumina between corpora lutea (CLa) of different bovine breeds, which is necessary to know when taking biopsies from a variety of breeds.

4) The fixative used after harvesting the sample has a significant effect on the nuclear density in luteal tissue. It is clear that if there is a significant influence of the used fixative, further research is necessary to find out the optimal fixation protocol to perform accurate studies.

Materials and methods

Selection of the corpora lutea

Maximal 8 hours before stunning, blood samples of clinically healthy cows were taken. At most 20 minutes after stunning and bleeding, genital tracts without macroscopic pathologies and bearing a clearly visible CL were isolated out of Holstein Friesian (HF; n=25) and Belgian Blue (BB; n=23) cows. To eliminate CLa in which the luteolytic process was already initiated, only CLa of cows with a peripheral progesterone concentration >5 ng/ml were included in the study. To avoid confounding by age, age distribution (two to six years) among both breeds was blocked. Based on the selection criteria described by Ireland et al. (1980), supplemented with those described by Miyamoto et al. (2000) and D'haeseleer et al. (2006), all 48 selected CLa belonged to either stage II (i.e. cycle day 5 to 10; n=24) or stage III (i.e. cycle day 11 to 17; n=24), equally divided among both breeds. In figure 1, a tree diagram is presented, illustrating the distribution of the selected CLa throughout the different subsets.

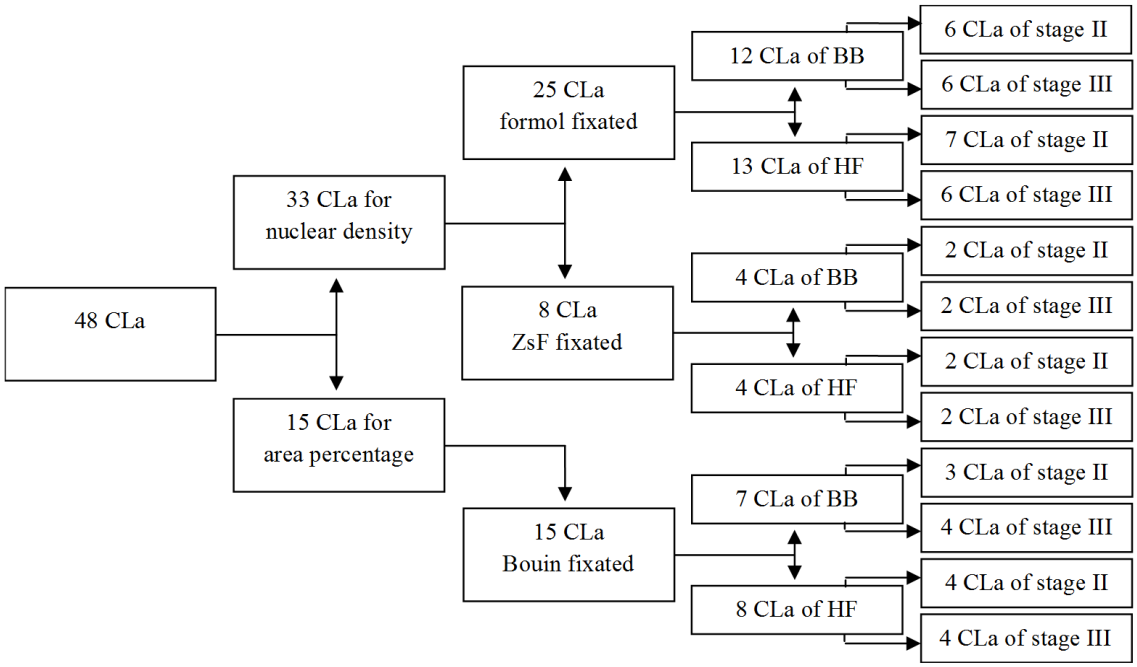


Figure 1: *a tree diagram of the distribution of the corpora lutea (CLa) used in the study. Forty eight CLa were divided into two subsets (1 and 2). In subset 1 (n=33), the nuclear density was analyzed. Two fixation protocols were applied: 1a) immersion during 48 hours in 3.5% neutral buffered formaldehyde (n=25; 'formol fixated') and 1b) immersion during 72 hours in zinc-salts fixative (n=8; 'ZsF fixated'). In subset 2 (n=15), the area percentage of collagen fibers and vascular tissue/lumina was determined. Those CLa were immersed during 48 hours in Bouin's solution ('Bouin fixated'). Due to the stratification, the contribution of both breeds (Belgian Blue, BB and Holstein Friesian, HF) and both luteal stages (II, day 5 to 10 and III, day 11 to 17) was equal within each subset*

Basically, the 48 CLa were randomly divided into two subsets (1 and 2). Blocking for breed and luteal stage was applied, resulting in an equal contribution of both parameters within each subset. In subset 1 (n=33) the nuclear density was assessed, while in subset 2 (n=15) the volume density of connective tissue (collagen fibers), and vascular tissue/lumina was assessed. Within subset 1, two different fixation protocols were tested, consequently this subset was split up into subset 1a (n=25; Formol fixation) and 1b (n=8; ZsF fixation) (figure 1). The CLa of subset 2 were fixated in Bouin's solution.

Sampling procedure and processing of the luteal tissue samples

A general overview of the sampling protocol used, is given in figure 2.

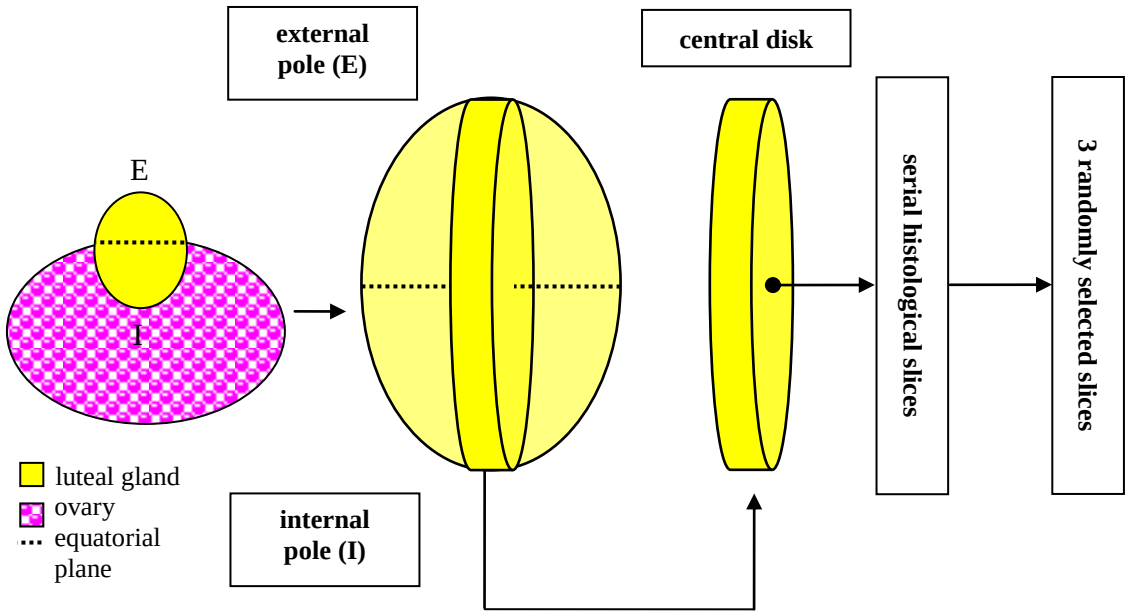


Figure 2: *a central disk, sectioned perpendicularly to the equatorial plane was isolated containing the ovulation point (i.e. the external pole; marked with a small needle before enucleation and present during the whole process) and the internal pole. A series of histological slices was made, starting from the plane giving origin to the arrow, coming out of the 'central disk'. Based on a systematic-random-sampling procedure (sampling interval of eight slices), three histological slices were chosen for further research*

Briefly, after identification of the external pole (i.e. the ovulation point) by inserting a small needle, lutectomy was done. At maximal diameter, a disk (containing both poles and the sagittal plane) defined as 'central disk' (CD) with a maximal thickness of 0.5 cm was isolated and immersed in fixative. During processing of the tissue samples, great attention was furthermore given to work atraumatically in order to avoid excessive shearing and tearing of the tissue. As afterwards it was not necessary to eliminate any micrographs because of significant artefacts, we are confident that the tissue handling was well performed.

To assess the effect of the used fixative on the nuclear density, the tissue samples of subset 1a were immersed in neutral-buffered 3.5 % formaldehyde (NBF) for 48 hours at room

temperature (RT), while those in subset 1b were immersed in Zinc-salts-fixative (ZsF) for 3 days at RT, with refreshment once after 24 hours (González et al. 2001). The difference in number of glands in subset 1a versus 1b has no statistical reason. The CLa of subset 2 were fixated by immersion during 24 hours in Bouin's fixative at RT (Hopwood 1977). After fixation, the CDs were dehydrated during 22 hours and embedded in paraffin following a standardized protocol (STP 420 D, Microm, Prosan, Merelbeke, Belgium).

Out of each CD, a series of histological sections (5 µm thickness) was made (HM 360, Microm) (figure 2). The sections were mounted on gelatin-coated slides and dried overnight at RT. Based on a systematic-random-sampling procedure (with a sampling interval of eight slices), three slices were selected from each CD, resulting in three histological slices for each CL.

The sections of the CLa of subset 1 were stained with *'Haematoxylin-Eosin'* (HE), following a standard protocol, as described by Stevens (1977) by means of the Linear Stainer II (Sakura Finetek Belgium, Berchem, Belgium). The sections originating from the CLa of subset 2 were stained by a modified *Masson* staining technique, using a 1% (v/v) Biebrich scarlet solution and a 2.5% (g/v) aniline Blue solution without nuclear stain (Bradbury and Gordon 1977).

Before micrographs were made, the inner and outer zone were defined in all selected histological sections (figure 3).

The central border of the inner zone was situated in the region closest to the center of the tissue still containing steroidogenic cells, while the peripheral border of the outer zone was situated in the region as close as possible to the luteal capsule, but still presenting progesterone producing cells. The peripheral boundary of the inner zone and the central border of the outer zone were determined geometrically, aiming to compare similar surfaces in both zones, preventing overlap of both zones. Since we were mainly interested in the variation within a CL, we preferred to analyze a higher number of micrographs within a lower number of glands instead of vice versa. We opted to make the micrographs with a high magnification (x1000) because of the acquisition of the highest resolution.

In each of the 48 CLa, 72 micrographs were taken evenly spread throughout the gland's CD. This number was based on the results of a power analysis, done in a preceding pilot study (Cools et al. 2013).

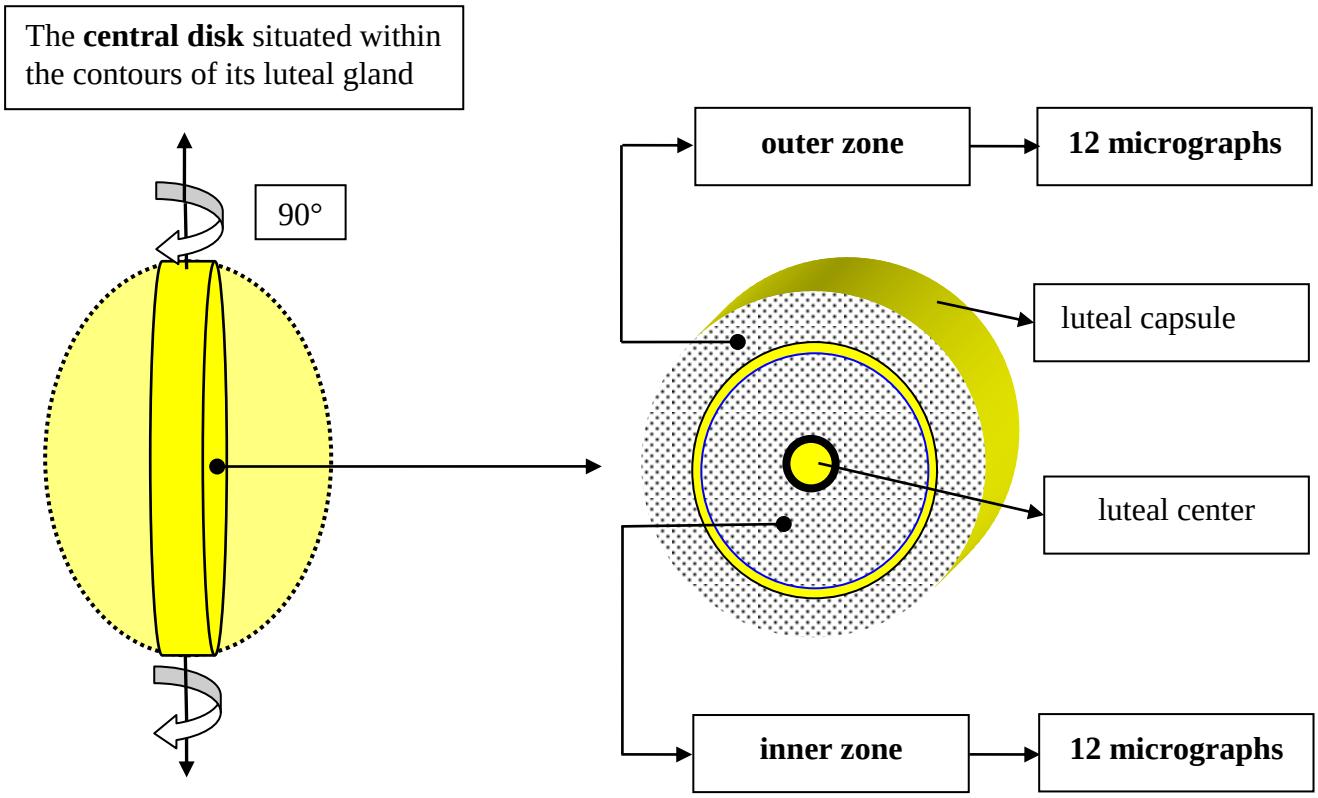


Figure 3: *schematic representation of the delineation of the outer and inner zone relative to the luteal capsule and luteal center in the histological slices. The central border of the inner zone was situated in the region, closest to the center of the tissue, that still contained steroidogenic cells, while the peripheral border of the outer zone was situated as close as possible to the luteal capsule, but still containing progesterone producing cells. The peripheral boundary of the inner zone and the central border of the outer zone were determined geometrically, preventing overlap of both zones and aiming to compare similar surfaces in both zones. Within each zone 12 micrographs were made, resulting in 72 observations for each CL*

Analysis of the micrographs

Nuclear Density

The digital micrographs were analyzed using the *'Cell F'* software program (Soft Imaging System, Olympus Belgium N.V.). By means of a touch-counting technique, the number of nuclei per micrograph (fixed area 11 875 µm²) was counted in the CLa of subset 1. Based on light microscopic characteristics described in literature (such as size, shape and morphology of both the nucleus and the cytoplasm) (Donaldson and Hansel 1965; Fields and Fields 1996;

O'Shea 1987; O'Shea et al. 1989; Wiltbank 1994), the cellular population ('Total') was differentiated into *'large luteal steroidogenic cells'* (LLC), *'small luteal steroidogenic cells'* (SLC) and *'non-steroidogenic cells'* (NSC) by the researcher himself and not by the computer. In figure 4 examples of the different cell types are visualized.

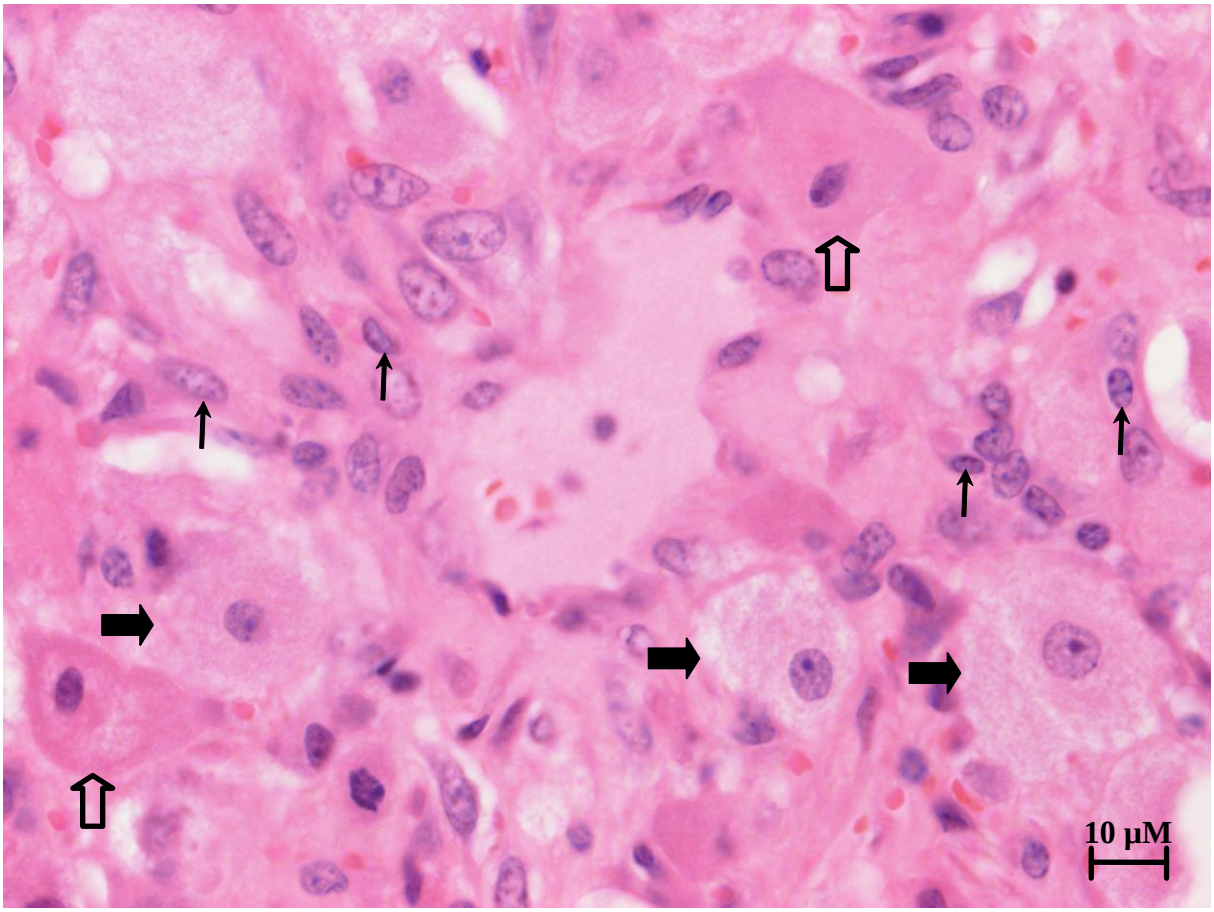


Figure 4: *a micrograph made by the camera mounted on the microscope, visualizing bovine luteal tissue stained with the HE technique (magnification 1000x). Examples of the different cell types are indicated by the arrows. The three thick, black arrows point LLC, the two thick, transparent arrows indicate SLC, the four small, black arrows are situated near NSC*

The nuclear density was expressed as the 'absolute nuclear density' (i.e. the number of nuclei within a micrograph) and the 'relative nuclear density' (i.e. the proportion of the different cell types in view of the total number of nuclei counted on a micrograph).

Volume density of the different morphological components

A point-counting technique was performed using the same software in the histological sections of subset 2. A grid (with squares of 5 µm by 5 µm) generating 475 cross-points, was projected on each photo. Each cross-point was studied, whether it was situated in *'collagen fibers'* or *'vascular tissue/lumina'*. The number of points situated in collagen fibers or vascular tissue/lumina was expressed as a percentage relative to the total number of points, resulting in a volume fraction for both CL components (Dharmarajan et al. 1988). In figure 5 an image of luteal tissue stained by the modified *Masson* technique is visualized.

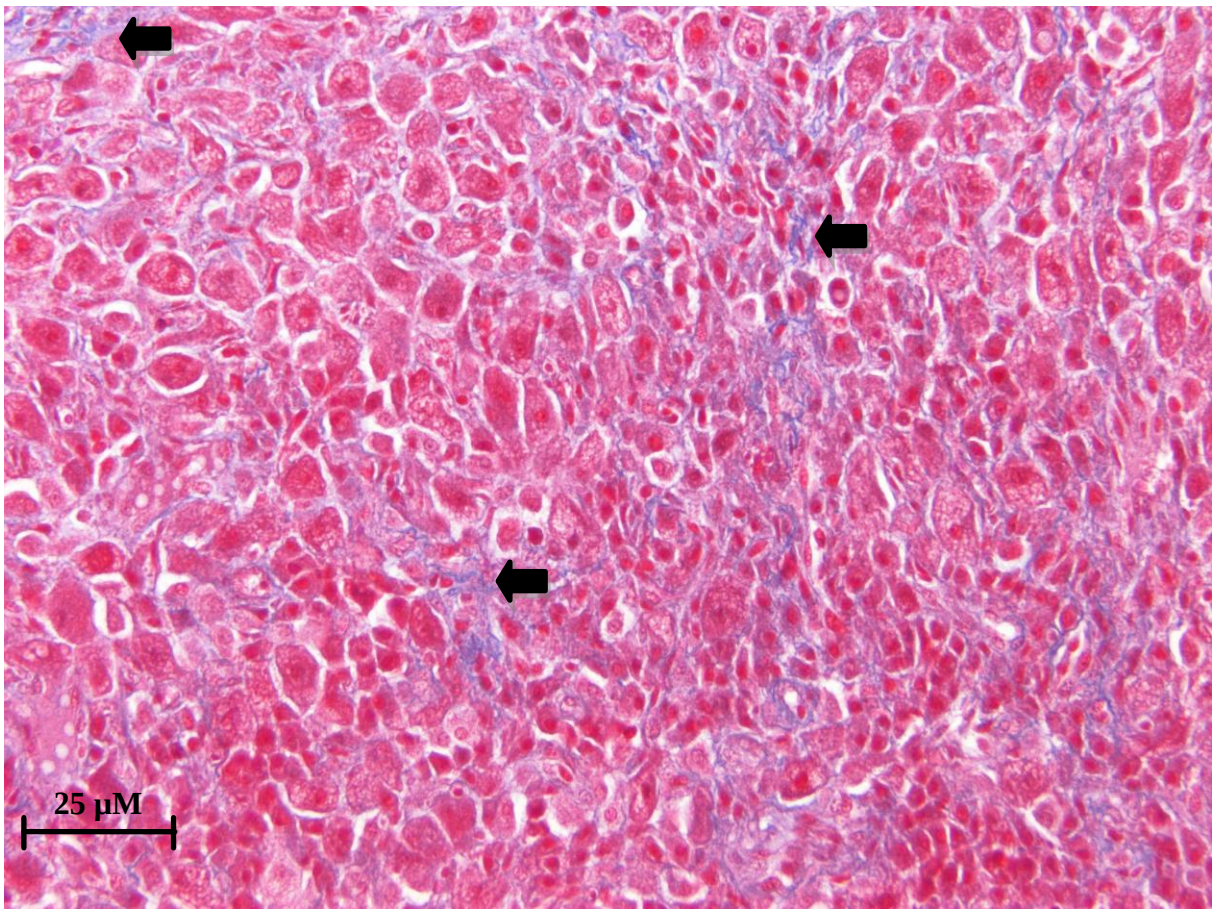


Figure 5: *a micrograph made by the camera mounted on the microscope, visualizing bovine luteal tissue stained with the modified Masson technique (magnification 400x). The arrows indicate some zones with collagen fibers, stained in blue*

Statistics

The results of the inner zone were compared with the data of the outer zone concerning the *'nuclear density'* and the *'area percentage'* in all 48 CLa. Statistical analysis of the results in subset 1 and 2 was carried out by means of the SPSS software package version 19 (IBM company, New York, USA). Based on the results of the normality tests (Kolmogorov-Smirnov, normal Q-Q-plot, detrended normal Q-Q plot, assessment of the boxplot, the skewness and kurtosis of the histogram), square root transformation was necessary for the nuclear density numbers and natural logarithm transformation for the collagen fibers and vascular tissue/lumina.

In subset 1, a mixed model was constructed with 4 outcome variables (sqrtTotal (square root of the Total nuclear density); sqrtLLC (Large Luteal Cells); sqrt SLC (Small Luteal Cells) and sqrtNSC (Non Steroidogenic Cells) and four independent fixed factors ('breed', n=2; 'luteal stage', n=2; 'fixation', n=2 and 'location', n=2). In subset 2, the same procedure was applied, but the model contains only two outcome variables (lnCOLLAGEN and lnVASCULAR TISSUE/LUMINA) and three independent fixed factors ('breed', n=2; 'luteal stage', n=2 and 'location', n=2). In all models, the factor 'CL' was included as a random effect to account for multiple observations within the same gland. The independent fixed factors were incorporated into the multivariable model when their significance level (P-value) in the univariable model was smaller than 0.15. Only those factors persisted in the final model when their P-value was smaller than or equal to 0.05 in the multivariable model. The final model was also tested for possible interactions.

Results

Topographic variation of the nuclear density (subset 1)

The effect of the four fixed factors ('breed', 'luteal stage', 'fixation' and 'location') on the four outcome variables ('sqrtTotal', 'sqrtLLC', 'sqrtSLC' and 'sqrtNSC') was assessed. When the independent variables were individually tested in an univariable model with CL included as random factor, the factors 'breed' and 'stage' had no significant influence on the outcome variables ($P > 0.15$). The effect of 'fixation' was significant for 'sqrtTotal' ($P=0.02$), 'sqrtLLC' ($P=0.005$) and 'sqrtNSC' ($P=0.005$). In the NBF fixated CLa, a higher nuclear density was seen concerning the LLC, NSC; compared to the ZsF-fixated samples. The fixation effect was the strongest on the NSC (table 1).

The effect of 'location' was significant for all cell types (sqrtTotal: $P < 0.001$; sqrtLLC: $P < 0.001$; sqrtSLC: $P < 0.001$; sqrtNSC: $P < 0.001$). This results in the following general linear model:

sqrtCell Type=β_f*fixation + β_l*location + β_{f*l}*(fixation*location) + ε, with

sqrtCell Type: the square root transformed nuclear density of each cell type

β: this factor describes the direction and magnitude of the association of each individual fixed factor (fixation, (f); location, (l); interaction between location and fixation, (f*l)) with sqrtCell Type

ε: the random error

Table 1. *estimators of the cell type specific mixed models*

cell type	**SqrtTotal**			**SqrtLLC**			**SqrtSLC**			**SqrtNSC**		
variable	**β[1]**	**SE[2]**	**P[3]**	**β**	**SE**	**P**	**B**	**SE**	**P**	**β**	**SE**	**P**
constant	5.37	0.14	<0.001	1.21	0.1	<0.001	2.45	0.12	<0.001	4.44	0.13	<0.001
breed												
HF	Ref[4]	…	0.8	Ref	…	0.5	Ref	…	0.9	Ref	…	0.5
BB	0.03	0.11		-0.06	0.08		0.004	0.1		0.08	0.1	
stage												
II	Ref	…	0.4	Ref	…	0.9	Ref	…	0.6	Ref	…	0.2
III	0.11	0.11		-0.01	0.08		-0.05	0.1		0.1	0.1	
fixation												
ZsF	Ref	…	0.019	Ref	…	0.005	Ref	…	0.9	Ref	…	0.005
NBF	0.33	0.13		0.29	0.09		0.01	0.12		0.4	0.1	
location												
Inner	Ref	…	<0.001	Ref	…	<0.001	Ref	…	<0.001	Ref	…	<0.001
Outer	0.72	0.03		0.39	0.03		0.54	0.03		0.5	0.03	
fixation*location	-0.05	0.03	0.1	-0.08	0.03	0.017	-0.05	0.04	0.1	-0.04	0.04	0.4

[1]the β-value (slope) of the different fixed factors; [2]the standard error of the variance estimate of the parameter; [3]the estimated significance level; [4]reference category

As the contribution of each independent factor was not significant for each individual cell type, the cell type specific models were expressed in table 1.

In table 2, the nuclear densities were expressed for each CL, differentiated for the three described cell types. The absolute nuclear density is higher in the outer zone when compared to the inner zone (as indicated in the linear model). Nevertheless, in most CLa, analysis of the relative nuclear densities revealed another tendency: the relative nuclear density of the NSC was higher in the inner zone, while the relative density of the steroidogenic cells (LLC and SLC) was lower in the inner zone when compared with the outer zone (table 2; $P < 0.05$).

Table 2. *comparison of the absolute and relative (%) number of nuclei of each cell type within the inner and outer zone, differentiated for each luteal gland (CL), breed and stage*

			absolute and relative (%) number of cells per micrograph (11 875 µm²)							
CL	**breed**	**stage**	LLC[a] (%)		SLC[b] (%)		NSC[c] (%)		Total[d]	
			inner	**outer**	**inner**	**outer**	**inner**	**outer**	**inner**	**outer**
1	**BB**	**III**	3 (8)	3 (7)	5 (15)	9 (22)	24 (77)	29 (71)	32	41
2	**HF**	**III**	2 (6)	2 (5)	5 (17)	7 (20)	23 (77)	27 (75)	30	36
3	**BB**	**II**	1 (4)	2 (5)	5 (15)	9 (23)	26 (81)	28 (72)	32	39
4	**BB**	**III**	1 (4)	2 (4)	5 (14)	6 (15)	27 (82)	35 (81)	33	43
5	**HF**	**II**	1 (4)	3 (7)	6 (21)	8 (21)	21 (75)	27 (72)	28	38
6	**HF**	**II**	2 (8)	2 (6)	5 (22)	7 (19)	21 (70)	21 (75)	28	30
7	**HF**	**III**	4 (12)	6 (17)	6 (18)	6 (17)	22 (70)	24 (66)	32	36
8	**HF**	**II**	4 (14)	4 (10)	5 (19)	8 (21)	18 (67)	25 (69)	27	37
9	**BB**	**II**	2 (8)	4 (11)	4 (15)	6 (19)	21 (77)	22 (70)	27	32
10	**BB**	**II**	2 (10)	4 (13)	5 (20)	7 (25)	16 (70)	18 (62)	23	29
11	**BB**	**II**	1 (4)	2 (5)	5 (15)	10 (22)	25 (81)	33 (73)	31	45
12	**BB**	**III**	1 (4)	3 (7)	8 (26)	14 (34)	21 (70)	24 (59)	30	41
13	**BB**	**II**	1 (4)	3 (8)	7 (26)	10 (28)	17 (70)	22 (64)	25	35
14	**BB**	**III**	1 (6)	3 (9)	7 (27)	11 (31)	16 (67)	20 (60)	24	34
15	**HF**	**II**	3 (9)	3 (7)	9 (31)	11 (29)	18 (60)	25 (64)	30	39
16	**HF**	**II**	2 (6)	4 (11)	8 (25)	11 (30)	22 (69)	22 (59)	32	37
17	**HF**	**III**	2 (7)	3 (8)	7 (23)	9 (24)	21 (70)	26 (68)	30	38
18	**HF**	**III**	2 (7)	3 (8)	5 (19)	7 (20)	20 (74)	26 (72)	27	36
19	**HF**	**II**	3 (9)	4 (10)	7 (20)	10 (24)	25 (71)	28 (66)	35	42
20	**HF**	**II**	3 (8)	5 (11)	7 (19)	10 (22)	26 (73)	30 (67)	36	45
21	**HF**	**II**	3 (9)	4 (9)	7 (21)	11 (24)	23 (70)	30 (67)	33	45
22	**HF**	**III**	3 (9)	4 (10)	8 (24)	12 (29)	22 (67)	25 (61)	33	41
23	**HF**	**II**	2 (6)	4 (9)	8 (23)	11 (24)	25 (71)	31 (67)	35	46
24	**HF**	**III**	3 (9)	4 (9)	7 (21)	11 (26)	23 (70)	28 (65)	33	43
25	**HF**	**III**	3 (9)	4 (10)	7 (22)	10 (24)	22 (69)	27 (66)	32	41
26	**HF**	**III**	3 (9)	4 (10)	7 (20)	10 (23)	25 (71)	29 (67)	35	43
27	**BB**	**III**	3 (8)	4 (9)	7 (19)	11 (24)	26 (73)	30 (67)	36	45
28	**BB**	**III**	2 (6)	4 (9)	7 (19)	11 (23)	27 (75)	32 (68)	36	47
29	**BB**	**III**	3 (9)	4 (9)	7 (20)	11 (25)	25 (71)	29 (66)	35	44
30	**BB**	**II**	3 (8)	4 (10)	8 (23)	11 (26)	24 (69)	27 (64)	35	42
31	**BB**	**II**	2 (6)	4 (10)	7 (21)	10 (25)	24 (73)	26 (65)	33	40
32	**BB**	**III**	2 (6)	4 (9)	6 (18)	10 (23)	25 (76)	30 (68)	33	44
33	**BB**	**II**	3 (9)	5 (11)	8 (23)	13 (30)	24 (68)	26 (59)	35	44

[a]: large luteal cells; [b]: small luteal cells; [c]: cells other than LLC and SLC (endothelial cells, pericytes, fibroblasts, -cytes); [d]: the sum of LLC, SLC and NSC; HF: Holstein Friesian, BB: Belgian Blue; stage II: cycle day 5 to 10, stage III: cycle day 11 to 17.

The volume density occupied by the different morphological components in the CL (subset 2)

Table 3. *comparison of the volume density occupied by collagen fibers and vascular tissue/lumina between the inner zone and the outer zone: medians are expressed, together with minimal and maximal value [min; max], differentiated for each luteal gland (CL), breed and stage*

CL	breed	stage	collagen		vessels	
			outer	inner	outer	inner
1	**BB**	**II**	0 [0;13.1]	1.1 [0;8.6]	11.8 [1.7;23.6]	12.2 [2.7;23.2]
2	**HF**	**II**	0.4 [0;11.6]	0.9 [0;7.2]	8.6 [3.8;19.6]	8.4 [1.5;17.9]
3	**HF**	**II**	0.6 [0;19.4]	0.8 [0;9.7]	8.8 [1.7;35.2]	9.7 [2.9;57.7]
4	**HF**	**II**	1.3 [0;12]	1.9 [0.2;11.4]	13.9 [2.7;24.6]	11.8 [4.4;25.3]
5	**HF**	**III**	0.4 [0;14.1]	1.3 [0;35.2]	9.7 [3.6;23.4]	10.7 [4.2;33.9]
6	**HF**	**II**	1.1 [0;9.7]	3.3 [0.2;8]	9.5 [4.6;50.5]	11.2 [3.2;29.9]
7	**HF**	**III**	0.6 [0;14.1]	2.5 [0.2;18.7]	11.6 [5.7;36.6]	10.3 [2.7;21.1]
8	**HF**	**II**	1.3 [0;10.5]	1.5 [0;12.2]	9.3 [1.3;39.6]	8.4 [1.7;31.8]
9	**HF**	**III**	0.6 [0;21.1]	3.2 [0.2;37.7]	9.7 [0.6;23.2]	10.1 [4.4; 23.4]
10	**BB**	**III**	0.0 [0;21.5]	0.8 [0;17.3]	11.2 [2.3;28.8]	12.6 [4.8;24.4]
11	**BB**	**III**	0.8 [0;20]	1.3 [0;30.9]	9.3 [0.6;21.9]	11.1 [4.2;22.5]
12	**BB**	**III**	1.9 [0;15.6]	2.5 [0;14.1]	11.2 [1.5;38.3]	12.2 [4.2;26.3]
13	**BB**	**II**	0.2 [0-4.2]	0.2 [0;4.6]	10.7 [3.4;24.6]	12.6 [2.1;26.3]
14	**BB**	**II**	0.2 [0;6.5]	0.4 [0;3.6]	11.0 [4;19.4]	9.5 [3.6;19.8]
15	**BB**	**III**	0.4 [0;12.4]	1.1 [0;33.9]	10.1 [5.5;20.2]	9.9 [3.4;28.8]

HF: Holstein Friesian, BB: Belgian Blue; stage II: cycle day 5 to 10, stage III: cycle day 11 to 17

In view of these analyses, two different 'morphological components' were defined: *'collagen fibers'* and *'vascular tissue/lumina'*. Table 3 gives an overview of the volume density occupied by the different components in the inner and outer zone.

The median amount of collagen fibers and vessels in the inner zone was higher when compared with the outer zone ($P < 0.05$).

Again, a general model could be constructed as follows:
morphological component=β_b*breed + β_s*stage + β_l*location + β_{b*s}*(breed*stage) + β_{s*l}*(stage*location) + β_{b*l}*(breed*location).

In table 4, the component-specific estimators are visualized.

Table 4. *estimators of the morphological component specific mixed models*

morphological component	**LnCollagen fibers**			**LnVascular tissue/lumina**		
variable	**β[1]**	**SD[2]**	**P[3]**	**β**	**SD**	**P**
constant	2.60	0.1	<0.001	3.97	0.05	<0.001
breed						
HF	Ref[4]	…	0.01	Ref	…	0.3
BB	-0.33	0.11		-0.081	0.08	
stage						
II	Ref	…	0.003	Ref	…	0.7
III	0.40	0.11		0.036	0.08	
location						
Inner	Ref	…	<0.001	Ref	…	0.001
Outer	-0.29	0.03		-0.093	0.03	
breed*stage	0.18	0.23	0.4	-0.15	0.11	0.18
stage*location	-0.017	0.06	0.8	0.072	0.03	0.03
breed*location	0.091	0.06	0.1	0.062	0.03	0.06

[1]the β-value (slope) of the different fixed factors; [2]the standard error of the variance estimate of the parameter; [3]the estimated significance level; [4]reference category

Corpora lutea of HF contain more collagen, while there is no significant difference concerning vascular tissue/lumina. Younger CLa (stage II) contain more collagen fibers, while the surface occupation of vascular tissue/lumina is not different between both luteal stages. Within a CL, the outer zone contains less collagen fibers and vascular tissue/lumina in comparison with the inner zone.

Discussion

The main purpose of the present study was to assess the variation in the topographic distribution of the total and cell type dependent nuclear density number, and the amount of collagen fibers and vascular tissue/lumina within a bovine CL in order to get an idea about the stringency of the sample site location. This question was analyzed based on 3 main hypotheses, questioning the effect of 'location', 'luteal age' and 'breed'. Also the effect of the fixation protocol on the topographic spread of the different cell types was analyzed.

There is an abundance of studies describing the histology of bovine CLa. But literature data describing the topographic variation of the luteal histology within the bovine CL are scarce. To the best of our knowledge, we are aware of only one study reporting some small differences in cellular composition between the 'inner and outer region' of ovine CLa (Rodgers et al. 1984), and one study analyzing the amount of collagen type 1 in a bovine CL (Silvester and Luck 1999), but no studies assessing the topographic variation of the nuclear density in bovine CLa. Nevertheless, this knowledge is strictly necessary when one wants to design a sampling procedure to monitor luteal histology in living cows based on one or more biopsies.

We selected nuclear density, area percentage of collagen fibers and vascular tissue/lumina as parameters for further assessment, since we aimed to analyze the histological parameters most decisive for progesterone production. When we simplify the progesterone production process to its most essential steps, the level of progesterone production is basically depending on: 1) the acquisition of constituents by the blood supply, 2) the production of progesterone in the 'production units' (i.e. the steroidogenic cells), and 3) the evacuation of residues and progesterone by the luteal circulation. So, by visualization of the volume density of collagen fibers and vascular tissue/lumina, the level of acquisition and evacuation was analyzed. The nuclear density, on its turn, is an histological trait useful for the assessment of the level of progesterone production. Moreover, these parameters are straightforward and relatively easy and cheap to analyze by light microscopy and hence suitable to analyze on a large scale.

But does it make sense to analyse nuclear density as a possible explanatory factor for progesterone production? Based on the current knowledge this is difficult to answer. There are several studies reporting a positive correlation between the CL-size and the progesterone blood concentration in dairy or beef cattle, indicating that an increased amount of steroidogenic tissue is associated with a higher blood progesterone level (Assey et al.1993; Peters et al. 1994; Segerson et al. 1984; Spell et al. 2001; Yung et al. 1996). Smaller

ovulatory follicles give lower progesterone plasma concentrations on day 7 and 14 and give lower luteal weight on day 7 and 14 (Vasconcelos et al. 2001). Poor follicular development may result in an inadequate corpus luteum (Wathes et al. 2003). Smaller ovulatory follicles give lower luteal weights ($r=0.73$; $p < 0.1$) and lower plasma progesterone concentrations ($r=0.64$; $p < 0.01$) on day 5= (Robinson et al. 2005).

Green et al. (2007) found no correlation between the plasma progesterone concentration and the progesterone production (in vivo/in vitro) per unit tissue in cycle day 5 luteal glands of non-lactating and late lactation dairy cows. But there was a correlation between plasma progesterone concentration and total luteal in vivo progesterone content ($r^2==0.69$; $p < 0.001$), and total synthetic in vitro capacity ($r^2==0.43$; $p < 0.001$). The luteal mass in cows in late lactation was higher compared to the non lactating cows, leading to a higher synthetic capacity.

The relation between luteal mass and plasma progesterone is not always that consistent.

Yung et al. (1996) found no relationship between luteal mass and plasma progesterone in case of a negative energy balance in one year old Holstein Friesian heifers on cycle day 10.

Lucy et al. (1995) found no relationship between CL size and plama progesterone on cycle day 17 after treatment with bovine somatotropin hormone (bST).

A lot of these studies analyzed the relationship between CL size and plasma progesterone production during the period that both factors are increasing significantly. This could confound the assessment of this relationship. So, Mann (2009) reanalyzed different studies to compare CL-weight and plasma progesterone across the luteal phase. From cycle day 5 to 8, there is a significant increase in CL-weight, while there was no further increase from cycle day 8 to 16. But plasma progesterone is increasing during this whole period. As a consequence, there is a discrepancy between the moment of maximal luteal diameter (day 8) and the maximal plasma progesterone concentration (day 13; Taylor and Rajamahendran, 1991).

Sartori et al. (2002) found a positive correlation between the luteal mass and the plasma progesterone concentration in heifers and lactating cows on day 7, but not in dry cows. Lactating cows had a higher CL volume versus heifers, but showed a lower progesterone concentration in the serum on day 7. They concluded that lactating cows have similar or lower progesterone in serum versus dry cows or heifers, despite larger CL. Could there be an effect of luteal nuclear density on the plasma progesterone concentration? Studies dealing with this aspect are rather scarce. Milvae et al. (1991) give the most extreme example of a possible link between progesterone production and nuclear density. They

removed granulosa cells out of preovulatory follicles of heifers and reinjected those cells again in some of them. Progesterone production by the CL, originating from those follicles was compared with a control group. The animals, in which the granulosa cells were given back showed the same plasma progesterone concentration as the control animals. The animals, which lost their granulosa cells showed a significantly reduced progesterone concentration from cycle day 7 to 17 (7 ng/ml versus 1.5 ng/ml). Approximately 61 to 86% of the total number of granulosa cells were removed. There was no significant difference in the cycle length between the different groups. Garcia et al. (1981) stated that the more the granulosa cells are removed at aspiration, the worse the luteal disruption will be. They did not analyze the luteal histology, but hypothesized a reduction in LLC. In ewes, O'Shea et al. (1984) found that luteal weight and plasma progesterone concentration were lower in ewes in which ovulation was induced during anoestrus than in cyclic control ewes in the breeding season. The luteal volume was positively correlated with total number of cells per CL (r==0.87; p=0.01) but negatively correlated with number of cells per mm^3 luteal tissue (r=-0.68; p=0.05). So, smaller CL contained fewer cells, but more cells per unit volume. The cellular diameter of the small and large luteal cells was smaller in induced CL. Maciel et al. (1992) reported that when CLa of superovulated heifers were compared to control heifers, there was a positive correlation of plasma progesterone on day 7 with mass and volume of the CL (r=0.97; $p < 0.005$). But the histology (volume density) was in both groups the same. Mee et al. (1993) reported that GnRH-supplementation at estrus increased the proportion of LLC with an increment in plasma progesterone concentrations, which increased earlier. So, it seems that nuclear density could be influenced. Robinson et al. (2006) compared macroscopic and cellular characteristics of cows with an early progesterone rise versus a delayed rise. The CL weight is smaller in the delayed group, but the total CL progesterone content was the same. The volume occupied by steroidogenic cells and endothelial cells showed no difference between both groups. The mean cell diameter of the small and large luteal cells did not show a group effect. In the cows with a delayed progesterone rise a higher luteal cell density was seen, eventually because of hypertrophy or proliferation of supporting cells, possibly leading to a less developmentally advanced CL. There was no correlation between luteal cell density or gland's diameter and plasma progesterone. So, when literature is explored, there are several studies justifying our assumption that differences in luteal histology can be used as a trade mark for luteal progesterone production. But the relationship between nuclear density and plasma progesterone concentration is not always that obvious and needs further research.

As luteogenesis is a dynamic process, defining the luteal cycle stage was necessary before inclusion of the glands. First of all, only those CLa were selected that showed active progesterone production (peripheral blood progesterone concentration >5ng/ml). To further classify the included CLa, we applied the methodology described by Miyamoto et al. (2000) and D'haeseleer et al. (2006), taking into account the gross appearance of the CL, and the appearances of both the accompanying follicles and the uterus. We only selected CLa of stage II and III. Stage I CLa were not selected as tissue samples taken by a biopsy needle from such young CLa would be difficult to process because of their loose structure and hemorrhagic character. Stage IV CLa were not taken into account, as they were not of interest for our research because they are approaching luteolysis.

We are fully aware that the morphometric methods applied in our study were subject to some caveats. As the sample size was high, an HE-overview staining in combination with an optimal preservation, fixation and embedding was preferred above the much more expensive immunohistochemical techniques. Classification of the nuclei was based on a set of light microscopic criteria generally accepted in literature (Donaldson and Hansel 1965; Fields and Fields 1996; O'Shea 1987; O'Shea et al. 1989; Wiltbank 1994), and was performed in a standardized way. The latter implies that, even when there was a certain but limited degree of misclassification, it was still possible to assess the effect of other factors as was done in the study.

Fixation may have caused significant cell shrinkage and may have affected the measurement of vascular space (Dharmarajan et al. 1983). Perfusion fixation of the CLa was however not possible in our experiment. Because of fixation by immersion, the volume fraction of the vascular tissue/lumina was probably slightly underestimated. The level of underestimation is however very likely to be identical in the different subsets, still allowing us to accurately estimate the effect of the fixed factors. The shrinkage level is depending on the fixation protocol. Schwall et al. (1986) fixed dispersed ovine luteal cells in 1% paraformaldehyde-0.1 M plosphate buffer (pH 7.4) for 20 min at 4°C. Fixation induced a reduction of 11% of the cell diameter of both steroidogenic and non-steroidogenic luteal cells. O'Shea et al. (1989) calculated a shrinkage factor of 0.85 by measuring weight, and volume by fluid displacement of slices of luteal tissue before and after fixation. Fixation was done with Bouin's fluid (no further details about the protocol were given). Assessment of a shrinkage factor is not done in our study. But the effect of fixation on the nuclear density was always positive (with ZsF fixation as reference), indicating that the shrinkage factor of NBF-fixation was always higher than that of the ZsF-fixation. Based on this observation and based on the fact that the luteal

glands are randomly assigned to both fixation protocols, we can conclude that the shrinkage factor for NBF-fixation is probably higher than for ZsF-fixation. So, nuclear densities assessed on ZsF-fixed samples will probably better represent the real, original situation in fresh tissue.

Based on the results of our study, breed and luteal stage were not significantly associated with nuclear density, meaning that there is no significant influence of the breed (HF vs. BB) or the luteal stage (II vs. III) on the nuclear density of the different cell types (LLC, SLC, and NSC). The latter is in conflict with the results of Lei et al. (1991), who concluded that the NSC% is significantly associated with the luteal stage (stage II: 74%; stage III: 60%; $P < 0.05$). However, their results were based on just one CL per mentioned stage, which is, in our opinion, insufficiently taking into account the rather high inter cow variation as was found in the present study. Looking to the square-root-transformed total nuclear density number, expressed as the sqrtTotal, we can state that the location (b=0.72) has a greater effect on the total nuclear density than the type of fixation (b=0.33). This trend can be extrapolated to the individual cell types, but the difference between both effects is not that obvious as for the sqrtTotal. Concerning the cellular composition of a bovine CL, it is difficult to compare our nuclear density data with other studies, as they mostly assessed the volume density by point counting (Fields and Fields 1996; Neves and Marques 2006; O'Shea et al. 1989), a technique not used in our experiment.

The relative collagen fiber content (volume density) was situated in the same range as was found in the study of Parkinson et al.(1994): 1.5 - 2% on day 7 or 8 of the cycle. Based on our results, it is clear that the relative collagen fiber content in bovine CLa is higher in CLa of stage III, in the inner zone of the gland and in CLa of HF cows. The effect of stage (b=0.40) was greatest, followed by breed (b=-0.33) and location (-0.29). As in our results, Silvester and Luck (1999) stated that the inner zone of the CL contains significantly more collagen type 1 than the outer zone. The relative vessel content generally follows the same trends as the collagen fiber content, but the effect of luteal stage and breed was not significant. So, for the relative vessel content, only the factor location (P=0.001) had a significant influence.

The fact that a CL is originating from a collapsed follicle partially explains the described luteal topographic architecture. The higher collagen fiber content and area occupied by vessels in the inner zone could at least partly be explained by the increasing density of collagenous/vascularized trabeculae in the direction of the center (like the spokes in a wheel). Besides that, Silvester and Luck (1999) stated that within a luteal lobe, the amount of collagen type I increased in the inner parenchymal tissue, while there was no significant

increase detected in the outer region. The latter means that as well between the parenchymal lobes as within one lobe, the chance of attendance of collagen type 1 is higher in the inner zone when compared to the outer. The latter phenomenon explains on his turn the decreasing trend in nuclear density within the lobes going from the outer to the inner region. Furthermore, based on the constitution of a follicle, the inner zone of the collapsed follicle contains relatively more granulosa cells (remnants of the avascular membrana granulosa) while the theca interna cells (which are accompanied by a vascular network) were more abundant in the outer zone. During luteogenesis, hypertrophy is more important than hyperplasia in the granulosa cell population, while hyperplasia is more important for theca interna cells (Donaldson and Hansel 1965; McClellan et al. 1975). Keeping this in mind and supposing that there is only a partial migration of the steroidogenic cells, this leads to a heterogenic 'cell mixture': an inner zone with a lower nuclear density (relatively more LLC) and an outer zone with a higher nuclear density because of the presence of a relatively higher number of small luteal cells. It is furthermore known that after ovulation the vascularisation is migrating from the theca interna into the membrana granulosa (Irving-Rodgers et al. 2006). This could initially give rise to a higher number of NSC in the outer region. The topographic spread of the NSC was in our results not that obvious as the spread of the steroidogenic cells. Based on the fact that: 1) luteogenesis starts from a collapsed follicle; 2) the fibrillar collagen content is higher in the inner zone; 3) cellular mixing by migration is rather limited; 4) hypertrophy is more important for LLC and hyperplasia for SLC; 5) vascularisation is invading the granulosa layer, starting from the theca layer; we can explain the higher absolute nuclear density of SLC and NSC in the outer zone. Following this reasoning, one should expect a higher nuclear density of LLC in the inner zone. Nevertheless, the opposite is true in our data. In their study, Alila and Hansel (1984) suggest the possibility of SLC transforming to LLC in bovine CLa. As the SLC are more abundant in the outer when compared to the inner zone, this could explain the higher number of LLC in the outer zone in the present study.

A final and most important point of discussion is the stringency of the sampling site when taking one single biopsy sample in order to get an overview of the complete CL. In our study, we found a general trend that the nuclear density of all examined cell types reduced when moving from the outer to the inner zone. The latter was especially the case for the steroidogenic cells, and among the steroidogenic cells for the SLC. But, the behavior in topographic distribution of all the cell types was basically the same. The latter implies that in view of taking a biopsy of a bovine CL in order to analyze cell-associated traits, the direction

of the biopsy does not really matter, as long as it goes through the inner zone of the CL and contains the total diameter of the CL. Concerning the morphological components, the collagen fiber content and the amount of vessels are increasing from the outer to the inner zone. Consequently, the same sampling criteria are applicable for assessing these histological parameters.

As a conclusion we can state that the topographic difference in nuclear density for the most prominent cell types within a bovine CL is only significant when comparing the inner versus the outer zone. This trend is independent of luteal stage (stage II vs. III) and breed (HF vs. BB), but significantly depends on fixation protocol. The lower nuclear density in the inner zone was compensated by a higher amount of collagen fibers and vascular tissue/lumina. For future purposes, our results imply that if a sample representative for the whole gland has to be taken, it is necessary that the biopsy goes through the inner zone and includes the total diameter of the gland.

Acknowledgements

The BOF is thanked for their financial support (05B01906). Also Lobke De Bels and Liliana Standaert of the laboratory of the Department of Morphology are thanked for their technical support and advice.

Reference List

Abecia JA, Lozano JM, Forcada F, Zarazaga L, 1997: Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim Reprod Sci **48**, 209-218.

Alila HW, Hansel W, 1984: Origin of different cell types in the bovine corpus luteum as characterized by specific monoclonal antibodies. Biol Reprod **31**, 1015-1025.

Assey RJ, Purwantara B, Greve T, Hyttel P, Schmidt MH, 1993: Corpus-luteum size and plasma progesterone levels in cattle after cloprostenol-induced luteolysis. Theriogenology **39**, 1321-1330.

Bradbury P, Gordon K, 1977: Connective tissues and stains. In: Bancroft JD, Stevens A (eds), Theory and practice of histological techniques. Churchill Livinstone New York, p. 104.

Cools S, Van den Broeck W, de Kruif A, Opsomer G, 2009: Is taking a biopsy a relevant technique to monitor bovine luteal function in vitro? Reprod Domest Anim. **44** (Suppl. 3), 61.

Cools S, Van den Broeck W, De Vliegher S, Piepers S, Opsomer G, 2012: The bovine luteal histological composition: a topographic point of view. Reprod Domest Anim **47** (Accepted)

D'haeseleer M, Cocquyt G, Van Cruchten S, Simoens P, Van den Broeck W, 2006: Cell-specific localisation of apoptosis in the bovine ovary at different stages of the estrous cycle. Theriogenology **65**, 757-772.

Dharmarajan AM, Mastroyannis C, Yoshimura Y, Atlas SJ, Wallach EE, Zirkin BR, 1988: Quantitative light microscopic analysis of corpus luteum growth during pseudopregnancy in the rabbit. Biol Reprod **38**, 863-870.

Dharmarajan AM, Meyer GT, Bruce NW, 1983: Morphometric analysis of the corpus luteum of 16-day pregnant rats: The effect of preparative procedures on volume of luteal cell, interstitial, and vascular components. Am. J Anat **168**, 51-65.

Donaldson L, Hansel W, 1965: Histological study of bovine corpora lutea. J Dairy Sci **48**, 905-909.

Fields MJ, Fields PA, 1996: Morphological characteristics of the bovine corpus luteum during the estrous cycle and pregnancy. Theriogenology **45**, 1295-1325.

Garcia J, Jones GS, Acosta AA, Wright GL Jr, 1981: Corpus luteum function after follicle aspiration for oocyte retrieval. Fertil Steril **36**, 565-572.

González L, Anderson I, Deane D, Summers C, Buxton D, 2001: Detection of immune system cells in paraffin wax-embedded ovine tissues. J Comp Pathol **125**, 41-47.

Green MP, Mann GE, Hunter MG, 2007: Luteal characteristics and progesterone production on day 5 of the bovine oestrous cycle. Reprod Domest Anim **42**, 643-647.

Hopwood D, 1977: Fixation fixatives. In: Bancroft JD, Stevens A (eds.), Theory and practice of histological techniques. Churchill Livingstone New York, p. 26.

Ireland JJ, Murphee RL, Coulson PB, 1980: Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J Dairy Sci **63**, 155-160.

Irving-Rodgers HF, Catanzariti KD, Aspden WJ, D'Occhio MJ, Rodgers RJ, 2006: Remodeling of extracellular matrix at ovulation of the bovine ovarian follicle. Mol Reprod Dev **73**, 1292-1302.

Kot K, Anderson LE, Tsai SJ, Wiltbank MC, Ginther OJ, 1999: Transvaginal, ultrasound-guided biopsy of the corpus luteum in cattle. Theriogenology **52**, 987-993.

Lei ZM, Chegini N, Rao CV, 1991: Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol Reprod **44**, 1148-1156.

Lucy MC, Thatcher WW, Collier RJ, Simmen FA, Ko Y, Savio JD, Badinga L, 1995: Effects of somatotropin on the conceptus, uterus, and ovary during maternal recognition of pregnancy in cattle. Domest Anim Endocrinol **12**, 73-82.

Maciel M, Rodriguezmartinez H, Gustafsson H, 1992: Fine-structure of corpora-lutea in superovulated heifers. ZBL Vet Med A **39**, 89-97.

Mann GE, 2009: Corpus luteum size and plasma progesterone concentration in cows. Anim Reprod Sci **115**, 296-299.

Mann GE, Lamming GE, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction **121**, 175-180.

McClellan MC, Dieckman MA, Abel Jr, JH, Niswender GD, 1975: Luteinizing hormone, progesterone and the morphological development of normal and superovulated corpora lutea in sheep. Cell Tissue Res **164**, 291-307.

Mee MO, Stevenson JS, Alexander BM, Sasser RG, 1993: Administration of GnRH at estrus influences pregnancy rates, serum concentrations of LH, FSH, estradiol-17beta, pregnancy-specific protein B, and progesterone, proportion of luteal cell types, and in vitro production of progesterone in dairy cows. J Anim Sci **71**, 185-198.

Milvae RA, Alila HW, Bushmich SL, Hansel W, 1991: Bovine corpus luteum function after removal of granulosa cells from the preovulatory follicle. Domest Anim Endocrin **8**, 439-443.

Miyamoto Y, Skarzynski DJ, Okuda K, 2000: Is tumor necrosis factor alpha a trigger for the initiation of endometrial prostaglandin F(2alpha) release at luteolysis in cattle? Biol Reprod **62**, 1109-1115.

Neves MM, Marques Jr, AP, 2006: Volumetric proportions of the corpus luteum constituents of Nelore. Arq Bras Med Vet Zootec **58**, 944-946.

Niswender GD, Reimers TJ, Diekman MA, Nett TM, 1976: Blood flow: a mediator of ovarian function. Biol Reprod **14**, 64-81.

O'Shea JD, 1987: Heterogeneous cell types in the corpus luteum of sheep, goats and cattle. J Reprod Fertil Suppl **34**, 71-85.

O'Shea JD, Rodgers RJ, D'Occhio MJ, 1989: Cellular composition of the cyclic corpus luteum of the cow. J Reprod Fertil **85**, 483-487.

O'Shea JD, Rodgers RJ, Wright PJ, 1984: Morphometric analysis and fuction in vivo and in vitro of corpora lutea from ewes treated with LHRH during seasonal anoestrus. J Reprod Fertil **72**, 75-85.

Parkinson TJ, Turvey A, Jenner LJ, 1994: A morphometric analysis of the corpus luteum of the cow during the estrous cycle and early pregnancy. Theriogenology **41**, 1115-1126.

Parr RA, Davis IF, Miles MA, Squires TJ, 1993: Liver blood flow and metabolic clearance rate of progesterone in sheep. Res Vet Sci **55**, 311-316.

Peters KE, Bergfeld EG, Cupp AS, Kojima FN, Mariscal V, Sanchez T, Wehrman ME, Grotjan HE, Hamernik DL, Kittok RJ, 1994: Luteinizing hormone has a role in development of fully functional corpora lutea (CL) but is not required to maintain CL function in heifers. Biol.Reprod **51**, 1248-1254.

Rabiee AR, Macmillan KL, Schwarzenberger F, 2001: The effect of level of feed intake on progesterone clearance rate by measuring faecal progesterone metabolites in grazing dairy cows. Anim Reprod Sci **67**, 205-214.

Robinson RS, Hammond AJ, Hunter MG, Mann GE, 2005: The induction of a delayed post-ovulatory progesterone rise in dairy cows: a novel model. Domest Anim Endocrinol **28**, 285-295

Robinson RS, Hammond AJ, Nicklin LT, Schams D, Mann GE, Hunter MG, 2006: Endocrine and cellular characteristics of corpora lutea from cows with a delayed post-ovulatory progesterone rise. Domest Anim Endocrinol **31**, 154-172.

Rodgers RJ, O'Shea JD, Bruce NW, 1984: Morphometric analysis of the cellular composition of the ovine corpus luteum. J Anat **138**, 757-770.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC, 2002: High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle. J Dairy Sci **85**, 2831-2842.

Sartori R, Rosa GJ, Wiltbank MC, 2002: Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci **85**, 2813-2822.

Schwall RH, Gamboni F, Mayan MH, Niswender GD, 1986: Changes in distribution of sizes of luteal cells during the estrous cycle. Biol Reprod **34**, 911-918.

Segerson EC, Hansen TR, Libby DW, Randel RD, Getz WR, 1984: Ovarian and uterine morphology and function in Angus and Brahman cows. J Anim Sci **59**, 1026-1046.

Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE, 1990: Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil **90**, 1-10.

Silvester LM, Luck MR, 1999: Distribution of extracellular matrix components in the developing ruminant corpus luteum: a wound repair hypothesis for luteinization. J Reprod Fertil **116**, 187-198.

Spell AR, Beal WE, Corah LR, Lamb GC, 2001: Evaluating recipient and embryo factors that affect pregnancy rates of embryo transfer in beef cattle. Theriogenology **56**, 287-297.

Sreenan JM, Diskin MG, 1983: Early embryonic mortality in the cow: its relationship with progesterone concentration. Vet Rec **112**, 517-521.

Stevens A, 1977: The haematoxylins. In: Bancroft JD, Stevens A (eds.), Theory and practice of histological techniques. Churchill Livingstone New York, p. 89.

Taylor C, Rajamahendran R, 1991: Follicular dynamics and corpus-luteum growth and function in pregnant versus nonpregnant dairy-cows. J Dairy Sci **74**, 115-123.

Vasconcelos JLM, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC, 2001: Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology **56**, 307-314.

Virolainen JV, Love RJ, Tast A, Peltoniemi OA, 2005a: Plasma progesterone concentration depends on sampling site in pigs. Anim Reprod Sci **86**, 305-316.

Virolainen JV, Peltoniemi OA, Munsterhjelm C, Tast A, Einarsson S, 2005b: Effect of feeding level on progesterone concentration in early pregnant multiparous sows. Anim Reprod Sci **90**, 117-126.

Virolainen JV, Tast A, Sorsa A, Love RJ, Peltoniemi OA, 2004: Changes in feeding level during early pregnancy affect fertility in gilts. Anim Reprod Sci **80**, 341-352.

Wathes DC, Taylor VJ, Cheng Z, Mann GE, 2003: Follicle growth, corpus luteum function and their effects on embryo development in postpartum dairy cows. Reprod.Suppl **61**, 219-237.

Wiltbank MC, 1994: Cell types and hormonal mechanisms associated with mid-cycle corpus luteum function. J Anim Sci **72**, 1873-1883.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A, 2006: Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology **65**, 17-29.

Yung MC, VandeHaar MJ, Fogwell RL, Sharma BK, 1996: Effect of energy balance and somatotropin on insulin-like growth factor I in serum and on weight and progesterone of corpus luteum in heifers. J Anim Sci **74**, 2239-2244.

Chapter 4.3

Validation of a luteal biopsy technique to assess bovine luteal morphology

Cools S[1], Van den Broeck W[2], De Vliegher S[1], Piepers S[1], Opsomer G[1]

[1]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

[2]Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

To be submitted

Abstract

The present paper describes a sampling procedure to monitor the luteal histology via a biopsy of the corpus luteum in cows. Also, different fixation protocols were tested. In total, 46 luteal glands (23 in luteal stage II and 23 in stage III), were randomly assigned to three groups and fixated by different immersion protocols: subset 1a (N=19; neutral-buffered 3.5 % formaldehyde) and 1b (N=8; Zinc salts Fixative) to assess the nuclear density, and subset 2 (N=19; Bouin's fixative) to analyze the volume density of collagen fibers and vascular tissue. To validate the biopsy technique, two biopsies were taken out of the glands, one perpendicular to the equatorial plane, containing the ovulation point and one along the equatorial plane. A tissue disk ('Central Disk'), containing both prelevation sites of the biopsies and the luteal center was isolated.

The results obtained from biopsy sections were compared with those obtained from sections made out of the whole luteal gland (i.e. the Central Disk; overview sections). The results obtained from the central disk are assumed to be the gold standard. For nuclear density, a good agreement was observed between the biopsy sections and the overview sections. The highest agreement index was obtained for the ZsF-fixation (0.94; P-value < 0.001). Regarding the volume density of the vessels and collagen fibers (analyzed in the biopsies and central disks of subset 2), the agreement between biopsies and overviews was only high enough (more than 0.90) for the distribution of vascular lumina.

In conclusion, this biopsy technique allows us to analyze histological and eventually histophysiological characteristics of the complete luteal gland, based on one single biopsy sample. Still, a prerequisite is that these histological traits are correlated with the parameter 'nuclear density' (for example intracellular steroidogenic enzymes) or the parameter 'vessels' (such as endothelial specific lectines). The representativeness for the results of a biopsy taken through the luteal gland's central point and containing the total diameter of the luteal gland with the gold standard depends on the fixative that is used.

Key words: cow; corpus luteum; morphology; validation; biopsy

Introduction

The increasing level of milk production in modern high yielding dairy cows has been associated with a significant decrease in the cows' reproductive performances. Besides the delayed resumption of postpartal cyclicity (Opsomer and de Kruif, 1999), embryonic death has been demonstrated to be a major contributor to the globally mentioned fertility decline (Sreenan and Diskin, 1983; Diskin and Morris, 2008). One of the currently most striking hypotheses is the one claiming that lower peripheral progesterone levels during the first days after insemination are associated with significantly higher levels of early embryonic death (Shelton et al., 1990; Mann and Lamming, 2001). Cows with reduced postovulatory concentrations of progesterone suffer from an impaired embryonic development ending up with an unsatisfactory interferon-τ (IFNτ) production and a higher risk to return in heat because of the inability to prevent uterine prostaglandin secretion (Mann and Lamming, 2001). Several authors demonstrated a positive correlation between the size of the ovulatory follicle and the postovulatory plasma progesterone rise in both dairy heifers (Moreira et al., 2000) and dry as well as lactating dairy cows (Sartori et al., 2002). The increase in peripheral progesterone concentration expressed per cubic millimeter luteal tissue up to day seven following ovulation was however markedly lower in lactating dairy cows in comparison with their non-lactating counterparts, which let the authors to conclude that lactation significantly hampers the postovulatory progesterone rise (Sartori et al., 2002) . In a recent study, Pretheeban et al. (2009) found that the IFNτ-mRNA expression in pre-implantation embryos of lactating cows was lower than in pre-implantation embryos of heifers, indicating another level at which lactation may negatively interfere with fertility in dairy cattle.

Currently, there is a lot of debate concerning the underlying reasons for the reduced peripheral progesterone concentrations in lactating dairy cows. Whether or not the luteal progesterone production is impaired and can hence be seen as a major contributor to the unsatisfactory progesterone levels, has not yet been fully elucidated. Because several animal and management-associated factors are known to influence the peripheral plasma progesterone concentration (Parr et al., 1993; Abecia et al., 1997; Rabiee et al., 2001; Sangsritavong et al., 2002; Virolainen et al., 2004; 2005a; 2005b; Wiltbank et al., 2006), it is difficult to monitor the luteal progesterone production in an objective, non-confounded way.

An innovative and more straightforward approach compared to the assessment of the peripheral plasma progesterone concentration is the analysis of the *in vivo* histology and eventually the histophysiology of the luteal gland (corpus luteum, CL) by examination of

luteal tissue samples obtained by transvaginal biopsy prelevation (Kot et al., 1999; Tsai et al., 2001; Beg et al., 2005).

Until now, several studies described the bovine luteal histology by means of light and ultra structural microscopy of intact or enzymatic dispersed luteal tissue (Donaldson and Hansel, 1965; O'Shea, 1987; O'Shea et al., 1989; Lei et al., 1991; Parkinson et al., 1994; Fields and Fields, 1996; Neves and Marques, 2006; Cools et al., 2013). To what extent the histology of a complete CL can accurately be assessed just by taking biopsies *in vivo* is however not clear yet. In a previous study (Cools et al., 2013a), we already demonstrated the necessity to take the samples through the center of the CL containing the gland's total diameter, when histological parameters such as nuclear density, collagen fiber content, and the amount of vessels need to be assessed.

The main goal of the present study was to analyze whether parameters such as nuclear density, collagen content, and the amount of vessels of the intact luteal gland can be monitored in an accurate way by means of luteal biopsy samples.

Materials and methods

Selection of the CL

In a slaughterhouse, genital tracts without macroscopic pathologies and bearing a CL were isolated out of clinically healthy Holstein Friesian (HF) and Belgian Blue (BB) heifers and cows maximally 20 minutes after stunning and bleeding. Age distribution (two to six years) was not significantly different between both breeds, because of blocking. In total, 46 corpora lutea (CLa) equally distributed among both breeds were selected (23 CL in luteal stage II, i.e. cycle day 5 to 10, and 23 in stage III, i.e. cycle day 11 to 17), based on the selection criteria described by Ireland et al. (1980), modified with those of Miyamoto et al. (2000) and D'haeseleer et al. (2006). From each cow, a blood sample was taken less than 8 hours before stunning to assess the peripheral blood progesterone concentration. Cows with a blood progesterone concentration of less than 5 ng/mL were eliminated as in those cows the luteolytic process was expected to be already initiated. The CLa were randomly divided into two subsets (1 and 2), blocked for breed and luteal stage. In subset 1 (number of CLa, N=27) the nuclear density was assessed, while in subset 2 (N=19) the volume density of both the connective tissue (collagen fibers; Collagen) and vascular tissue/lumina (Lumina) was analyzed. Within subset 1 two different fixation protocols were tested. Consequently, subset

1 was split up into subset 1a (N=19; neutral-buffered 3.5% formol fixation; NBF) and 1b (N=8; zinc salts fixation; ZsF), while the CLa of subset 2 were fixated in Bouin's solution (further details will be given in the next section).

Prelevation and processing of the biopsies

Two biopsies were taken out of a CL: one in a sagittal plane, perpendicular to the equatorial plane, starting near the ovulation stigma and containing both the internal and external pole of the gland, and one following the equatorial plane (figure 1). Both biopsy sections crossed the luteal center. The biopsies were taken by means of a biopsy system, named *'CoaxialQuick-Core® Biopsy Echotip® Needle Set'* QCS-18-9.0-20T of Cook (William Cook Europe, Bjaeverskov, Denmark). Subsequently, a disk (='Central Disk, CD') with a maximal thickness of 0.5 cm containing both biopsy sites was isolated from the CL at the maximal diameter of the CL (figure 1).

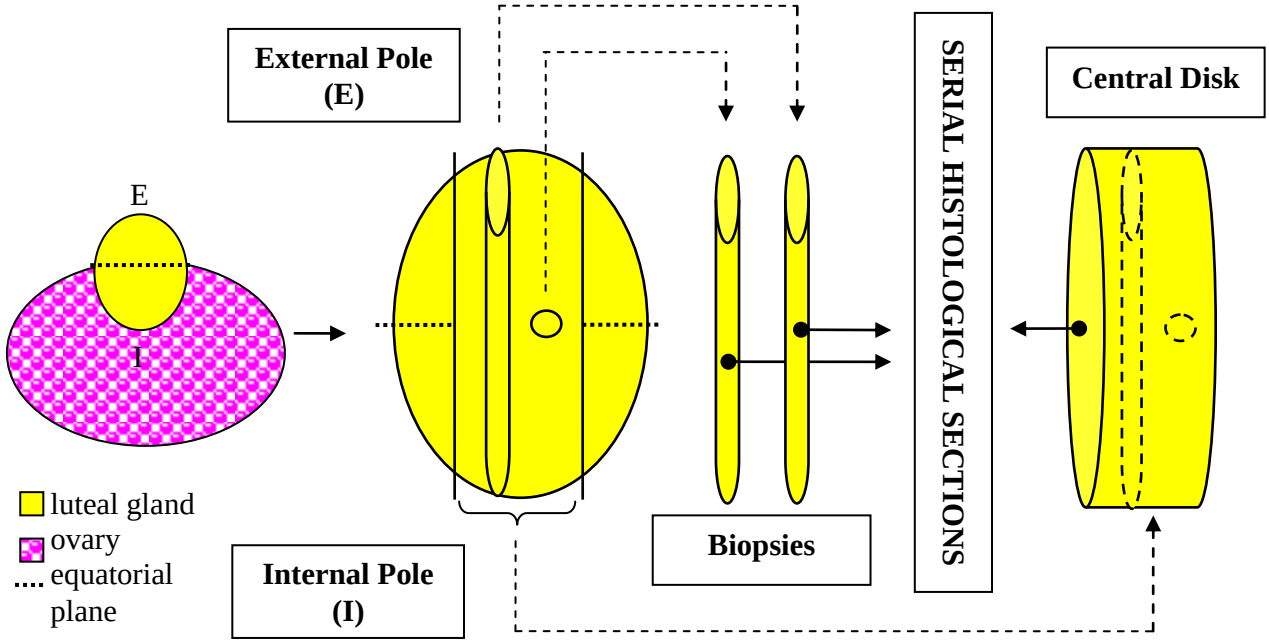


Figure 1: *two biopsies were taken out of the luteal gland (CL): one as central as possible in a sagittal plane perpendicular to the equatorial plane (starting near the ovulation stigma); and one following the equatorial plane. Both had to pass the luteal center. Subsequently, at the maximal diameter of the CL, a central disk, containing the biopsy sites, was isolated. The serial histological sections were denominated as 'overviews' (OV; originating from the central disk) and as 'biopsy views' (BV, made out of the biopsies)*

To assess the effect of the used fixative on the representativeness of a biopsy for determination of the nuclear density, the tissue samples (CD and biopsies) of subset 1a were immersed in NBF at room temperature (RT) during 48 hours and 8 hours for the CD and the biopsies, respectively. The samples of subset 1b were fixated in ZsF during 3 days at RT, with refreshment after the first 24 hours (Stevens, 1977; Gonzalez et al., 2001). The samples of the CL of subset 2 were fixated by immersion in Bouin's solution during 24 hours at RT (Bradbury and Gordon, 1977; Hoflack et al., 2008).

The CD and biopsies were subsequently dehydrated during 22 hours and embedded in paraffin following a standardized protocol (STP 420 D, Microm, Prosan, Merelbeke, Belgium).

Out of each CD, a series of histological sections (5 µm thickness) was made (HM 360, Microm) (figure 1). These sections were denominated as 'overviews' (OV), because they give a total cross-sectional image of the disk. The biopsies were cut lengthwise into histological sections, further referred to as 'biopsy views' (BV). The sections were mounted on gelatin-coated slides and dried overnight at RT. By means of a 'systematic random sampling procedure' (with a sampling interval of eight sections), three sections were selected out of each CD and biopsy, resulting in nine sections per CL for further research (three OV within each CD and three BV within each of the two biopsies).

The sections of the CL of subset 1a and b were stained with 'Haematoxylin-Eosin' (HE) following a standardized protocol (Stevens, 1977) by means of the Linear Stainer II (Sakura Finetek Belgium, Berchem, Belgium). In subset 2, the selected sections were stained by the modified 'Masson trichrome' (MMT) staining protocol (Bradbury and Gordon, 1977; Hoflack et al., 2008) .

Before the micrographs were made, an inner and outer zone were defined in the tissue sections (figure 2).

Within the three overview sections, 12 micrographs were randomly taken in each zone (magnification x 1000) by means of an Olympus DP 50 digital camera mounted on a motorized Olympus BX61 light microscope (Olympus Belgium N.V., Aartselaar, Belgium), resulting in 72 images per CD. Within the three sections of both biopsies, 6 photos were taken in each zone, again resulting in 72 micrographs. Consequently, in total, 144 micrographs per CL were analyzed.

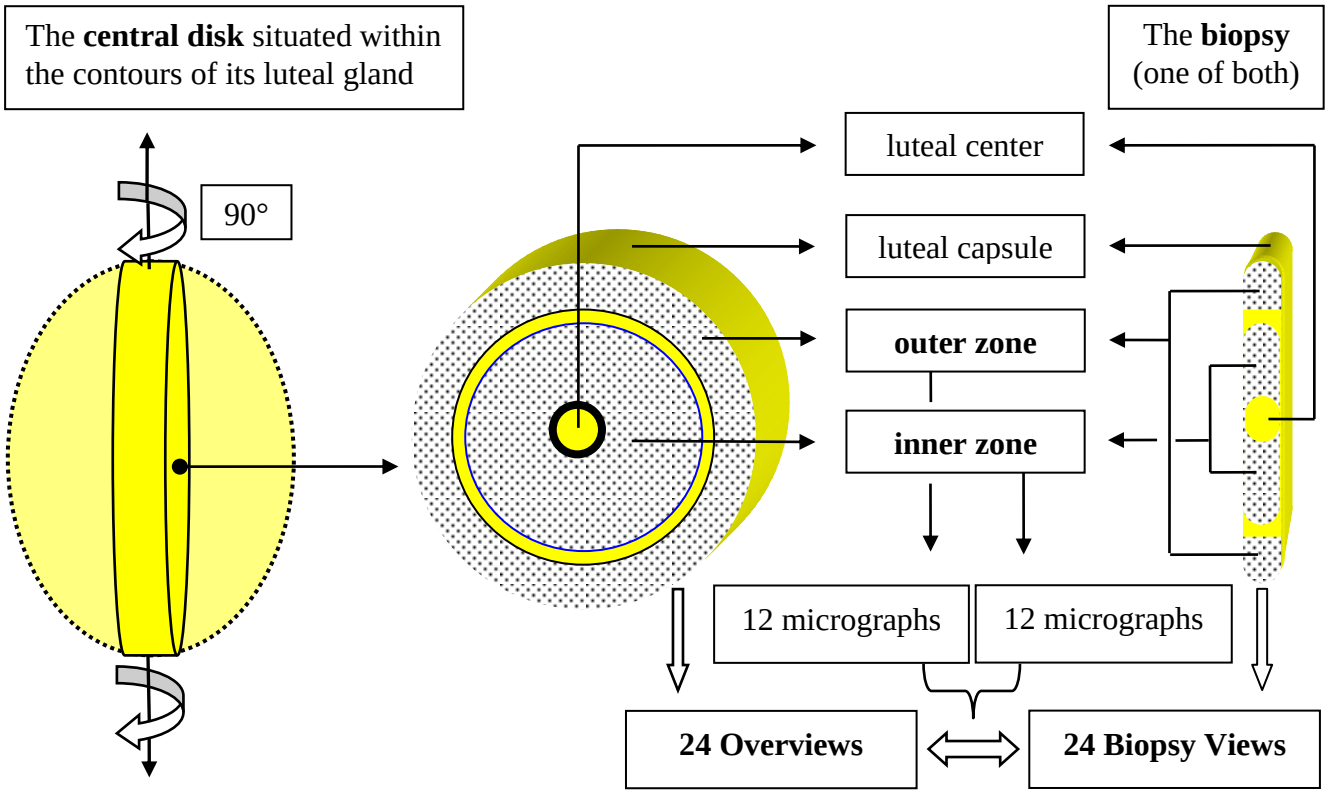


Figure 2: *this figure presents the delineation of the outer and inner zone relative to the luteal capsule and luteal center in the overview sections and biopsy sections. The central border of the inner zone was the region closest to the center of the tissue that still contained cells with the morphology of steroidogenic cells, while the peripheral border of the outer zone was defined as the region adjacent to the luteal capsule still containing steroidogenic cells. The peripheral boundary of the inner zone and the central border of the outer zone were determined geometrically preventing overlap of both zones. Within both zones of the central disk 12 micrographs were made. This was done for the three overview sections, resulting in 72 micrographs. In each zone of the sections of both biopsies six micrographs were taken, again resulting in 72 observations. Altogether, 144 micrographs were made for each luteal gland*

Analysis of the micrographs

The digital micrographs were analyzed using the software *'Cell F'* (Soft Imaging System, Olympus Belgium N.V.). The researcher counts by means of a touch-counting technique the number of nuclei per micrograph (i.e. 'the absolute nuclear density') in subset 1 and differentiates them into 'large luteal cells' (LLC), 'small luteal cells' (SLC) and 'non steroidogenic cells' (NSC) (Wiltbank, 1994). Criteria for the differentiation of the different

cell types were given in the pilot study (Cools et al., 2013b). By means of a point counting technique, the volume occupied by the two functional components Collagen and Lumina was assessed (i.e. 'the volume density') as previously described (Cools et al., 2013a.

Statistics

Statistical analysis of the results was carried out by means of the SPSS software package version 19 (IBM company, New York, USA). Normalization of the data was obtained by performing a square root transformation or natural logarithm transformation for the absolute nuclear density (SqrtTotal) and both collagen fibers (LnCollagen) and lumina (LnLumina), respectively.

The agreement between biopsy and overview sections for SqrtTotal and volume density for both LnCollagen and LnLumina was evaluated by agreement analysis of the parallel samples (i.e. the samples, biopsy versus CD, originating from the same CL) as decribed by Bland and Altman. First, the mean difference between biopsy 1 or 2(X) and the central disk (Y) for the different histological parameters was calculated and tested against zero using a one sample T-test. Significance was detected at $P < 0.05$. Also, the regression line for deviation was drawn and tested to detect significant deviation from the line of equality, and the different Pearson correlation coefficients were calculated. In subset 1, this correlation coefficient was calculated between the mean values of biopsy 1 and the CD within the same CL for both fixation protocols. The same was done between biopsy 2 and the CD. For subset 2 as well, the coefficient of correlation for each biopsy was assessed.

In all analyses, the standardized agreement index (AI) was determined by the following formula: $AI=1 - (2SD_{diff}/Mean_{XY})$ where SD_{diff} =standard deviation of the differences between the parallel samples X and Y, and $Mean_{XY}$=the overall mean of the parallel X and Y samples. A positive AI supports agreement and a value larger than 0.5 indicates good agreement. The agreement limits were defined as $Diff_{XY} \pm 2SD_{diff}$ with $Diff_{XY}$ as the difference between the parallel samples X and Y. An agreement plot of the difference against the mean of X and Y was used to spot outliers, defined as differences lying outside of the agreement limits.

In a final step, the coefficient of variation (CV) for the repeated measurements (CD versus biopsy 1 versus biopsy 2) within the same CL was analyzed. This CV was multiplied with 100 to express it as a percentage.

Results

The biopsies as representatives of the whole luteal gland: nuclear density

In the OV of the 27 CL of subset 1, 1944 micrographs were analyzed (1368 in subset 1a and 576 in subset 1b) and compared with the results of the BV. In figure 3, the results (mean and standard deviation) of the OV and BV are visualized.

The one sample T-test demonstrated for all four comparisons a very small but significant mean difference in nuclear density between sqrtTotal of the OV and the BV ($0.001 < P < 0.01$; Table 1). The agreement indices were all higher than 0.5 (Table 1). Also, data points lying outside the limits of agreement in the Bland-Altman plots were limited to a maximum of 2 data points (10.53%) (Table 1 and figure 4).

The CV for the repeated measurements in the ZsF-fixated CLa was 2% and in the NBF-fixated CLa 3%, concerning the nuclear density.

The scatter plots were constructed for the sqrtTotal of the OV versus the BV (figure 4). The best fitting linear function was presented with the corresponding R^2.
The Pearson correlation coefficient for subset 1a (NBF) was 0.52 (P=0.021) and 0.51 (P=0.027) for biopsy 1 and 2 respectively, while this was 0.80 (P=0.016) and 0.86 (P=0.006) in subset 1b (ZsF).

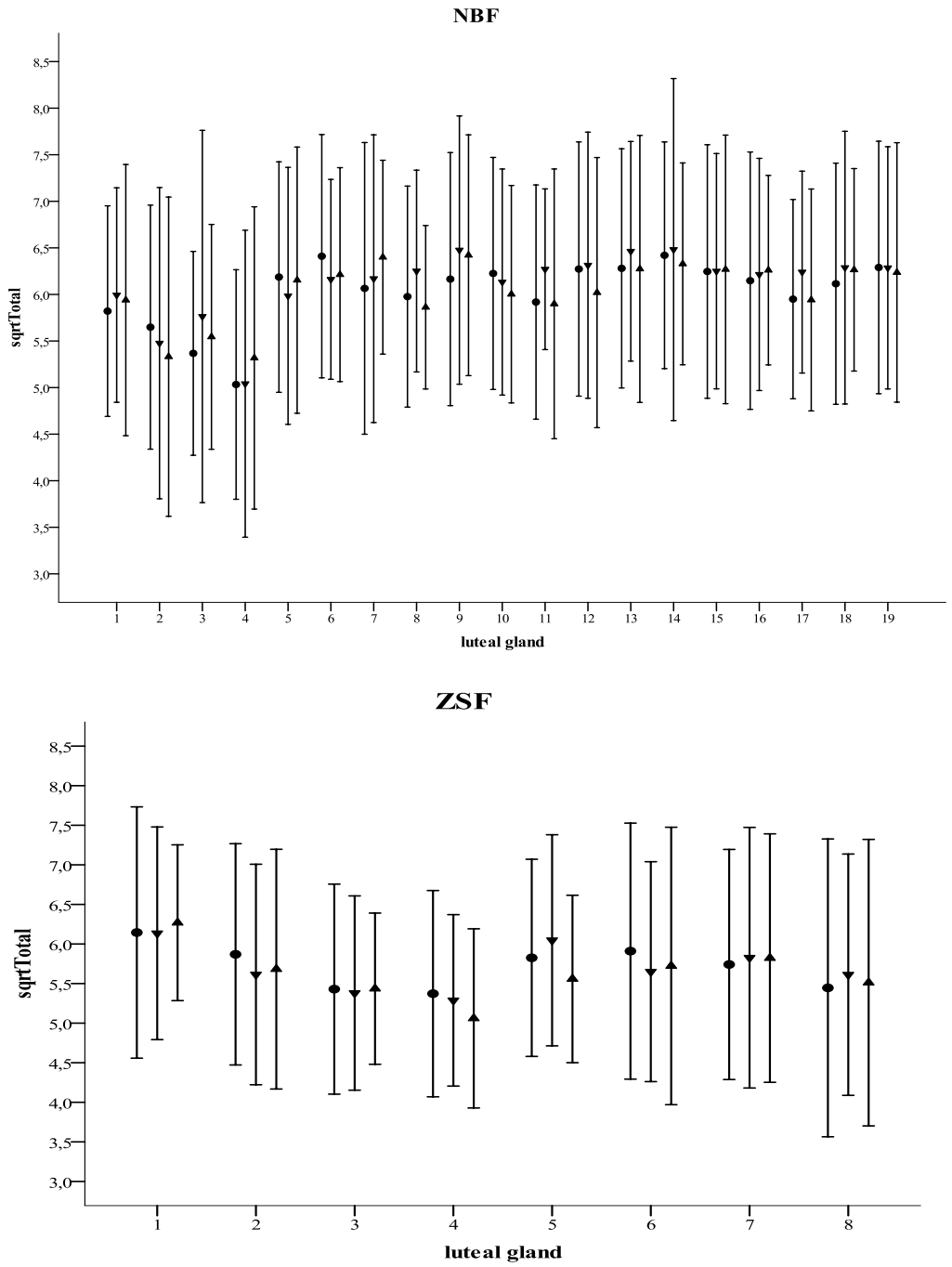


Figure 3: *comparison of the average square root transformed total nuclear density (sqrt Total) of the 'overviews' (●) with the 'biopsy views' of biopsy 1 (▼) and 2 (▲) in combination with the standard deviation (StDev) multiplied by 2 for subset 1a (NBF; upper figure) and 1b (ZsF; lower figure) for each luteal gland (NBF: n= 19; ZsF: n=8)*

Table 1. *overview of the Bland Altman agreement indices with the corresponding 95% agreement limits and correlation coefficients for evaluating the agreement between overview sections and biopsy sections for the nuclear density of corpora lutea fixated with either NBF or ZsF*

	mean diff[1]	**SD[2]**	**lower limit[3]**	**upper limit[4]**	**AI[5]**	**% outliers[6]**
NBF						
biopsy 1	-0.20	0.31	-0.82	0.42	0.90	5.26
biopsy 2	-0.11	0.30	-0.72	0.50	0.90	10.53
ZsF						
biopsy 1	-0.04	0.18	-0.32	0.40	0.94	0.00
biopsy 2	0.07	0.18	-0.28	0.42	0.94	0.00

[1]mean diff: mean difference in nuclear density between overview sections and biopsy sections

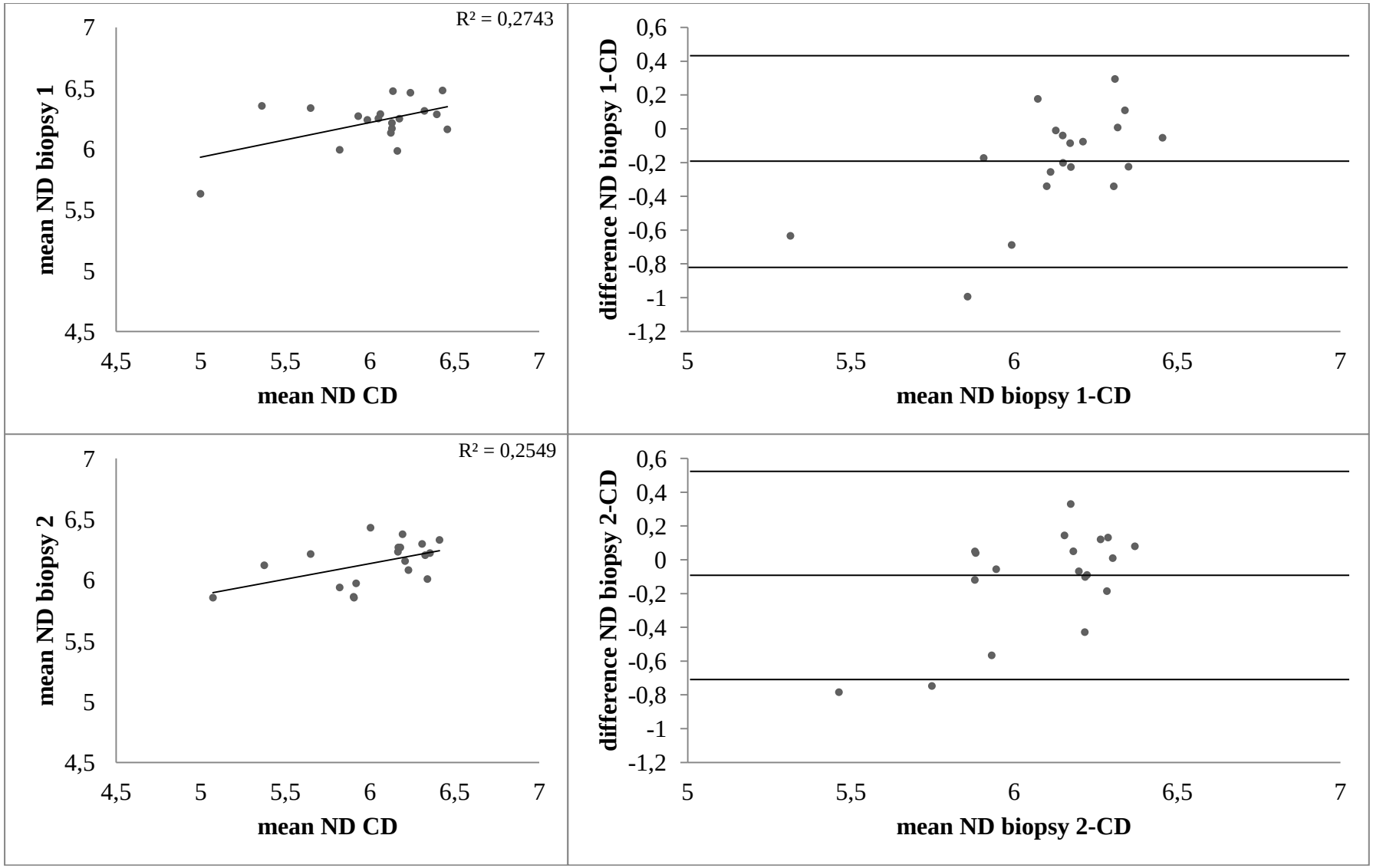
[2]SD: standard deviation of the differences

[3]lower limit: mean difference - 2 times the standard deviation

[4]upper limit: mean difference + 2 times the standard deviation

[5]AI: agreement index

[6]% outliers: data pairs situated beyond the constructed interval


R² = 0,2743
mean ND biopsy 1
mean ND CD
7
6,5
6
5,5
5
4,5
4,5
5
5,5
6
6,5
7
difference ND biopsy 1-CD
mean ND biopsy 1-CD
0,6
0,4
0,2
0
-0,2
-0,4
-0,6
-0,8
-1
-1,2
R² = 0,2549
mean ND biopsy 2
mean ND CD
difference ND biopsy 2-CD
mean ND biopsy 2-CD

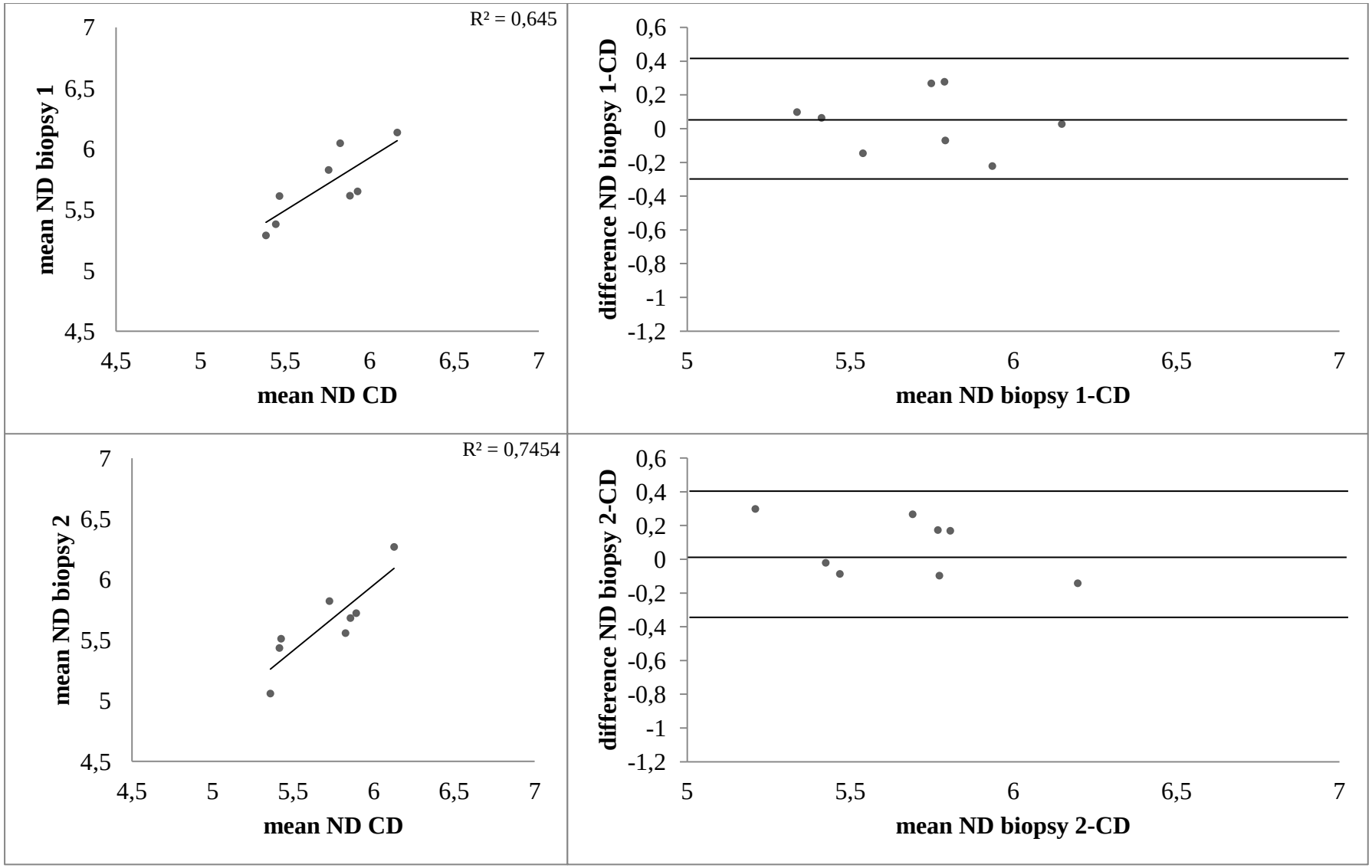

R² = 0,645
mean ND biopsy 1
mean ND CD
7
6,5
6
5,5
5
4,5
4,5
5
5,5
6
6,5
7
difference ND biopsy 1-CD
mean ND biopsy 1-CD
0,6
0,4
0,2
0
-0,2
-0,4
-0,6
-0,8
-1
-1,2
R² = 0,7454
mean ND biopsy 2
mean ND CD
difference ND biopsy 2-CD
mean ND biopsy 2-CD

Figure 4: *the scatter plot (on the left side) and the corresponding Bland Altman plot (on the right side) were established for both fixation protocols (NBF (upper part) and ZsF-fixation (lower part)) and within each protocol for both biopsies (biopsy 1 and 2). On the Bland Altman plot, the mean difference and the interval 'mean difference ± 2 times the standard deviation' of the nuclear density (ND) was expressed*

The biopsies as representatives of the whole luteal gland: volume density occupied by collagen fibers (Collagen) and vascular tissue/lumina (Lumina)

In subset 2, 19 luteal glands were analyzed, resulting in 1560 overview micrographs and the same number of biopsy micrographs, spread over two biopsies within each luteal gland.
In figure 5 the mean LnCollagen (i.e. the natural logarithm of the volume density occupied by collagen fibers) of the *'overviews'* was expressed together with those of both biopsies for every CL. Also, the standard deviation was visualized. The same was done for LnLumina.

The Pearson correlation coefficient between the OV (coming from the CD) and the BV (coming from both biopsies) was calculated for both parameters. The correlation in case of LnCollagen was 0.62 ($P=0.005$; biopsy 1) and -0.12 ($P > 0.05$; biopsy 2); in case of LnLumina it was 0.73 ($P < 0.0001$; biopsy 1) and 0.65 ($P=0.003$; biopsy 2).
The one sample T-test demonstrated a variable situation. For both comparisons of the parameter Lumina no significant mean difference could be detected, while a significant mean difference was detected in the area percentage occupied by collagen fibers between the OV versus the BV in case of biopsy 1($P < 0.001$), but not in case of biopsy 2 ($P > 0.05$) (Table 2).

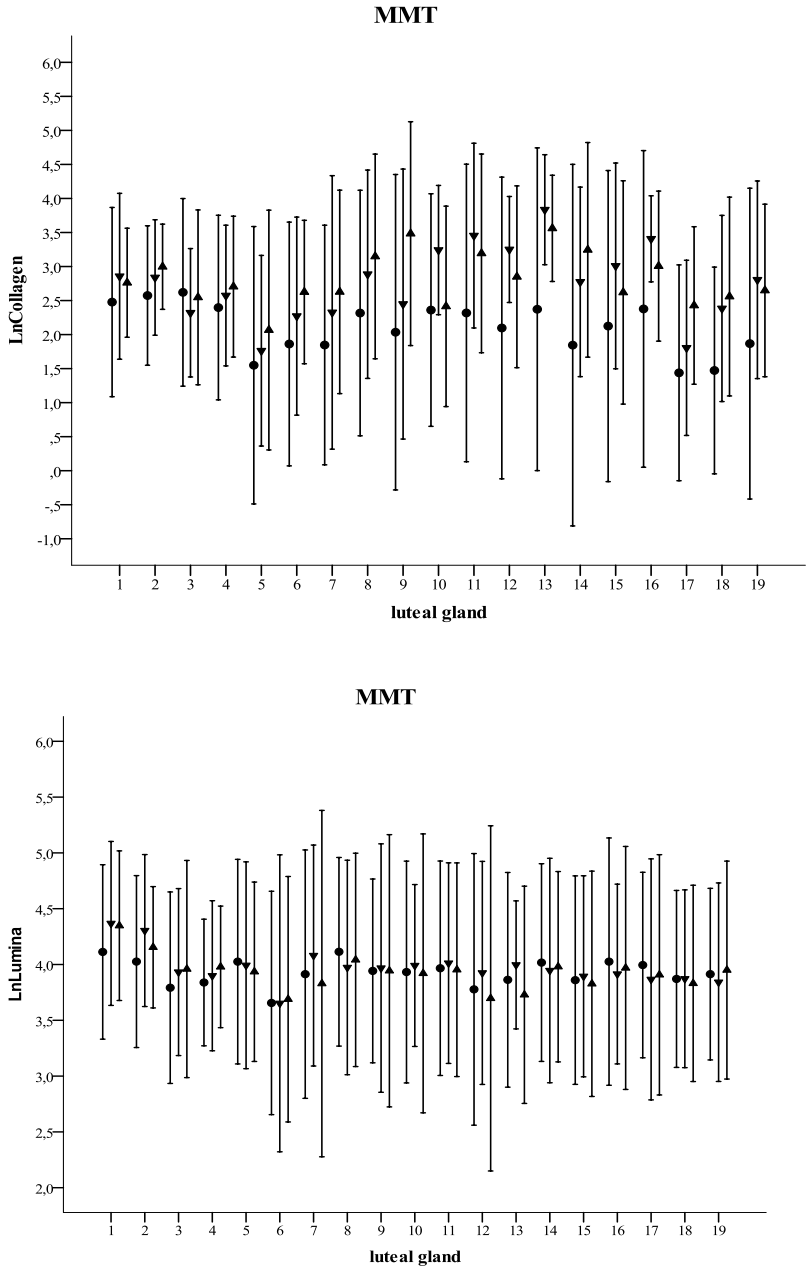


Figure 5: *comparison of the average natural logarithm transformed volume density occupied by collagen fibers (LnCollagen; upper) and vascular tissue/lumina (LnLumina; lower) of the 'overviews' (●) with the 'biopsy views', respectively, of biopsy 1 (▼) and 2 (▲) in combination with the standard deviation (StDev) multiplied by 2 for each luteal gland (n= 19). Luteal tissue is stained by the modified masson trichrome (MMT) technique*

Table 2. *overview of the Bland Altman agreement indices with the corresponding 95% agreement limits and correlation coefficients for evaluating the agreement between overview sections and biopsy sections for the volume density of collagen and vessels of corpora lutea*

	mean diff[1]	**SD[2]**	**lower limit[3]**	**upper limit[4]**	**AI**	**% outliers[5]**
collagen						
biopsy 1	-0.28	0.23	-0.75	0.18	0.68	10.53
biopsy 2	-0.20	0.60	-1.40	1.00	0.27	5.26
vessels						
biopsy 1	-0.0037	0.081	-0.17	0.16	0.94	5.26
biopsy 2	-0.037	0.11	-0.25	0.18	0.91	5.26

[1]mean diff: mean difference in volume density between overview sections and biopsy sections

[2]SD: standard deviation of the differences

[3]lower limit: mean difference – 2 times the standard deviation

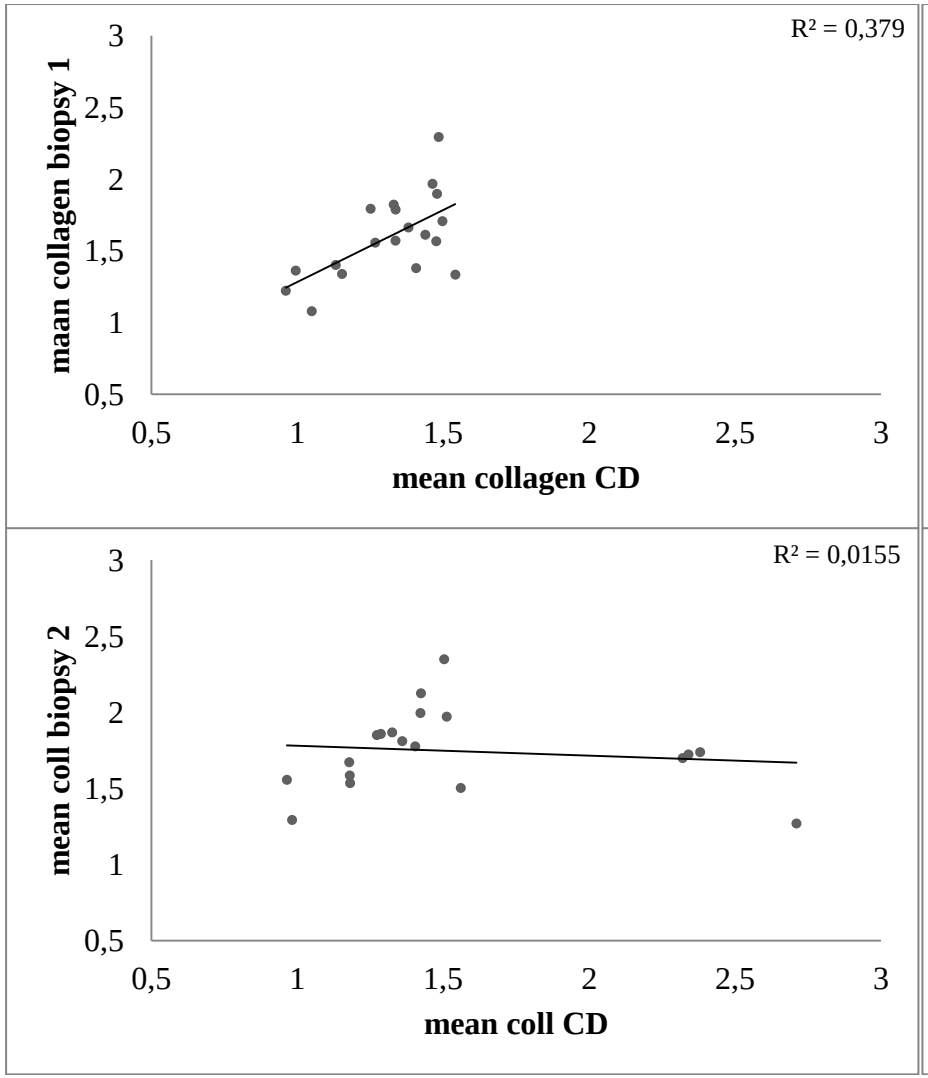
[4]upper limit: mean difference + 2 times the standard deviation

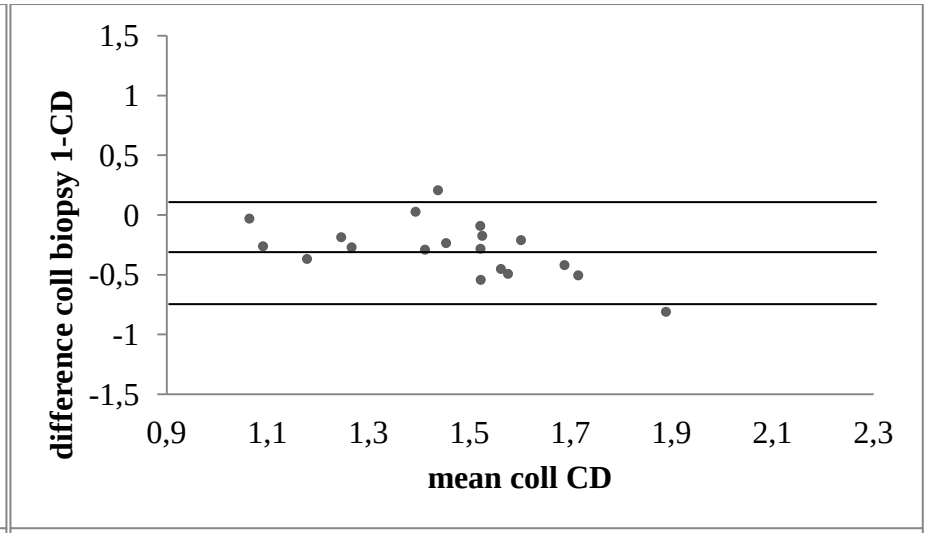
[5]AI: agreement index

[6]% outliers: data pairs situated beyond the constructed interval

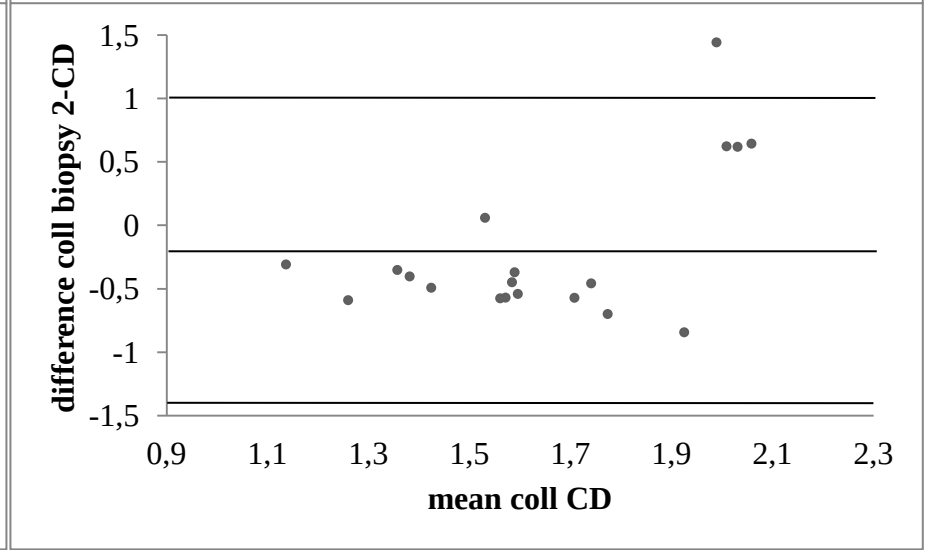
Scatter plots were constructed for both parameters LnCollagen and LnLumina, plotting OV against BV, differentiated for biopsy 1 and 2. The best fitting linear function was presented with the corresponding R^2 (figure 6). The corresponding Bland Altman plots were realized for both parameters LnCollagen and LnLumina, differentiated for both biopsies (figure 6). Index numbers of these plots are listed in Table 2.The agreement indices (AI) were all positive, but were remarkably higher for the parameter Lumina versus Collagen. For biopsy 2, the AI for Collagen was beneath 0.5. The relative amount of outliers was always above 5%.

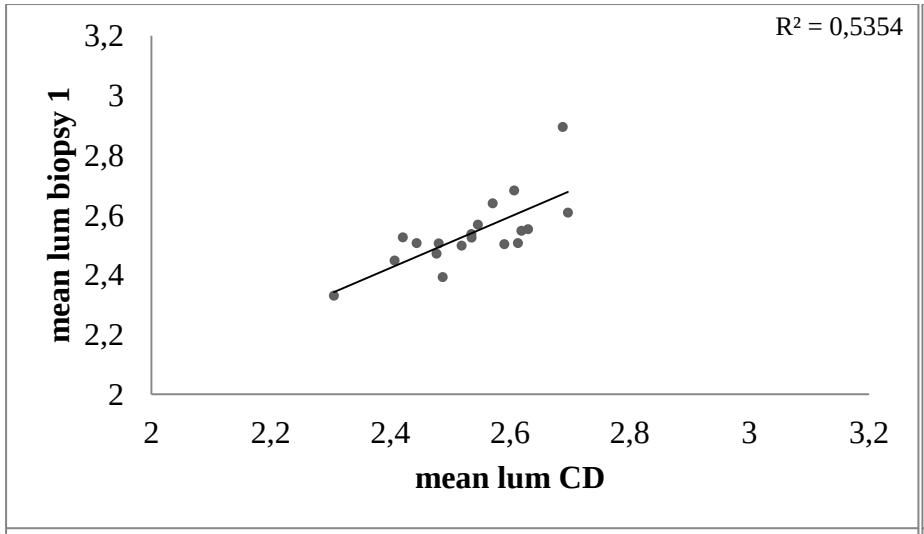
The coefficient of variation in the measurements for Collagen was 0.15, while it was 0.02 for the parameter Lumina.

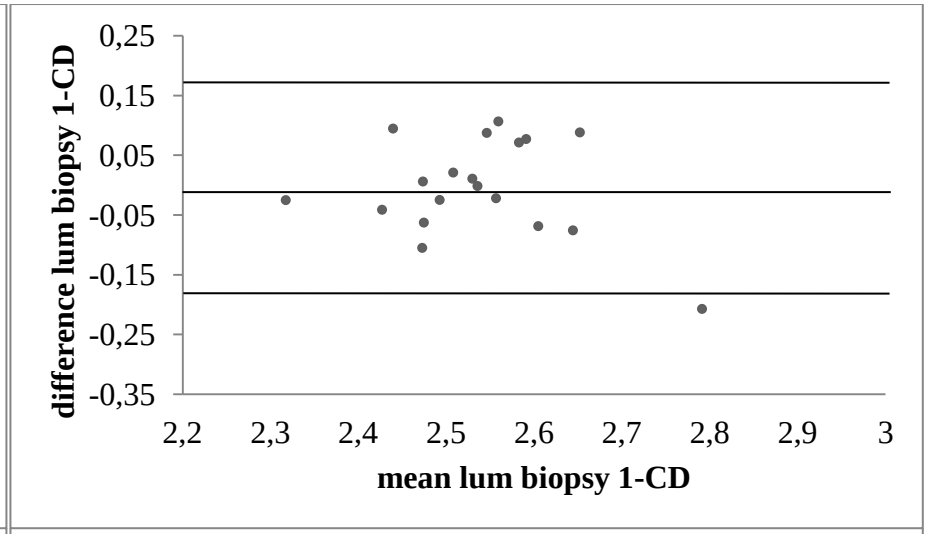

R² = 0,379
maan collagen biopsy 1
mean collagen CD
3
2,5
2
1,5
1
0,5
0,5
1
1,5
2
2,5
3

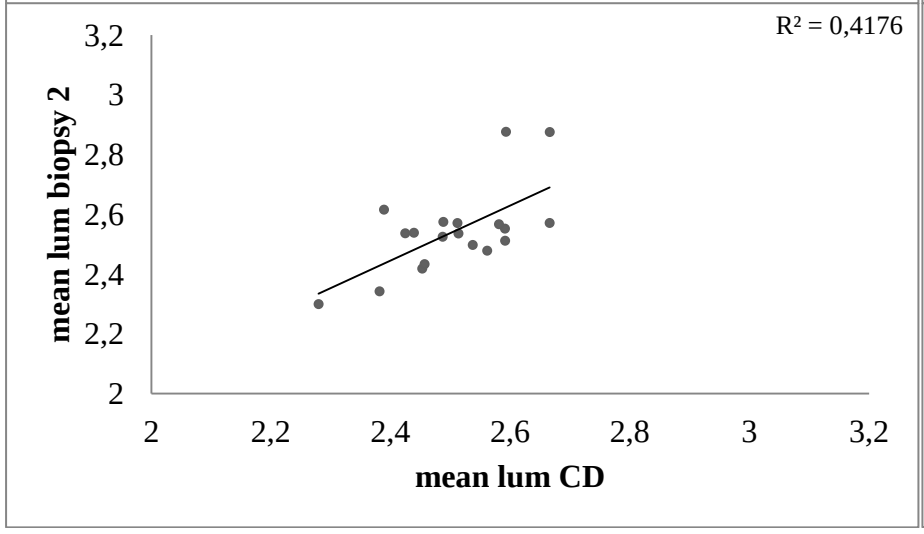

difference coll biopsy 1-CD
mean coll CD
1,5
1
0,5
0
-0,5
-1
-1,5
0,9
1,1
1,3
1,5
1,7
1,9
2,1
2,3

R² = 0,0155
mean coll biopsy 2
mean coll CD
3
2,5
2
1,5
1
0,5
0,5
1
1,5
2
2,5
3


difference coll biopsy 2-CD
mean coll CD
1,5
1
0,5
0
-0,5
-1
-1,5
0,9
1,1
1,3
1,5
1,7
1,9
2,1
2,3


R² = 0,5354
mean lum biopsy 1
mean lum CD
3,2
3
2,8
2,6
2,4
2,2
2
2
2,2
2,4
2,6
2,8
3
3,2


difference lum biopsy 1-CD
mean lum biopsy 1-CD
0,25
0,15
0,05
-0,05
-0,15
-0,25
-0,35
2,2
2,3
2,4
2,5
2,6
2,7
2,8
2,9
3


R² = 0,4176
mean lum biopsy 2
mean lum CD
3,2
3
2,8
2,6
2,4
2,2
2
2
2,2
2,4
2,6
2,8
3
3,2

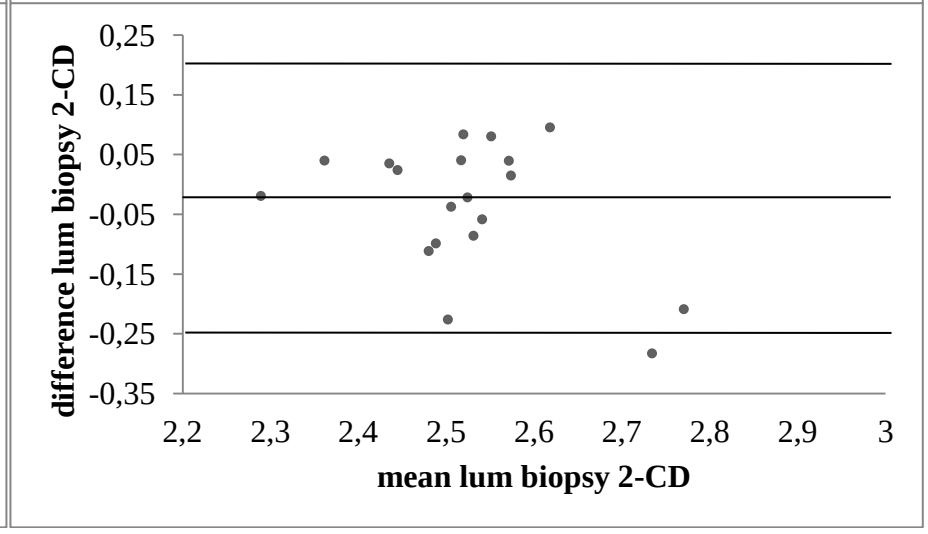

difference lum biopsy 2-CD
mean lum biopsy 2-CD
0,25
0,15
0,05
-0,05
-0,15
-0,25
-0,35
2,2
2,3
2,4
2,5
2,6
2,7
2,8
2,9
3

Figure 6: *the scatter plot (on the left side) and the corresponding Bland Altman plot (on the right) were established for the logarithm transformed volume density of Collagen and Lumina; and within each parameter for both biopsies (biopsy 1 and 2). On the Bland Altman plots the mean difference and the interval 'mean difference ± 2 times the standard deviation' are visualized*

Discussion

In the present study, we aimed to analyze the possibility to investigate in a reliable way some important morphological characteristics of a bovine CL by taking biopsies. If one could find a histological characteristic, which is distributed homogeneously or varies in a predictive way, it could be possible to extrapolate results, obtained by a luteal tissue sample, to the whole central disk, approximating the whole luteal gland. Supposing these morphological traits can be linked to the luteal histophysiology, biopsies can be used to study the luteal activity in a more straightforward manner, compared with blood progesterone assessment, which is known to be subjected to several confounding factors.

Feasibility studies concerning the *in vivo* biopsy technique were already carried out (Kot et al., 1999; Tsai et al., 2001). Kot et al. (2001) acquired in 92% of the attempts a luteal tissue specimen with minimal disturbance of the luteal function, as determined by measuring the life span and progesterone production. However, up till now, there are to our best knowledge no studies scrutinizing the representativeness of the biopsies with respect to the whole CL.

Because of its transient and dynamic nature, luteal stage influences the luteal histological features. Staging criteria were based on the gross appearance of the CL (Ireland et al., 1980), combined with the morphological characteristics of the follicles and uterus (Miyamoto et al., 2000; D'Haeseleer et al., 2006). More details are described in another study (Cools et al., 2013a).

Morphological parameters, useful for testing the representativeness of biopsies had to be determined in advance. Aptness of the parameters was defined and tested in another article (Cools et al., 2013a). Briefly, the preferable histological trait should be visualizable in a practical way by light microscopy and should be related to the luteal histophysiology of progesterone production. Based on the conclusions of our other paper (Cools et al., 2013a), a biopsy protocol was designed.

As blood progesterone concentration is depending on the cellular progesterone production, we chose to monitor nuclear density. But blood progesterone concentration is also depending

on the supply of components to and removal of progesterone from the CL. Consequently, also the degree of vascularization needs to be assessed (amount of vessels and collagen fibers).

The results of both biopsies originating from the same CL were analyzed independently, to test whether the same conclusions could be drawn based on the results of each biopsy on his own.

In another study, the topographic variation of the nuclear density within a bovine luteal gland was explained in detail (Cools et al., 2013a). Briefly, the nuclear density is significantly lower in the inner zone when compared to the outer, even when differentiation was made into the three different cell types.

Concerning the nuclear density, the correlations between the OV and BV were higher for the ZsF fixated biopsies (around 0.8) compared to the NBF-fixated ones (around 0.5). So, the correlation is higher when ZsF fixation is used. Nevertheless, the degree of agreement (i.e. the agreement index) was for the ZsF-fixated samples comparable to that of the NBF-fixation. The amount of outliers is situated under the proposed 5% for the ZsF fixated biopsies. But for the NBF fixated samples the number of outliers exceeded the limit. The remark has to be made, that the presence of one outlier resulted already in exceeding the limit of 5%. The mean differences in sqrtND are significantly different from zero. Nevertheless, this statistical difference has no biological relevance. The percentage of the variation we observed between the different CLa caused by the measuring technique is comparable for both fixation protocols (2 versus 3%).

The accuracy of the results of the ND obtained in the biopsies, when they are ZsF fixated, is high enough to correctly assess the ND of the central disk, approximating the whole luteal gland. The results of the biopsies, fixated in NBF are less accurate, compared to the ZsF immersed biopsies. We have to be careful with the interpretation of the parameter ‘percentage of outliers’, as the presence of one outliers already results in a overrunning of the limit. This brings us to the conclusion that the most reliable results concerning the nuclear density are obtained by the fixation of biopsies based on the ZsF fixation protocol.

When we look to the results for Collagen and Lumina, we can see that the correlation for the parameter Lumina is rather high. The correlation in case of the parameter Collagen is more difficult to interpret as the situation is completely different for biopsy 1 versus biopsy 2. The agreement index between the OV and the BV was remarkably lower in case of the functional compartment Collagen compared to Lumina. This lower congruence in Collagen was accompanied by a systematic overestimation in the BV. A possible explanation could be that the vast majority of the connective tissue was situated in trabeculae, which were distributed

more heterogeneously than Lumina. The overestimation could be due to the fact that the impact of the presence of these trabeculae was much greater in the BV than in the OV. Consequently, in case there was connective tissue in the histological section, the chance of attendance of connective tissue on a randomly taken micrograph in the biopsies was greater than on a micrograph in the OV. This discrepancy between the results obtained in the biopsies versus the CD was also visualized by the high coefficient of variation calculated for the parameter Collagen. Fifteen percent of the variation is caused by the measurement technique, while this was only 2% for the parameter Lumina.

In conclusion, we can state that the usefulness of biopsies in the assessment of the luteal morphology is depending on the selected fixative and the chosen histological characteristic. Orientation of the biopsy (parallel or perpendicular to the equatorial plane) had no significant effect on the nuclear density, nor on the presence of collagen fibers or vascular lumina. Based on the correlations found in our dataset, the preference should be given to assessment of the nuclear density on ZsF-fixated sections and the volume density occupied by Lumina. Yet further research is needed to identify histological parameters, preferably cell-associated traits or characteristics linked to the vascular tissue, which translate in an appropriate way the histophysiology of the luteal tissue.

Acknowledgements

The BOF is thanked for their financial support (05B01906). Also Lobke De Bels and Liliana Standaert of the laboratory of the department of morphology are thanked for their technical support.

Reference List

Abecia JA, Lozano JM, Forcada F, Zarazaga L, 1997: Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim Reprod Sci **48**, 209-218.

Beg MA, Gastal EL, Gastal MO, Ji S, Wiltbank MC, Ginther OJ, 2005: Changes in steady-state concentrations of messenger ribonucleic acids in luteal tissue during prostaglandin F2alpha induced luteolysis in mares. Anim Reprod Sci **90**, 273-285.

Bradbury P, Gordon K, 1977: Connective tissues and stains. In: Theory and practice of histological techniques, Bancroft JD, Stevens A (Ed), Churchill Livinstone, New York, p. 104.

Cools S, Van den Broeck W, De Vliegher S, Piepers S, Hostens M, Opsomer G, 2013a: Topographic distribution of the different cell types, connective tissue and vascular tissue/lumina within a functional bovine corpus luteum, and the influence of breed, fixative and luteal stage on it. Reprod Domest Anim **48**, 627-635.

Cools S, Van den Broeck W, De Vliegher S, Piepers S, Opsomer G, 2013b The bovine luteal histological composition: a topographic point of view. Reprod Domest Anim **48**, e29-32.

Diskin MG, Morris DG, 2008: Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim **43** (suppl 2), 260-267.

D'haeseleer M, Cocquyt G, Van Cruchten S, Simoens P, Van den Broeck W, 2006: Cell-specific localisation of apoptosis in the bovine ovary at different stages of the estrous cycle. Theriogenology **65**, 757-772.

Donaldson L, Hansel W, 1965: Histological study of bovine corpora lutea. J Dairy Sci **48**, 905-909.

Fields MJ, Fields PA, 1996: Morphological characteristics of the bovine corpus luteum during the estrous cycle and pregnancy. Theriogenology **45**, 1295-1325.

González L, Anderson I, Deane D, Summers C, Buxton D, 2001: Detection of immune system cells in paraffin wax-embedded ovine tissues. J Comp Pathol **125**, 41-47.

Hoflack G, Van den Broeck W, Maes D, Van Damme K, Opsomer G, Duchateau L, de Kruif A, Rodriguez-Martinez H, Van Soom A, 2008: Testicular dysfunction is responsible for low sperm quality in Belgian Blue bulls. Theriogenology **69**, 323-332.

Ireland JJ, Murphee RL, Coulson PB, 1980: Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J Dairy Sci **63**, 155-160.

Kot K, Anderson LE, Tsai SJ, Wiltbank MC, Ginther OJ, 1999: Transvaginal, ultrasound-guided biopsy of the corpus luteum in cattle. Theriogenology **52**, 987-993.

Lei ZM, Chegini N, Rao CV, 1991: Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol Reprod **44**, 1148-1156.

Mann GE, Lamming GE, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction **121**, 175-180.

Miyamoto Y, Skarzynski DJ, Okuda K, 2000: Is tumor necrosis factor alpha a trigger for the initiation of endometrial prostaglandin F(2alpha) release at luteolysis in cattle? Biol Reprod **62**, 1109-1115.

Moreira F, de la Sota RL, Diaz T, Thatcher WW, 2000: Effect of day of the estrous cycle at the initiation of a timed artificial insemination protocol on reproductive responses in dairy heifers. J Anim Sci **78**, 1568-1576.

Neves MM, Marques Jr AP, 2006: Volumetric proportions of the corpus luteum constituents of Nelore. Arq Bras Med Vet Zootec **58**, 944-946.

Opsomer G, de Kruif A,1999: Postpartum anestrus in dairy cattle-a review. Tierärztl Prax Ausg G Grosstiere Nutztiere **27**, 30-35.

O'Shea JD, 1987: Heterogeneous cell types in the corpus luteum of sheep, goats and cattle. J Reprod Fertil Suppl **34**, 71-85.

O'Shea JD, Rodgers RJ, D'Occhio MJ, 1989: Cellular composition of the cyclic corpus luteum of the cow. J Reprod Fertil **85**, 483-487.

Parkinson TJ, Turvey A, Jenner LJ, 1994: A morphometric analysis of the corpus luteum of the cow during the estrous cycle and early pregnancy. Theriogenology **41**, 1115-1126.

Parr RA, Davis IF, Miles MA, Squires TJ, 1993: Liver blood flow and metabolic clearance rate of progesterone in sheep. Res Vet Sci **55**, 311-316.

Pretheeban T, Gordon M, Singh R, Perera R, Rajamahendran R, 2009: Differential mRNA expression in *in vivo* produced pre-implantation embryos of dairy heifers and mature cows. Mol Reprod Dev **76**, 1165-1172.

Rabiee AR, Macmillan KL, Schwarzenberger F, 2001: The effect of level of feed intake on progesterone clearance rate by measuring faecal progesterone metabolites in grazing dairy cows. Anim Reprod Sci **67**, 205-214.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC, 2002: High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle. J Dairy Sci **85**, 2831-2842.

Sartori R, Rosa GJ, Wiltbank MC, 2002: Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci **85**, 2813-2822.

Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE, 1990: Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil **90**, 1-10.

Sreenan JM, Diskin MG, 1983: Early embryonic mortality in the cow: its relationship with progesterone concentration. Vet Rec **112**, 517-521.

Stevens A, 1977: The haematoxylins. In: Theory and practice of histological techniques, Bancroft JD, Stevens A (Ed.), Churchill Livingstone, New York, p. 89.

Tsai SJ, Kot K, Ginther OJ, Wiltbank MC, 2001: Temporal gene expression in bovine corpora lutea after treatment with PGF2alpha based on serial biopsies *in vivo*. Reproduction **121**, 905-913.

Virolainen JV, Love RJ, Tast A, Peltoniemi OA, 2005a: Plasma progesterone concentration depends on sampling site in pigs. Anim Reprod Sci **86**, 305-316.

Virolainen JV, Peltoniemi OA, Munsterhjelm C, Tast A., Einarsson S, 2005b: Effect of feeding level on progesterone concentration in early pregnant multiparous sows. Anim Reprod Sci **90**, 117-126.

Virolainen JV, Tast A, Sorsa A, Love RJ, Peltoniemi OA, 2004: Changes in feeding level during early pregnancy affect fertility in gilts. Anim Reprod Sci **80**, 341-352.

Wiltbank MC, 1994: Cell types and hormonal mechanisms associated with mid-cycle corpus luteum function. J Anim Sci **72**, 1873-1883.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A, 2006: Changes in reproductive physiology on lactating dairy cows due to elevated steroid metabolism. Theriogenology **65**, 17-29.

Chapter 5

In vivo research:

Monitoring of the luteal histophysiology

Chapter 5.1

Soybean meal, as protein source, increases the blood level of isoflavones and reduces the steroidogenic capacity in bovine corpora lutea, without affecting peripheral progesterone concentrations.

Cools S[a], Van den Broeck W[b], Vanhaecke L[c], Heyerick A[d], Bossaert P[e], Hostens M[a], Opsomer G[a]

[a]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[b]Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[c]Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[d]Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium
[e]Department of Veterinary Sciences, Campus Drie Eiken, Antwerp University, Universiteitsplein 1, 2610 Wilrijk, Belgium

Animal Reproduction Science 2014, **144**, 79-89

Abstract

Thirty three Holstein Friesian cows were followed from 14 days pre partum until the fourth ovulation post partum. Housing conditions and basic ration were identical for all animals. Concentrates were individually supplemented according to the daily milk production level, using two different types of protein rich concentrates: soybean meal and rapeseed meal. Soybean and rapeseed meal are known to be respectively high and low in isoflavones. Cows were randomly divided into three groups and blocked for parity. Group I (n=11) was supplemented with soybean meal and acted as control group. Groups II (n=11) and III (n=11) were respectively supplemented with soybean and rapeseed meal and were subjected to a biopsy sampling of the corpus luteum at day 9 of the first three postpartal estrous cycles.

Soybean meal supplementation to lactating dairy cows (1.72 kg on average) induced an increase in the blood concentration of equol, dihydrodaidzein, o-desmethylangolensin in both soy groups and resulted in a reduced area occupied by steroidogenic (P=0.012) and endothelial cells (P=0.0007) in the luteal biopsies. Blood concentrations of equol and glycitein were negatively correlated with the areas occupied by steroidogenic (r=-0.410 with P=0.0002, respectively r=-0.351 with P=0.008) and endothelial cells (r=-0.337 with P=0.01, respectively r=-0.233 with P=0.085) in the 3 first estrous cycles. The latter however did not affect the diestrous peripheral blood progesterone concentration.

Introduction

The *Papilionoideae*, with soy as a member, are rich in isoflavones (Coward et al., 1993; Mazur and Adlercreutz, 1998), making soybeans and their dietary derivates a major nutritional source of phyto-estrogens for humans (Bingham et al., 1998; Hollman, 2001). Epidemiologic studies in humans have shown that the prevalence of aggressive mammary and prostate cancers is lower in those countries, where soy products are a common ingredient of the regular diet (Dhom, 1991; Mills et al., 1989; Shimizu et al., 1991). These cancer preventive properties were credited to several isoflavonic molecules and metabolites present in soy products (Adlercreutz, 1990). Isoflavones and their metabolites are generally recognized as a class of molecules with a high estrogenic potency (COT, 2003) compared to other members of the phyto-estrogen group. The mechanism suggested to be behind these molecules' anti-cancer effect, is the significant impairment of angiogenesis (Chapin et al., 1996; Ying et al., 2001; Zhou et al., 1999) through inhibition of cell proliferation and interruption of the breakdown of extracellular matrix surrounding growing vessels and tumors (Su et al., 2005).

High yielding dairy cows are frequently supplemented with protein rich concentrates, such as soybean meal, above their basic ration to meet their requirements in terms of protein intake (Steinfeld et al., 2006), resulting in increased blood concentrations of certain isoflavone molecules and metabolites (Piotrowska et al., 2006; Trinacty et al., 2009; Zdunczyk et al., 2006). Furthermore, isoflavone consumption has been described to increase the incidence of irregular estrous cycles and silent heats, to reduce conception rates and to increase embryonic losses in cattle (Kallela, 1984; Zdunczyk et al., 2005). While at least some of these effects can be attributed to a decreased sensitivity of the pituitary gland to gonadotropin releasing hormone (Nwannenna et al., 1994), Kaplanski et al. (1981) showed *in vitro* a dose dependent positive or negative effect of some isoflavones on the progesterone synthesis of bovine luteinized granulosa cells. In two recent articles (Cools et al., 2013a, 2013b) we described an innovative technique to histologically analyze bovine corpus luteum (CL) samples. We analyzed the topographic variation of the histological traits 'nuclear density' of steroidogenic and non-steroidogenic cells; and 'density' of blood vessels and collagen fibers. We concluded that parameters based on nuclear density or blood vessels in one CL biopsy are reliable characteristics to create a representative image of the whole luteal gland, if this biopsy is taken through the luteal gland's center and contains the total luteal diameter.

In the present study, we hypothesized that supplementation of soybean meal to high yielding dairy cows post partum increases the peripheral blood concentrations of isoflavones which

subsequently affects angio- and steroidogenesis in the developing luteal gland, resulting in an inferior progesterone secretion.

Materials and methods

Animals and housing

The present study took place from September 2008 to May 2009 in the university dairy herd of the Biocenter Agri-Vet (Melle, Belgium). The experiment was approved by the ethical committee of the Faculty of Veterinary Medicine, University of Ghent, Belgium (EC2008/084).

Table 1. *contents of the different feed components in the rations of dry and lactating cows and composition of the basic ration of the lactating cows*

nutrient	**contents**				**kg DM**	
	DM[1]	**fiber[2]**	**NE_L[3]**	**DIP[4]**	**lactation**	**dry period**
concentrate	87	105	1.584	115	individual	…
soybean meal	87	100	1.535	200	individual	1.40
rapeseed meal	87	125	1.257	160	individual	…
corn silage	30	190	1.520	48	7.98	8.00
grass silage	38	250	1.485	68	3.63	…
sugar beet pulp	21	197	1.733	104	1.5	…
corn cob mix	61	28	1.988	69	0.675	…
hay	83	260	1.363	82	0.855	…
wheat straw	84	420	0.713	3	…	*ad libitum*
minerals	100	…	…	…	0.300	0.200
magnesium oxide	100	…	…	…	…	0.025

[1]DM: dry matter, %

[2]fiber: fiber/kg DM (i.e. amount of fiber per kilogram of DM)

[3]NE_L: NE_L/kg DM (i.e. net energy for lactation per kilogram of DM)

[4]DIP: DIP/kg DM (i.e. amount of digestible intestinal protein per kilogram of DM)

In the dry period, cows were daily fed 8 kg dry matter (DM) of corn silage, 1.4 kg DM of soybean meal (Aveve, Merksem, Belgium) and wheat straw *ad libitum*. The dry period ration was supplemented with 25 g of magnesium oxide (Nutreco, Ghent, Belgium) and 200 g of a

mineral mixture specifically designed for dry cows (Nutreco, Ghent, Belgium). During the last 7 to 10 days before the expected calving date, heifers and multiparous cows were introduced into the lactating group and offered the ration for lactating cows supplemented with 2 kg of concentrates and, depending on the group, 1.4 kg DM of soybean meal or 1.7 kg DM of rapeseed meal (Aveve, Merksem, Belgium).

Thirty three Holstein Friesian primi- (n=13) and multiparous cows (n=20) (average 305-days milk yield of 9,350 kg (SD=1,919 kg), 4.11% fat (SD=0.61%), 3.41% protein (SD=0.24%)) were randomly divided into 3 groups while blocking was done based on lactation number. The three groups were housed together in the same loose housing barn with cubicles and had *ad libitum* access to the same basic TMR ration (corn silage, grass silage, sugar beet pulp, corn cob mix, hay and minerals; 53.2, 24.2, 10.0, 4.5, 5.7, 2.0% on kg DM basis). The composition of the basic lactation and dry ration is given in more detail in table 1.

Multiparous cows producing > 25 kg and primiparous cows producing > 20 kg of milk additionally received both an energy and protein rich concentrate (PROT) depending on the actual amount of milk produced per day. This surplus amount of concentrates was individually provided via automatic concentrate dispensers in the stable and in the milking robot. These dispensers were calibrated weekly. Group I (n=11) was supplemented with oil-extracted, toasted soybean meal and was used as control group (named as 'soy, control group'). Group II (n=11) and III (n=11) were respectively provided with the same oil-extracted, toasted soybean meal versus a mixture based on oil-extracted rapeseed meal as main protein source. The latter two groups were subjected to an *in vivo* prelevation of luteal biopsies and were therefore respectively named 'soy, puncture group' and 'rape, puncture group'. The ratio of energy versus protein content was identical for both protein concentrates, 1 kilogram of soybean meal being equivalent to 1.2 kg of the rapeseed mixture. The mean consumption of concentrates was not significantly different between the three groups (5.1 kg). The mean soybean meal consumption was 1.72 kg and the mean rapeseed meal consumption 2.2 kg.

Production parameters such as daily milk production (MP) and body condition score (BCS; assessment 2 times a week) were assessed. Monitoring of BCS was based on the system of Edmonson et al. (1989). The difference in BCS between the moment of calving and the sampling moment (i.e. 'BCS difference') was calculated. The cows were on average milked 2.68 (± 0.03) times per day by a robotic milking system (VMS, DeLaval NV, Ghent, Belgium). The animal caretaker was alerted automatically when a cow had not been milked for more than 10 hours.

In figure 1, a schematic overview is given of the sampling protocol during the whole study period.

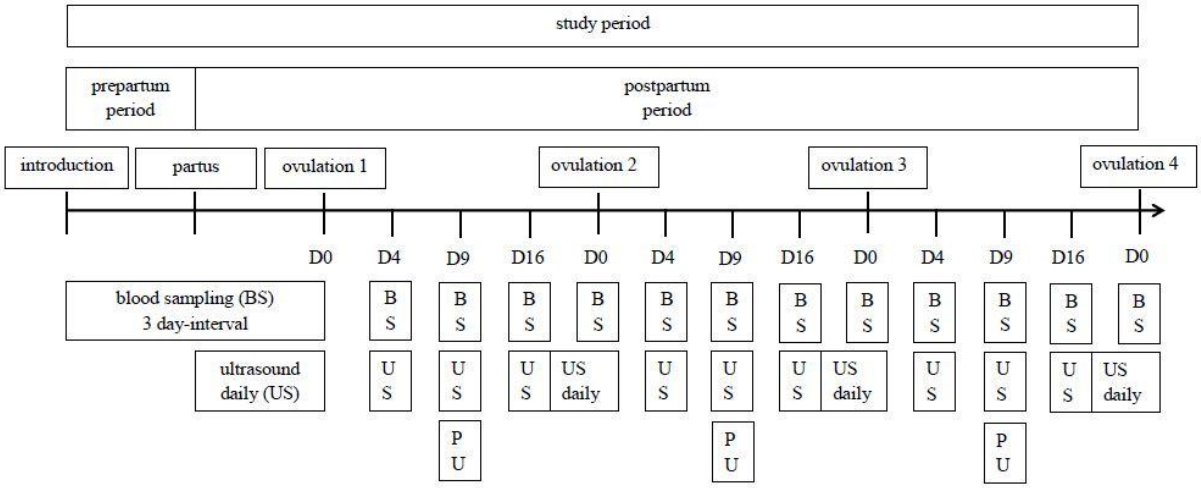


Figure 1: *schematic sampling protocol during the experimental period (from day 14 before expected calving date till fourth ovulation). D: cycle day; BS: blood sampling during that specific cycle day; US: trans rectal ultrasound research of the genital tract during that specific cycle day; US daily: daily trans rectal ultrasound research of the genital tract from cycle day 17 till the next ovulation; during the interval calving till first ovulation examination of the ovaries during the first days post partum was not possible because of insufficient uterine involution; PU: in vivo biopsy prelevation of luteal tissue samples*

Cows were included in the study from 14 days before the expected calving date till the fourth ovulation. After parturition, the cows' genital tracts were examined daily by trans rectal ultrasound examination (Tringa, linear, Esaote, 7.5 MHz) to monitor uterine health and ovarian dynamics. The moment of the first ovulation (day of ovulation indicated as day 0 of the cycle) was assessed retrospectively by ultrasonographic determination of a dominant follicle followed by the appearance of a CL in place of that follicle. Cows were subsequently examined on cycle days 4, 9, and 16. On days 9 and 16, the maximal and minimal orthogonal diameter of the maximal luteal cross sectional surface was measured. To calculate this maximal luteal cross-sectional surface, we approximated the actual surface of the CL as the surface of an ellipse: *area*=π*[(*maximal diameter***minimal diameter*)/4]. Also the difference between both surfaces (D16 and D9) was calculated. During the period between cycle day 17

and the next ovulation, ultrasound examination was done on a daily basis to assess the day of ovulation. The same protocol was repeated during estrous cycles two and three, till the fourth ovulation was reached. We defined the start of an estrous cycle as the day of ovulation and its end as the day before the next ovulation. The regularity of the subsequent estrous cycles (i.e. the period between two subsequent ovulations) is also calculated.

From the moment of inclusion in the study till the first ovulation post partum (i.e. day 0 of cycle 1), blood samples were taken with an interval of three days, whereupon blood samples were taken on cycle days 0, 4, 9, and 16 of the first three cycles and on day 0 of cycle four to confirm ovulation. To avoid stress, cows were automatically segregated in a separate pen after they had been milked in the morning by the VMS. Before the blood samples were taken, a rest period of 15 minutes was included and cows were always handled by the same person.

On day 9 of the estrous cycle, five luteal biopsies were harvested out of the cows of groups II and III. Cows were sedated (with 0.02 mg xylazine/kg body weight; Xyl-M® 2%; VMD) after the blood sampling procedure and an epidural anesthesia between the second and third caudal vertebra (using 120 mg procaine hydrochloride; procaine hydrochloride® 4% without adrenalin; VMD) was executed. A device equipped with a multiple angle probe (MAP) and a needle guidance system was inserted intra vaginally. On top of this needle guidance system, a sterile needle (14 gauge (G)) was fixated to puncture through the vaginal wall into the peritoneal cavity ipsilaterally to the ovary bearing the CL. Finally, the biopsy needle was guided through this needle guidance system and the 14 G-needle, and punctured into the CL. The biopsy needle was a modified semi-automatic high-speed Quick Core biopsy needle (QC-19-60.0; Cook Medical, Bloomington, IN, USA), with a length of 60.0 cm, a diameter of 19 G, a biopsy chamber and an echogenic tip. The ultrasound transducer was equipped with software visualizing the path followed by the biopsy needle. As demonstrated earlier (Cools et al., 2013a, 2013b), it was important to take the biopsy through the center of the luteal gland. Two of these samples were immediately immersed in Zinc-salts fixative (ZsF) at room temperature.

Laboratory analyses

a) Analysis of isoflavone molecules and metabolites in the PROT and the serum

Samples of both PROT (soy versus rape) (2 subsets of 7 samples; from each new batch (n=7) during the experimental period one representative sample was taken) were analyzed for isoflavone content. Extraction procedure was optimized by the laboratory of Pharmacognosy

and Phytochemistry (Faculty of Pharmaceutical Sciences, Ghent University, Belgium). Briefly, 1 g product was extracted with 25 ml MeCN, 20 ml H_2O and 5 ml HCL (1M); sonication during 2 times 15 min; centrifugation during 10 min at 3,000 rpm; filtration (20 µm) (not published). Analyses for the aglycones daidzein, glycitein and genistein, including their β-glycosides were done by HPLC executed by a standardized protocol as described by Maubach et al. (2008).

Isoflavones were also quantified in the blood serum. A sample treatment preceding the quantitative UHPLC-MS/MS analysis of isoflavone aglycones in hydrolyzed serum was based on the protocol of Bolca et al. (2010). Serum samples (200 µL), with 20 µL internal standard, were mixed with sodium acetate buffer (0.2 mol/L, pH=5.2; 50:50, vol:vol), incubated with 50 µL *Helix pomatia* type H-2 for 24 h at 37°C, and treated with 800 µL of 200 mmol/L hydrochloric acid in methanol. After centrifugation (5 min at 12,500 x g, 4°C), the supernatant fluid was stored at -20°C until analysis.

Quantification of genistein, daidzein, dihydrodaidzein (DHD), equol, O-desmethylangolensin (O-DMA), glycitein, and internal standard in hydrolyzed serum was performed by UHPLC-MS/MS. Ionization and detection parameters were optimized during infusion experiments with standards of genistein, daidzein, glycitein, DHD, equol, O-DMA, and 4-hydroxybenzophenone. The MS/MS data were collected in multiple reaction monitoring (MRM) mode by monitoring specific transitions of parent and product ions for each analyte.

b) Analysis of other blood parameters

Blood sampling was performed by puncture of the coccygeal vessels using evacuated gel-coated (urea, cholesterol), and heparinized (progesterone) tubes (Vacutainer, Terumo Europe N.V., Leuven, Belgium). Blood samples were centrifuged and stored at -20 °C. Analyses were performed by using a quantitative colorimetric method: urea, cholesterol (Cobas c system; Roche Diagnostics GmBH, Mannheim, Germany), and progesterone (Roche Diagnostics GmBH, Mannheim, Germany). Detection limits and coefficients of variation are published on the instructions for use.

c) Analysis of the luteal biopsies

The luteal biopsies, immersed in Zinc-salts fixative (ZsF), were fixed for 3 days at room temperature (RT), with refreshment of the fixative after the first 24 hours. The biopsies were subsequently dehydrated during 22 hours and embedded in paraffin following a standardized

protocol (STP 420 D, Microm, Prosan, Merelbeke, Belgium). Out of each biopsy, a series of histological sections (5 µm thickness) was made (HM 360, Microm). The biopsies were cut lengthwise. The sections were mounted on APES-coated slides and dried overnight at RT. The even numbered sections were selected for visualization of steroidogenic cells (staining of 3β-hydroxysteroid dehydrogenase (3β-HSD); based on the protocol of Conley et al., 1995), the uneven numbered sections were used for staining of endothelial cells (*Bandaereia simplicifolia* isolectin (BSI); based on Augustin et al., 1995). In both biopsies, three respectively four sections were selected for both stainings, resulting in seven evenly numbered and seven unevenly numbered stained sections. An inner and outer zone were defined geometrically in the tissue sections. Five micrographs were randomly taken in each zone of each section (magnification x 1000) by means of an Olympus DP 50 digital camera mounted on a motorized Olympus BX61 light microscope (Olympus Belgium N.V., Aartselaar, Belgium), resulting in 70 images per staining, per cow. In total we analyzed 4,620 micrographs per staining. The digital micrographs were analyzed using the *'Cell F'* software program (Soft Imaging System, Olympus Belgium N.V.). Based on specific cut off levels for the primary colors red, green and blue, the stained areas could be delineated and quantified in an objective and standardized way. Data collection was done in a single blinded way.

Statistics

a) Normality testing

The blood concentrations of the six monitored parent isoflavone molecules or metabolites needed to be ln-transformed to meet normality conditions. The blood concentration of urea and progesterone were log-transformed. The surface occupation results of angio- and steroidogenesis were both sqrt-transformed.

b) Effect of the biopsy protocol on the luteal function and major luteal characteristics

To examine the effect of the biopsy protocol on luteal function, data of the soy, puncture group were analyzed against those of the soy, control group. Several models were composed to analyze the effect of the biopsy sampling on the luteal function and major luteal characteristics: linear mixed models were constructed by PROC MIXED from SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA, 2010) with plasma progesterone concentration, luteal dimensions or cyclic regularity as dependent variables and group (n=2; soy, control versus soy, puncture) as independent fixed factor. We corrected for the

fact that measures were repeated within the same cow in 3 subsequent estrous cycles using the repeated measures module.

c) Isoflavone concentrations in the two types of PROT and their effect on blood levels of isoflavone molecules and their metabolites

First, descriptive statistics describing the concentration of the different isoflavone molecules were assessed based on the seven subsamples for each PROT using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA, 2010). Then, we tested the effect of PROT type and estrous cycle number (1, 2, 3) on the blood isoflavone concentrations. Therefore, a linear mixed effects model was created (PROC MIXED from SAS version 9.3), including Group (II, III) and estrous cycle number (1, 2, 3) as fixed variables and isoflavone concentrations (glycitein, genistein, equol, O-DMA, DHD and daidzein) as the outcome variable. The group*cycle number interaction was also computed. Other independent factors (cycle day, lactation number, BCS difference, sqrt daily MP, log urea and cholesterol) were included in the model to correct for confounding effects, but were not visualized. Repeated measures within the cow were taken into account by adding cow within PROT in the RANDOM statement with the unstructured covariance structure and 'estrous cycle number' in the REPEATED statement with an autoregressive order 1 covariance structure.

d) Effects of soy versus rape supplementation on luteal angio- and steroidogenesis during the first 3 estrous cycles post partum

We tested the hypothesis that soy in comparison with rape supplementation impairs the angiogenesis and consequently also the steroidogenesis in the developing luteal gland. Therefore, a linear mixed effects model (PROC MIXED from SAS version 9.3) was used to investigate whether the dependent variables 'area occupied by BSI-positive cells' and 'area occupied by 3β-HSD-positive cells' were significantly different in the soy versus rape supplemented groups. As in the model described above, this model was corrected for possible confounding effects and for the repeated measurements within the same cow.
To examine the association between the isoflavone concentrations in the blood and the luteal angio- and steroidogenetic capacity of the CLa, Pearson correlation coefficients were calculated between the isoflavone blood concentrations and the luteal area occupied by BSI and 3β-HSD positive cells respectively.

e) Effect of soy versus rape supplementation on the peripheral progesterone concentration during the first 3 estrous cycles post partum

Again, a linear mixed model (PROC MIXED from SAS version 9.3) was constructed with peripheral progesterone concentration as dependent variable and PROT as independent fixed variable. Again, this model was corrected for possible confounding effects and for the repeated measurements within the same cow.

Results

a) Effect of the biopsy sampling on luteal function and major luteal characteristics.

There was no significant difference in the log-transformed blood progesterone concentration between both soy groups (soy, control 1.70 versus soy, puncture 1.51) indicating that puncturing the CL at day 9 of the cycle did not affect the progesterone secretion by the CL. Furthermore, no significant differences could be found between the maximal luteal surface of the CLs at day 9 and 16 of the cycle (respectively soy, control 5.28 cm^2 vs. soy, puncture 5.72 cm^2 and soy, control 5.34 cm^2 vs. soy, puncture 6.64 cm^2), nor were there significant differences in cycle regularity between the non-punctured versus punctured soy cows (24.93 days vs. 25.74 days).

Based on these results, we concluded that puncturing the CL on day 9 of the estrous cycle does not affect luteal morphometrics and progesterone secretion. Therefore, the data of the soy control and the soy puncture group were merged to analyze the effect of soy supplementation versus rape supplementation.

b) Isoflavone concentrations in the PROT and in the blood of the cows in relation to the type of PROT supplemented

In table 2, the results of the isoflavone analysis of the soybean meal are visualized.

Table 2. *descriptive statistics (mean, median, standard deviation (SD), minimum (min), and maximum (max)) of the concentration (mg/g) of the aglycones: daidzein, glycitein and genistein, including their β-glycosides, their acetyl- and malonylglucosides in the soybean meal (n=7)*

molecule	mean	median	SD	min	Max
daidzin	0.6186	0.6490	0.09445	0.4985	0.7505
glycitin	0.2076	0.1863	0.05661	0.1353	0.2958
genistin	1.1555	1.1547	0.1498	0.9864	1.4081
malonyldaidzin	0.2810	0.2807	0.03497	0.2360	0.3217
malonylglycitin	0.09181	0.07880	0.02999	0.0650	0.1415
malonylgenistin	0.5853	0.5902	0.04769	0.5151	0.6487
acetyldaidzin	0.1190	0.1227	0.01444	0.1025	0.1341
acetylglycitin	0.06617	0.0641	0.01161	0.0525	0.0830
acetylgenistin	0.2159	0.2188	0.0171	0.1941	0.2461
daidzein	0.0101	0.0104	0.0006997	0.0090	0.0109
glycitein	0.05597	0.05200	0.0102	0.0440	0.0702
genistein	0.0414	0.0382	0.007669	0.0354	0.0548
total daidzin equivalents	**0.9757**	**1.0400**	**0.1325**	**0.80**	**1.14**
total genistin equivalents	**1.9071**	**1.9600**	**0.2043**	**1.68**	**2.25**
total glycitin equivalents	**0.4229**	**0.3900**	**0.10062**	**0.30**	**0.57**
total isoflavones	**3.3057**	**3.4200**	**0.3979**	**2.78**	**3.82**

The results of the rapeseed meal analysis were systematically situated beneath the detection limit (for aglycones: < 5 µg/g; for glycosides: < 7.5-10 µg/g, depending on the molecule). In table 3, the estimated b-values for the time effect (estrous cycle number) and PROT effect (soy versus rape) on the different isoflavone blood concentrations are expressed together with their significance levels.

Table 3. *the estimated effects (b-values (β)), standard error (SD) and p-values (P) for the time effect (estrous cycle number) and protein rich concentrate (PROT; soy versus rape) effect on the concentration of different isoflavone molecules in the peripheral circulation*

effect	**intercept**			**estrous cycle number**						**PROT**				
				cycle 1		**cycle 2**		**cycle 3**[1]			**soy**		**rape**[1]	
molecule	**β**	**SD**	**P**	**β**	**SD**	**β**	**SD**	**β**	**SD**	**P**	**β**	**SD**	**β**	**SD**	**P**
glycitein	-0.18	2.08	0.68	-0.74	0.29	0.18	0.25	0	…	0.0036	-0.49	0.38	0	…	0.20
genistein	8.38	0.10	<0.0001	0.017	0.015	0.0043	0.013	0	…	0.50	-0.0019	0.014	0	…	0.89
equol	2.49	3.05	0.40	-0.80	0.42	-0.28	0.37	0	…	0.040	2.39	0.61	0	…	0.0001
O-DMA	2.47	0.56	0.0001	-0.22	0.085	-0.15	0.075	0	…	0.027	0.36	0.081	0	…	<0.0001
DHD	3.13	0.95	0.0007	0.11	0.13	-0.21	0.11	0	…	0.032	0.36	0.18	0	…	0.050
daidzein	4.41	0.19	<0.0001	-0.0019	0.029	0.0067	0.026	0	…	0.94	0.062	0.027	0	…	0.022

[1]estrous cycle 3 and rape-group are the reference levels.

Concentrations of the molecules equol, O-DMA, and DHD increased with time and were systematically higher in soy-fed cows. Also the glycitein concentration increased over time, while for daidzein a discrete but significant increase in the blood content was detected in soy-fed cows. A non-significant PROT*time interaction indicates that the isoflavone concentrations change similarly over time in both supplemented groups.
In table 4, the estimated mean blood concentrations are expressed (together with the 95%-confidence intervals), differentiated for estrous cycle number and PROT.
The results were obtained by back-transformation of the outcomes of the LSMESTIMATE tool of SAS (version 9.3).

c) Effect of the type of PROT on angio- and steroidogenesis of the CL at day 9 of the estrous cycle

In table 5, the estimated b-values for estrous cycle number and PROT effect on luteal angio- and steroidogenesis are expressed together with their significance levels. The interaction between estrous cycle number and PROT was significant for the surface occupation by HSD positive cells: the increase from cycle 1 to cycle 2 was significantly higher for the rape group compared to the soy group. Results show that both angio- and steroidogenesis are increasing with estrous cycle number, while they are both significantly lower in the soy-supplemented cows.
In table 5, the estimated relative mean area occupied by endothelial or steroidogenic cells is expressed (together with the 95%-confidence intervals), differentiated for PROT (soy versus rape). The results were obtained by back-transformation of the outcomes of the LSMESTIMATE tool of SAS (version 9.3).
In figure 2, a scatter plot is designed, expressing the correlation between sqrtareaBSI and sqrtareaHSD.

Table 4. *the estimated mean blood concentrations (µg/L) and [95%-confidence interval; C.I.] for the six parent isoflavone molecules or metabolites, differentiated for estrous cycle number and protein rich concentrate ('PROT')*

effect	**estrous cycle number**						**PROT**			
	cycle 1		**cycle 2**		**cycle 3**		**Soy**		**rape**	
molecule	**mean**[1]	**C.I.**[1]	**mean**	**C.I.**	**mean**	**C.I.**	**mean**	**C.I.**	**mean**	**C.I.**
glycitein	0.2[1]	[0.1;0.3]	0.4[2]	[0.3;0.7]	0.3[1,2]	[0,2;0.6]	0.2	[0.2;0.3]	0.4	[0.2;0.7]
genistein	4,492	[4,403;4,583]	4,403	[4,316;4,492]	4,403	[4,316;4,492]	4,447	[4,359;4,492]	4,403	[4,316;4,492]
equol	0.2	[0.1;0.4]	0.3	[0.2;0.7]	0.45	[0.2;1.0]	1.0[1]	[0.6;2.0]	0.1[2]	[0.03;0.3]
O-DMA	13.6	[12.1;15.3]	14.4	[12.9;16.1]	16.8	[14.9;18.9]	17.8[1]	[16.4;19.3]	12.4[2]	[10.9;14.2]
DHD	8.3[1]	[6.6;10.5]	6.1[2]	[4.9;7.6]	7.54[1,2]	[6.0;9.4]	8.7[1]	[7.2;10.5]	6.1[2]	[4.5;8.2]
daidzein	79.84	[76.71;83.10]	80.6	[77.5;83.9]	79.84	[76.7;83.1]	82.3[1]	[80.6;84.8]	77.5[2]	[74.4;80.6]

[1]Based on the back-transformation of LSMESTIMATE: mean with 95%-confidence intervals (C.I.)

[1] and [2] indicate differences ($P < 0.05$) of the estimated mean concentrations between the different categories within a fixed factor.

Table 5. *the effects of estrous cycle number ('cycle') and protein rich concentrate ('PROT') on areaBSI and areaHSD; and the estimated relative mean area occupied by endothelial and steroidogenic cells differentiated for cycle number and PROT*

effect	areaBSI[2]					areaHSD[2]				
	β[3]	SD[4]	mean[6]	[C.I.][6]	P[4]	β	SD	mean	[C.I.]	P
intercept	3.08	0.64			…	8.37	1.42			…
cycle										
1	-0.39[1]	0.087	6.43	[5.81;7.07]		-1.33[1]	0.25	20.11	[17.73;22.65]	
2	-0.038[2]	0.072	8.34	[7.72;8.99]	<0.0001	-0.18[2]	0.21	27.44	[24.94;30.05]	<0.0001
3	0[2]	…	8.54	[7.89;9.22]		0[2]	…	30.68	[27.97;33.51]	
PROT[1]										
soy	-0.22[1]	0.076	7.16	[6.60;7.74]	0.012	-0.87[1]	0.26	22.12	[19.72;24.66]	0.0007
rape	0[2]	…	8.35	[7.74;8.98]		0[2]	…	29.94	[27.07;32.95]	
cycle*PROT[5]										0.025

[1]PROT: 'soy' represents the cows fed soybean meal; 'rape' the cows fed rapeseed meal

[2]sqrtareaBSI and sqrtareaHSD: the measured area occupied by endothelial cells or steroidogenic cells were sqrt-transformed for further analysis

[3]β: the β-factor was estimated based on the LS-Means-method

[4]SD: standard deviation; P: P-value for each fixed factor

[5]categorized data not shown

[6]based on back transformation of LSMESTIMATES

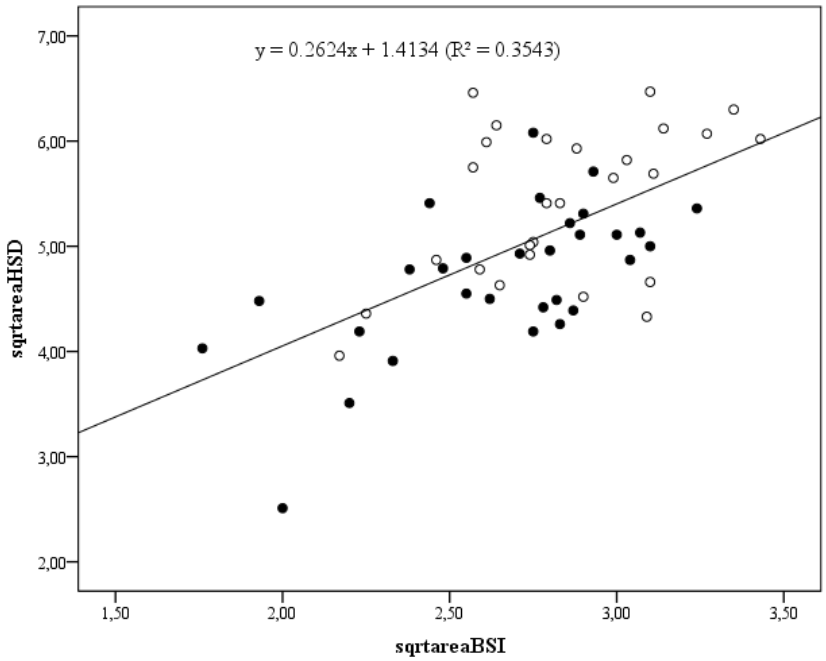


Figure 2: *scatter plot visualizing the correlation between sqrtareaBSI and sqrtareaHSD (black circles represent cows of the soy group, white circles are representatives of the rape group)*

In this image, one can see that the results of group II (= soy) are situated more in the lower left quadrant, while those of group III (= rape) are situated more in the upper right quadrant indicating that luteal glands of cows supplemented with soybean meal contained a lower concentration of steroidogenic and vascular cells versus the CLa of cows receiving rapeseed meal.

When the correlations between the blood concentrations of the different isoflavone molecules and sqrtareaHSD (= steroidogenesis) or sqrtareaBSI (= angiogenesis) were assessed, a significantly negative correlation was found between equol and sqrtareaBSI and sqrtareaHSD respectively -0.337 (P=0.01) and -0.410 (P=0.002). The same was found for glycitein, respectively -0.233 (P=0.085; trend) and -0.351 (P=0.008).

d) Effect of type of PROT on the peripheral progesterone concentration

No significant effect (P=0.55) of the PROT on the (log transformed) serum progesterone concentrations could be found. As no significant associations with the peripheral

progesterone concentrations could be detected, detailed data from the different models are not presented.

Discussion

In the present field study, we examined whether the use of soybean meal as protein source for high yielding dairy cows negatively affects angio- and steroidogenesis of the cows' CL since this could contribute to declined fertility results. The rationale to perform the study was the fact that in humans, soy based diets are associated with a lower risk to suffer from aggressive tumors based on the anti-angiogenetic effects of the isoflavone compounds present in soy products.

To increase the strength of the results in terms of 'usability for practice', the study was performed on the dairy herd of the Faculty of Veterinary Medicine of the Ghent University (Belgium) which is commercially run, and specific interventions for the study were strictly minimized to dividing the participating cows into three groups based on the supplementation of a specific PROT (soy versus rapeseed based) and on the fact that cows were subjected to biopsy sampling of their CL or not. The amount of PROT offered to the cows was not specifically adapted for the study and was calculated according to the cows' needs for maintenance and milk production using the Dutch protein evaluation system (Tamminga et al., 1994). On average, cows were supplemented with approximately 2 kg soybean meal per animal, which is a realistic amount and reflects what is currently offered to cows in many European dairy herds.

First of all, we assessed whether the biopsy procedure influenced luteal morphology and function. To do so, the peripheral blood progesterone concentration, the dimensions of the maximal luteal surface, and cycle length were compared between a punctured versus a non-punctured control group both receiving soybean meal as protein source. As no differences could be detected between these two groups, we concluded that biopsy sampling at day 9 of the cycle does not significantly interfere with the luteal function during that estrous cycle. The latter is in agreement with Kot et al. (1999) and Tsai et al. (2001) who performed similar studies based on biopsy sampling of the CL. Although their sampling protocol was even more intense than ours, they also could not detect a significant effect on subsequent blood progesterone, luteal area and the interval biopsy-next ovulation.

Generally, genistein is known to be the isoflavone molecule most abundantly present in soybeans and to have the strongest estrogenic effect (Shutt and Cox, 1972). Daidzein and

glycitein are also present in soybeans and like genistein, they are estrogenic active. In literature, a substantial variation in isoflavone concentrations in soybeans has been described. One can find that soybeans contain 1.2 to 4.2 mg isoflavones per g dry matter (Bingham et al., 1998; Eldridge and Kwolek, 1983; Mazur and Adlercreutz, 1998), with mostly genistein, daidzein and their conjugates, and in lesser amounts glycitein (Kurzer and Xu, 1997; Tham et al., 1998). Isoflavones are associated with the protein fraction of the soybeans, while the oil fraction only contains negligible isoflavone amounts (Mazur, 1998). The latter may result in an enrichment of the amount of isoflavones present in soybean meal versus their amount in the oil fraction or in whole soybeans as such. Notwithstanding this enrichment, only moderate concentrations of 2.16 to 3.0 mg/g fresh product have been mentioned in soybean meal products used as a dietary protein source for dairy cows (Coward et al., 1993; Farmakalidis and Murphy, 1985; Seo and Morr, 1984). Also other factors such as growth rate of the plants, stress caused by acute changes in temperature, humidity, insects, plant variety (influences the topographic distribution of isoflavones within the plant), the harvest- and conservation method have been mentioned to affect the isoflavone concentration in soy compounds (Ibarreta et al., 2001; Liggins et al., 2000; Price and Fenwick, 1985; Tsukamoto et al., 1995). Moreover, besides the variations described, heat treatment is known to significantly increase the bio-availability of the bio-active compounds by facilitating the decarboxylation from malonyl- to acetylglucosides and further to aglycones (Barnes et al., 1994). Seo and Morr (1984) found 2.88 mg isoflavones per g fresh soybean meal, of which 2.16 mg was genistein. Zdunczyk et al. (2006) found 3 mg isoflavones per g fresh soybean meal. In our samples, we found 3.14 mg/g, which is in the same range. Isoflavone molecules found in the soybean meal of the present study were mostly genistin equivalents, followed by daidzin equivalents and glycitin.

Trinacty et al. (2009) analyzed the isoflavone content (genistein and daidzein) in extruded rapeseed cake. The concentrations were below the sensitivity level of the used analytical method. The limit of detection (LOD) for daidzein was 3.5 µg/g and 2.8 µg/g for genistein. The isoflavone content in the rapeseed meal of our study was also situated beneath the detection limits which were < 5 µg/g for aglycones, and < 7.5-10 µg/g for glycosides, depending on the molecule.

The most prominent isoflavone molecules detectable in the peripheral circulation are daidzein, genistein, equol and O-desmethylangolensin (O-DMA). Genistein, daidzein and glycitein are inactive glucosides, which after consumption are partially metabolized to heterocyclic phenols, known to have estrogen like activities. Soybeans themselves do not

contain equol, nor O-DMA and dihydrodaidzein (DHD). The latter three are known as isoflavone metabolites detectable in the blood, among which equol is known to be more estrogenic than daidzein, while there is a lot of debate concerning the estrogenic activity of O-DMA and DHD. We did not analyze para-ethylphenol, as this molecule is not supposed to be estrogenically active (Lundh, 1995; Setchell et al., 2002).

Watzkova et al. (2010) fed lactating dairy cows extruded full fat soy (4 kg on DM-basis) versus extruded rapeseed cake. The plasma concentrations for daidzein, genistein and equol were significantly higher in the soy group. Woclawek-Potocka et al. (2005) and Piotrowska et al. (2006) fed cows soybeans and found also significant concentrations of equol in the plasma, but were not able to detect considerable concentrations of daidzein or genistein. Reason could be the difference in dynamics of these molecules in the peripheral circulation: the interval between consumption of the isoflavone molecules and their appearance in the blood is molecule specific. Also the half-life is mentioned to be rather specific for each individual isoflavone molecule. Woclawek-Potocka et al. (2008) described these dynamics more in detail, although this is of less importance for the interpretation of our data, since in our study the PROT was delivered by an automated system giving the daily amount spread over an interval of 24 hours.

Trinacty et al. (2009) found significantly lower blood concentrations for daidzein, genistein and equol in cows fed extruded rapeseed (58.0 mg/day) versus cows fed extruded full fat soybeans (3,297.0 mg/day): daidzein respectively 13.5 ng/ml and 49.3 ng/ml, genistein 42.9 ng/ml and 78.7 ng/ml, equol 18.3 ng/ml and 218.8 ng/ml. Nevertheless, the spread of these observations is too open to sustain our present observation. Zdunczyk et al. (2006) did a similar experiment as we did. They fed 40 postpartum cows (till 70 days post partum) an identical basic ration supplemented with 3 kg soybean meal (20 cows) versus 0.7 kg soybean meal (20 cows). During a period of 10 weeks, a significant difference in the blood plasma concentration of isoflavones (sum of daidzein, genistein, equol and para-ethylphenol) was seen: 5 µmol/l and 2 µmol/l respectively.

Based on the results of the present study, it can be concluded that the blood concentration of the parent isoflavone molecules is not strongly influenced by the type of PROT, while the concentration of their metabolites (equol, DHD and O-DMA) is significantly higher in soybean versus rapeseed meal-supplemented cows. In the present study, the blood isoflavone metabolite concentration increased with increasing time post partum. The latter may be contributed to a facilitation of the metabolization to O-DMA and equol, or to the increasing amount of supplemented soy concomitant with the increasing level of milk production. But

these are just speculative thoughts and need further research. Piotrowska et al. (2006) found a significant increase of equol and para-ethylphenol in bovine plasma and luteal tissue after consumption of 2.5 kg of bruised soybeans per day. The achievable blood concentration is much higher than the concentration of the endogenous estrogens, but their activity is lower (factor 0.01-0.001) (Benassayag et al., 2002). Postpartal fat mobilization may also have contributed to the gradual increase in blood concentration of isoflavones, since the fat concentration of these molecules could be 20 times higher than the plasma concentration. In addition, genistein and daidzein induce an inhibition of lipogenesis and an activation of lipolysis (Nowicka et al., 2006).

Several studies described detrimental effects of isoflavones on cows' fertility (*in vivo* and *in vitro*) (Kallela et al., 1984; Kaplanski, 1981; Nwannenna et al., 1994; Zdunczyk et al., 2005). To the best of our knowledge however, no study has analyzed the effect on the luteal histological constitution and histophysiology. The progesterone concentration in the plasma did not differ significantly between both PROT groups in our study. This is in accordance with the study of Woclawek-Potocka et al. (2005) and Watzkova et al. (2010). Kaplanski et al. (1981) stated that the suppressing effect of the isoflavones (biochanin A and genistein) on the *in vitro* progesterone synthesis of bovine granulosa cells was dose dependent and biphasic. Below a certain concentration, progesterone synthesis was stimulated; above this threshold progesterone synthesis was suppressed. This threshold concentration was shown to be dependent on the LH-concentration. The more LH a cow secretes, the higher the isoflavone concentration should be in order to efficiently suppress progesterone synthesis. Wong and Keung (1999) found that genistein, daidzein, biochanin and formononetin inhibit the 3β-HSD/isomerase complex.

Apart from luteal steroidogenesis, blood progesterone concentrations also depend on the hematogenic supply of constituents towards the CL and removal of residues and progesterone out of the CL (Arfuso et al., 2005). In a mature bovine CL, 40 to 75% of the cells are vascular. The result is a dense capillary network with 2 to 4 capillaries for each steroidogenic cell (Goede et al., 1998). In comparison to other organs, the blood flow per tissue unit is the highest for the CL: on day 14, an ovine CL receives 1,122 ml blood/(min*100g) (Bruce and Moor, 1976). In the present study, CLa of cows supplemented with soybean meal have a capillary network that is significantly sparser in comparison to CLa of rape supplemented cows. Some studies have described a detrimental effect of isoflavones on angiogenesis. Fotsis et al. (1993, 1997) stated that pathologic angiogenesis (i.e. in aggressive tumors) was decreased following soy supplementation. They furthermore demonstrated that genistein

inhibits the basic fibroblast growth factor (bFGF)-induced invasion into gels and the formation of capillary like structures. Chapin et al. (1996), Kurzer and Xu (1997), and Tham et al. (1998) describe an anti-angiogenic effect of genistein. Zhou et al. (1999) found a reduction in microvessel density in a human prostate tumor transplanted in mice, when soy was consumed. Li and Sarkar (2002) stated that genistein downregulates the transcription and translation of genes controlling angiogenesis in human prostate tumors. This effect was dose- and time dependent. In our study, we found that the blood concentrations of equol and glycitein were negatively correlated with sqrtareaBSI and sqrtareaHSD representing respectively luteal angio- and the steroidogenesis. Furthermore, we showed that feeding soybean meal was associated with a significantly smaller area of the CL being occupied by 3β-HSD and BSI positive cells. A positive correlation between the area occupied by 3β-HSD positive cells and that occupied by BSI positive cells (data not shown), which is in agreement with the results of Yamashita et al. (2008), indicates that luteal steroidogenesis and angiogenesis are linked with each other at the level of the molecular regulation. Nevertheless, we were not able to detect a significant correlation between the blood progesterone concentration and the luteal vascularization at day 9 of the estrous cycle. The latter is in agreement with Lüttgenau et al. (2011) who concluded that plasma progesterone concentrations in the mid luteal phase are independent of luteal blood flow.

Conclusions

In the present study we demonstrated that commercially available soybean meal contains isoflavones in concentrations that are able to induce increases in the peripheral concentration of estrogenically active isoflavone metabolites (equol, O-DMA, DHD) in high yielding dairy cows post partum, even when supplemented in relatively low amounts (1.72 kg per day on average). When compared with rapeseed meal, soy supplementation was furthermore associated with a decreased angio- and steroidogenesis at the level of the CL at day 9 of the estrous cycle.

However, we were not able to demonstrate any effect on the peripheral progesterone concentration during the first 3 estrous cycles after calving. Therefore, although the results of our study suggest negative effects of soy feeding on CL activity in recently calved dairy cows, the contribution of this effect on the peripheral progesterone concentration and consequently on overall fertility of these cows, warrants further research.

Acknowledgements

The BOF is thanked for their financial support (05B01906). Lobke De Bels and Liliana Standaert of the laboratory of the Department of Morphology are thanked for their technical support and advice.

Reference List

Adlercreutz H, 1990: Diet, breast cancer, and sex hormone metabolism. Ann NY Acad Sci **595**, 281-290.

Arfuso F, McGeachie JK, Meyer GT, 2005: A quantitative study of blood capillary formation (angiogenesis) concomitant with parenchymal tissue differentiation. Endothelium **12**, 171-177.

Augustin HG, Braun K, Telemenakis I, Modlich U, Kuhn W, 1995: Ovarian angiogenesis. Phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. Am J Pathol **147**, 339-351.

Barnes S, Kirk M, Coward L, 1994: Isoflavones and their conjugates in soy foods - extraction conditions and analysis by HPLC Mass-Spectrometry. J Agric Food Chem **42**, 2466-2474.

Benassayag C, Perrot-Applanat M, Ferre F, 2002: Phytoestrogens as modulators of steroid action in target cells. J Chromatogr B Analyt Technol Biomed Life Sci **777**, 233-248.

Bingham SA, Atkinson C, Liggins J, Bluck L, Coward A, 1998: Phyto-oestrogens: where are we now? Br J Nutr **79**, 393-406.

Bolca S, Li J, Nikolic D, Roche N, Blondeel P, Possemiers S, De Keukeleire D, Bracke M, Heyerick A, van Breemen RB, Depypere H, 2010: Disposition of hop prenylflavonoids in human breast tissue. Mol Nutr Food Res **54** (suppl. 2), S 284-S294.

Bruce NW, Moor RM, 1976: Capillary blood flow to ovarian follicles, stroma and corpora lutea of anaesthetized sheep. J Reprod Fertil **46**, 299-304.

Chapin RE, Stevens JT, Hughes CL, Kelce WR, Hess RA, Daston GP, 1996: Endocrine modulation of reproduction. Fundam Appl Toxicol **29**, 1-17.

Committee on toxicity of chemicals in food, consumer products and the environment (COT), 2003: COT-report, Phytoestrogens and health. Food standards agency.

Conley AJ, Kaminski MA, Dubowsky SA, Jablonka-Shariff A, Redmer DA, Reynolds LP, 1995: Immunohistochemical localization of 3 beta-hydroxysteroid dehydrogenase and P450 17 alpha-hydroxylase during follicular and luteal development in pigs, sheep, and cows. Biol Reprod **52**, 1081-1094.

Cools S, Van den Broeck W, DeVliegher S, Piepers S, Hostens M, Opsomer G, 2013a: Topographic distribution of the different cell types, connective tissue and vascular tissue/lumina within a functional bovine corpus luteum and its association with breed, type of fixation protocol and stage during the cycle. Reprod Domest Anim **48**, 627-635.

Cools S, Van den Broeck W, DeVliegher S, Piepers S, Opsomer G, 2013 b: The bovine luteal histological composition: a topographic point of view. Reprod Domest Anim **48**, e29-e32.

Coward L, Barnes NC, Setchell KDR, Barnes S, 1993: Genistein, daidzein, and their beta-glycoside conjugates - antitumor isoflavones in soybean foods from American and Asian diets. J Agr Food Chem **41**, 1961-196.

Dhom G, 1991: Epidemiologic and morphogenetic aspects of prostate cancer. Urologe A **30**, 3-4.

Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G, 1989: A body condition scoring chart for Holstein dairy cows. J Dairy Sci **72**, 68-78.

Eldridge AC, Kwolek WF, 1983: Soybean Isoflavones - Effect of Environment and Variety on Composition. J Agric Food Chem **31**, 394-396.

Farmakalidis E, Murphy PA, 1985: Isolation of 6"-O-Acetylgenistin and 6"-O-Acetyldaidzin from toasted defatted soyflakes. J Agric Food Chem **33**, 385-389.

Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L, 1993: Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis. Proc Natl Acad Sci U.S.A. **90**, 2690-2694.

Fotsis T, Pepper MS, Aktas E, Breit S, Rasku S, Adlercreutz H, Wahala K, Montesano R, Schweigerer L, 1997: Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. Cancer Res **57**, 2916-2921.

Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG, 1998: Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest **78**, 1385-1394.

Hollman PCH, 2001: Evidence for health benefits of plant phenols: local or systemic effects? J Sci Food Agricult **81**, 842-852.

Ibarreta D, Daxenberger A, Meyer HHD, 2001: Possible health impact of phytoestrogens and xenoestrogens in food. Apmis **109**, S402-S425.

Kallela K, Heinonen K, Saloniemi H, 1984: Plant oestrogens; the cause of decreased fertility in cows. A case report. Nord Vet Med **36**, 124-129.

Kaplanski O, Shemesh M, Berman A, 1981: Effects of phyto-oestrogens on progesterone synthesis by isolated bovine granulosa cells. J Endocrinol **89**, 343-348.

Kot K, Anderson LE, Tsai SJ, Wiltbank MC, Ginther OJ, 1999: Transvaginal, ultrasound-guided biopsy of the corpus luteum in cattle. Theriogenology **52**, 987-993.

Kurzer MS, Xu X, 1997: Dietary phytoestrogens. Annu Rev Nutr **17**, 353-381.

Li YW, Sarkar FH, 2002: Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. Cancer Lett **186**, 157-164.

Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA, 2000: Daidzein and genistein contents of vegetables. Br J Nutr **84**, 717-725.

Lundh T, 1995: Metabolism of estrogenic isoflavones in domestic-animals. Proc Soc Exp Biol Med **208**, 33-39.

Lüttgenau J, Ulbrich SE, Beindorff N, Honnens A, Herzog K, Bollwein H, 2011: Plasma progesterone concentrations in the midluteal phase are dependent on luteal size, but independent of luteal blood flow and gene expression in lactating dairy cows. Anim Reprod Sci **125**, 20-29.

Maubach J, Bracke M, Depypere H, De Keukeleire D, Heyerick A, 2008: Cellular uptake of soy-derived phytoestrogens *in vitro* and in human whole blood. JPCCR **2**, 064-070.

Mazur W, 1998: Phytoestrogen content in foods. Baillieres Clin. Endocrinol. Metab **12**, 729-742.

Mazur W, Adlercreutz H, 1998: Naturally occurring oestrogens in food. Pure Appl Chem **70**, 1759-1776.

Mills PK, Beeson WL, Phillips RL, Fraser GE, 1989. Cohort study of diet, lifestyle, and prostate cancer in Adventist men. Cancer **64**, 598-604.

Nowicka E, Szkudelski R, Nogowski L, 2006: Influence of phytoestrogens on humans and animals. Medycyna Weterynaryjna **62**, 736-738.

Nwannenna AI, Madej A, Lundh TJ, Fredriksson G, 1994: Effects of oestrogenic silage on some clinical and endocrinological parameters in ovariectomized heifers. Acta Vet Scand **35**, 173-183.

Piotrowska K, Woclawek-Potocka I, Bah MM, Piskula M, Pilawski W, Bober A, Skarzynski DJ, 2006: Phytoestrogens and their metabolites inhibit the sensitivity of the bovine corpus luteum on luteotrophic factors. J Reprod Dev **52**, 33-41.

Price KR, Fenwick GR, 1985: Naturally-occurring estrogens in foods - A Review. Food Addit Contam **2**, 73-106.

Seo A, Morr CV, 1984: Improved high-performance liquid-chromatographic analysis of phenolic-acids and isoflavonoids from soybean protein products. J Agric Food Chem **32**, 530-533.

Setchell KDR, Brown NM, Lydeking-Olsen E, 2002: The clinical importance of the metabolite equol - A clue to the effectiveness of soy and its isoflavones. J Nutr **132**, 3577-3584.

Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM, 1991: Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. Br J Cancer **63**, 963-966.

Shutt DA, Cox RI, 1972: Steroid and phytoestrogen binding to sheep uterine receptors *in vitro*. J Endocrinol **52**, 299-310.

Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M, de Haan C, 2006: Livestock's long shadow, FAD, Rome 2006.

Su SJ, Yeh TM, Chuang WJ, Ho CL, Chang KL, Cheng HL, Liu HS, Cheng HL, Hsu PY, Chow NH, 2005: The novel targets for anti-angiogenesis of genistein on human cancer cells. Biochem Pharmacol **69**, 307-318.

Tamminga S, Van Straalen WM, Subnel APJ, Meijer RGM, Steg A, Wever CJG, Blok MC, 1994: The Dutch protein evaluation system: the DVE/OEB-system. Livest Prod Sci **40**, 139-155.

Tham DM, Gardner CD, Haskell WL, 1998: Clinical review 97 - Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. J Clin Endocr Metab **83**, 2223-2235.

Trinacty J, Krizova L, Schulzova V, Hajslova J, Hanus O, 2009: The effect of feeding soybean-derived phytoestogens on their concentration in plasma and milk of lactating dairy cows. Arch Tierernahr **63**, 219-229.

Tsai SJ, Kot K, Ginther OJ, Wiltbank MC, 2001: Temporal gene expression in bovine corpora lutea after treatment with PGF2α based on serial biopsies *in vivo*. Reproduction **121**, 905-913.

Tsukamoto C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K, 1995: Factors affecting isoflavone content in soybean seeds - changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J Agric Food Chem **43**, 1184-1192.

Watzkova J, Krizova L, Pavlik A, Schulzova V, Hajslova J, Lojza J, 2010: The effect of soybean-derived phytoestrogens on concentrations of plasma isoflavones, 15-keto-13,14-dihydroprostaglandin F_{2alfa} and progesterone in dairy cows. Acta Vet Brno **79**, 525-532.

Woclawek-Potocka I, Bah MM, Korzekwa A, Piskula MK, Wiczkovski W, Depta A, Skarzynski DJ, 2005: Soybean derived phytoestrogens regulate prostaglandin secretion in endometrium during the oestrous cycle and early pregnancy in cattle. Exp Biol Med **230**, 189-199.

Woclawek-Potocka I, Piskula MK, Bah MM, Siemieniuch MJ, Korzekwa A, Brzezicka E, Skarzynski DJ, 2008: Concentration of isoflavones and their metabolites in the blood of pregnant and non-pregnant heifers fed soy bean. J Reprod Dev **54**, 358-363.

Wong CK, Keung WM, 1999: Bovine adrenal 3 beta-hydroxysteroid dehydrogenase (EC 1.1.1.145)/5-ene-4-ene isomerase (EC 5.3.3.1): characterization and its inhibition by isoflavones. J Steroid Biochem Mol Biol **71**, 191-202.

Yamashita H, Kamada D, Shirasuna K, Matsui M, Shimizu T, Kida K, Berisha B Schams D, Miyamoto A, 2008: Effect of local neutralization of basic fibroblast growth factor and vascular endothelial growth factor by a specific antibody on the development of the corpus luteum in the cow. Mol Reprod Dev **75**, 1449-1456.

Ying CW, Hsu JT, Shieh SC, 2001: Growth inhibition of human endothelial cells by the phyto-oestrogen biochanin A, a metabolite of genistein. Br J Nutr **85**, 615-620.

Zdunczyk S, Janowski T, Baranski W, Ras M, 2006: Studies on the effect of isoflavones on postpartum ovarian activity in cows. Tieraerztl Umschau **61**, 127-132.

Zdunczyk S, Piskula M, Janowski T, Baranski W, Ras M, 2005: Concentrations of isoflavones in blood plasma of dairy cows with different incidence of silent heat. B Vet I Pulawy **49**, 189-191.

Zhou JR, Gugger ET, Tanaka T, Guo YP, Blackburn GL, Clinton SK, 1999: Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. J Nutr **129**, 1628-1635.

Chapter 5.2

A field study to unravel factors that are significantly associated with the activity of the corpus luteum during the first three postpartum cycles in high yielding dairy cows, based on the amount of steroidogenic and endothelial cells present in the luteal tissue

Cools S[a], Van den Broeck W[b], Bossaert P[c], Hostens M[a], Opsomer G[a]

[a]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

[b]Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

[c]Department of Veterinary Sciences, Campus Drie Eiken, Antwerp University, Universiteitsplein 1, 2610 Wilrijk, Belgium

Reproduction in Domestic Animals

Accepted

Abstract

In the present study, 22 high yielding Holstein-Friesian cows (14 multi- and 8 primiparous) were followed from the first till the fourth ovulation post partum. Housing conditions and basic ration were identical for all animals. Concentrates were individually supplemented according to the daily milk production level, using two different types of protein rich concentrates: soybean and rapeseed meal. Cows were randomly divided into two groups and blocked for parity. Group I (n = 11) was supplemented with soybean meal, while Group II (n = 11) was provided with rapeseed meal. Both groups were subjected to a biopsy sampling of the corpus luteum at day 9 of the estrous cycle. Several production and blood parameters, linked with the typical homeorrhetic adaptations during a period of negative energy balance, were monitored. In the luteal biopsies, endothelial cells were visualized by *Bandeiraea simplicifolia* isolectin and the steroidogenic cells by 3β-hydroxysteroiddehydrogenase. Daily milk yield, changes in body condition score and blood concentrations of non-esterified fatty acids and β-hydroxy-buturic acid were not significantly different between the soy- and the rape-supplemented groups, leading to the conclusion that the difference in protein rich concentrate did not induce group specific differences in energy status.

Total blood cholesterol concentration increased significantly with days post partum and with days within the estrous cycle (day 0-4 versus day 9-16). Blood urea concentrations were systematically higher in the soy-supplemented and multiparous cows.

The vascular network within the corpus luteum was significantly denser in cycles 2 and 3 in comparison to the first postpartum cycle (P = 0.0005). An identical evolution was seen for the surface occupied by steroidogenic cells (P = 0.0001).

In comparison to multiparous cows, primiparous animals had larger luteal glands on cycle day 9. No cycle number effect was detected on the size of the corpus luteum at day 9 of the cycle, while on day 16 luteal glands became larger with increasing cycle number.

The peripheral blood progesterone concentration showed besides the predictable effect related to the day of the estrous cycle, an increasing trend with increasing cycle number. Furthermore, the peripheral progesterone concentration was overall higher in primiparous cows (P = 0.013).

The area occupied by endothelial cells was positively correlated with the area occupied by steroidogenic cells (r = 0.59; P < 0.0001). Nevertheless, there was no significant correlation between both areas and the blood progesterone concentration. Both the areas occupied by endothelial and by steroidogenic cells were negatively correlated with the blood

concentration of non-esterified fatty acids (respectively r = -0.377; P = 0.004 and r =-0.355; P = 0.007).

Based on the results of the present study, we conclude that primiparous cows generally have higher peripheral progesterone levels during the first 3 cycles after calving which is associated with a bigger corpus luteum. In comparison to those of the first postpartum cycle, corpora lutea of cycles 2 and 3 have a denser capillary network and a higher number of steroidogenic cells while these are only associated with a trend of higher peripheral progesterone concentrations.

Introduction

In present-day dairy cows, the embryonic and fetal mortality rate is stated to reach up to 56% (Diskin et al, 2012). Early embryonic death seems in lactating high-yielding dairy cows more prevalent in comparison to non-lactating heifers and non-lactating dairy cows (Sartori et al, 2002; Cerri et al, 2009 a; b; Diskin et al, 2012).

Suboptimal peripheral progesterone concentrations during the diestrous period both preceding insemination, as well as during early pregnancy have been suggested to be at least co-responsible for a substantial amount of these early embryonic losses (Niswender and Nett, 1994; Mann and Lamming, 2001, Diskin and Morris, 2008; Diskin et al, 2012; Wiltbank et al, 2012). Staples et al (1997) found that the conception rate rose with 1.44%, when plasma progesterone 3 days before timed artificial insemination increased with 1 ng/mL ($r^2 = 0.11$). Progesterone supplementation shortly after insemination resulted in higher pregnancy rates in some studies (Robinson et al, 1989; Morris and Diskin, 2008), although the positive effect of exogenous progesterone supplementation is far from consistent (Wiltbank et al, 2012).

Reduced peripheral progesterone concentrations may result from an increased metabolization of progesterone or an insufficient production by the corpus luteum (CL) (Davis et al, 1992; Lopez et al, 2005; Wiltbank et al, 2006). In lactating dairy cows, progesterone is partly drained by the udder via the milk fat. Nevertheless, progesterone is mainly metabolized by the liver. The hepatic metabolic clearance rate of progesterone (MCR; l/(min*kg $BW^{0.75}$)) is depending to a large extent on the liver blood flow (Sangsritavong et al, 2002; Rhinehart et al, 2009), which on his turn is mainly dependent on the dry matter intake (DMI; Cumming et al, 1971; Sangsritavong et al, 2002). One can conclude that there is a general agreement nowadays that lactating high yielding dairy cows are dealing with a higher progesterone 'loss' mainly via an increased metabolization rate in the liver and via some extra loss via the milk.

The role of an inadequate progesterone production by the CL, which has been described as 'luteal inadequacy', in the phenomenon of insufficient peripheral progesterone levels in lactating dairy cows, has not yet been completely cleared although some studies suggest this as a significant contributing factor (Shelton et al, 1990). Progesterone production is mainly depending on two physiologic processes: luteal steroido- and angiogenesis (Bollwein et al, 2013). Luteal steroidogenesis refers to the progesterone production by the steroidogenic cells of the CL, and mainly depends on the total number of these cells and the individual activity of each of these 'production units' (Robinson et al, 2006).

The regulation of the luteal steroid production is largely dependent on the pulsatile secreted luteinizing hormone (LH; Morris and Richards, 1993; Niswender et al, 2000). A peak of LH is necessary for the initiation of the luteinization, only a follicle rupture is not sufficient (Hayashi et al, 2006). This hormone is the most important regulator of the progesterone production in small luteal cells (Tsafriri and Reich, 1999) and is necessary for the luteal maintenance and survival (Hansel and Blair, 1996). On the contrary, LH has no enhancing effect on the steroidogenesis of large luteal cells (Hoyer et al, 1984; Wiltbank et al, 1989). The luteal sensitivity for LH increases from cycle day 5 till 8 (Robinson et al., 2006). Luteinizing hormone has direct effects on the small luteal cells by the LH-receptor, but modulates also indirectly the luteal steroidogenesis via growth factors, cytokines, and others. It also directly stimulates the synthesis of vascular endothelial growth factor, leading to a stimulation of the angiogenesis (Christenson and Stouffer, 1997). The negative energy balance, typical for the postpartum period of the present-day high yielding dairy cows, induces a lowered plasma level of glucose, insulin and IGF-1 (Butler, 2003) and even ketonemia (Butler and Smith, 1989), leading to a suppression of the LH-pulse frequency (Butler and Smith, 1989), possibly affecting the subsequent luteogenesis (Villa-Godoy et al., 1988, Butler, 2000). Shelton et al. (1990) found a delayed or impaired progesterone increase in repeat breeding cows, a phenomenon which is described nowadays more frequently in high yielding dairy cows as 'luteal inadequacy'. They proposed, together with Mann and Lamming (2001), that an impaired luteinization or an inadequate LH-support of the steroidogenesis could be the reason.

As blood cholesterol is the main source of cholesterol for steroidogenesis in bovines, the blood cholesterol availability also affects the luteal progesterone production (Gwynne and Strauss, 1982; Grummer and Carroll, 1988; Niswender and Nett, 1994).

Luteal angiogenesis on the other hand, refers to the physiologic process through which new blood vessels form. The latter being a prerequisite to obtain an intense luteal steroidogenesis as it is responsible for the supply of materials towards the luteal gland and the conveyance of progesterone towards the peripheral circulation (Acosta et al, 2002). Nevertheless, the relation between luteal blood flow measured by Doppler ultrasound and plasma progesterone levels is not that unambiguous: Herzog et al (2010) found a significant correlation of 0.71 between the luteal blood flow and plasma progesterone levels, while in a subsequent study of the same group (Lüttgenau et al, 2011) this correlation could not be confirmed in bovine mid cycle luteal glands. But possibly, this could learn us more about the accuracy of the Doppler technology and does not necessarily informs us about the luteal blood flow perse. Both

processes, steroidogenesis and angiogenesis, however, are very complicated and strictly regulated (Augustin et al, 1995; Diaz et al, 2002; Shirasuna et al, 2012). Luteal volume is positively correlated with the peripheral plasma progesterone concentration indicating that the number of luteal cells is at least co-decisive for the final amount of progesterone present in the blood (Ribadu et al, 1994; Vasconcelos et al, 2001; Bollwein et al, 2013).

Modern dairy cows with a high genetic merit for milk production have to overcome a period of negative energy balance (NEB) in the immediate postpartum period (Butler, 2003; Pryce et al, 2003; Veerkamp et al, 2003). This period of NEB is characterized by elevated blood levels of non-esterified fatty acids (NEFAs) and ketobodies, and relatively low levels of glucose (Oldenbroek et al, 1997; Gutierrez et al, 1999; Snijders et al, 2001). Previous studies have shown that during this period of NEB, NEFAs can also reach the follicular fluid of the ovary (Leroy et al, 2004) at levels high enough to hamper granulosa- and theca cell proliferation (Vanholder et al, 2005, 2006). Some studies indicated that the NEB has a carry-over effect on blood progesterone concentrations during the postpartum period. Progesterone in the peripheral circulation increases during the first two or three postpartum ovulatory cycles and the rate of increase in progesterone levels is reduced or moderated by NEB early post partum (Villa-Godoy et al, 1988; Spicer et al, 1990; Staples et al, 1990). In the study of Villa-Godoy et al (1988), it was stated that the NEB had no effect on the luteal progesterone production during the first cycle, but in the second and third cycle its negative effect was obvious. Cows experiencing the severest NEB during the first 9 days post partum, still showed decreased serum progesterone levels during their third estrous cycle in comparison to cows experiencing a moderate or no NEB during that period (Villa-Godoy et al, 1988). Luteal life span was normal, but the progesterone production was reduced. Possible explanations could be a reduced luteal development or a decreased secretory activity per luteal cell, or both.

Different methods have been used to examine *in vivo* the capacity of the luteal gland to secrete progesterone (Abecia et al, 1997; Virolainen et al, 2005; Thijssen et al, 2011). In three earlier manuscripts we have proposed an innovative technique to harvest tissue samples of a CL in dairy cows (Cools et al, 2009; 2013 a; b), and demonstrated that this method can be used to accurately examine the histophysiology of the luteal gland in living animals. The latter allows examining in a more detailed manner the functionality of different cell types present in the CL and the factors that are associated with it.

The main aim of the present study was to unravel the effect of a number of typical homeorrhetic adaptations present during the postpartum period (increased blood concentration of NEFA and urea) on the angiogenetic and steroidogenetic capacity of bovine

luteal cells harvested under field conditions. To do so, we used an optimized sampling technique to harvest tissue samples of the CL of the first three estrous cycles post partum. Nevertheless, the level of those homeorrhetic adaptations are influenced or confounded by several cow- and management-linked factors. That is why we analyzed also the effect of cycle day, cycle number, type of protein rich concentrate (PROT) and lactation number. Furthermore, we also examined the effect of these independent factors on the blood cholesterol and progesterone concentration.

Materials and methods

Animals, housing and luteal gland sampling

The present study took place in the commercially run dairy herd of the Faculty of Veterinary Medicine, University of Ghent in Belgium (Biocenter Agri-Vet, Melle, Belgium) from September 2008 till May 2009 and was approved by the ethical committee of the Faculty of Veterinary Medicine, University of Ghent, Belgium (EC2008/084).

Twenty two Holstein Friesian heifers (n = 8) and cows (n = 14) (average 305-day milk yield of 9,350 kg (S.D. = 1,919 kg), 4.11% fat (S.D. = 0.61%), 3.41% protein (S.D. = 0.24%)) were randomly divided into 2 groups. Blocking was done based on lactation number (1 till 4). The purpose of this blocking effect was to create 2 groups with a comparable distribution concerning lactation number (or age). In figure 1 an overview of the general study design is visualized. All 22 animals were housed in the same loose housing stable with cubicles, where both groups were not physically separated from each other. Every cow had *ad libitum* access to the same basic total mixed ration (TMR) (corn silage, grass silage, sugar beet pulp, corn cob mix, hay and minerals; 53.2, 24.2, 10.0, 4.5, 5.7, 2.0% on kg dry matter (DM) basis). The surplus concentrate gift (both energy and protein rich) depended on the cow-specific level of daily milk production (MP) and was provided via automatic concentrate dispensers both in the stable and in the milking robot (VMS, Delaval NV, Ghent, Belgium). These automates were calibrated weekly. The first group, 11 cows, was provided with soybean meal as main protein source, while the second group (n = 11) was supplemented with rapeseed meal as main protein source. The ratio of energy versus protein content was identical for both protein concentrates, but the equivalent of one kilogram of soybean meal, was 1.2 kg of the mixture based on rapeseed meal. More information about the different concentrates and roughages is summarized in table 1. Both groups were subjected to an *in vivo* prelevation of luteal biopsies (named respectively ‘soy, puncture’ and ‘rape, puncture group’).

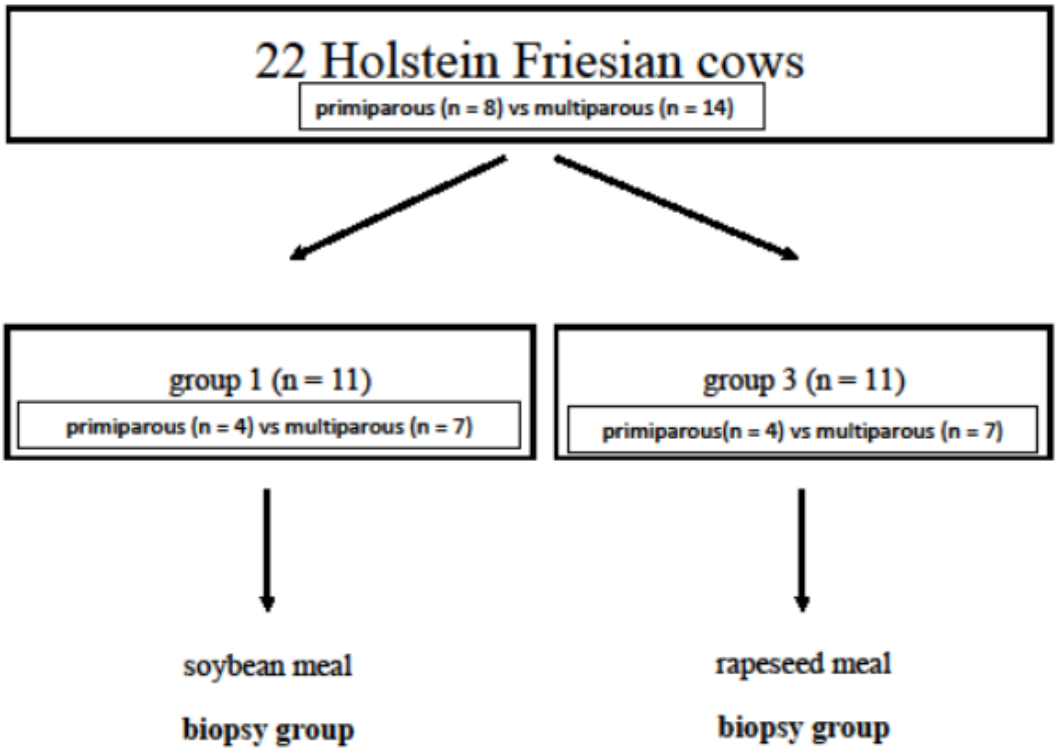


Figure 1: An overview of the general study design is visualized.

***Table 1**. Contents on dry matter basis of the different feed components in the ration of the lactating dairy cows.*

nutrient	**DM, %**	**crude fibre, g/kg**	**NE_L, MCal/kg**	**DIP, g/kg**
concentrate	87.0	105	1.584	115
soybean meal	87.0	100	1.535	200
rapeseed meal	87.0	125	1.257	160
corn silage	30.1	190	1.520	48
grass silage	38.0	250	1.485	68
sugar beet pulp	20.9	197	1.733	104
corn cob mix	61.0	28	1.988	69
hay	83.0	260	1.363	82

DM = dry matter; NE_L = Netto Energy for lactation; DIP = Digestible intestinal protein

Cows were included in the experiment from the first till the fourth ovulation post partum. To identify the first ovulation, the cows' genital tract was examined daily by trans rectal

ultrasound (Tringa®, linear, Esaote), starting as soon as the ovaries were accessible after parturition. The moment of first ovulation (day of ovulation indicated as day 0 of the cycle) was assessed retrospectively by ultrasonographic determination of a dominant follicle followed by the appearance of a CL in place of that follicle. From the moment of the first ovulation on, cows were also examined on cycle days 4, 9, and 16. On cycle days 9 and 16, the maximal and minimal orthogonal diameter of the maximal luteal cross sectional surface were measured and the luteal surface was calculated by the formula used to calculate the area of an ellipse: $Area = \pi * \left\{\frac{maximal\ diameter * minimal\ diameter}{4}\right\}$.

After cycle day 16, ovarian ultrasound examination was done again on a daily basis to define the day of the next ovulation. The same protocol was repeated during estrous cycles two and three.

Cow health was monitored during the whole experiment by means of close observation by the animal caretaker and regular clinical examinations performed by the researcher. Close observation of the individual milk yield and concentrate intake was done to follow up the animals' health status. Mastitis, (endo)metritis, cystic ovarian follicles (COF), extended luteal phase (> 20 d) and abomasal displacement were reasons to exclude animals from the experiment.

The cows were on average milked 2.68 (±0.03) times per day by a robotic milking system. Production parameters as daily MP and body condition score (BCS; assessment based on 3 day intervals) were assessed. To measure the BCS, a 5 point scale with 0.25 increments was used (Edmonson et al, 1989). The difference in BCS between the sampling moment and the day of parturition was also calculated and termed as 'BCSdifference'.

Until the first ovulation post partum (i.e. day 0 of estrous cycle 1), blood samples were taken with an interval of three days, whereupon blood samples were taken on cycle days 0, 4, 9, and 16 of the first three estrous cycles and on day 0 of cycle four to confirm the fourth ovulation (i.e. the end point of the experiment). Measures were taken to minimize stress during blood sampling, preventing a stress induced increase in blood NEFAs (Leroy et al, 2011). Cows' health was monitored daily during the whole experiment by means of follow-up of the individual daily MP, concentrate intake and regular clinical examinations. On cycle day 9, cows of Groups I and II were sedated (with 0.02 mg xylazine/kg body weight; Xyl-M® 2%; VMD) after the blood sampling procedure. To reduce rectal contractions and to anesthetize the perivaginal region during the biopsy procedure, an epidural anesthesia between the second and third caudal vertebra (using 120 mg procaine hydrochloride; procaine

hydrochloride® 4% without adrenalin; VMD) was executed. A device equipped with a multiple angle probe (MAP) was inserted intra vaginally. This device contains a needle guidance system, situated above the MAP. On top of this needle guidance system, a sterile injection needle of 14 gauge (G) was fixated to puncture through the vaginal wall into the peritoneal cavity ipsilateral to the ovary bearing the CL. The inner wall of this needle guidance system was coated with Teflon to prevent damaging of the tip of the biopsy needle, which was guided through this needle guidance system and through the 14 G-needle, going through the vaginal wall and subsequently puncturing into the CL. The biopsy needle used was a modified semi-automatic high-speed Quick Core biopsy needle (QC-19-60.0; Cook Medical, Bloomington, IN, USA), with a length of 60.0 cm, a diameter of 19 G and a biopsy chamber. This biopsy needle contained an echogenic tip, visible by ultrasound. To ascertain the sample was correctly taken, the ultrasound transducer was equipped with software visualizing the path followed by the biopsy needle. As demonstrated earlier (Cools et al, 2009; 2013a; b), it was important to take the biopsy through the center of the luteal gland. When a CL with a cavity was sampled, the cavity collapsed during puncturing but was still detectable on the ultrasound. The sampling protocol was the same for these glands. From each luteal gland, 5 biopsies were taken. Two of these samples were immediately immersed in Zinc-salts fixative (ZSF) at room temperature (RT), while the other 3 were prepared for other analyses, which are beyond the scope of the present paper. Sampling of the glands with a cavity leads to a biopsy consisting of two parts. The capsular endings were identified by means of Chinese ink. And during embedding in paraffin both pieces were oriented in the original position, reconstructing the initial biopsy as it was in the biopsy chamber.

Laboratory analyses

Blood profiles

Blood serum was analyzed for urea, cholesterol, NEFAs, and beta-hydroxybutyrate (BOHB)-levels. Urea was analyzed by the test kit *ureal* (detection limit 0.5 mmol/L (or 3.0 mg/dL); inter-assay coefficient of variation ≤ 1.3%; intra-assay coefficient of variation ≤ 1.0%). Cholesterol was measured by *chol2* (0.1 mmol/L (or 3.86 mg/dL); ≤ 1.6%; ≤ 0.8%). Both test kits are based on a quantitative colorimetric method (Cobas c system; Roche Diagnostics GmBH, Mannheim, Germany). Non esterified fatty acids and BOHB were analyzed by the quantitative colorimetric method respectively named *NEFA FS* (0.01 mmol/L; ≤ 1.15%; ≤ 1.07%) and *β-hydroxybutyrate FS* (0.01 mmol/L; ≤ 3.78%; ≤ 1.31%) (Diagnostic Systems GmBH, Holzheim, Germany). The plasma progesterone concentration was analyzed using

the colorimetric method *progesteron II kit* (0.095 nmol/L (or 0.03 ng/mL); ≤ 5.5%; ≤ 2.9%) (Roche Diagnostics GmBH, Mannheim, Germany).

Luteal biopsies

The luteal biopsies, immersed in Zinc-salts fixative (ZSF), were fixed for 3 days at room temperature (RT), with refreshment of the fixative after the first 24 hours. The biopsies were subsequently dehydrated during 22 hours and embedded in paraffin following a standardized protocol (STP 420 D, Microm, Prosan, Merelbeke, Belgium). Out of each biopsy, a series of histological sections (5 µm thickness) was made (HM 360, Microm). The biopsies were cut lengthwise. The sections were mounted on 3-aminopropyltriethoxysilane (APES)-coated slides and dried overnight at RT. The even numbered sections were selected for 3-beta-hydroxysteroiddehydrogenase (3β-HSD) staining, the uneven numbered sections were used for *Bandeiraea simplicifolia* isolectin (BSI) staining.

Staining for progesterone production (based on 3β-HSD) was done based on the protocol described by Conley et al (1995), but some modifications were necessary. Briefly, we included an antigen retrieval step; non-specific background staining was reduced by 10% (v/v) normal rabbit serum in phosphate buffered saline (PBS) with 3% (v/v) Tween 20; primary antibody was polyclonal goat anti-human 3β-HSD (1/150 (v/v) in PBS with 1% (v/v) normal rabbit serum and 0.3% (v/v) Tween20); secondary antibody was biotin-labeled rabbit anti-goat immunoglobulin (1/500 (v/v) in PBS combined with 5% (v/v) normal bovine serum and 1% (v/v) normal rabbit serum). Adrenal tissue of a calf was used as a positive control. To determine specificity of the antibodies, negative controls were achieved by incubating the luteal tissue without the different antibodies.

Staining procedure for angiogenesis (based on BSI) was based on the study of Augustin et al (1995) with some modifications. In a few words: antigen retrieval was done; 10% (v/v) normal goat serum (in PBS and 0.3% (v/v) Tween 20) was used to reduce aspecific background staining; biotin-labeled BSI was used (1/30,000 (v/v) with 5% (v/v) bovine serum). Negative controls were achieved by incubating the sections in advance with lactose 0.4 M).

In both biopsies, three respectively four sections were selected for both stainings, resulting in seven evenly numbered and seven unevenly numbered sections. An inner and outer zone was defined geometrically in the tissue sections. Five micrographs were randomly taken in each zone of each section (magnification x 1,000) by means of an Olympus DP 50 digital camera mounted on a motorized Olympus BX61 light microscope (Olympus Belgium N.V.,

Aartselaar, Belgium), resulting in 70 images per staining, per cow. In total we analyzed 4,620 micrographs per staining. The digital micrographs were analyzed using the 'Cell F' software program (Soft Imaging System, Olympus Belgium N.V.). Based on specific cutoff levels for the primary colors red, green and blue, the stained areas could be delineated and quantified in an unbiased and standardized way. The stained area represents respectively the surface occupied by steroidogenic cells (areaHSD) or endothelial cells (areaBSI). Both surfaces are expressed relatively to the total area of the analyzed micrograph as percentages.

Statistics

Data handling and descriptives

To reach normality, blood concentration data of NEFAs, BOHB, urea and progesterone were log-transformed, while daily milk production data and the surface-occupation-results for angio- and steroidogenesis were square root-transformed.

To acquire better knowledge about the functional capacity of the CL during the first 3 postpartal estrous cycles, the Pearson correlation coefficients (SAS version 9.3; SAS Institute, Inc., Cary, NC, USA, 2010) between the following variables were calculated: areaBSI and areaHSD; areaBSI or areaHSD and the peripheral blood progesterone or cholesterol concentration; the blood cholesterol and the blood progesterone concentration; the blood concentration of NEFAs or BOHB and the areaBSI or areaHSD.

Model building for luteal characteristics, blood progesterone and cholesterol concentration

The effect of cycle number (first, second and third) on angiogenesis (areaBSI) and steroidogenesis (areaHSD) was determined by constructing linear mixed models (PROC MIXED; SAS version 9.3; SAS Institute, Inc., Cary, NC, USA, 2010). Cycle number, lactation number and PROT were included as main effects. Lactation number was recoded into primiparous (1) and multiparous cows (>1). In all models, 'cow' was included as random factor while the REPEATED statement was used to account for correlated data within each cow. Least square means were computed with the LSMESTIMATE statement for categorical variables. Similar linear models were built to assess variables being associated with luteal maximal surface on day 9 and 16 including difference in luteal growth from day 9 till 16 between the first 3 consecutive cycles post partum.

Finally, comparable models were constructed for peripheral progesterone and cholesterol concentration including 'cycle day' as a supplementary main effect.

Results

Descriptive statistics

In table 2, the descriptive data of the studied parameters are visualized. The daily MP, BCS and BCSdifference were not significantly different between both nutritional groups. Also NEFAs and BOHB were not different between the soy and rape group. Urea blood concentration was marginally higher in the soy group, while blood cholesterol was significantly ($P < 0.0001$) higher in the rape group. These results indicate that differences in the outcome of the dependent variables will not be induced by differences in the energy balance of the cows.

Table 2. *Descriptive statistics of the different analyzed parameters.*

	mean ±S.D.	**median**	**range**
area HSD, %	26.61 ± 10.97	26.41	1.19-64.27
area BSI, %	7.80 ± 3.14	7.57	0.71-23.80
NEFA, mg/dL	6.74 ± 4.56	5.0	0.50-30.20
BOHB, mg/dL	8.35 ± 4.07	7.65	0.20-31.57
urea, mg/dL	22.83 ± 6.70	22.02	3.05-56.02
cholesterol, mg/dL	137.53 ± 27.00	134.50	3.86-214.00
progesterone, ng/mL	3.55 ± 3.57	2.56	0.032-20.48
BCS	2.68± 0.47	2.53	1.50-3.75
BCSdifference	1.05 ± 0.60	0.98	-0.25-2.75
milk yield, L	36.50 ± 10.03	35.03	14.45-71.23

Correlations between relevant variables

Figure 2 shows the correlation between areaBSI and areaHSD. In this figure, the results of the first estrous cycle are more clustered in the lower, left quadrant, while those of the second and third cycle are mainly situated in the upper right quadrant. A cycle number effect is graphically indicated. When the data of both puncture groups were taken together, the correlation was 0.59 ($P < 0.0001$).

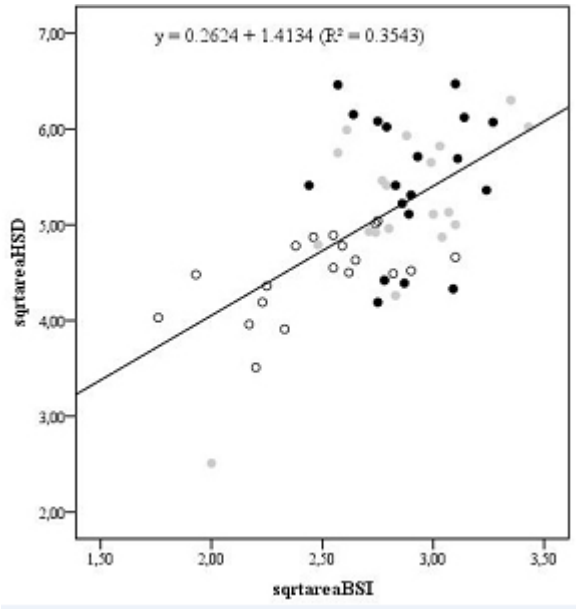


Figure 2: Scatter plot visualizing the correlation between sqrtareaBSI and sqrtareaHSD (white circles represent results from the first oestrous cycle, grey and black circles from respectively the second and third oestrous cycle post partum).

As progesterone is depending on both luteal angio- and steroidogenesis, we could expect a correlation between areaBSI or areaHSD and the peripheral progesterone level. Nevertheless, the Pearson correlation coefficients between areaHSD and progesterone and between areaBSI and progesterone were not significant ($P > 0.05$) (0.189 with $P = 0.160$; respectively 0.177 with $P = 0.188$).

In Table 3, the Pearson correlation coefficients between cholesterol on the one hand and progesterone, areaHSD and areaBSI on the other are displayed. Results show a positive correlation between the cholesterol and progesterone and areaHSD, but no significant ($P > 0.05$) correlation between cholesterol and areaBSI.

Furthermore, a significantly negative correlation between NEFAs and areaHSD and areaBSI was found, while no significant correlation ($P > 0.05$) between these area parameters and BOHB could be detected (Table 3). Also in Table 3, the significantly negative correlations between progesterone on the one hand and NEFAs respectively BOHB are remarkable.

Table 3. *Pearson correlation coefficients (pearson r) between progesterone, area HSD or area BSI on the one hand and cholesterol, NEFA or BOHB on the other.*

parameter	progesterone	area HSD	area BSI
	pearson r	pearson r	pearson r
cholesterol	0.195****	0.310*	0.229
NEFA	-0.276****	-0.355**	-0.377**
BOHB	-0.166***	-0.208	-0.053

Level of significance: **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05

Luteal angiogenesis and steroidogenesis

In Table 4 the results of the final multivariable linear mixed model are given, showing the effect (β-value and significance) of each independent variable on areaBSI (=angiogenesis) and areaHSD (=steroidogenesis). In figures 3a till 3d, the estimated values of areaBSI and areaHSD within the different classes of the categorized factors are plotted. A significant cycle number effect was detected (P = 0.0005 for BSI and P = 0.0001for HSD) indicating that steroido- and angiogenesis both increase with cycle number. Both were also higher in the rape-supplemented group. Angiogenesis furthermore tends to decrease with lactation number (P = 0.08).

Table 4. *Effects of cycle number and other variables on BSI area occupation (area BSI) and HSD area occupation (area HSD).*

	category	area BSI[3]			area HSD[3]		
		β[4]	**S.D.[5]**	**P[6]**	**β**	**S.D.**	**P**
intercept		1.3	0.86	0.1	9.27	1.82	0.0001
cycle number	1	-0.36[b8]	0.082		-1.46[b]	0.27	
	2	0.014[a]	0.082	0.0005	-0.35[a]	0.22	0.0001
	3	0[a]	…		0[a]	…	
PROT[1]	soy	-0.23[b]	0.076	0.0087	-1.05[b]	0.29	0.001
	rape	0[a]	…		0[a]	…	
lactation number[2]	1	1.72[b]	0.92	0.081	0.23[a]	0.24	0.35
	>1	0[a]	…		0[a]	…	
cycle number*PROT[7]							0.021

[1] PROT: 'soy' represents the cows fed soybean scrap; 'rape' the cows fed rapeseed scrap

[2] lactation number: heifers are code 1; multiparous cows are code >1

[3] area BSI and area HSD: the measured area occupied by endothelial cells or steroidogenic cells was sqrt-transformed for further analysis

[4] β: the β-factor was estimated based on the LS-Means-method

[5] S.D.: standard error

[6] p-value for fixed factors

[7] categorized data not shown

[8] values with different superscripts in the same column within the same variable differ significantly $P < 0.05$

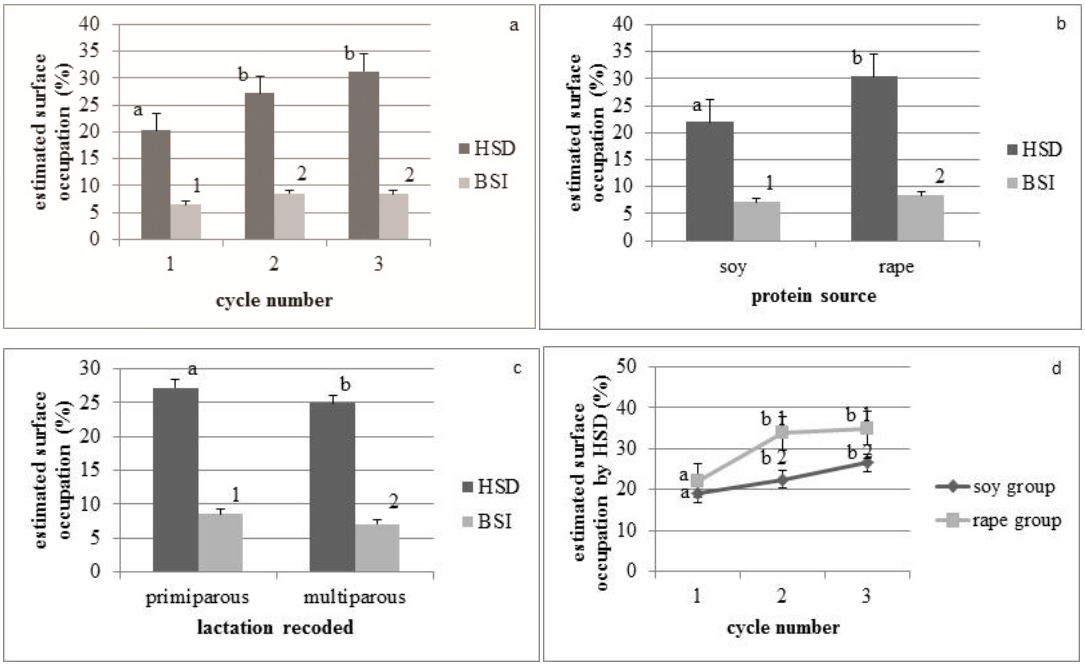


Figure 3 a-c: The estimated surface occupations of HSD and BSI in different cycle numbers (1 till 3; a), nutritional groups (soy and rape; b), or lactation numbers (primi- and multiparous; c) correcting for the other factors in the final multivariable linear mixed model. The letters a and b (for HSD) and the number 1 and 2 (for BSI) indicate differences (P < 0.05) of estimated surface occupations between respectively cycle numbers (a), nutritional groups (b), .and lactation number (c).

Figure 3d: The effect of the interaction of cycle number and nutritional group on the surface occupation by HSD in the final multivariable model. The differences (P < 0.05) of estimated surface occupation by HSD are shown, with a and b indicating differences between cycle number within the same nutritional group and 1 and 2 indicating the differences between nutritional groups within the same cycle number.

In Table 5, the final model for the luteal surface on day 9 and on day 16 is shown. The luteal surface on day 9 is significantly larger in heifers versus multiparous cows (P = 0.046), but there is no significant effect of cycle number. The model for the luteal surface on day 16 is more complicated. Besides the three main effects, an interaction between cycle number and PROT was found (P < 0.042) and is visualized in figure 4a. The luteal surface area on cycle day 16 increases with cycle number (P = 0.009) (figure 4b). Soy fed cows tend to have a smaller maximal luteal surface on day 16 versus rape fed cows. The luteal growth between day 9 and 16 is larger for CLa of the third versus the first cycle (P= 0.028; data not shown).

Table 5. Effects of cycle number and other relevant variables on maximal luteal surface on cycle day 9 and on cycle day 16.

	category	luteal surface day 9			luteal surface day 16		
		β[3]	**S.D.[4]**	**P[5]**	**β[3]**	**S.D.[4]**	**P[5]**
intercept		5.96	0.32	< 0.0001	1.60	2.67	0.55
cycle number	1	-0.12	0.29		-3.00[a]	1.24	
	2	0.090	0.28	0.73	-0.13[b]	1.33	0.009
	3	0	…		0[b]	…	
lactation number[1]	1	0.25[a7]	0.27	0.046	0.72	0.51	0.162
	>1	0[b]	…		0	…	
PROT[2]	soy	-0.40	0.29	0.18	0.16	0.80	0.058
	rape	0	…		0	…	
cycle number*PROT[6]							0.042

[1]lactation number: heifers are code 1; multiparous cows are code >1

[2]PROT: protein rich concentrate: 'soy' represents the cows fed soybean scrap; 'rape' the cows fed rapeseed scrap

[3]β: the β-factor was estimated based on the LS-Means-method

[4]S.D.: standard error

[5]p-value for each fixed factor

[6]categorized data not shown

[7]values with different superscripts in the same column within the same variable differ significantly $P < 0.05$

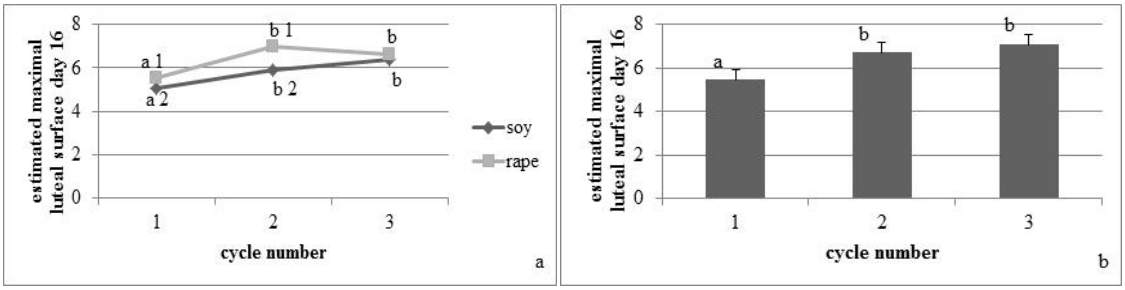


Figure 4a: The effect of the interaction of cycle number and nutritional group on the maximal luteal surface in the final multivariable model. The differences (P < 0.05) of estimated maximal luteal surface are shown, with a and b indicating differences between cycle number within the same nutritional group and 1 and 2 indicating the differences between nutritional groups within the same cycle number.

Figure 4b: The estimated maximal luteal surface in different cycle numbers correcting for other factors in the final multivariable linear mixed model. The letters a and b indicate differences (P < 0.05) of estimated maximal luteal surface between cycle numbers.

Postpartum blood progesterone and cholesterol concentration

The variables 'cycle day', 'cycle number', 'lactation', and 'PROT' remained in the model as main factors. The model is expressed in Table 6.

Figures 5a till d visualize the estimated blood progesterone concentration within the classes of the independent categorized factors based on the final model. Results illustrate that progesterone keeps increasing from day 0 till day 16, with the strongest increase from day 0 till day 9, followed by a lowered increase from day 9 till 16. The interaction term 'cycle number*cycle day' remained in the final model. The cycle day effect was significant ($p < 0.0001$). Estrous cycle effect showed an increasing trend ($p = 0.072$), meaning that progesterone levels tend to be higher with increasing cycle number post partum. Primiparous cows showed a higher blood progesterone concentration compared to multiparous cows ($P = 0.013$). This difference is mainly situated in the first oestrous cycle.

Table 6. *Effects of cycle number and other relevant fixed variables on the blood progesterone concentration.*

	category	progesterone		
		β[3]	**S.D.[4]**	**P[5]**
intercept		1.45	0.40	< 0.0001
cycle day	0	-2.14[a7]	0.18	< 0.0001
	4	-1.52[b]	0.17	
	9	-0.64[c]	0.16	
	16	0[d]	0	
cycle number	1	0.012[a]	0.47	0.072
	2	1.21[b]	0.53	
	3	0[a]	0	
lactation number[1]	1	0.088[a]	0.082	0.013
	>1	0[b]	0	
PROT[2]	soy	-0.026	0.055	0.65
	rape	0	0	
cycle number*cycle day[6]				0.002

[1]lactation number: heifers are code 1; multiparous cows are code >1

[2]protein source: 'soy' represents the cows fed soybean scrap; 'rape' the cows fed rapeseed scrap

[3]β: the β-factor was estimated based on the LS-Means-method

[4]S.D.: standard error

[5]p-value for each fixed factor

[6]categorized data not shown

[7] values with different superscripts in the same column within the same variable differ significantly P < 0.05

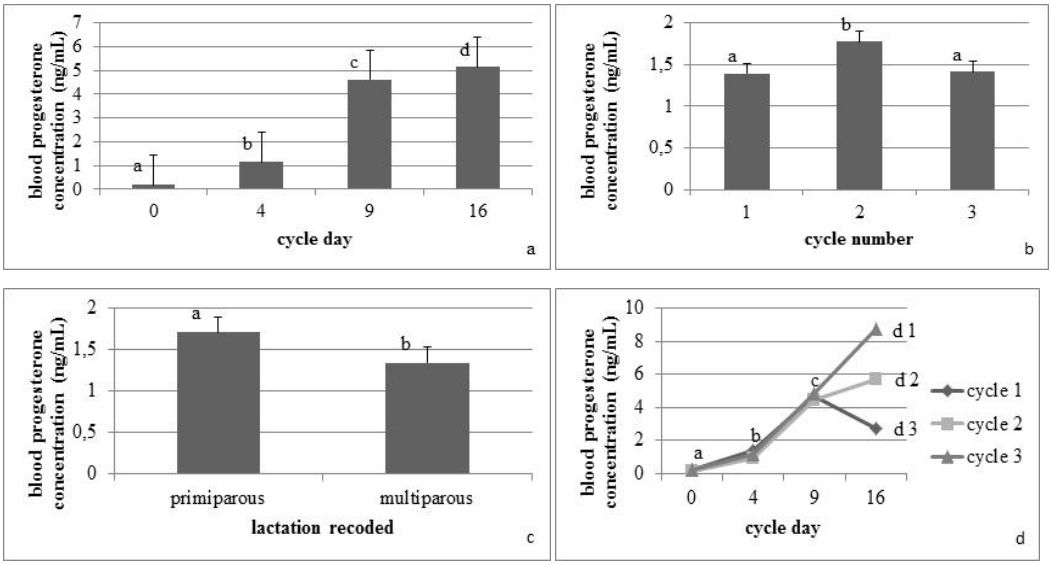


Figure 5a-c: The estimated blood progesterone concentration on/in different cycle days (0 till 16; a), cycle numbers (1 till 3; b), or lactation numbers (primi- and multiparous; c) correcting for other factors in the final multivariable linear mixed model. The letters a, b, c, and d indicate differences (P < 0.05) of estimated blood progesterone concentration between respectively cycle days (a), cycle numbers (b), and lactation numbers (c).
Figure 5d: The effect of the interaction of cycle day and cycle number on the blood progesterone concentration in the final multivariable model. The differences (P < 0.05) of blood progesterone concentration are shown, with a, b, c, and d indicating differences between cycle days within the same cycle number and 1, 2, and 3 indicating the differences between cycle numbers within the same cycle day.

In Table 7, the results of the model analyzing the factors being associated with the post partum blood cholesterol concentration are shown. Blood cholesterol concentrations increase with increasing cycle number, while they decrease from cycle day 0 to day 4, after which they increase towards day 16. The rape-fed animals showed an increased blood cholesterol concentration versus the soy-supplemented animals and the concentration is higher in multiparous versus primiparous cows. Figures 6a till d show the estimated blood cholesterol concentration within the different classes of the independent categorized factors.

Table 7. *Effects of cycle number and other variables on cholesterol blood concentration.*

	category	cholesterol		
		β[3]	**S.D[4].**	**P[5]**
intercept		173.08	3.07	< 0.0001
cycle number	1	-23.48[a6]	2.52	
	2	-4.76[b]	2.52	< 0.0001
	3	ref[b]	…	
cycle day	0	-7.32[a]	2.91	
	4	-9.22[b]	2.91	0.007
	9	-3.00[c]	2.91	
	16	ref[c]	…	
PROT[1]	soy	-19.15[a]	2.27	< 0.0001
	rape	ref[b]	…	
lactation[2]	1	-20.77[a]	2.08	< 0.0001
	>1	ref[b]	…	

[1]protein source: 'soy' represents the cows fed soybean scrap; 'rape' the cows fed rapeseed scrap

[2]lactation: heifers are code 1; multiparous cows are code >1

[3]β: the β-factor was estimated based on the LS-Means-method

[4]S.D.: standard error

[5]p-value for each fixed factor

[6] values with different superscripts in the same column within the same variables differ significantly $P < 0.05$

Discussion

Aiming to elucidate the effect of the typical homeorrhetic adaptations, induced by the negative energy balance (NEB) during the postpartum period on the luteal steroido-, angiogenesis and the blood progesterone concentration during the first 3 postpartum estrous cycles in modern dairy cows, CL biopsies and blood samples were taken from 22 high yielding dairy cows. As the degree of the NEB is depending on several cow- and management-linked factors, we selected some relevant factors to study their effect on the luteal steroido-, angiogenesis and blood progesterone concentration. These factors were cycle day, cycle number, lactation number and protein rich concentrate (PROT).

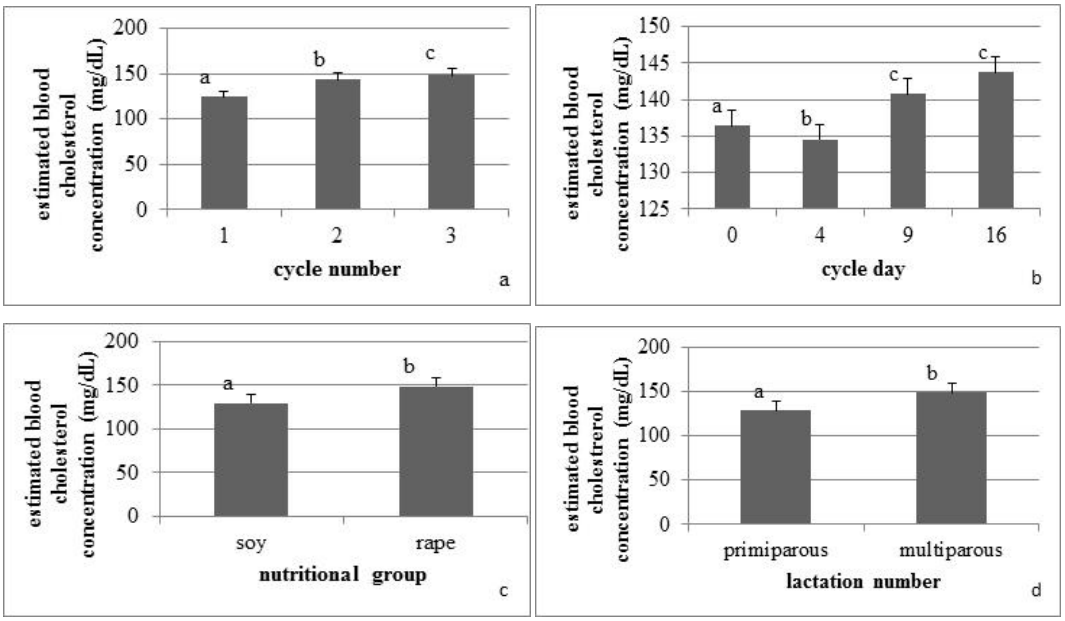


Figure 6a-d: The estimated blood cholesterol concentration in/on different cycle numbers (1 till 3; a), cycle days (0 till 16; b), nutritional groups (soy and rape; c), and lactation numbers (primi- and multiparous; d) correcting for other factors in the final multivariable linear mixed model. The letters a, b, and c indicate differences ($P < 0.05$) of estimated blood cholesterol concentration between respectively cycle numbers (a), cycle days (b), nutritional groups (c), and lactation numbers (d).

A negative correlation was found between the blood progesterone concentration on the one hand and the blood NEFA and BOHB level on the other. This negative correlation was also seen within the luteal gland itself, namely between the blood concentration of NEFA and areaHSD (= luteal area occupied by steroidogenic cells) or areaBSI (= luteal area occupied by angiogenetic cells). The increased blood level of urea, seen in multiparous and soy supplemented cows during the postpartum period, had no significant effect on the peripheral progesterone concentration. On the contrary, a higher blood cholesterol level resulted in a higher blood progesterone concentration, and even the amount of steroidogenic (area HSD) and angiogenetic cells (area BSI) present in the CL was increased. Blood cholesterol is furthermore higher in the rape- versus the soy-supplemented cows. Nevertheless, unless the positive effect of cycle number on the luteal steroidogenesis and angiogenesis, the progesterone concentration merely tended to be higher in the consecutive cycles post partum. However, primiparous cows showed in the present study a significantly higher progesterone concentration in the blood versus multiparous cows and this effect was mainly situated during the first cycle. Moreover, the luteal glands on day 9 were larger for primiparous in

comparison to multiparous cows in our study. Size of the CL on day 16 of the cycle increased with cycle number post partum and tended to be smaller in soy supplemented animals.

According to the literature, the negative correlation between blood progesterone on the one hand and blood NEFA and BOHB on the other could be related to two different levels: the hepatic level (i.e. progesterone clearance) or the luteal level (i.e. progesterone production). In healthy cows, the period of elevated NEFA and BOHB blood concentrations coincides with the period of increasing DMI in an attempt to compensate the physiological NEB. This means that the blood concentrations of NEFA and BOHB are expected to be still higher around the period of the first estrous cycle (in comparison to cycle 2 and 3), which is still accompanied by an elevated DMI. This increased level of DMI induces an increased hepatic blood flow, leading to a higher metabolic clearance rate of progesterone. This could be the base of the negative correlation between the blood concentration of NEFA and BOHB and the blood concentration of progesterone. On the other hand, we also detected a significantly negative correlation between the blood concentration of NEFA and areaHSD (= luteal area occupied by steroidogenic cells) or areaBSI (= luteal area occupied by angiogenetic cells). Consequently, it could be possible that an increased blood NEFA concentration impairs luteal angio- and steroidogenesis. Vanholder et al (2005) described an impaired *in vitro* proliferation of granulosa cells due to an increased level of apoptosis when NEFA concentration was elevated. Knowing that peripherally elevated NEFA- and BOHB-levels in the postpartum cow are reflected at the ovarian level (Leroy et al, 2004), this effect could *in vivo* possibly result in a CL with a reduced number of steroidogenic cells. Several studies dealing with the effect of energy restriction on tumor growth, describe an inhibitory effect on cell growth and angiogenesis (Mukherjee et al, 1999; Thompson et al, 2004). As early luteal development is comparable with the growth rate of some malign human tumors (Eberhard et al, 2000), NEFA and BOHB can hypothetically also jeopardize luteal angiogenesis.

Some studies indicate that rations high in crude protein (17-19%) are associated with impaired fertility (Westwood et al, 1998; Butler, 1998). In this context, others showed a negative correlation between urea and fertility (Wittwer et al, 1999; Hojman et al, 2004). Blood urea levels generally reflect dry matter intake and metabolic activity of the liver. Consequently, the higher urea levels noticed in multiparous cows in the present study, may simply be a reflection of the higher dry matter intake of both the basic TMR as well as of the amount of supplemented concentrates of the multiparous animals that produce higher amounts of milk.

Cholesterol, mainly originating from high density lipoproteins, is indispensable for progesterone production (Gwynne and Strauss, 1982; Grummer and Carroll, 1988; Niswender and Nett, 1994). Supplementation of PROT (like soybeans and rapeseed) increases the blood cholesterol concentration in bovines (Gagliostro et al, 1991; Schauff et al, 1992; Damgaard et al, 2013). As the MP in multiparous cows was higher compared to primiparous cows, the PROT amount supplemented to these animals was also higher. Consequently, it seems logical that the cholesterol concentration is higher in the blood of multiparous cows. The higher blood cholesterol level in the rape-group seems contradictory, as the lipid content between both protein rich concentrates is not significantly different. The reason could be found in human nutrition: the serum total cholesterol concentration is reduced by soy consumption, accompanied by a shift from non-HDL towards HDL-cholesterol (Tham et al, 1998; van der Schouw et al, 2000). Anderson et al (1995) found the same trends. Soy contains molecules lowering serum cholesterol, such as isoflavones (genistein), saponins, and sterols (Cassidy et al, 1994; Nowicka et al, 2006). The effect of cycle day on the blood cholesterol concentrations did not found confirmation in the literature. In the study of Talavera et al (1985), total cholesterol was negatively correlated with progesterone ($r = -0.4$; $P < 0.01$) in the period from cycle day 2 through cycle day 9, the nadir being situated in the mid luteal phase. This is in contrast with our findings as we found an increase in cholesterol with increasing cycle day.

The progesterone production is besides the biochemistry of the luteal steroidogenic cells also depending on the blood supply/removal (Arfuso *et al.*, 2005). In our study, we found a positive correlation between the area occupied by angiogenetic and the area occupied by steroidogenetic cells, indicating that both are closely linked with each other. Augustin et al (1995) compared two endothelial markers to visualize angiogenesis: von Willebrand factor (vWF) VIII and BSI (*Bandeiraea simplicifolia* isolectin). They concluded that BSI, that binds alpha-N-acetylgalactosamin and alfa-galactose, stains more and especially younger blood vessels than vWF VIII. This is the reason why we opted for BSI as a marker for angiogenesis in the present study. Based on our model indicating factors that are significantly associated with areaBSI, we can conclude that the degree of angiogenesis within a bovine CL is increasing throughout the postpartum period. During the first three consecutive estrous cycles, the density of the vascular network is increasing. Rape supplementation is associated with an increased angiogenesis in comparison to soy supplementation. Major indicators for energy balance like NEFA and BOHB are not directly associated with the luteal angiogenetic capacity. Bagavandoss and Wilks (1991) stated that progesterone itself could have anti-

angiogenetic effects. Nevertheless, we found no significant correlations between blood progesterone and areaBSI.

The model, created for areaHSD, was basically the same as for areaBSI, except for the interaction term cycle number*PROT. The behavior of the estimated surface occupation throughout the consecutive cycles was significantly different between both nutrition groups.

According to the literature, progesterone levels increase during the first three cycles post partum (Staples et al, 1990). In the present study however, progesterone merely tended to be higher in the consecutive cycles post partum. The peripheral blood progesterone concentration is lower during a period of NEB (Spicer et al, 1990). A severe NEB during the first 9 days still gives lowered blood progesterone concentrations in the third cycle. Villa-Godoy et al (1988) stated that the progesterone production during the first cycle post partum was normal, followed by the second and third, which were dealing with residual impairing effects of NEB. In the present study however, instead of a higher progesterone concentration during the first in comparison to the subsequent cycles, we detected an increasing trend in progesterone concentration with increasing cycle number. The fact that primiparous cows showed in the present study a significantly higher progesterone concentration in the blood versus multiparous cows and that this effect is mainly situated during the first cycle, suggests that this could be an effect of an increased NEB or of an increased hepatic metabolization of progesterone in multiparous cows. Furthermore, a lowered insulin like growth factor (IGF)-1 concentration usually concomitant with NEB after calving, could lead to a lowered progesterone synthesis as has been shown in ewes (Juengel et al, 1997).

Also on macroscopic level the NEB could influence the luteal morphometrics (i.e. luteal size). Britt (1992) hypothesized that the NEB could induce inferior follicles leading to inferior CLa. The NEB could be responsible for the ovulation of a smaller dominant follicle, leading to a smaller CL, whose ability to secrete progesterone could be compromised (Roche et al, 2000). Mann (2009) reported a strong increase of the luteal diameter from day 5 till 8. From day 8 through 16 there was no further significant increase anymore, while blood progesterone was still rising in this period. Taylor and Rajamahendran (1991) found the same trends: maximal luteal diameter on day 8, and maximal plasma progesterone on day 13. In our study, luteal glands of the third cycle were still more growing from cycle day 9 to 16 compared to glands of the first cycle.

Conclusions

Results of the present study show that in comparison to multiparous cows, primiparous ones have larger luteal glands together with a higher peripheral progesterone concentration. Within the same cow, the luteal vascular network becomes denser, the number of luteal steroidogenic cells and the blood cholesterol concentration higher throughout the consecutive postpartal estrous cycles. These phenomena are not resulting in a significant increase in luteal diameter or blood progesterone concentration, although for the latter a positive trend could be detected. The blood cholesterol level is positively correlated with the blood progesterone concentration, and the luteal steroido- and angiogenic process. On the other hand, the blood NEFA- and BOHB- concentrations are negatively correlated with the luteal angio- and steroidogenetic process and the attendant blood progesterone concentration. Based on the results of the present study, it is clear that both the luteal steroido- and angiogenesis are evolving throughout the postpartum period, and are influenced by several factors, reinforcing the hypothesis of 'luteal inadequacy' in high yielding dairy cows in the early postpartum period.

Acknowledgements

The BOF is thanked for their financial support (05B01906). Lobke De Bels and Liliana Standaert of the laboratory of the Department of Morphology are thanked for their technical support and advice.

References

Abecia JA, Lozano JM, Forcada F, Zarazaga L, 1997: Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim Reprod Sci **48**, 209-218.

Acosta TJ, Yoshizawa N, Ohtani M, Miyamoto A, 2002: Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F(2 alpha) injection in the cow. Biol Reprod **66**, 651-658.

Acosta TJ, Hayashi KG, Ohtani M, Miyamoto A, 2003: Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. Reproduction **125**, 759-767.

Amselgruber WM, Schafer M, Sinowatz F, 1999: Angiogenesis in the bovine corpus luteum: an immunocytochemical and ultrastructural study. Anat Histol Embryol **28**, 157-166.

Anderson JW, Johnstone BM, Cooknewell ME, 1995: Meta-analysis of the effects of soy protein-intake on serum-lipids. New Engl J Med **333**, 276-282.

Arfuso F, McGeachie JK, Meyer GT, 2005: A quantitative study of blood capillary formation (angiogenesis) concomitant with parenchymal tissue differentiation. Endothelium **12**, 171-177.

Augustin HG, Braun K, Telemenakis I, Modlich U, Kuhn W, 1995: Ovarian angiogenesis. Phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. Am J Pathol **147**, 339-351.

Bagavandoss P, Wilks JW, 1991: Isolation and characterization of microvascular endothelial cells from developing corpus luteum. Biol Reprod **44**, 1132-1139.

Bao B, Thomas MG, Williams GL, 1997: Regulatory roles of high-density and low-density lipoproteins in cellular proliferation and secretion of progesterone and insulin-like growth factor I by enriched cultures of bovine small and large luteal cells. J Anim Sci **75**, 3235-3245.

Bollwein H, Lüttgenau J, Herzog K, 2013: Bovine luteal blood flow: basic mechanism and clinical relevance. Reprod Fertil Dev **25**, 71-79.

Britt JH, 1992: Influence of nutrition and weight loss on reproduction and early embryonic death in cattle. Proc XVII World Buiatrics Congress **2**, 143-149.

Butler WR, 1998: Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. J Dairy Sci **81**, 2533-2539.

Butler WR, 2000: Nutritional interactions with reproductive performance in dairy cattle. Anim Reprod Sci **60**, 449-457.

Butler WR, 2003: Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest Prod Sci **83**, 211-218.

Butler WR, Calaman JJ, Beam SW, 1996: Plasma and milk urea nitrogen in relation to pregnancy rate in lactating dairy cattle. J Anim Sci **74**, 858-865.

Butler WR, Smith RD, 1989: Interrelationships between energy balance and postpartum reproductive function in dairy cows. J Dairy Sci **72**, 767-783.

Cassidy A, Bingham S, Setchell KDR, 1994: Biological effects of a diet of soy protein-rich in isoflavones on the menstrual-cycle of premenopausal women. Am J Clin Nutr **60**, 333-340.

Cerri RLA, Rutigliano HM, Chebel RC, Santos JEP, 2009a: Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reproduction **137**, 813-823.

Cerri RLA, Juchem SO, Chebel RC, Rutigliano HM, Bruno RGS, Galvao KN, Thatcher WW, Santos JEP, 2009b: Effect of fat source differing in fatty acid profile on metabolic parameters, fertilization, and embryo quality in high-producing dairy cows. J Dairy Sci **92**, 1520-1531.

Christenson LK, Stouffer RL, 1997: Follicle-stimulating hormone and luteinizing hormone chorionic gonadotropin stimulation of vascular endothelial growth factor by macaque granulosa cells from pre- and periovulatory follicles. J Clin Endocr Metab **82**, 2135-2142.

Conley AJ, Kaminski MA, Dubowsky SA, Jablonka-Shariff A, Redmer DA, Reynolds LP, 1995: Immunohistochemical localization of 3 beta-hydroxysteroid dehydrogenase and P450 17 alpha-hydroxylase during follicular and luteal development in pigs, sheep, and cows. Biol Reprod **52**, 1081-1094.

Cools S, Van den Broeck W, de Kruif A, Opsomer G, 2009: Is taking a biopsy a relevant technique to monitor bovine luteal function in vitro? Reprod Domest Anim **44** (Suppl. 3), 61.

Cools S, Van den Broeck W, DeVliegher S, Piepers S, Hostens M, Opsomer G, 2013a: Topographic distribution of the different cell types, connective tissue and vascular tissue/lumina within a functional bovine corpus luteum and its association with breed, type of fixation protocol and stage during the cycle. Reprod Domest Anim DOI: 10.1111/rda.12136.

Cools S, Van den Broeck W, DeVliegher S, Piepers S, Opsomer G, 2013 b: The bovine luteal histological composition: a topographic point of view. Reprod Domest Anim 48, e29-e32.

Cools S, Van den Broeck W, Vanhaecke L, Heyerick A, Bossaert P, Hostens M, Opsomer G, 2013c: Feeding soybean meal increases the blood levels of isoflavones and reduces the steroidogenic capacity in bovine corpora lutea without affecting peripheral progesterone concentrations. Anim Reprod Sci (*Accepted).*

Cumming IA, Mole BJ, Obst J, Blockey MA, Winfield CG, Goding JR, 1971: Increase in plasma progesterone caused by undernutrition during early pregnancy in the ewe. J Reprod Fertil **1**, 146-147.

Damgaard BM, Weisbjerg MR, Larsen T, 2013: Priming the cow for lactation by rapeseed supplementation in the dry period. J Dairy Sci **96**, 3652-3661.

Davis IF, Parr RA, Squires TJ, Miles MA, Christodoulou C, Hughes CJ, 1992: Progesterone, milk production, and fertility in dairy cows. Proc Aust Soc Anim Prod **19**, 113-115.

Diaz FJ, Anderson LE, Wu YL, Rabot A, Tsai SJ, Wiltbank MC, 2002: Regulation of progesterone and prostaglandin F2alpha production in the CL. Mol Cell Endocrinol **191**, 65-80.

Dickson SE, Fraser HM, 2000: Inhibition of early luteal angiogenesis by gonadotropin-releasing hormone antagonist treatment in the primate. J Clin Endocr Metab **85**, 2339-2344.

Diskin MG, Morris DG, 2008: Embryonic and early fetal losses in cattle and other ruminants. Reprod Domest Anim **43** (suppl 2), 260-267.

Diskin MG, Parr MH, Morris DG, 2012: Embryo death in cattle: an update. Reprod Fertil Dev **24**, 244-251.

Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG, 2000: Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res **60**, 1388-1393.

Edmonson AJ, Lean D, Weaver ID, Farver T, Webster G 1989: A body condition scoring chart for Holstein dairy cows. J Dairy Sci **72**, 68-78.

Gagliostro G, Chilliard Y, Davicco MJ, 1991: Duodenal rapeseed oil infusion in early and midlactation cows. 3. Plasma hormones and mammary apparent uptake of metabolites. J Dairy Sci **74**, 1893-1903.

Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG, 1998: Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest **78**, 1385-1394.

Grummer RR, Carroll DJ, 1988: A review of lipoprotein cholesterol metabolism: importance to ovarian function. J Anim Sci **66**, 3160-3173.

Grummer RR, Davis CL, 1984: Plasma concentration and lipid composition of lipoproteins in lactating dairy cows fed control and high grain diets. J Dairy Sci **67**, 2894-2901.

Gutierrez CG, Gong JG, Bramley TA, Webb R, 1999: Effects of genetic selection for milk yield on metabolic hormones and follicular development in postpartum dairy cattle. J Reprod Fertil **24**, 16.

Gwynne JT, Strauss JF, 1982: The role of lipoprotein in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocr Rev **3**, 299.

Hansel W, Blair RM, 1996: Bovine corpus luteum: a historic overview and implications for future research. Theriogenology **45**, 1267-1294.

Hayashi KG, Matsui M, Acosta TJ, Kida K, Miyamoto A, 2006: Effect of the dominant follicle aspiration before or after luteinizing hormone surge on the corpus luteum formation in the cow. J Reprod Develop **52**, 129-135.

Herzog K, Brockhan-Ludemann M, Kaske M, Beindorff N, Paul V, Niemann H, Bollwein H, 2010: Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. Theriogenology **73**, 691-697.

Hojman D, Kroll O, Adin G, Gips M, Hanochi B, Ezra E, 2004: Relationships between milk urea and production, nutrition, and fertility traits in Israeli dairy herds. J Dairy Sci **87**, 1001-1011.

Hoyer PB, Fitz TA, Niswender GD, 1984: Hormone-independent activation of adenylate-cyclase in large steroidogenic ovine luteal cells does not result in increased progesterone secretion. Endocrinology **114**, 604-608.

Juengel JL, Nett TM, Anthony RV, Niswender GD, 1997: Effects of luteotrophic and luteolytic hormones on expression of mRNA encoding insulin-like growth factor I and growth hormone receptor in the ovine corpus luteum J Reprod Fertil **110**, 291-298.

Kenny DA, Boland MP, Diskin MG, Sreenan JM, 2001: Effect of pasture crude protein and fermentable energy supplementation on blood metabolite and progesterone concentrations and on embryo survival in heifers. Anim Sci **73**: 501-511.

Leroy JL, Bossaert P, Opsomer G, Bols PE, 2011: The effect of animal handling procedures on the blood non-esterified fatty acid and glucose concentrations of lactating dairy cows. Vet J **187**: 81-84.

Leroy JL, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PE, Dewulf J, de Kruif A, 2004: Metabolic changes in follicular fluid of the dominant follicle in high yielding dairy cows early post partum. Theriogenology **62**, 1131-1143.

Lopez H, Caraviello DZ, Satter LD, Fricke PM, Wiltbank MC, 2005: Relationship between level of milk production and multiple ovulations in lactating dairy cows. J Dairy Sci **88**, 2783-2793.

Lüttgenau J, Ulbrich SE, Beindorff N, Honnens A, Herzog K, Bollwein H, 2011: Plasma progesterone concentrations in the mid-luteal phase are dependent on luteal size, but independent of luteal blood flow and gene expression in lactating dairy cows. Anim Reprod Sci **125**, 20-29.

Mann GE, 2009: Corpus luteum size and plasma progesterone concentration in cows. Anim Reprod Sci **115**, 296-299.

Mann GE, Lamming GE, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction **121**, 175-180.

Martinez-Chequer JC, Stouffer RL, Hazzard TM, Patton PE, Molskness TA, 2003: Insulin-like growth factors-1 and-2, but not hypoxia, synergize with gonadotropin hormone to promote vascular endothelial growth factor-A secretion by monkey granulosa cells from preovulatory follicles. Biol Reprod **68**, 1112-1118.

Morris D, Diskin M, 2008: Effect of progesterone on embryo survival. Animal **2**, 1112-1119.

Morris JK, Richards JS, 1993: Hormone induction of luteinization and prostaglandin endoperoxide synthase-2 involves multiple cellular signaling pathways. Endocrinology **133**, 770-779.

Mukherjee P, Sotnikov AV, Mangian HJ, Zhou JR, Visek WJ, Clinton SK, 1999: Energy intake and prostate tumor growth, angiogenesis, and vascular endothelial growth factor expression. J Natl Cancer Inst **91**, 512-523.

Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW, 2000: Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev **80**, 1-29.

Niswender GD, Nett TM, 1994: Corpus luteum and its control in primate species. In: Knobil E, Neill JD (ed), The physiology of Reproduction. Raven Press Ltd New York, p 781.

Nowicka E, Szkudelski R, Nogowski L, 2006: Influence of phytoestrogens on humans and animals. Med Weter **62**, 736-738.

Oldenbroek JK, Galesloot PAJ, Pool MH, van der Werf JHJ, 1997: Effects of selection for milk yield on feed intake and metabolism of heifers in early lactation. In: van Arendonk JAM (ed), Book of Abstracts of the 48th EAAP, Vienna, Wageningen Pers, Wageningen.

Pryce JE, Royal MD, Garnsworthy PC, Mao IL, 2003: Fertility in the high-producing dairy cow. Livest Prod Sci **86**, 125-135.

Rhinehart JD, Starbuck-Clemmer MJ, Flores JA, Milvae RA, Yao J, Poole DH, Inskeep EK, 2009: Low peripheral progesterone and late embryonic/early fetal loss in suckled beef and lactating dairy cows. Theriogenology **71**, 480-490.

Ribadu AY, Ward WR, Dobson H, 1994: Comparative-evaluation of ovarian structures in cattle by palpation per rectum, ultrasonography and plasma progesterone concentration. Vet Rec **135**, 452-457.

Robinson NA, Leslie KE, Walton JS, 1989: Effect of treatment with progesterone on pregnancy rate and plasma concentrations of progesterone in Holstein cows. J Dairy Sci **72**, 202-207.

Robinson RS, Hammond AJ, Nicklin LT, Schams D, Mann GE, Hunter MG, 2006: Endocrine and cellular characteristics of corpora lutea from cows with a delayed post-ovulatory progesterone rise. Domestic Animal Endocrinol **31**, 154-172.

Roche JF, Mackey D, Diskin MD, 2000: Reproductive management of postpartum cows. Anim Reprod Sci **60-61**, 703-712.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC, 2002: High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle. J Dairy Sci **85**, 2831-2842.

Sartori R, Sartori-Bergfelt R, Mertens SA, Guenther JN, Parish JJ, Wiltbank MC, 2002: Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci **85**, 2803-2812.

Savion N, Laherty R, Cohen D, Lui GM, Gospodarowicz D, 1982: Role of lipoproteins and 3-hydroxy-3-methylglutaryl coenzyme A reductase in progesterone production by cultured bovine granulosa cells. Endocrinology **110**, 13-22.

Schauff DJ, Elliott JP, Clark JH, Drackley JK, 1992: Effects of feeding lactating dairy cows diets containing whole soybeans and tallow. J Dairy Sci **75**, 1923-1935.

Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE, 1990: Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil **90**, 1-10.

Shirasuna K, Nitta A, Sineenard J, Shimizu T, Bollwein H, Miyamoto A, 2012: Vascular and immune regulation of corpus luteum development, maintenance, and regression in the cow. Domestic Animal Endocrinol. **43**, 198-211.

Snijders SEM, Dillon PG, O'Farrell KJ, Diskin M, Wylie ARG, O'Callaghan D, Rath M, Boland MP, 2001: Genetic merit for milk production and reproductive success in dairy cows. Anim Reprod Sci **66**, 17-31.

Spicer LJ, Tucker WB, Adams GD, 1990: Insulin-like growth factor-I in dairy cows: relationships among energy balance, body condition, ovarian activity, and estrous behavior. J Dairy Sci **73**, 929-937.

Staples CR, Thatcher WW, Clark JH, 1990: Relationship between ovarian activity and energy status during the early postpartum period of high producing dairy cows. J Dairy Sci **73**, 938-947.

Staples CR, Thatcher WW, Burke JM, 1997: Influences of dietary energy, fat, and protein on reproductive performance of lactating dairy cows. In: Proc IX Int Conf on Prod Dis Farm Anim, Ferdinand Enke Verlag, Stuttgart, Germany, pp 204-221.

Talavera F, Park CS, Williams GL, 1985: Relationships among dietary lipid intake, serum cholesterol and ovarian function in Holstein heifers. J Anim Sci **60**, 1045-1051.

Taylor C, Rajamahendran R, 1991: Follicular dynamics and corpus-luteum growth and function in pregnant versus nonpregnant dairy-cows. J Dairy Sci **74**, 115-123.

Tham DM, Gardner CD, Haskell WL, 1998: Clinical review 97 - Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence J Clin Endocr Metab **83**, 2223-2235.

Thijssen JM, Herzog K, Weijers G, Brockhan-Luedemann M, Starke A, Niemann H, Bollwein H, de Korte CL, 2011: Ultrasound image analysis offers the opportunity to predict plasma progesterone concentrations in the estrous cycle in cows: a feasibility study. Anim Reprod Sci **127**, 7-15.

Thompson HJ, McGinley JN, Spoelstra NS, Jiang W, Zhu Z, Wolfe P, 2004: Effect of dietary energy restriction on vascular density during mammary carcinogenesis. Cancer Res **64**, 5643-5650.

Tsafriri A, Reich R, 1999: Molecular aspects of mammalian ovulation. Exp Clin Endocr Diab **107**, 1-11.

van der Schouw YT, de Kleijn MJJ, Peeters PHM, Grobbee DE, 2000: Phyto-oestrogens and cardiovascular disease risk. Nutr Metab Cardiovas **10**, 154-167.

Vanholder T, Leroy JL, Soom AV, Opsomer G, Maes D, Coryn M, de Kruif A, 2005: Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation *in vitro*. Anim Reprod Sci **87**, 33-44.

Vanholder T, Opsomer G, de Kruif A, 2006: Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. Reprod Nutr Dev **46**, 105-119.

Vasconcelos JL, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC, 2001: Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology **56**, 307-314.

Veerkamp RF, Beerda B, van der Lende T, 2003: Effects of genetic selection for milk yield on energy balance, levels of hormones and metabolites in lactating cattle and possible links to reduced fertility. Livest Prod Sci **83**, 257-265.

Villa-Godoy A, Hughes TL, Emery RS, Chapin LT, Fogwell RL 1988: Association between energy balance and luteal function in lactating dairy cows. J Dairy Sci **71**, 1063-1072.

Virolainen JV, Love RJ, Tast A, Peltoniemi OA, 2005: Plasma progesterone concentration depends on sampling site in pigs. Anim Reprod Sci **86**, 305-316.

Westwood CT, Lean IJ, Kellaway RC, 1998: Indications and implications for testing of milk urea in dairy cattle: a quantitative review. Part 2. Effect of dietary protein on reproductive performance. New Zeal Vet J **46**, 123-130.

Wiltbank MC, Knickerbocker JJ, Niswender GD, 1989: Regulation of the corpus-luteum by protein kinase-C. 1. Phosphorylation activity and steroidogenic action in large and small ovine luteal cells. Biol Reprod **40**, 1194-1200.

Wiltbank MC, Lopez H, Sartori R, Sangsritavong S, Gümen A, 2006: Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology **65**, 17-29.

Wiltbank MC, Souza AH, Carvalho PD, Bender RW, Nascimento AB, 2012: Improving fertility to timed artificial insemination by manipulation of circulating progesterone concentrations in lactating dairy cattle. Reprod Fertil Devel **24**, 238-243.

Wittwer FG, Gallardo P, Reyes J, Optiz H, 1999: Bulk milk urea concentrations and their relationship with cow fertility in grazing dairy herds in southern Chile. Prev Vet Med **38**, 159-166.

Chapter 6

General discussion

Introduction

The main question of this dissertation is: 'Is the luteal activity in high yielding dairy cows during the postpartum period compromised and does this contribute to a lowered peripheral progesterone concentration during the postpartum period?'

The initial challenge was the creation and optimization of a sampling technique allowing us to assess the luteal histophysiology in a representative way.

Secondly, the *in vivo* impact of the new sampling technique in high yielding dairy cows on luteal functionality was assessed.

And finally, the effect of soybean meal consumption and homeorrhetic adaptations, typical for the postpartum high yielding dairy cow, on luteal steroidogenesis and angiogenesis was analyzed, based on a longitudinal follow-up study.

Topographic variation of the luteal histology and the *in vitro* validation of the created sampling technique

The main function of the bovine luteal gland is progesterone production. This process is depending on steroidogenesis and angiogenesis. Steroidogenesis, on its turn, is depending on the number of steroidogenic cells and their steroidogenic activity. But steroidogenesis also depends on the acquisition of constituents (such as cholesterol, oxygen) and removal of residues (such as water and carbondioxide) and progesterone. The latter implies that progesterone production also depends on angiogenesis. This background information urged us to select histological traits which were cell (steroidogenesis) or vessel associated (angiogenesis). More specifically, 'nuclear density' and 'vascular lumina' were emphasized.

In **chapter 4.1**, the main purpose was to analyze the topographic behaviour of the aforementioned histological characteristic, the 'nuclear density' (ND) throughout the luteal gland. This histological trait is important, as it is a measure for the number of steroidogenic cells, and consequently also for the resultant progesterone secretion in the luteal gland. As this histological trait behaves dynamically during the luteal phase, it was necessary to select luteal glands of a well-defined luteal stage. Only luteal glands of stage II or III were included. Although stage I corpora lutea show a maximal angiogenic activity, they were not incorporated, because of the brittle texture, making them very difficult to handle in a systematic way. Also stage IV glands were not analyzed, as they are beyond the scope of the current research. Luteal glands of the latter stage are near to the initiation of luteolysis, while we are interested in CLa which are still in the luteogenic phase.Stage II and III luteal glands

have ceased to grow while the peripheral progesterone concentration still increases, assuming modifications in the luteal histology. In the ovine CL, Niswender et al. (1976) indicated a functional heterogeneity and Rodgers et al. (1984) found some small structural differences, depending on the sampling site within the gland. Several other studies mentioned a relation between follicular and luteal histology and blood progesterone concentration. A decreased follicular diameter just before ovulation or a reduction in the number of follicular granulosa cells led to a smaller CL and even to a lower blood progesterone concentration (Garcia et al., 1981; Milvae et al., 1991; Vasconcelos et al., 2001). Lactating cows had a larger CL compared to the CL of their non-lactating counterparts, while the plasma progesterone concentration was even lower in lactating animals (Sartori et al., 2002). Altogether, all these studies indicate some variation in luteal histophysiology. Nevertheless, none of these studies investigated the histological characteristics associated with these variations.

In our pilot study, only one structural trend was observed in the topographic variation of the ND within the bovine luteal gland: the outer zone showed a higher nuclear density compared to the inner zone. The latter can be explained using a metaphor. Imagine that the luteal gland is a forest. When you walk from the border towards the center of the forest, you will see that the number of trees per surface unit is decreasing. This trend is seen for each different cell type (large luteal, small luteal and non-steroidogenic cells). This trend was also prudently described by Niswender et al. (1976). No other studies are found mentioning this topographic behaviour of the ND. A sample size calculation revealed that, based on the variation of the ND in bovine luteal glands, 66 micrographs were necessary (evenly spread over the inner and outer zone) to reliably estimate the ND. Our estimated sample size approached the sample size assessed by the only study dealing with the estimation of the reliability of a sample for luteal histology (Neves and Marques, 2002). In further research, we always took into account this minimal number of observations.

This study contains some caveats, which will be discussed in more detail: 1) The fixation of our luteal tissue samples was done by immersion, while fixation by perfusion gives a more realistic assessment. We opted for immersion as perfusion should not be possible in biopsy samples. 2) The used staining technique -HE-staining- was not the most ideal visualization technique for steroidogenic and vascular cells. Nevertheless, first of all this technique was chosen above the immunohistochemical techniques, because one wanted to identify different cell types, making it necessary to visualize several cellular characteristics, other than the presence or absence of steroidogenic enzymes, and secondly because of the financial aspect as the sample size was rather high. 3) Cell type differentiation was mainly done based on

nuclear characteristics. Although these factors can indeed influence the outcome, they were systematic and the impact was the same for all samples, most likely not confounding the conclusions.

Based on conclusions of the pilot study, we were able to simplify the *in vitro* sampling protocol. As the only significant difference in ND was situated between the inner and outer zone, it was sufficient to prelevate one tissue disk out of the luteal gland. This disk needs to contain the luteal centre and the glands capsula (to be sure the total area is represented). This tissue disk will be called 'central disk'. Based on this new (reduced) sampling protocol, another *ex vivo* study was set up (**chapter 4.2**) in which, the number of histological traits for further research was expanded compared with the study described in chapter 4.1: besides ND, also 'the area occupied by vascular tissue and lumina' and 'by collagen fibers' were analyzed. Reason for the analysis of the supplementory traits is the fact that a luteal gland does not contain only steroidogenic cells, but it is a composition of steroidogenic cells, vascular tissue and connective tissue. In this study, the ND is the representative for steroidogenesis, the 'area occupied by vascular tissue and lumina' for angiogenesis, and the 'area occupied by collagen fibers' for the supportive compartment. Besides the expansion in the number of scrutinized histological traits (i.e. dependent variables), we also analyzed the effect of some independent variables on these traits. As the histological composition is dynamic, the luteal stage (stage II versus III) could have an effect on these traits. We compared also breeds with an opposite purpose, Holstein Friesian (HF, a breed selected for milk production) versus Belgian Blue (BB, a breed selected for meat production), to see whether breed effects could influence luteal histology. In view of the optimization of our sampling protocol for the *in vivo* study, we assessed also the effect of 2 frequently used fixatives: Neutral buffered 3.5% fomaldehyde (NBF) versus Zinc-salts fixative (ZsF). And again the topographic effect, inner zone versus outer zone is analyzed on a larger number of luteal glands (46 glands in this study versus 6 in the pilot study). As we said already, the analysis of the topographic variation was narrowed down to the comparison of the inner versus the outer luteal zone. As in the forest, where the lowered number of trees allows the growth of more herbs, the lowered nuclear density in the inner luteal zone is compensated by an increase in collagen fibers and vessels. In fact, as we did not measure the cellular diameters of the luteal cells, we cannot state that the lowered nuclear density in the inner luteal zone is completely compensated by collagen fibers and vessels, because we have to take into account that the cellular diameter could increase as a response to the lowered density. In the forest metaphore this means that the less densely

positioned trees in the centre of the forest should have larger crowns, obscuring (partly) the sunlight, allowing less bush and herbs to grow. This increasing trend in collagen from the outer to the inner zone was also seen by Silvester and Luck (1999). The inner zone was also significantly denser vascularized compared to the outer zone. The ND was not different between luteal glands of Belgian Blue (BB) (a breed selected for meat production) versus Holstein Friesian (HF) (a breed selected for milk production). Also, the older luteal glands (stage III) did not differ from the younger ones (stage II) concerning the ND. Besides the influence of the location (inner versus outer), the most important effect on ND was the fixation effect. Nuclear density was systematically overestimated based on tissue samples immersed in NBF compared to ZsF-fixation.

Comparison of the results of this chapter with other studies was difficult, because most have been using 'volume density' and the number of observations was frequently insufficient based on our sample size estimation. The estimation of luteal age had also evolved over time (Ireland et al. (1980) versus Miyamoto et al. (2000) versus D'haeseleer et al. (2006)), making it difficult to analyze whether or not one was comparing luteal glands of the same age. In bovine luteal glands, an extra complicating factor was that the difference between large luteal cells (LLC) and small luteal cells (SLC) was less obvious compared to ovine glands. Consequently, a cut off diameter was necessary in histological studies to differentiate LLC from SLC. This cut off value, used in histological studies, was variable for different studies. This could induce methodological differences in the volume occupation of LLC and SLC between studies only caused by the choice of another cut off diameter, and not induced by a biological variation. Besides that, studies using a dispersion technique induced a selective loss of luteal cells, leading to a lowered representativeness of the results (Chegini et al., 1984; Weber et al., 1987). Therefore, we had based our differentiation between LLC and SLC only on nuclear and cytoplasmic characteristics, without measuring diameters and we used intact tissue samples, no dispersion. And finally, when literature is reviewed about morphometric data concerning luteal histology, Nelore cows and Shorthorns were used (O'Shea et al., 1989; Neves and Marques, 2002; 2006), but data about Holstein Friesian and Belgian Blue were lacking.

Concerning connective tissue, the results are comparable with those of Parkinson et al. (1994). The luteal glands of Belgian Blue cows contain less collagen fibers versus CLa of Holstein Friesian cows. Surprisingly, an opposite trend was seen in the testes of the bulls of these breeds: the testes of BB bulls contained relatively more connective tissue compared to HF bulls (Hoflack et al., 2008). Stage III luteal glands contain more collagen fibers compared

to stage II. The luteal glands of HF cows tended to have a denser vascular bed compared to the BB, the same conclusion can be made for stage III CLa versus stage II, but without statistical significance. On the contrary, the inner zone has a denser vascular network compared to the outer zone.

To the best of our knowledge, this is the first study discussing the importance of the location of the sampling site on the representativeness of a biopsy. As we found a systematic, predictive topographic variation of the analyzed histological traits 'nuclear density', 'area occupied by vascular tissue' and 'by collagen fibers' in CLa of different breeds and luteal stages, the accuracy of the topographic location of the sampling site is very important in our opinion. Within a luteal gland, the topographic variation of those traits was only significant between the inner and outer zone. So the direction of the biopsy does not matter as long as it goes through the luteal center and contains the total diameter of the gland (i.e. the complete traject from the outer capsula to the inner centre and again to the outer capsula on the opposite site). Studies done during the past decades (Donaldson and Hansel, 1965; Weber et al., 1987; Lei et al., 1991; Parkinson et al., 1994), did not take into account these conditions or did not describe very detailed their sampling protocol (except for O'Shea et al., 1989; Neves and Marques, 2002; 2006), which may influence the representativeness of their morphometric results.

In the previous studies, we analyzed the topographic variation of three histological traits and found that these characteristics followed a well defined pattern. Based on these observations, we assume that the results obtained by means of one single sample could be representative for the intact luteal gland. In the next chapter (**chapter 4.3**), these assumptions were tested for the histological characteristics, described in chapter 4.2. Also, the effect of different fixation protocols on the representativeness of a biopsy was analyzed. A modified sampling protocol (based on the protocol of chapter 4.2) was set up. Two biopsies were taken perpendicular to each other (one following the polar axis, containing the ovulation stigma and one following the equatorial plane), both through the center of the luteal gland and including the entire width from capsula to capsula. After the prelevation of the biopsies, a 'central disk' is dissecated, containing both biopsy sites. The tissue samples were stained with haematoxylin-eosin for the visualization of the ND and vascular tissue/lumina, while another staining (Modified masson trichrome staining) was performed to visualize the collagen fibers. Microscopic photographs were made and analyzed in both biopsies and the central disk. The results coming from the biopsies were compared with those coming from the central disk.

The level of congruence between the results of the biopsies and the central disk was calculated based on the correlation coefficients and agreement indices. These coefficients and indices learned us that we can representatively assess the ND of the luteal gland based on one biopsy. But the representativeness was higher when luteal tissue was immersed in zinc salts fixative (ZsF) versus neutral buffered formaldehyde (NBF). The area occupied by vascular tissue and lumina can also be reliably measured based on one biopsy. In view of our *in vivo* study, it was interesting to know that one biopsy is sufficient to analyze ND and vascular tissue: keeping in mind that the lower the number of biopsies needed, the lesser the probable impact on the subsequent luteal function. On the contrary, we could not analyze the collagen fiber content by means of a biopsy. There was always a tendency to overestimate the collagen fiber content of the luteal gland based on a biopsy. But the absolute amount of the overestimation was too capricious to insert a systematic correction factor. Nevertheless, as we saw a significant breed effect on luteal collagen fiber content and as Hoflack et al. (2008) hypothesized in bulls a negative effect of the collagen fiber content on testicular performance, it could still be interesting to analyze in further research the same hypothesis at the female side. Since our research indicated that our sampling protocol was not suitable for the assessment of the collagen fiber content, we did not maintain this histological trait in further *in vivo* research. One extra comment had to be made. The mean difference of the ND was significantly different from 0, but this difference was too small to be biologically relevant.

The conclusions of the studies done in chapter 4.1, 4.2 and 4.3 can be summarized as follows: it is certainly possible to monitor luteal histology based on a single biopsy, provided that 1) a histological trait is chosen closely related with ND or vascular tissue; 2) the correct fixative is chosen in case of ND-related characteristics, namely Zinc-salts Fixative; 3) the correct sampling technique is applied: through the luteal center and containing the total gland's diameter; and 4) at least 66 observations are done (33 in the inner and 33 in the outer zone). Both histological traits 'ND' and 'vascular tissue' are closely related to both essential histophysiological traits, respectively steroidogenesis and angiogenesis, both melting together into the luteal progesterone production.

During the *in vivo* study, we also assessed the effect of the biopsy sampling technique on luteal functionality. No significant effect was seen on the blood progesterone concentration, nor on the maximal luteal surface on cycle day 9 and 16, nor on the cycle regularity. So, based on these findings, it can be concluded that the biopsy protocol had no significant

impairing effect on the luteal histophysiology, which was also confirmed by Kot et al. (1999) and Tsai et al. (2001).

In the following *in vivo* study, we will immunohistochemically quantify the area occupied by steroidogenic cells and by endothelial cells, both linked with respectively the ND or the area occupied by vascular tissue and lumina. This linkage between the histological traits of the *in vitro* and the *in vivo* study, allows us to extrapolate the sampling protocol of the *in vitro* study to the *in vivo* experiment.

In vivo study: Can soybean meal supplementation modify the luteal activity?

In **chapter 5.1** we assessed the effect of soy supplementation under realistic practical circumstances on luteal histophysiology. This luteal histophysiology was analyzed by visualization of steroidogenic and angiogenetic cells based on one luteal biopsy as mentioned earlier in the *in vitro* studies.

Humans are less likely to develop mammary and prostate cancers when soy products are implemented in the daily consumption pattern (Mills et al., 1989; Dhom, 1991; Shimizu et al., 1991; Kurzer and Xu, 1997; Setchell and Cassidy, 1999). This preventive role was ascribed to isoflavones, being present in high concentrations in soybeans (Adlercreutz, 1990; Bingham et al., 1998; Hollman, 2001). These molecules inhibit pathological angiogenesis by decreasing the cell proliferation and the breakdown of the extracellular matrix, which is necessary for the migration of endothelial cells (EC) (Ibarreta et al., 2001; Su et al., 2005). Based on the proliferation index and microvessel maturation index (MMI), luteal angiogenesis is comparable to tumor angiogenesis rates (Goede et al., 1998). This means that the vascular network stays relatively immature, giving the opportunity for remodelling. An important difference with tumoral angiogenesis is that the luteal angiogenesis is tightly regulated (Goede et al., 1998).

Besides luteal angiogenesis, a second important process is going on, namely steroidogenesis. This process is initially also depending on the intense proliferation and migration of steroidogenic cells. Moreover, Yamashita et al. (2008) stated that the regulation of angiogenesis and steroidogenesis are intertwined at the molecular level. From biochemical point of view, both processes relate on common stimulatory molecules, such as VEGF and bFGF. Physiologically, steroidogenic cells depend on the supply of constituents and the

removal of residues and progesterone by the vascular component to accomplish their steroidogenic task.

As high yielding dairy cows are also frequently supplemented with soybean meal to meet their protein requirements, the main hypothesis of the study in chapter 5.1 was as follows: soy supplementation under realistic practical conditions leads to an increase of the isoflavone blood concentration, affecting both the steroidogenic and angiogenic process during luteogenesis, resulting in a decreased progesterone secretion.

The study to test this hypothesis, was executed in one and the same commercial dairy herd. A first advantage is that the 33 HF cows are kept under identical conditions. A second advantage is the possibility to extrapolate the results or conclusions of this study towards practice.

In literature, an important variation is described in the isoflavone content of soybeans. To prevent confounding effects induced by a variation in the isoflavone content of the soybean meal, every new batch was sampled. The most important molecule was genistein, followed by daidzein and glycitein. No significant variation in the total isoflavone concentration was found between the different batches of soybean meal. The concentrations in the alternative protein concentrate, rapeseed meal, were systematically situated beneath the detection limits, as confirmed by Trinacty et al. (2009).

Our results reveal that glycitein and daidzein, two parent molecules, were increased by soy supplementation, but we found no increase of the third parent molecule genistein, described as the most important isoflavonic molecule (Kurzer and Xu, 1997). Soy supplementation also significantly increases blood concentrations of the estrogenic active metabolites equol, O-DMA and DHD. Moreover, a time effect was seen, meaning that the concentration was increasing with the length of the supplementation period. The latter could most probably be caused by a facilitation of the metabolism. We have not analyzed the most important metabolite of genistein, para-ethylphenol, because of its loss of biological activity in contrast to O-DMA and DHD, originating from daidzein (Lundh, 1995; Setchell et al., 2002)

So, consequently to soy supplementation, we found an increase in the blood concentration of those isoflavone molecules of which several effects on fertility are described in literature. And indeed, these higher blood concentrations of equol and glycitein, were both associated with a decreased area occupied by HSD-positive and BSI-positive cells in the luteal biopsies. This allowed us to conclude that soy supplementation had a negative impact on the luteal steroidogenesis and angiogenesis. Other studies, such as those from Fotsis et al. (1993; 1997) and Zhou et al. (1999) found similar effects on tumoral angiogenesis, while Kaplanski et al.

(1981) and Wong and Keung (1999) reported analogous effects on luteal steroidogenesis in cattle.

Nevertheless, these histophysiological effects seem not to have an impact on the peripheral progesterone concentration on cycle day 9. Lüttgenau et al. (2011) stated that the midluteal progesterone concentration is independent of the luteal blood flow. We did not analyze the progesterone concentration in the luteal tissue itself, nor in the central venous blood, draining the luteal tissue.

In this *in vivo* study, luteal steroidogenesis was assessed by measuring the surface occupied by 3β-HSD-positive cells, and not by counting the number of steroidogenic cells. As the staining intensity in the histological slices was not measured, we actually can not answer questions about their individual activity. Angiogenesis creates a vascular bed with a certain capillary density. That is what we can analyze by means of the BSI-staining. Staining intensity has no meaning in this case. The BSI stains significantly more blood vessels compared to vWF VIII during the angiogenic phase (Augustin et al., 1995). Therefore we preferred the BSI-staining.

We preferred to analyze the luteal angiogenesis by means of an *in vivo* model over the *in vitro* culture models, because of the importance of the interaction between different cell types, such as pericytes and EC. Also the presence of the 3-dimensional organization is necessary for the expression of certain proteins, such as collagen type IV, laminin. Several cells or molecules (such as pericytes and fibronectin) do have a different role depending on the time point in the angiogenic process: a stimulatory role in the induction of the angiogenesis, but also a stabilizing (or inhibitory) role further in the angiogenic process. This makes the reconstruction by means of *in vitro* models more complicated. We realized that the punctum maximum of the angiogenesis is earlier than day 9 (rather the first 5 days), but it was practically seen not feasible to sample younger luteal glands. These latter samples are too brittle, making them unsuitable for further preparation in a well standardized protocol. So, in our study, the CL was sampled in the early plateau phase of the angiogenesis, i.e. cycle day 9. This means that we cannot evaluate whether the early development of the vascular bed was delayed or suboptimal. As repeat breeders are often dealing with a delayed progesterone increase (Shelton et al., 1990), a delay in the initial phase of the angiogenesis could be a possible explanation and an interesting hypothesis for further research. As our sampling method was not suitable for stage I luteal glands, this hypothesis was beyond the scope of our research.

Both processes, steroidogenesis and angiogenesis seem to be interconnected. Based on the study of Herzog et al. (2010), luteal blood flow is positively correlated with the peripheral progesterone concentrations (r=0.71). Besides that, on the molecular level, both processes are intertwinned on the regulatory level. Shelton et al. (1990) found that subfertile cows showed a prolonged interval between LH-peak and initiation of the progesterone rise, a lower progesterone increasing rate and a lower progesterone concentration in the blood. This delayed progesterone rise, with even less than one day, should impair embryonic development (Mann and Lamming, 2001; Hommeida et al., 2004; Robinson et al,2005). So, there are indications for ar role for luteal inadequacy in the problem of (early) embryonic death. As we measured the peripheral progesterone concentration, we can not evaluate the luteal activity based on our progesterone results, because the latter is the result of a dynamic equilibrium between progesterone production and clearance (via milk, liver, and others). Less invasive should be the assessment of the milk progesterone. Nevertheless, we opted to measure the blood progesterone, because of the higher variation in the milk progesterone concentration (Dobson et al., 1975; Pope et al., 1976; Mather et al., 1978).
We want to stress once again an important advantage of this study. The experimental design allows us to approach as much as possible the practical conditions of that commercial dairy herd. The housing conditions, the ration, and the overall management were the same for all 33 HF animals. Even the supplemented amount of protein concentrate was individually modified to the daily milk production and was on average 2 kg soy per day, which is a realistic amount. Keeping our experimental interventions as small as possible, the daily herd management was disturbed as minimal as possible, and that makes our conclusions useful for practice.

So, we conclude that the supplementation of soybean meal in amounts typically used in practice, increases the blood concentration of equol, O-DMA and DHD, which leads to a decrease in capillary density and steroidogenic cells in the bovine luteal gland at cycle day 9, yet without a significant effect on the peripheral blood progesterone concentration. The latter implies that soy supplementation probably does not directly impair peripheral effects of progesterone, like the feedback effects towards hypothalamus and pituitary gland. But we did not monitor the central blood progesterone (i.e. the local concentration in the blood draining the luteal gland and going towards the uterus). This local concentration is higher compared to the peripheral progesterone concentration. This peripheral concentration underwent a certain degree of dilution, hepatic clearance and mammary excretion. So, actually as long as we do

not measure the central progesterone concentration at the level of the genital tract, it is not possible to state that the described decrease in angio- and steroidogenesis should not have a negative impact on the local progesterone concentration in the luteal gland or uterus. This interesting topic requires further research.

In vivo study: longitudinal follow up of the luteal histophysiology

In **chapter 5.2** a second part of the *in vivo* experiment was conducted. The luteal activity (steroidogenesis, angiogenesis) and maximal luteal surface during the first 3 estrous cycles post-partum were followed up longitudinally in 33 HF high yielding dairy cows on one commercial dairy farm. Also the progesterone, the cholesterol, the NEFA, the BOHB, and the urea concentrations in the peripheral blood were monitored. The effect of cycle number (1 to 3), cycle day (0, 4, 9, and 16), lactation number (primi- versus multiparous) and type of protein rich concentrate (rape versus soy) on the monitored dependent variables was analyzed. The dependent variables were areaHSD, areaBSI, progesterone, and maximal luteal surface. Afterwards, also the correlation between on the one hand NEFA, BOHB, or cholesterol and on the other HSD, BSI, or progesterone was analyzed.

We can conclude that primiparous cows had larger luteal glands and higher blood progesterone concentrations. Within the same cow we saw that the area occupied by BSI,-positive cells (areaBSI), the area occupied by HSD-positive cells (areaHSD) and blood cholesterol were increasing with cycle number, without a significant increase of the maximal luteal surface and only a positive increasing trend of the peripheral blood progesterone concentration. The blood cholesterol concentration was positively correlated with the blood progesterone concentration, the areaBSI and the areaHSD. On the contrary, NEFA and BOHB were negatively correlated with the blood progesterone concentration, the areaBSI and the areaHSD.

So, the luteal angio- and steroidogenesis evolve throughout the postpartum period and are influenced by several factors (cycle number, protein rich concentrate), reinforcing the hypothesis of 'luteal inadequacy' during the first oestrous cycles in high yielding dairy cows in the early post-partum period and establishing a link between NEB (NEFA and BOHB) and luteal activity.

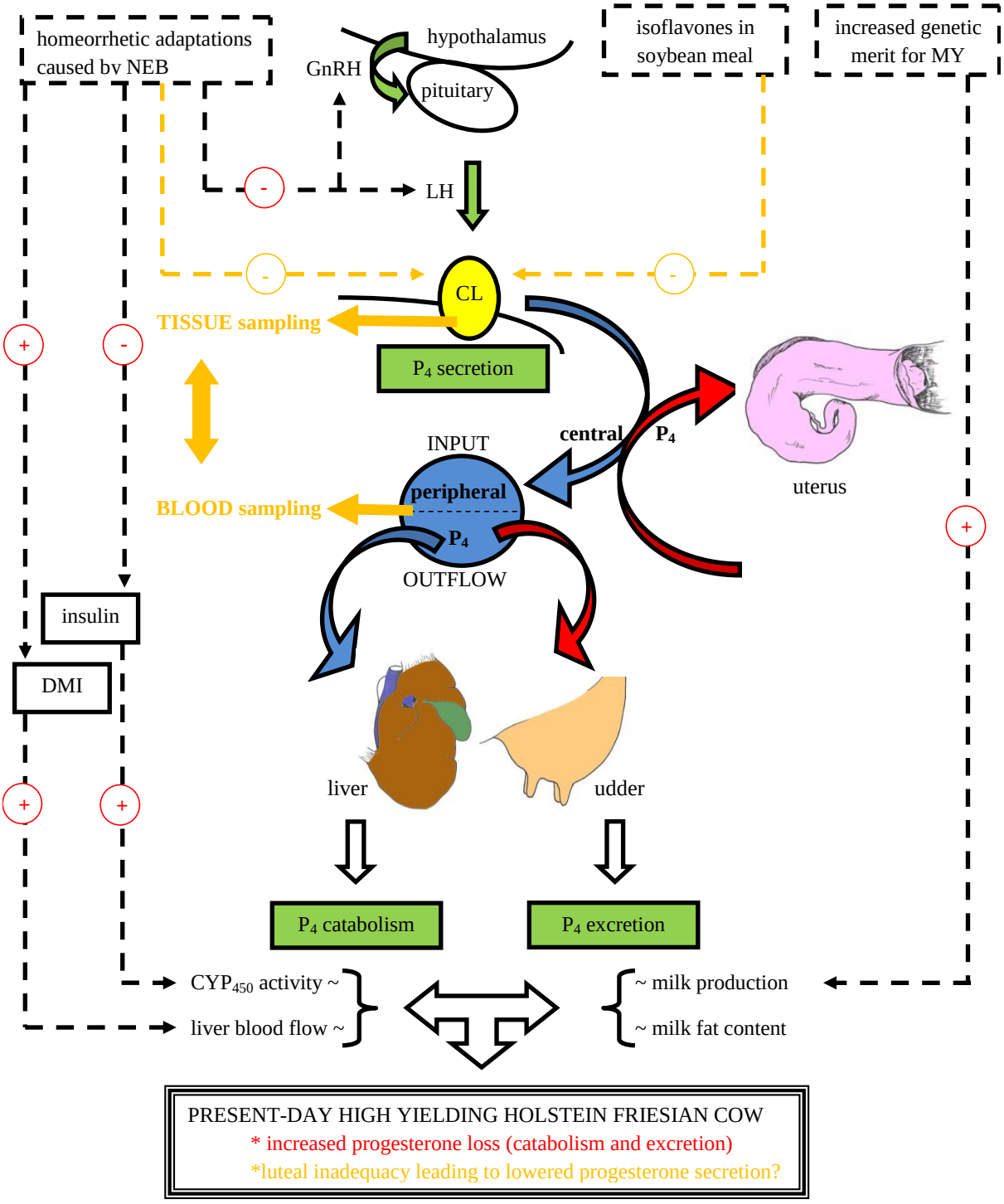


Figure 1: *the concluding overview scheme containing the research questions originating by exploring the literature*

So, when we return to the concluding overview scheme at the beginning of the aims of the thesis, we saw there were still three specific questions open (figure 1):
1) Can we monitor the luteal histophysiology by means of a biopsy prelevated *in vivo* directly out of the luteal gland and this without hampering the subsequent luteal function?
2) Does the homeorrhetic adaptations caused by the NEB have an impact on luteal performance?
3) Does soybean supplementation under practical circumstances have an influence on luteal activity.

The first question we can answer affirmative. Yes, we optimized a tissue sampling technique making it possible to monitor certain histological traits *in vivo* in high yielding dairy cows without deleterious effects on luteal physiology.
Concerning the second question we can conclude that the NEB (NEFA and BOHB) compromisesthe luteal activity (angiogenesis and steroidogenesis).
The same conclusion can be made for the soybean supplementation. The isoflavones, present in soyben meal, eventually after metabolization inhibits the angiogenic and steroidogenic process.
As a general conclusion, we can say that the main hypothesis could not be confirmed based on our research data. Nevertheless, we discovered that the angiogenesis and steroidogenesis in the bovine luteal gland during this period were compromised, we did not find negative effects on the peripheral blood progesterone concentrations in high yielding Holstein Friesian cows during this postpartum period. So, probably it is necessary to assess the central blood progesterone concentration to expose the discrete effects of the modified luteal histophysiology on the progesterone outflow towards the blood circulation.

Reference List

Adlercreutz H, 1990: Diet, breast cancer, and sex hormone metabolism. Ann N.Y. Acad Sci **595**, 281-290.

Augustin HG, Braun K, Telemenakis I, Modlich U, Kuhn W, 1995: Ovarian angiogenesis. Phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. Am J Pathol **147**, 339-351.

Bingham SA, Atkinson C, Liggins J, Bluck L, Coward A, 1998: Phyto-oestrogens: where are we now? Br J Nutr **79**, 393-406.

Chegini N, Ramani N, Rao CV, 1984: Morphological and biochemical characterization of small and large bovine luteal cells during pregnancy. Mol Cell Endocrinol **37**, 89-102.

D'haeseleer M, Cocquyt G, Van Cruchten S, Simoens P, Van den Broeck W, 2006: Cell-specific localisation of apoptosis in the bovine ovary at different stages of the estrous cycle. Theriogenology **65**, 757-772.

Dhom G, 1991: Epidemiologic and morphogenetic aspects of prostate cancer. Urologe A **30**, 3-4.

Dobson H, Midmer SE, Fitzpatrick RJ, 1975: Relationship between progesterone concentrations in milk and plasma during bovine estrous cycle. Vet Rec **96**, 222-223.

Donaldson L, Hansel W, 1965: Histological study of bovine corpora lutea. J Dairy Sci **48**, 905-909.

Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L, 1993: Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis. Proc Natl Acad Sci U.S.A. **90**, 2690-2694.

Fotsis T, Pepper MS, Aktas E, Breit S, Rasku S, Adlercreutz H, Wahala K, Montesano R, Schweigerer L, 1997: Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. Cancer Res **57**, 2916-2921.

Garcia J, Jones GS, Acosta AA, Wright GL Jr, 1981: Corpus luteum function after follicle aspiration for oocyte retrieval. Fertil Steril **36**, 565-572.

Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG, 1998: Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest **78**, 1385-1394.

Herzog K, Brockhan-Ludemann M, Kaske M, Beindorff N, Paul V, Niemann H, Bollwein H, 2010: Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. Theriogenology **73**, 691-697.

Hoflack G, Van den Broeck W, Maes D, Van Damme K, Opsomer G, Duchateau L, de Kruif A, Rodriguez-Martinez H, Van Soom A, 2008: Testicular dysfunction is responsible for low sperm quality in Belgian Blue bulls. Theriogenology **69**, 323-332.

Hollman PCH, 2001: Evidence for health benefits of plant phenols: local or systemic effects? J Sci Food Agricult **81**, 842-852.

Hommeida A, Nakao T, Kubota H, 2004: Luteal function and conception in lactating cows and some factors influencing luteal function after first insemination. Theriogenology **62**, 217-225.

Ibarreta D, Daxenberger A, Meyer HHD, 2001: Possible health impact of phytoestrogens and xenoestrogens in food. Apmis **109**, S402-S425.

Ireland JJ, Murphee RL, Coulson PB, 1980: Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J Dairy Sci **63**, 155-160.

Kaplanski O, Shemesh M, Berman A, 1981: Effects of phyto-oestrogens on progesterone synthesis by isolated bovine granulosa cells. J Endocrinol **89**, 343-348.

Kot K, Anderson LE, Tsai SJ, Wiltbank MC, Ginther OJ, 1999: Transvaginal, ultrasound-guided biopsy of the corpus luteum in cattle. Theriogenology **52**, 987-993.

Kurzer MS, Xu X, 1997: Dietary phytoestrogens. Annu Rev Nutr **17**, 353-381.

Lei ZM, Chegini N, Rao CV, 1991: Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol Reprod **44**, 1148-1156.

Lundh T, 1995: Metabolism of estrogenic isoflavones in domestic-animals. Proc Soc Exp Biol Med **208**, 33-39.

Lüttgenau J, Ulbrich SE, Beindorff N, Honnens A, Herzog K, Bollwein H, 2011: Plasma progesterone concentrations in the mid-luteal phase are dependent on luteal size, but independent of luteal blood flow and gene expression in lactating dairy cows. Anim Reprod Sci **125**, 20-29.

Mann GE, Lamming GE, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction **121**, 175-180.

Mather EC, Camper PM, Vahdat F, Whitmore HL, Gustafsson BG, 1978: Theriogenology **10**, 119-129.

Milvae RA, Alila HW, Bushmich SL, Hansel W, 1991: Bovine corpus luteum function after removal of granulosa cells from the preovulatory follicle. Domest Anim Endocrin **8**, 439-443.

Mills PK, Beeson WL, Phillips RL, Fraser GE, 1989: Cohort study of diet, lifestyle, and prostate cancer in Adventist men. Cancer **64**, 598-604.

Miyamoto Y, Skarzynski DJ, Okuda K, 2000: Is tumor necrosis factor alpha a trigger for the initiation of endometrial prostaglandin F(2alpha) release at luteolysis in cattle? Biol Reprod **62**, 1109-1115.

Neves MM, Marques Jr AP, 2002: Tamanho da amostra para estudo da proporção volumétrica dos constituintes do corpo lúteo bovino. Arq Bras Med Vet Zootec **7**, 81-85.

Neves MM, Marques Jr AP, 2006: Volumetric proportions of the corpus luteum constituents of Nelore. Arq Bras Med Vet Zootec **58**:944-946.

Niswender GD, Reimers TJ, Diekman MA, Nett TM, 1976: Blood flow: a mediator of ovarian function. Biol Reprod **14**, 64-81.

O'Shea JD, Rodgers RJ, D'Occhio MJ, 1989: Cellular composition of the cyclic corpus luteum of the cow. J Reprod Fertil **85**, 483-487.

Parkinson TJ, Turvey A, Jenner LJ, 1994: A morphometric analysis of the corpus luteum of the cow during the estrous cycle and early pregnancy. Theriogenology **41**, 1115-1126.

Pope GS, Majzlik I, Ball PJH, Leaver JD, 1976: Br Vet J **132**, 497-506.

Robinson RS, Hammond AJ, Hunter MG, Mann GE, 2005: The induction of a delayed post-ovulatory progesterone rise in dairy cows: a novel model. Domest Anim Endocrinol **28**, 285-295.

Rodgers RJ, O'Shea JD, Bruce NW, 1984: Morphometric analysis of the cellular composition of the ovine corpus luteum. J Anat **138**, 757-770.

Sartori R, Rosa GJ, Wiltbank MC, 2002: Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci **85**:2813-2822.

Setchell KD, Cassidy A, 1999: Dietary isoflavones: biological effects and relevance to human health. J Nutr **129**, 758-767.

Setchell KDR, Brown NM, Lydeking-Olsen E, 2002: The clinical importance of the metabolite equol - A clue to the effectiveness of soy and its isoflavones. J Nutr
132, 3577-3584.

Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE, 1990: Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil **90**, 1-10.

Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM, 1991: Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. Br J Cancer **63**, 963-966.

Silvester LM, Luck MR, 1999: Distribution of extracellular matrix components in the developing ruminant corpus luteum: a wound repair hypothesis for luteinization. J Reprod Fertil **116**, 187-198.

Su SJ, Yeh TM, Chuang WJ, Ho CL, Chang KL, Cheng HL, Liu HS, Cheng HL, Hsu PY, Chow NH, 2005: The novel targets for anti-angiogenesis of genistein on human cancer cells. Biochem Pharmacol **69**, 307-318.

Trinacty J, Krizova L, Schulzova V, Hajslova J, Hanus O, 2009: The effect of feeding soybean-derived phytoestogens on their concentration in plasma and milk of lactating dairy cows. Arch Tierernahr **63**, 219-229.

Tsai SJ, Kot K, Ginther OJ, Wiltbank MC, 2001: Temporal gene expression in bovine corpora lutea after treatment with PGF2alpha based on serial biopsies *in vivo*. Reproduction **121**, 905-913.

Vasconcelos JLM, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC, 2001: Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology **56**, 307-314.

Wong CK, Keung WM, 1999: Bovine adrenal 3 beta-hydroxysteroid dehydrogenase (EC 1.1.1.145)/5-ene-4-ene isomerase (EC 5.3.3.1): characterization and its inhibition by isoflavones. J Steroid Biochem Mol Biol **71**, 191-202.

Yamashita H, Kamada D, Shirasuna K, Matsui M, Shimizu T, Kida K, Berisha B, Schams D, Miyamoto A, 2008: Effect of local neutralization of basic fibroblast growth factor or vascular endothelial growth factor by a specific antibody on the development of the corpus luteum in the cow. Mol Reprod Dev **75**, 1449-1456.

Zhou JR, Gugger ET, Tanaka T, Guo YP, Blackburn GL, Clinton SK, 1999: Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. J Nutr **129**, 1628-1635.

Summary

Since several decades dairy cows have been selected towards a higher level of milk production. One of the consequences is that these cows are dealing with a period of negative energy balance, leading to the fact that dairy farmers are confronted nowadays with high yielding dairy cows struggling with low fertility at different levels: delayed resumption of ovarian activity, reduced estrus expression and pregnancy maintenance. The outcome is a prolonged calving interval of which the economic impact is far from negligible. In the **general introduction** two important causes of this extended calving interval are highlighted: firstly the increased (voluntary) waiting period and secondly the increased incidence of early embryonic death.

In the **literature review**, the most important cause of this high incidence of early embryonic death, insufficient peripheral progesterone concentration, is highlighted. First of all, the question is posed whether progesterone is indeed that important to maintain pregnancy (**Chapter 2.1**). When literature is reviewed an evolution is remarkable in the researchers' point of view. Initially, progesterone was seen as the absolute (and only) cut-off prerequisite to sustain pregnancy. Nowadays however, progesterone is still seen as an essential component, but it is presented more as one of the factors favouring the chance of maintaining pregnancy. Recent studies even identify an optimal progesterone concentration, being associated with a minimal chance of early embryonic death. In chapter 2.1, we also discuss the direct and indirect effects of progesterone on the embryonic interferon tau secretion and on the endometrial secretory activity.

The peripheral blood progesterone concentration is a resultant of different factors (**chapter 2.2**): luteal progesterone production, hepatic clearance of progesterone and mammary progesterone excretion. It has already been scientifically proven that, specifically in the present-day high yielding dairy cows, the progesterone loss via the milk and the hepatic clearance is increased because of the increasing trend in milk production leading to a decrease in the peripheral progesterone concentration. But, in literature there is no hard evidence available concerning the capacity of the luteal gland to produce progesterone in these high yielding dairy cows. Several studies suggest that 'luteal inadequacy' in these cows, also contributes to the insufficient peripheral progesterone concentration. In chapter 2.2, the available knowledge about the luteal morphometrics is reviewed. Besides, the physiological and biochemical processes of luteal angio- and steroidogenesis are highlighted, both being essential contributors to the peripheral progesterone concentration.

The periparturient high yielding dairy cow induces some homeorrhetic adaptations to cope with the negative energy balance, leading to several typical modifications in her metabolic

profile. Reviewing the literature (**chapter 2.3**), several indications appear supposing or proving deleterious effects on luteal and endometrial functionality, with negative effects on embryonic survival rate. A second aspect described in chapter 2.3 is the effect of isoflavones on luteal activity. Because of the high protein requirements of modern cows, farmers are obliged to supplement these animals with an extra protein source, mostly soybean meal, which is rich in isoflavones. These isoflavones are mainly metabolized by the ruminal bacterial flora leading to the absorption of several parent molecules (genistein, daidzein and glycitein) and metabolites (O-desmethyl-angolensin, dihydrodaidzein, and equol). These molecules seem *in vivo* to have a negative impact on luteal functionality. *In vitro,* inhibiting effects have been described on steroido- and angiogenesis, but these *in vitro* effects are not yet confirmed by *in vivo* studies.

In the final **chapter 2.4** of the literature review, the available information about biopsy sampling techniques of the luteal gland is reviewed, giving us the opportunity to optimize our sampling technique based on experiences of other researchers.

Although it is generally accepted that the peripheral blood progesterone concentration is suboptimal in present-day high yielding Holstein Friesian cows and that this contributes to an increased incidence of early embryonic death, the role of 'luteal inadequacy' in the story of the reduced blood progesterone is still a 'black hole'. Therefore, the **main hypothesis** of this thesis is: the luteal activity in present-day high yielding Holstein Friesian cows is compromised during the postpartum period leading to a suboptimal peripheral blood progesterone concentration (**chapter 3**).

In a first phase of our research (**chapter 4**) we investigate, based on *ex vivo* research, whether it is possible to analyze the luteal histophysiology based on one single luteal gland. tissue sample. A pilot study (**chapter 4.1**) is set up to assess the variation in cell density within a bovine luteal gland. Six luteal glands (stage II or III) are harvested out of just as many healthy cows at the slaughterhouse. The luteal cell density is assessed by calculating the nuclear density of the different luteal cell types on Haematoxylin-Eosin-stained histological sections from a number of topographic regions evenly spread throughout the glands, in order to give an overview of the pattern of cellular distribution within the whole gland. Cells are differentiated into ‘large luteal cells’, ‘small luteal cells’ and ‘non-steroidogenic cells’. Results show that the cellular density, within a tissue sample is not significantly influenced by its location in relation to the gland’s equatorial plane. However, the position with respect to the polar axis of the gland has a decisive effect, as the nuclear density is significantly

higher ($P < 0.05$) in the peripheral regions (outer zone) when compared with the central regions (inner zone) of the gland, and this counts for all three cell types.

In a next study (**chapter 4.2**), we analyze the effect of fixative, breed, luteal stage and location on the nuclear density, volume density of connective tissue and vascular tissue/lumina within a bovine luteal gland in view of the development of an *in vivo* sampling technique to longitudinally monitor luteal histophysiology. The inner zone defined as the zone geometrically closest to the center of the gland, shows a significantly lower nuclear density (for all cell types) and a higher volume density of collagen fibers and vessels when compared with the outer zone ($P < 0.001$). The nuclear density in luteal glands from Holstein Friesian cows is not significantly different from that in Belgian Blue cows, nor is it in stage II versus stage III glands. The collagen fiber content is significantly lower in glands of Belgian Blue cows ($P=0.01$) and in younger glands ($P=0.003$). Hence, it seems that the lower nuclear density in the inner zone is compensated by a higher amount of collagen fibers. As the type of fixative applied has a significant effect on the nuclear density of the different cell types, the present study warrants future research to further optimize the fixation protocol. As a conclusion, we can state that the topographic difference in nuclear distribution for the different cell types in a bovine luteal gland is only significant when comparing the inner versus the outer zone. This implies that, if a sample representative for the whole gland has to be taken, e.g. when taking an *in vivo* sample, it is necessary that the biopsy goes through the inner zone and contains the total diameter of the gland.

Finally (**chapter 4.3**), a sampling procedure is described and validated to monitor luteal histology via a biopsy of the corpus luteum in cows. Also, different fixation protocols are tested. In total, 46 luteal glands (23 in luteal stage II and 23 in stage III), are randomly assigned to three groups and fixated by different immersion protocols: subset 1a (N=19; neutral-buffered 3.5 % formaldehyde) and 1b (N=8; Zinc salts Fixative) to assess the nuclear density, and subset 2 (N=19; Bouin's fixative) to analyze the volume density of collagen fibers and vascular tissue. To validate the biopsy technique, two biopsies are taken out of the glands, one perpendicular to the equatorial plane, containing the ovulation point and one along the equatorial plane. A tissue disk ('central disk'), containing both prelevation sites of the biopsies and the luteal center is isolated.

The results obtained from biopsy sections are compared with those obtained from sections made out of the whole luteal gland (i.e. the central disk; overview sections). The results obtained from the central disk are assumed to be the gold standard. For nuclear density, a good agreement is observed between the biopsy sections and the overview sections. The

highest agreement index is obtained for the ZsF-fixation (0.94; P-value < 0.001). Regarding the volume density of the vessels and collagen fibers (analyzed in the biopsies and central disks of subset 2), the agreement between biopsies and overviews is only high enough (more than 0.90) for the distribution of lumina.

In conclusion, this biopsy technique allows us to analyze histological and eventually histophysiological characteristics of the complete luteal gland, based on one single biopsy sample. Still, a prerequisite is that these histological traits are correlated with the parameter 'nuclear density' (for example intracellular steroidogenic enzymes) or the parameter 'vessels' (such as endothelial specific lectines). The representativeness for the results of a biopsy taken through the luteal gland's central point and containing the total diameter of the luteal gland with the gold standard depends on the fixative that is used.

In the second phase, we made the step to *in vivo* research (**chapter 5**). A field study is done and described into two parts, executed on the same animals. In the first part (**chapter 5.1**), thirty three Holstein Friesian cows are followed from 14 days pre partum until the fourth ovulation post partum. Housing conditions and basic ration are identical for all animals. Concentrates are individually supplemented according to the daily milk production level, using two different types of protein rich concentrates: soybean meal and rapeseed meal. Soybean and rapeseed meal are known to be respectively high and low in isoflavones. Cows are randomly divided into three groups and blocked for parity. Group I (n=11) is supplemented with soybean meal and acted as control group. Groups II (n=11) and III (n=11) are respectively supplemented with soybean and rapeseed meal and are subjected to a biopsy sampling of the corpus luteum at day 9 of the first three postpartal estrous cycles. In the luteal biopsies, endothelial cells are visualized by *Bandeiraea simplicifolia* isolectin and the steroidogenic cells by 3β-hydroxysteroiddehydrogenase.

Soybean meal supplementation to lactating dairy cows (1.72 kg on average) induces an increase in the blood concentration of equol, dihydrodaidzein, o-desmethylangolensin in both soy groups and results in a reduced area occupied by steroidogenic (P=0.012) and endothelial cells (P=0.0007) in the luteal biopsies. Blood concentrations of equol and glycitein are negatively correlated with the areas occupied by steroidogenic (r=-0.410 with P=0.0002, respectively r=-0.351 with P=0.008) and endothelial cells (r=-0.337 with P=0.01, respectively r=-0.233 with P=0.085) in the 3 first estrous cycles. The latter however do not affect the diestrus peripheral blood progesterone concentration.

In the second part (**chapter 5.2**), several production and blood parameters are monitored on the same cows during the same experiment. Daily milk yield, changes in body condition

score and blood concentrations of non-esterified fatty acids and β-hydroxy-buturic acid are not significantly different between the soy- and the rape-supplemented groups, leading to the conclusion that the difference in protein rich concentrate do not induce group specific differences in energy status.

Total blood cholesterol concentration increases significantly with days post partum and days within the oestrous cycle (day 0-4 versus day 9-16). Blood urea concentrations are systematically higher in the soy-supplemented and multiparous cows.

The vascular network within the corpus luteum is significantly denser in cycles 2 and 3 in comparison to the first postpartum cycle (P=0.0005). An identical evolution is seen for the surface occupied by steroidogenic cells (P=0.0001).

In comparison to multiparous cows, primiparous animals have larger luteal glands on cycle day 9. No cycle number effect is detected on the size of the corpus luteum at day 9 of the cycle, while on day 16 luteal glands become larger with increasing cycle number.

The peripheral blood progesterone concentration shows besides the predictable effect related to the day of the oestrous cycle, an increasing trend with increasing cycle number. Furthermore, the peripheral progesterone concentration is overall higher in primiparous cows (P=0.013).

The area occupied by endothelial cells is positively correlated with the area occupied by steroidogenic cells (r=0.59; P < 0.0001). Nevertheless, there is no significant correlation between both areas and the blood progesterone concentration. Both the areas occupied by endothelial and by steroidogenic cells are negatively correlated with the blood concentration of non-esterified fatty acids (respectively r=-0.377; P=0.004 and r =-0.355; P=0.007).

Based on the results of the present study, we conclude that primiparous cows generally have higher peripheral progesterone levels during the first 3 cycles after calving which is associated with a bigger corpus luteum. In comparison to those of the first postpartum cycle, corpora lutea of cycles 2 and 3 have a denser capillary network and a higher number of steroidogenic cells, while this is associated with a trend of higher peripheral progesterone concentrations.

In **chapter 6** the results obtained in our experiments or field studies are discussed in view of the current knowledge in literature, leading to the following conclusions:

* The assumption of topographic homogeneity in the bovine luteal histology is incorrect as nuclear density and collagen fibers are increasing, respectively decreasing from the luteal center towards the luteal capsula. The vascular density follows the trend of collagen.

* Based on the variation in the nuclear density in bovine luteal glands, at least 66 observations, evenly distributed over the inner and outer zone are necessary to reliably estimate the nuclear density within a CL.
* The luteal glands of Belgian Blue cows contain less collagen fibers versus CLa from Holstein Friesian cows. This is the opposite compared to the testes of the male counterparts.
* It is possible to monitor the histology of a bovine luteal gland based on one single biopsy, provided that

    --> the chosen histological traits are closely related with nuclear density or vascular tissue;

    --> Zinc salts fixative is used in case of nuclear density related traits;

    --> the specimen contains the total luteal diameter and goes through the luteal center.

* Soybean meal supplementation under representative Belgian circumstances, leads to a significant increase of the blood concentration of the metabolites equol, O-desmethylangolensin, dihydrodaidzein and the parent molecules glycitein and daidzein, but not of genistein.
* The higher blood concentrations of equol and glycitein are associated with a decreased area of HSD-positive and BSI-positive cells, leading to the conclusion that soybean supplementation, even under Belgian circumstances where this supplementation is rather limited, impairs luteal steroidogenesis and angiogenesis in bovines, without a significant effect on the peripheral blood progesterone concentration.
* AreaBSI, areaHSD and blood cholesterol are increasing with cycle number, resulting in a positive trend of the peripheral blood progesterone concentration.
* The blood cholesterol concentration is positively correlated with the peripheral blood progesterone concentration, the areaBSI and areaHSD, while the blood NEFA and BOHB concentrations are negatively correlated with the peripheral blood progesterone concentration, the areaBSI and the areaHSD, indicating respectively a positive effect of cholesterol and a negative effect of the negative energy balance on the luteal histophysiology.

Samenvatting

Decennia lang worden koeien geselecteerd op melkproductie. Eén van de gevolgen is dat deze koeien bij het opstarten van de melkproductie een periode van NEB ondergaan, wat leidt tot het feit dat melkveehouders de dag van vandaag geconfronteerd worden met hoogproductieve dieren die worstelen met een slechtere vruchtbaarheid en dit op verschillende niveaus: uitgestelde hervatting van de ovariële activiteit, verminderde bronstexpressie, daling in fertilisatiegraad en een kleinere kans op het behoud van dracht. Het resultaat is een verlenging van de tussenkalftijd, waarvan de economische impact allerminst verwaarloosbaar is. In de **algemene inleiding** worden 2 belangrijke oorzaken van deze verlengde tussenkalftijd in het licht gezet: vooreerst de toename van de (vrijwillige) wachtperiode en ten tweede de toename in de prevalentie van vroeg embryonale sterfte.

In het literatuuroverzicht wordt de voornaamste oorzaak van de verhoogde prevalentie van vroeg embryonale sterfte, namelijk de verlaagde progesteronconcentratie besproken. Eerst en vooral wordt de vraag gesteld of progesteron inderdaad zo belangrijk is voor het behoud van de dracht (**hoofdstuk 2.1**). Wanneer de literatuur hieromtrent bekeken wordt, is er een duidelijke evolutie te zien in het standpunt van de onderzoekers. Initieel werd progesteron aanzien als de absolute (en enige) vereiste voor het behoud van de dracht, terwijl men momenteel progesteron nog steeds als essentieel beschouwt, zij het als één van de vele factoren die de kans op het behoud van dracht beïnvloeden. Recente studies wijzen zelfs op het bestaan van een optimale progesteronconcentratie, die gepaard gaat met een minimale kans op vroeg embryonale sterfte. In dit hoofdstuk worden ook de directe en indirecte effecten van luteaal progesteron op de embryonale interferon tau secretie en de secretorische activiteit van het endometrium beschreven.

De progesteronconcentratie, gemeten in de perifere bloedvaten (verder kortweg de progesteronconcentratie genoemd), is een resultante van tal van factoren (**hoofdstuk 2.2**): de luteale progesteronproductie, de hepatische klaring van progesteron en de mammaire progesteronexcretie. Wetenschappelijk gezien staat het reeds vast dat in de hedendaagse hoogproductieve melkkoeien, het progesteronverlies via melk en lever verhoogd is omwille van de stijgende trend in de melkproductie, wat leidt tot een gedaalde progesteronconcentratie. Daarentegen is er in de literatuur geen hard bewijs te vinden aangaande een eventueel gereduceerd prestatievermogen van het gele lichaam bij deze hoogproductieve melkkoeien. Een aantal studies suggereren wel een tekortkoming in de luteale functie van deze koeien, dewelke op zijn beurt zou bijdragen aan de verlaagde progesteronconcentratie. In dit hoofdstuk wordt bovendien de beschikbare en relevante kennis inzake de luteale morfometrie besproken. Daarnaast wordt ook de fysiologie en biochemie

van de luteale angiogenese en steroïdogenese belicht, beide essentieel voor de toestroom van progesteron in de bloedcirculatie.

In de periode rondom het afkalven induceren hoogproductieve melkkoeien een aantal homeorrhetische aanpassingen om het hoofd te kunnen bieden aan de negatieve energiebalans. Deze veroorzaken een aantal kenmerkende wijzigingen in hun metabool profiel, die, althans volgens de geraadpleegde literatuur (**hoofdstuk 2.3**), een hypothetisch of bewezen negatief effect kunnen hebben op de luteale en endometriale functionaliteit, met bijgevolg negatieve effecten op de embryonale overlevingskansen. Een tweede aspect in dit hoofdstuk is het effect van de isoflavones op de luteale functie. Omwille van een verhoogde eiwitbehoefte, wordt de moderne melkveehouder verplicht om zijn hoogproductieve melkkoeien een extra eiwitbron te supplementeren. Meestal is dit sojaschroot, dat rijk is aan isoflavones. Eerst en vooral worden een aantal relevante aspecten van de biologische beschikbaarheid van de voornaamste isoflavones besproken. *In vitro* zijn er inhiberende effecten op de steroido- en angiogenese aangetoond, dewelke kort worden beschreven. Deze molecules lijken ook *in vivo* een negatief effect te hebben op de luteale functionaliteit, echter de gekende *in vitro* effecten werden nog niet ondubbelzinnig bewezen in *in vivo* studies.

In een finaal **hoofdstuk 2.4** van de literatuurstudie wordt de beschikbare informatie verzameld aangaande de technieken voor het *in vivo* bemonsteren van het corpus luteum. Dit geeft ons de gelegenheid om op basis van deze gegevens, onze staalnametechniek te optimalizeren.

Vermits het enerzijds algemeen is aanvaard dat de progesteronconcentratie suboptimaal is bij de hedendaagse hoogproductieve Holstein Friesian koeien en dat dit leidt tot een verhoogde prevalentie van vroeg embryonale sterfte en vermits anderzijds de rol van een gereduceerd prestatievermogen van het gele lichaam in deze theorie nog steeds vaag is, wordt de **hoofdhypothese** als volgt opgesteld: "De luteale progesteronproductie in de hedendaagse hoogproductieve Holstein Friesian koeien is verslechterd gedurende de vroege postpartum periode, wat leidt tot een reductie in de progesteronconcentratie. In een eerste fase van ons onderzoek (**hoofdstuk 4**) onderzoeken we, gebaseerd op *ex vivo* onderzoek, of het mogelijk is de luteale histofysiologie te analyseren op basis van één enkel weefselstaal. Een pilootstudie (**hoofdstuk 4.1**) wordt opgezet om de variatie in celdensiteit te bepalen in een bovien geel lichaam. Zes corpora lutea (luteaal stadium II en III) worden verzameld uit evenveel koeien aan de slachtlijn. De luteale celdensiteit wordt bepaald door de kerndensiteit van de verschillende luteale celtypes te berekenen op hematoxyline-eosine gekleurde weefselcoupes. Het aantal waarnemingen wordt gelijk verdeeld over een aantal topografisch omlijnde

gebieden van de klier, zodat we een overzicht krijgen van het verdelingspatroon van de verschillende celtypes over het gehele corpus luteum. Onderscheid wordt gemaakt in 'grote luteale cellen', 'kleine luteale cellen' en 'niet steroidogene cellen'. De resultaten laten zien dat de kerndensiteit binnen een weefselstaal niet significant beïnvloed wordt door zijn localisatie ten opzichte van het evenaarsvlak van de klier. Daarentegen is de positie ten opzichte van de polaire as wel belangrijk, vermits de kerndensiteit significant hoger ($P < 0,05$) is in de perifere regio's (buitenste zone) in vergelijking met de centrale regio's (binnenste zone) van de klier. Dit geldt voor elk van de drie celtypes.

In een volgende studie (**hoofdstuk 4.2**) onderzoeken we het effect van het soort fixatief, het ras, het luteaal stadium en de locatie binnen de klier op de kerndensiteit en de oppervlakte ingenomen door bindweefsel, dan wel vasculair weefsel in een bovien corpus luteum. Dit onderzoek heeft tot doel kennis in te zamelen met het oog op de ontwikkeling van een *in vivo* staalnametechniek voor de opvolging van de boviene luteale histofysiologie. De binnenste zone, gedefinieerd als de zone die geometrisch gezien het dichtst bij het centrum van de klier ligt, vertoont een significant lagere kerndensiteit (voor elk celtype) en een grotere oppervlakte ingenomen door collageenvezels en bloedvaten, in vergelijking met de buitenste zone ($P < 0,001$). De kerndensiteit in een corpus luteum van een Holstein Friesian koe is niet significant verschillend van deze van een Belgisch Witblauwe koe, noch was dit het geval voor stadium II versus stadium III corpora lutea. Het gehalte aan collageenvezels is significant lager in gele lichamen van Belgisch Witblauwe koeien ($P=0,01$) en in jongere klieren ($P=0,03$). Bijgevolg lijkt het dus dat de lagere kerndensiteit in de binnenste luteale zone gecompenseerd wordt door een hoger gehalte aan collageenvezels. Vermits het soort fixatief een significant effect had op de kerndensiteit van de verschillende celtypes, wijst deze studie erop dat het fixatieprotocol verder geoptimaliseerd dient te worden in verder onderzoek. Als conclusie kunnen we stellen dat het topografisch verschil in kerndensiteit van de verschillende celtypes van een bovien geel lichaam enkel significant is wanneer de binnenste en buitenste zone vergeleken worden. Dit betekent dat, wanneer een staal, representatief voor de gehele klier, dient te worden genomen, het noodzakelijk is dat de biopsie door het luteale centrum gaat en de hele diameter van de klier bevat.

Finaal wordt er een staalnamemethode beschreven en gevalideerd (**hoofdstuk 4.3**) om de boviene luteale histologie te monitoren middels een biopsie. Er worden in deze studie ook verschillende fixatieprotocols getest. In totaal worden 46 corpora lutea (23 in luteaal stadium II en 23 in stadium III) at random toegewezen aan 3 groepen en gefixeerd gebruik makend van verschillende fixatieprotocols (gebaseerd op onderdompeling): groep 1a ($N=19$; neutraal

gebufferde 3,5% formaldehyde) en 1b (N=8; fixatief gebaseerd op zinkzouten), beiden om de kerndensiteit te bepalen, en groep 2 (N=19; fixatief van Bouin) om de oppervlakte te bepalen ingenomen door collageenvezels en bloedvaten. Om deze biopsietechniek te valideren worden twee biopten uit de klier genomen, één loodrecht op het evenaarsvlak, doorheen het vroegere ovulatiestigma en één volgens het evenaarsvlak. Er wordt een weefselschijf ('Centrale Schijf') uit de klier geïsoleerd, die beide biopsieplaatsen omvat. De resultaten die verkregen worden uit de biopsiecoupes worden vergeleken met deze van de coupes gemaakt uit de gehele klier (d.w.z. uit de 'Centrale Schijf'; overzichtscoupes), dewelke beschouwd worden als de gouden standaard. Voor de kerndensiteit wordt een hoge graad van overeenkomst verkregen tussen de biopsiecoupes en de overzichtscoupes. De hoogste graad van overeenkomst wordt bekomen voor de ZsF-fixatie (0,94; P-waarde < 0,001). Wat betreft de oppervlakte ingenomen door bloedvaten en collageenvezels (geanalyseerd op de biopsies en 'Centrale Schijven' van groep 2), is de overeenkomst tussen biopsiecoupes en overzichtscoupes enkel hoog genoeg (meer dan 0,90) voor de bloedvaten.

Als besluit kunnen we stellen dat deze biopsietechniek ons in staat stelt om histologische en eventueel histofysiologische kenmerken van een intact geel lichaam te analyseren op basis van één enkele biopsie. Doch, een vereiste is wel dat deze histologische kenmerken gecorreleerd zijn met de parameter 'kerndensiteit' (bijvoorbeeld intracellulair gehalte aan steroidogene enzymes) of met de parameter 'bloedvaten' (zoals endotheelspecifieke lectines). De representativiteit van de resultaten bekomen uit één biopsie, genomen doorheen het centrale punt van de klier en de volledige diameter van de klier omvattend, hangt af van het gebruikte fixatief.

In een tweede fase zetten we de stap naar het *in vivo* onderzoek (**hoofdstuk 5**). Deze veldstudie wordt verdeeld in 2 delen, uitgevoerd op dezelfde dieren. In een eerste deel (**hoofdstuk 5.1**) worden 33 Holstein Friesian koeien opgevolgd van 14 dagen voor de verwachte afkalfdatum tot de vierde ovulatie post partum. Huisvesting en basisrantsoen zijn identiek voor alle dieren. Krachtvoeders worden individueel gesupplementeerd in functie van de dagelijkse melkproductie. Er worden 2 verschillende types eiwitkern gebruikt: sojaschroot en raapzaadschroot. Van soja- en raapzaadschroot is geweten dat ze respectievelijk veel en weinig isoflavones bevatten. De koeien worden at random verdeeld in 3 groepen en geclusterd voor pariteit. Groep I (N=11) wordt gesupplementeerd met sojaschroot en fungeert als controlegroep. Groep II (N=11) en III (N=11) worden respectievelijk gesupplementeerd met soja- en raapzaadschroot en worden onderworpen aan een staalname van het corpus luteum op dag 9 van de eerste 3 cycli post partum . In de luteale biopsies worden endotheelcellen

gevisualiseerd met *Bandeiraea simplicifolia* isolectine en de steroidogene cellen met 3β-hydroxysteroiddehydrogenase.

Supplementatie van sojaschroot aan lacterende melkkoeien (gemiddeld 1,72 kg per dag) veroorzaakt een verhoging van de bloedconcentratie van equol, dihydrodaidzein en O-desmethylangolensin in beide sojagroepen en resulteert in een reductie van de oppervlakte ingenomen door steroidogene cellen (P=0,012) en endotheelcellen (P=0,0007) in de luteale biopsies. De bloedconcentraties van equol en glycitein zijn negatief gecorreleerd met de oppervlakte ingenomen door steroidogene cellen (r=-0,410 met P=0,0002, respectievelijk r=-0,351 met P=0,008) en endotheelcellen (r=-0,337 met P=0,01, respectievelijk r=-0,233 met P=0,085) in de eerste 3 cycli na afkalven. Dit laatste echter heeft geen effect op de perifere bloedprogesteronconcentratie gedurende de dioestrus faze.

In het tweede deel van de veldstudie (**hoofdstuk 5.2**) worden verschillende productie- en bloedparameters gemonitord op dezelfde koeien gedurende dezelfde experimentele periode in hetzelfde experimentele opzet. De dagelijkse melkproductie, de veranderingen in de lichaamsconditiescore en de bloedconcentraties van niet-veresterde vetzuren en β-hydroxyboterzuur zijn niet significant verschillend tussen de soja- en de raapzaad gesupplementeerde groepen. Dit leidt tot de conclusie dat het verschil in type eiwitkern niet de oorzaak kan zijn van de groepspecifieke verschillen in energiestatus.

De concentratie van het totale cholesterol in het bloed stijgt significant met het aantal dagen post partum en met het cyclusstadium (dag 0-4 versus dag 9-16). De ureumconcentraties in het bloed zijn systematisch hoger in de soja-gesupplementeerde groep en bij de multipare koeien.

Het vaatnetwerk binnen het corpus luteum iss significant compacter in cyclus 2 en 3 in vergelijking met de eerste cyclus post partum (P=0,0005). Een identieke evolutie is te zien voor de oppervlakte die ingenomen wordt door de steroidogene cellen (P=0,0001).

Primipare dieren hebben, in vergelijking met de multipare koeien grotere corpora lutea op cyclusdag 9. Het cyclusnummer heeft geen significant effect op de omvang van het corpus luteum op cyclusdag 9, terwijl op dag 16 de gele lichamen groter worden met het cyclusnummer.

De progesteronconcentratie vertoont, naast het voorspelbare effect cyclusdag, een stijgende trend met het stijgend cyclusnummer. Bovendien is deze progesteronconcentratie gemiddeld gezien steeds hoger bij de primipare dieren (P=0,013).

De oppervlakte die bezet wordt door endotheelcellen is positief gecorreleerd met de oppervlakte ingenomen door steroidogene cellen (r=0,59; P < 0,0001). Nochtans is er geen

significante correlatie tussen beide oppervlaktes en de progesteronconcentratie. Beide oppervlakten zijn negatief gecorreleerd met de NEFA concentratie in het perifere bloed (respectievelijk r=-0,377; P=0,0004 en r=-0,355; P=0,007).

Gebaseerd op de resultaten van deze studie, kunnen we besluiten dat primipare koeien gedurende de eerste 3 cycli na afkalven gemiddeld gezien hogere progesteronconcentraties vertonen in hun bloed, wat ook geassocieerd is met een groter geel lichaam. In vergelijking met de corpora lutea van de eerste cyclus post partum, is het vasculaire netwerk in de corpora lutea van cyclus 2 en 3 compacter en vertonen deze gele lichamen ook een grotere oppervlakte ingenomen door steroidogene cellen, terwijl deze vaststellingen zich enkel reflecteren in een tendens tot een hogere perifere bloedprogesteronconcentratie.

In **hoofdstuk 6** worden de resultaten, bekomen via onze experimenten en veldstudies, kritisch bekeken en getest aan de kennis beschikbaar in de literatuur. Dit leidt tot de volgende conclusies:

* De aanname van een topografische homogeniteit in de boviene luteale histologie is niet correct, vermits de kerndensiteit en de collageenvezels stijgen, respectievelijk dalen gaande van het luteale centrum naar het luteale kapsel. De vasculaire densiteit volgt de tendens van het collageen.

* Gebaseerd op de variatie in de kerndensiteit in het boviene gele lichaam, zijn er minimaal 66 waarnemingen nodig, gelijkwaardig verdeeld over de binnenste en buitenste zone, om op een betrouwbare manier de kerndensiteit in te schatten.

* De corpora lutea van Belgisch Witblauwe koeien bevatten minder collageenvezels in vergelijking met deze van Holstein Friesian koeien. Dit in tegenstelling van wat men ziet in de testes aan mannelijke zijde.

* Het is mogelijk de histologie van een bovien geel lichaam op te volgen op basis van één enkel biopt, gesteld dat:

> het gekozen histologisch kenmerk sterk gerelateerd is met de kerndensiteit of het vasculair weefsel;

> het fixatief met zinkzouten wordt gebruikt in geval van kerndensiteit gerelateerde kenmerken;

> het staal de totale luteale diameter omvat en door het luteale centrum gaat.

* Supplementatie van sojaschroot onder typische Belgische praktijkomstandigheden resulteert in een significante stijging van de bloedconcentratie van de metabolieten equol, O-desmethylangolensine, dihydrodaidzeine en de moedermolecules glycitein en daidzein, maar niet van genistein.

* De verhoogde bloedconcentratie van equol en glyciteine resulteert in een reductie van de oppervlakte ingenomen door HSD en BSI-positieve cellen. Dit leidt tot de conclusie dat sojaschrootsupplementatie, zelfs onder Belgische omstandigheden, de luteale steroido- en angiogenese in runderen verslechtert, zonder een significant effect te hebben op de perifere progesteronconcentratie.
* De oppervlakte ingenomen door BSI en HSD-positieve cellen en het totale bloedcholesterol stijgen met het cyclusnummer, resulterend in een positieve tendens van de perifere progesteronconcentratie.
* De cholesterolconcentratie in het bloed is positief gecorreleerd met de perifere progesteronconcentratie en de oppervlakte ingenomen door BSI en HSD-positieve cellen. De bloedconcentraties van NEFA en BOHB daarentegen zijn negatief gecorreleerd met de perifere progesteronconcentratie en de oppervlakte ingenomen door BSI en HSD-positieve cellen, indicatief voor respectievelijk een positief effect van cholesterol en een negatief effect van de negatieve energiebalans (NEFA en BOHB) op de luteale histofysiologie.

Acknowledgments - Dankwoord

Toen ik 1 oktober 2006 met mijn doctoraat begon, had ik de intentie om dit binnen de gebruikelijke vier jaar af te werken. Toen ik eind 2009 echter het aanbod kreeg om de praktijk in te gaan, heb ik die kans met twee handen gegrepen. Met een onderzoek dat praktisch afgerond was en het idee dat ik naast de praktijk wel tijd zou vinden om mijn artikels en het bijhorende " boek" vlotjes af te werken, startte ik vol vertrouwen aan de combinatie. Niets bleek minder waar. Na mezelf tientallen deadlines opgelegd te hebben en een hoop slapeloze nachten doorgebracht te hebben achter de computer tussen wat sneetjes door is het eindelijk zover.

Vandaag, 20 mei 2014, staat dan ook al even in mijn en menig andere agenda's aangeduid. Iets later dan gepland, maar de eindmeet is nabij. Met het bereiken van deze dag besef ik meer dan eens dat het voltooien van een doctoraatsonderzoek niet het werk is van één persoon. Dit is dan ook dé gelegenheid om een aantal mensen, die me op een of andere manier geholpen hebben, te bedanken. Het grootste gevaar is om hier iemand te vergeten, wat bij mij niet onoverkomelijk is, maar ik ga mij er toch aan wagen.

Op de eerste plaats zou ik mijn promotoren willen bedanken. Geert en Wim, dankzij jullie ben ik in het jaar 2006 niet direct in de praktijk ‘gedropt'. Met het idee dat ik nog mijn hele leven tijd had om "de baan op te gaan", heb ik via jullie de mogelijkheid gekregen om mij gedurende 4 jaar onder de vleugels van de Faculteit volop in te zetten voor mijn onderzoek: "het ontrafelen van één specifiek puzzelstukje in het hele verhaal aangaande de voortplanting en het metabolisme van melkvee." Dit ging natuurlijk gepaard met het nodige vallen en opstaan, maar jullie bleven steeds in me geloven.

Wim, bedankt voor de vele uren dat je me de microscoop ter beschikking stelde, soms op de meest onmogelijke momenten van de dag of de nacht. Moest die microscoop kunnen spreken…ik denk dat hij veel geheimen van me heeft ontfutseld.

Geert, het feit dat ik ‘een moeilijke schrijver’ ben, heeft je waarschijnlijk talrijke keren in je haar laten krabben. Het heeft je er echter niet van weerhouden om telkens een nieuwe versie van een artikel onder handen te nemen. Dank daarvoor. Daarnaast gaf je me ook de gelegenheid om me tijdens die jaren nog toe te leggen op de kliniek en de buitenpraktijk. Dit was vaak een welgekome afwisseling of uitlaatklep. Dit apprecieer ik ten zeerste. Toen ik de faculteit verliet, maakte ik de belofte dat ik mijn doctoraat zou afwerken. En

belofte maakt schuld. Het resultaat zien we vandaag. Bedankt aan beide promotoren voor jullie geduld en jullie steun, bedankt om dit mee te realiseren.

Bovendien zou ik ook een woordje van dank willen richten aan Professor de Kruif, destijds de voorzitter van onze vakgroep, die toen al uitgegroeid was tot een kleine onderneming. Ondanks het feit dat we met velen waren, maakte je er toch een punt van om iedereen zo individueel mogelijk te benaderen. De vele korte babbels en de zogenaamde officiële 'functioneringsgesprekken' waren hiervan het resultaat. Conflicten werden altijd diplomatisch opgelost, een kunst op zich. De vele kilometers, die ik met u heb doorgebracht hetzij in de TGV, hetzij in de auto op weg naar één of ander congres hebben me toch ook wat 'levenswijsheid' opgeleverd.

Verder gaat mijn dank uit naar de leden van de examen- en leescommissie. Professor Simoens, bedankt om te willen fungeren als voorzitter van de examencommissie. Het is ook van uw artistieke hand dat ik een aantal figuren gekregenb heb. Profesor Bollwein, I appreciate that you accepted the invitation for the exam committee. Thank you for coming all the way to Gent for my PhD defense. Professor Bossaert, bedankt om te willen zetelen in mijn begeleidingscommissie en vooral om tijd vrij gemaakt te hebben om mijn proefschrift deftig na te lezen en me met een heleboel tips bij te staan in het verbeteren ervan. Ik besef maar al te goed, dat dit voor jou, naast je functie als professor te Antwerpen, praktijkdierenarts in Nederland en kersverse vader aan het thuisfront, niet altijd even vanzelfsprekend zal zijn geweest. Als er dan nog eens 'flikken' en 'platte batterijen' bij kwamen kijken om het strak dagschema te verstoren, dan besef ik maar al te goed welke offers er af en toe voor nodig waren. Professor De Vliegher, een welgemeende dankjewel om te zetelen als lid van de begeleidingscommissie en dan ook je steentje bij te dragen aan dit eindresultaat. Professor Vanhaecke, ook jou wil ik om dezelfde redenen bedanken. Maar daarenboven is er op jullie dienst veel tijd en moeite gespendeerd aan het analyseren van de vele bloedstalen. Professor Cornillie en dr. Mirjan Thijs, ook jullie hebben je, als lid van de examencommissie, deze taak ter harte genomen. Ik heb er werk aan gehad… maar het resultaat mag er wezen, denk ik. Mirjan, ik ben ook dat kleintje in je buik enorm dankbaar. Hij of zij zorgde er namelijk voor dat we kozen voor de korte pijn en dat mijn verdediging niet nog eens uitgesteld werd. Ik wens jou en Hans dan ook alle geluk toe met jullie gezinnetje.

Er zijn tal van mensen, waarmee ik in de loop der jaren heb samengewerkt. Sta me toe ook deze persoonlijk te bedanken.

Van het Departement Morfologie zou ik Lobke en Liliana willen bedanken. Zij hebben vele uren gespendeerd achter de snijtafel om met de grootste zorg deftige weefselcoupes van mijn

stalen te maken. Ook de discussies over het verbeteren van kleurprotocollen waren schering en inslag.
Mieke, als laborante aan de Vakgroep Veterinaire Volksgezondheid en Voedselveiligheid, heb je met veel toewijding de analyses van een deel van de bloedstalen uitgevoerd. Soms zal je ongetwijfeld wel eens gedacht hebben: "Is hij daar nu weer?" Maar daarvan heb ik dan alleszinds niets gemerkt.
Ook een welgemeende dank aan Arne, destijds verbonden aan het 'Laboratory of Pharmacognosy and Phytochemistry' van de Faculteit Farmaceutische Wetenschappen, voor het analyseren van mijn voederstalen.
Het mag dus gezegd zijn, ik heb beroep kunnen doen op een heleboel deskundige handen.

Gelukkig was er tijdens die jaren meer dan alleen maar mijn onderzoek. Niet dat ik het niet graag deed, maar als de moed je eens in de schoenen zonk, dan konden we onze gedachten eens verzetten op de Kliniek Voortplanting en Verloskunde en de Buitenpraktijk. Catharina, Jan, Maarten, merci voor raad en daad op de kliniek; Jef en Marcel, hetzelfde voor jullie, maar dan op de buitenpraktijk.
De hele groep van 'den Di 08' is echter veel groter dan de personen die ik hier al bedankte. Om echter niemand voor het hoofd te stoten, ga ik me er niet op wagen om iedereen bij naam te noemen. Maar ook aan jullie, merci allemaal om me te helpen waar nodig. Voor elk probleem, groot of klein, kon ik wel bij iemand op de vakgroep terecht.
Na wat omzwervingen op de vakgroep, kreeg ik uiteindelijk mijn vaste stek op de bureau met telefoonnummer 7528. "Den 28", de bewuste bureau waar alles gebeurde. Miel, Philippe (professor Bossaert voor de vrienden), Tim, Mohamed en Wendy; moesten de Plecostoma's nog in leven zijn en moesten de meubels kunnen spreken, ze zouden vele boeken, dikker dan dit exemplaar, kunnen schrijven over de 'mémoires van den 28'. Voor mij zal dit de rest van mijn levensjaren alvast een begrip blijven.
Karel, André en Lydia van de proefhoeve te Melle. Bedankt voor de hulp en het advies tijdens mijn veldproef. De vele uren die ik daar heb doorgebracht, werden vaak doorspekt met gesprekken die praktisch relevant waren. Ook dat deed soms deugd, eens een goede discussie voeren met een veehouder met 'nuchter boerenverstand' over de zaken die hem dagdagelijks bezig hielden. Het zorgde ervoor dat ik zelf met beide voeten op de grond bleef, en het gaf me het nodige perspectief om mijn 'realisaties' te relativeren.
De studenten diergeneeskunde zou ik ook niet willen vergeten De overgrote meerderheid van hen zal reeds gaan vliegen zijn, vermits we 4 jaar later zijn dan gepland. Door de bezoeken

aan het slachthuis, zodat jullie het rectaal onderzoek onder de knie konden krijgen, de hechtpractica op baarmoeders, de casusnamiddagen op verloskunde en de scripties die ik mocht begeleiden, had ik het idee dat ik toch iets bijdroeg aan de veeartsen van de toekomst. Dat jeugdig enthousiasme werkte aanstekelijk. Ook bedankt aan alle studenten die mij geholpen hebben bij mijn veldproef . En blijkbaar kan daar al eens iets moois uit voortvloeien...

Maar wat is een mens zonder vrienden of familie?
Jan en Han, ook jullie zou ik willen bedanken voor de jaren samen op de unief. Han, we zijn mekaar wat uit het oog verloren sinds je vertrek naar Frankrijk, maar ik hoop ooit nog eens bij je te passeren en te zien hoe je het daar stelt. Jan, ook wij zien elkaar sinds ons afstuderen een pak minder. Maar vermits de Limburgse grens oversteken toch nog iets vanzelfsprekender is dan de Franse grens, slagen we er nog in om elkaar af en toe te ontmoeten. Het klassieke verhaal, allebei een druk leven, een eigen gezin 'in opbouw', maken het er niet makkelijker op; maar toch, we gaan één van de komende … nog eens passeren, beloofd. Dan nemen we zeker de boeken over Peru terug mee.
Geert, Raf, Els, Jan, Veerle, Tom en zo vele anderen; jullie hebben er voor gezorgd dat het leven op het negende verdiep van 'den Boudewijn' veel aangenamer werd, al dan niet met behulp van de bar op het gelijkvloers.... Vermits ik 6 jaar moest studeren heb ik een aantal van jullie zien toekomen en een aantal van jullie met spijt in het hart zien vertrekken. Die reünies blijven een goed idee en moeten we dan ook proberen vol te houden.
Doctoren Bossaert, Hostens en Vanhoorebeke ofwel Philippe, Miel en Sebastiaan; Hans en Diedrich, jullie verdienen een extra woordje van dank. Ik weet dat ik niet altijd een 'modelvoorbeeld' ben als het op vriendschap aan komt. Ik pleit schuldig aan gebrek aan initiatief. Ondanks dit alles waren jullie er wanneer de zorgen de bovenhand haalden en het onbezorgde leven iets minder rooskleurig was. Tussen pot en pint wisten we wat we aan elkaar hadden. Ook toen onze levens elk hun eigen gangetje gingen, probeerden we elkaar niet uit het oog te verliezen. Met de verschillende "voorjaarswendes" die we reeds gehad hebben, zijn we een mooie traditie gestart. Ik hoop dat we die in ere kunnen houden en ik sta erop om de volgende te organiseren. Maar help mij er op tijd en stond misschien toch nog maar eens aan denken...
Ook mijn collega's en het personeel van Dierenkliniek Venhei wil ik zeker niet vergeten. Bedankt dat ik bij jullie, in een aangename sfeer, de kans kreeg om hetgeen te doen wat ik het liefste doe en me verder kon en kan ontplooien tot rundveedierenarts.

Guido en Randy, voor jullie een extra woordje van dank; jullie geloofden in ons allebei, mijn vrouw Lieselot en ikzelf, en dankzij jullie kreeg ook Lieselot een plaatsje binnen de praktijk. Haar liefde voor de kalversector en mijn passie voor het melkvee zorgen ervoor dat de Stille Kempen professioneel net iets meer in hun mars hebben dan de mooie westhoek. Bovendien bedankt dat jullie er altijd in geloofden dat ik vandaag wel zou halen. Merci voor het vertrouwen.
Ook een merci aan de collega's van 'de zuidoost', Danny, Geert, Pieter en Kris, als de nood echt hoog was en de tijd begon te dringen om de deadline te halen, kon ik er toch een dagje verlof tussengepropt krijgen om de achterstand wat in te halen. Ik ontken ook niet dat ik hier en daar een extra uurtje heb meegepikt om mij achter de computer te zetten, ook hier zal af en toe wel eens een oogje voor dichtgeknepen geweest zijn.

Mama en papa, mijn jeugdjaren zijn zorgeloos verlopen. Ik denk dat ik, samen met Anke, Wim en Jinse, een jeugd gehad heb waarin we enerzijds konden doen wat we graag deden (waaronder paardrijden), maar waarin we anderzijds ook geleerd hebben wat werken is en dat je niets voor niets krijgt. Jullie hebben mij doorzettingsvermogen bijgebracht, en dat is de laatste tijd zeker van pas gekomen. Zonder jullie had ik nooit gestaan waar ik nu stond.
Ook mijn schoonfamilie verdient een speciaal woordje van dank. Jullie hulp en steun zijn van onschatbare waarde. Maria en José, jullie "reizen" naar de Kempen zijn al lang niet meer op handen en voeten te tellen. Jullie hulp in en rond ons huis heeft er al dikwijls voor gezorgd dat ik weer maar eens een taak van mijn "to-do" list kon schrappen zonder dat ik er zelf iets voor moest doen. En als je dan na een lange werkdag thuis komt in een opgeruimd huis vol lekkers, daar kan een mens alleen maar blij van worden. Om maar van de vrije zondagen te zwijgen dat ik in de Bernikkewallestraat aan de bureau gesleten heb voor dit doctoraat en alleen maar mijn voeten onder tafel moest schuiven, terwijl jullie mijn vrouwtje entertainden.

En als laatste zou ik nog de belangrijkste persoon in mijn leven willen bedanken. Mijn bollie, mijn Lotje, mijn vrouwtje. Toen ik je leerde kennen, jij studente, ik doctoraatsstudent, hadden we allebei nog een redelijk onbezorgd leven. Toen jij afstudeerde en de praktijk in wou en ik enkele maanden later eveneens de praktijkmicrobe voelde kriebelen kwam ons leven in een stroomversnelling. Samen werken, samen wonen, twee drukke jobs en de weinige vrije tijd die ik had ging grotendeels naar het doctoraat. Echt makkelijk hebben we het onszelf niet gemaakt. We stelden heel veel deadlines maar het duurde net iets langer voor het af raakte. Maar dan nog bleven we niet stil zitten. Ons eigen huisje, trouwen, een kindje op komst... Ik

heb echt bewondering voor je. De manier hoe jij je fulltime job als dierenarts combineert met het runnen van onze bvba, de perfectie nastreeft in ons huishouden, ons prachtige trouwfeest organiseerde en dan nu nog alles in de gaten houdt tijdens de verbouwingen van ons huis zijn niet te schatten. Ik kan nog iets leren van je organisatorisch talent. Misschien dat ik dit op den duur wat vanzelfsprekend ben gaan vinden, omdat je alles precies met het grootste gemak doet. Echter de laatste maanden waren toch niet evident voor je. Ons groeiende kleintje in je buik maakt het er voor jou niet makkelijker op en ik was iets te veel gefocust op mijn doctoraat. Ik wou dat ik wat meer tijd had gehad om wat taken van je over te nemen. Ik denk dat we vandaag des te meer als een keerpunt kunnen zien. Onze jobs zullen veel van ons blijven vragen, maar ik wil zeker proberen om de vrije tijd die we hebben optimaal in ons en ons kleintje te investeren. Ik denk dat jij meer dan wie ook weet hoe hard ik hiernaar uitkijk. Ik hoop een goeie papa te worden en wil mijn best doen om je de rest van ons leven gelukkig te maken. Mijn liefste bollie, je bent het beste wat mij ooit overkomen is en ik zie je doodgraag.

Steven

Curriculum Vitae

Steven Cools werd geboren op 10 mei 1982 te Geel. Na het behalen van het diploma secundair onderwijs aan het Sint-Aloysiuscollege te Geel (Latijn-Wetenschappen), startte hij in 2000 met de studie Diergeneeskunde aan de universiteit Gent. Hij verwierf het diploma Dierenarts (optie Herkauwers) in 2006 met grote onderscheiding en dit werd bekroond met de prijs van de Faculteit.

Nadien trad hij in dienst van de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde als doctoraatsstudent van het BOF Vlaanderen en verrichtte onderzoek met de titel 'Een studie handelend over de kwaliteit van het geel lichaam bij hoogproductieve melkkoeien gedurende de postpartum fase'. Dit onderzoek heeft uiteindelijk geleid tot dit proefschrift. Naast het onderzoek was hij ook betrokken bij de opleiding van de laatstejaarsstudenten en bij de klinische dienstverlening in de kliniek Voortplanting en Verloskunde van de Grote Huisdieren en op de Buitenpraktijk.

Na de afronding van het eigenlijke onderzoek, besloot hij in 2010 onder de vleugels van de universiteit te vertrekken en ‘de praktijk’ in te stappen als dierenarts Grote Huisdieren bij Dierenkliniek Venhei in Kasterlee. Nu, 4 jaar later, is het dan zover en ligt het exemplaar klaar om verdedigd te worden.

Steven Cools is auteur en mede-auteur van meerdere wetenschappelijke publicaties in (inter)nationale tijdschriften en gaf meermaals presentaties op (inter)nationale congressen.

Bibliography

Publications in international scientific journals

- Bossaert P, Leroy J, **Cools S**, Opsomer G, 2007: Impact of metabolic and endocrine parameters on ovulation in high yielding dairy cows. Reprod Domest Anim **42**, supplement 2, 118.
- Cornillie P, **Cools S**, Vandaele L, de Kruif A, Simoens P, 2007: Ichthyosis in the Belgian White and Red cattle breed. V D T **76**, 345-351.
- Opsomer G, Leroy J, Bossaert P, **Cools S**, de Kruif A, 2007: Early embryonic death in cattle. Reprod Domest Anim **42**, supplement 2, 63.
- Bossaert P, **Cools S**, Van Loo H, Leroy JLMR, de Kruif A, Opsomer G, 2008: Pathogenesis in clinical ketonemia practice and fatty liver in high-productive dairy cowsDe pathogenese en kliniek van ketonemie en leververvetting bij hoogproductieve melkkoeien. V D T **77**, 283-289.
- **Cools S**, Bossaert P, Caluwaerts T Hostens M, Opsomer G, de Kruif A, 2008: The economic consequences of extending the calving interval in high producing dairy cows. V D T **77**, 402-409.
- Bossaert P, Leterme L, Caluwaerts T, **Cools S**, Hostens M, Kolkman I, de Kruif A, 2009: Teaching transrectal palpation of the internal genital organs in cattle. J Vet Med Educ **36**, 451-460.
- Opsomer G, **Cools S**, de Kruif A, 2009: Corpus Luteum Function in the Bovine. Reprod Domest Anim **44**, 61.
- **Cools S**, Van den Broeck W, De Vliegher S, Piepers S, Hostens M, Opsomer G, 2013: Topographic Distribution of the Different Cell Types, Connective Tissue and Vascular Tissue/Lumina Within a Functional Bovine Corpus Luteum and its Association with Breed, Type of Fixation Protocol and Stage During the Cycle. Reprod Domest Anim **48**, 627-635.
- **Cools S**, Van den Broeck W, De Vliegher S, Piepers S, Opsomer G, 2013: The bovine luteal histological composition: a topographic point of view. Reprod Domest Anim **48**, e29-e32.
- **Cools S**, Van den Broeck W, Vanhaecke L, Heyerick A, Bossaert P, Hostens M, Opsomer G, 2014: Feeding soybean meal increases the blood level of isoflavones and reduces the steroidogenic capacity in bovine corpora lutea, without affecting peripheral progesterone concentrations. Anim Reprod Sci **144**, 79-89.

- Cools S, Van den Broeck W, Bossaert P, Hostens M, Opsomer G, 2014: A field study to unravel factors that are significantly associated with the activity of the corpus luteum during the first three postpartum cycles in high yielding dairy cows, based on the amount of steroidogenic and endothelial cells present in the luteal tissue. Reprod Domest Anim (Accepted).
- **Cools S**, Bossaert P, Caluwaerts T, Hostens M, de Kruif A, Opsomer G. (submitted) Importance of bacterial infections in early embryonic death in cattle. V D T.
- **Cools S**, Van den Broeck, De Vliegher S, Piepers S, Opsomer G (to be submitted): Validation of a luteal biopsy technique to assess bovine luteal morphology.

Publications in national scientific journals

- Bossaert P, Leroy JLMR, **Cools S**, Van Loo H, de Kruif A, Opsomer G, 2008: De metabole adaptatiemechanismen bij hoogproductief melkvee. V D T **77**, 207-218.
- **Cools S**, Bossaert P, Van Loo H, de Kruif A, Opsomer G, 2008: De behandeling en preventie van ketonemie en leververvetting bij hoogproductieve melkkoeien. V D T **77**, 363-375.
- Hostens M, Bossaert P, **Cools S**, de Kruif A, Opsomer G, 2010: Het gebruik van glucogene precursoren in de voeding van hoogproductief melkvee. V D T **79**, 247-258.

Proceedings, oral presentations and poster presentations on international conferences

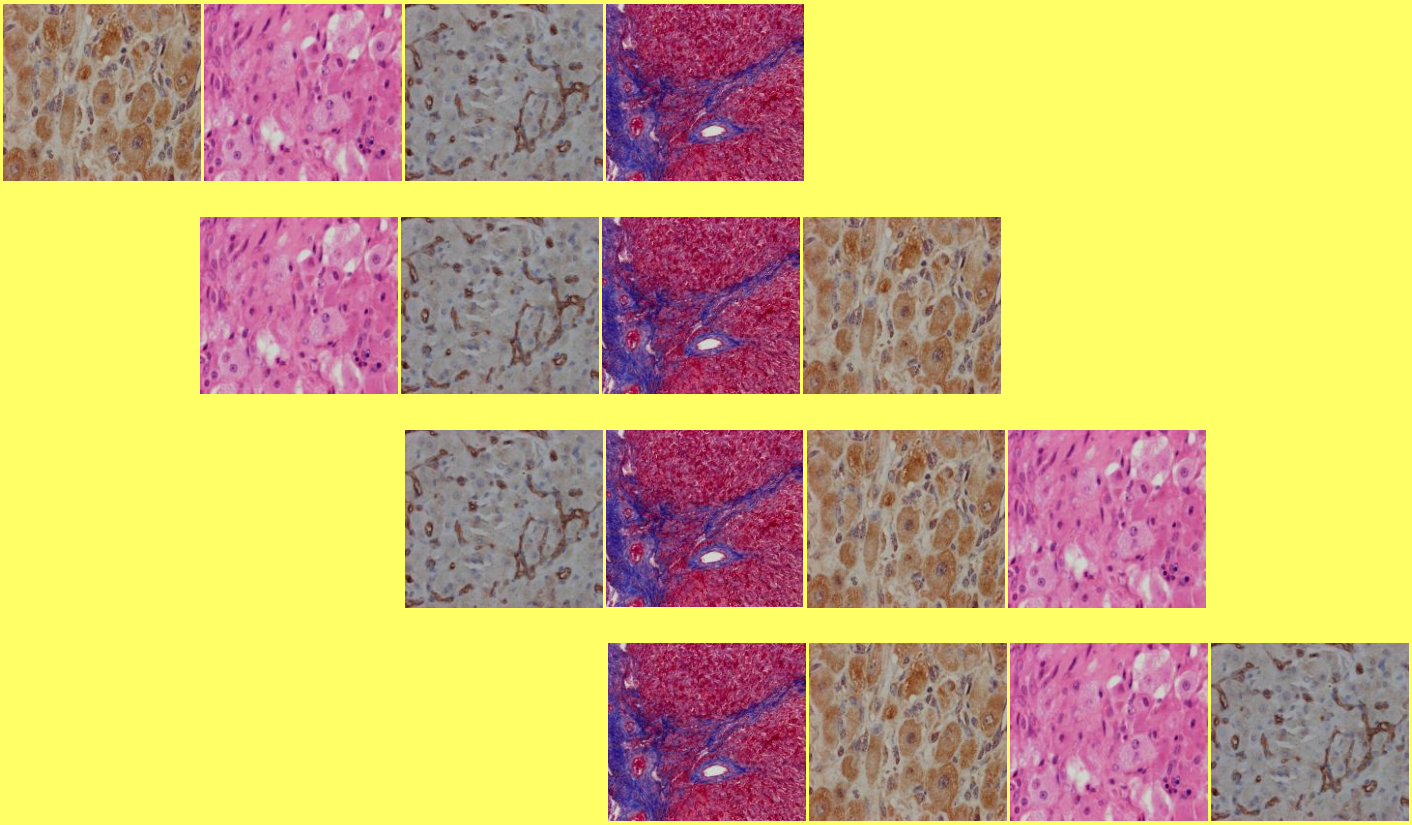
- Bossaert P, Leroy JLMR, **Cools S**, Opsomer G, 2007: "Pancreatic hypofunction of high-yieling dairy cattle: causes and effects". Oral presentation, International conference on production diseases in farm animals, Leipzig, Germany. 29/07-04/08/2007.
- **Cools S**, Catry B, Vanrobaeys M, de Kruif A, Opsomer G, 2007: "Antimicrobial resistance of staphylococci isolated out of subclinical and clinical cases of mastitis." Poster presention, Heifer Mastitis Conference, Ghent, Belgium. 24-26/06/2007.
- **Cools S**, Van den Broeck W, Piepers S, de Kruif A, Opsomer G, 2008: "Analysis of the topographic distribution of the different cell types, collagen and blood vessels in a bovine corpus luteum." Poster presentation, 41. Jahrestagung Physiologie und Pathologie der Fortpflanzung. 28-29/02/2008, Giessen, Germany
- **Cools S**, Van den Broeck W, Piepers S, de Kruif A, Opsomer G, 2008: "Reliability of the morphological characteristics (nuclear density, collagen and blood vessels) of two

biopsies, taken out of a bovine corpus luteum". Poster, 41. Jahrestagung Physiologie und Pathologie der Fortpflanzung. 28-29/02/2008, Giessen, Germany

- Bossaert P, Leterme L, Caluwaerts T, **Cools S**, Hostens M, Kolkman I, de Kruif A, 2009: "Teaching rectal palpation of the genital apparatus in cows: depiction of the learning process in live cows and evaluation of a simulated training model". Oral presentation, European Buiatrics Forum. 01-03/12/2009, Marseille, France.
- **Cools S**, 2009: "Is taking a biopsy a relevant technique to monitor bovine luteal function *in vivo*?", Workshop: Bovine corpus luteum function. Oral presentation. 13th conference of the European society for domestic animal reproduction, Ghent, Belgium. 09-12/09/2009.
- **Cools S**, Vanhaecke L, Heyerick A, Opsomer G, 2009: "Possible implications of feeding soybean meal on fertility and milk production of high yielding dairy cows in the early post partum period: preliminary results". Poster presentation, XIth international symposium on ruminant physiology, Clermont-Ferrand, France. 06-09/09/2009.

µ

UNIVERSITEIT GENT



ISBN nummer