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Induced short estrous cycles in cyclic dairy heifers and cows

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ACADEMIC DISSERTATION

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ABSTRACT

In the earlier studies of this research group, short estrous cycles were noted in a small number of heifers and cows when estrus and ovulation were induced with agonistic analogues of prostaglandin $F_{2\alpha}$ (PG) and gonadotropin-releasing hormone (GnRH) treatments administered to cyclic animals 24 h apart. This premature ovulation induced a shortened luteal phase in some animals, and the premature luteal regression was confirmed to be caused by premature, endogenous release of $PGF_{2\alpha}$, resembling the release during spontaneous luteal regression. Follicular size before and at ovulation, and the subsequent luteal size, were both unaffected by the treatment. Induction of ovulation 24 h after PG also significantly weakened estrous signs. Possible causes for such induced short estrous cycles in dairy cattle were further elucidated in the four experiments described in this thesis.

In Experiment I, estrus and ovulation were induced in heifers with PG and GnRH given 24 h apart during early (Day 7 after ovulation) or late (Day 14 after ovulation) diestrus, and the occurrence of induced short estrous cycles was compared between the groups. The preovulatory release of LH during the hour before and 6 h after GnRH administration and the basal release of LH on Days 1, 3 and 5 after ovulation were compared between the above-mentioned groups and an unmanipulated control group. Short estrous cycles occurred similarly when PG and GnRH were given either during early or late diestrus. The preovulatory and basal post-ovulatory release of LH on Days 1, 3 and 5 after ovulation were similar for early and late diestrus, and also for short and normal length estrous cycles. Lower basal LH concentration after ovulation coincided with higher progesterone concentration. The size of the preovulatory follicle during the three days before ovulation was significantly different for early and late diestrus, and also for short and normal length cycles three days and one day before ovulation ($P < 0.05$).

In Experiment II, the effect of gonadorelin doses of 0.1 mg or 0.5 mg given 24 h after PG during early diestrus on the occurrence of short estrous cycles, and on the preovulatory release of LH during 6 h following gonadorelin administration was investigated in cyclic dairy heifers. The dose of gonadorelin did not have a significant effect on the occurrence

of induced short estrous cycles. The preovulatory release of LH was similar irrespective of the gonadorelin dose, as was the size of the preovulatory follicle.

In Experiment III, the effect of the time interval between PG and GnRH (0 vs. 24 h) given during early diestrus (Day 7 after ovulation) on the occurrence of short estrous cycles was investigated in cyclic dairy heifers and cows. Short estrous cycles occurred more frequently after simultaneous administration of PG and GnRH in heifers, in comparison with administration 24 h apart ($P < 0.01$). The number of excluded cases due to unresponsiveness to GnRH appeared to be higher when the time interval between treatments was decreased. The size of the preovulatory follicle was similar in both groups.

In Experiment IV, the expression of endometrial receptors oxytocin, estrogen-α and progesterone and enzymes 20α-hydroxysteroid-dehydrogenase and cyclo-oxygenase-II, on Days 2 and 5 after ovulation was analyzed with real-time quantitative reverse transcriptase-polymerase chain reaction and immunohistochemistry. PG and GnRH were given 24 h apart to dairy cows during early diestrus (Day 8 after ovulation). Also peripheral blood estradiol-17β concentration was compared between induced short and normal length estrous cycles. No significant difference on Days 2 and 5 after ovulation in any of these receptors or enzymes were recorded between induced short and normal length cycles. The size of the preovulatory follicle was similar for short and normal length cycles, and was not related to the concentration of estradiol-17β.

According to the literature, events during follicular development and ovulation as well as during formation, support and regression of the CL could all lead to a shortened inter-estrous interval. The work reported in this thesis focused mainly on the events during the periovulatory period and also on the beginning of the luteal phase (until Day 5 post-ovulation). In summary, the occurrence of induced short estrous cycles was significantly increased with simultaneous administration of PG and GnRH, but was neither related to the size of the preovulatory follicle nor to the GnRH-induced preovulatory release of LH. Also the basal postovulatory release of LH on Days 1, 3 and 5 after ovulation was similar for induced short and normal length estrous cycles. The size of the preovulatory follicle was significantly larger when PG and GnRH were given 24 h apart during early diestrus in comparison with late diestrus, but the occurrence of short estrous cycles was similar for the groups. The size of the preovulatory follicle in cows did not correlate with the

preovulatory secretion of estradiol-17β. The endometrial expressions of receptors ER, OR and PR and enzymes 20α-HSD and COX-II were similar for short and normal length estrous cycles. The exact cause of induced short estrous cycles remains to be established. The results described should be taken into account in estrus synchronization protocols utilizing sequential treatments with PG and GnRH in order to avoid reduced fertility due to induced short estrous cycles.

ORIGINAL PUBLICATIONS

This thesis is based on the following original articles. In the text they will be referred to with Roman numerals, as below.

I Rantala M.H., Taponen J., 2015.
LH secretion around induced ovulation during early and late diestrus and its effect on the appearance of short estrous cycles in cyclic dairy heifers.
Theriogenology 83, 497-503.

II Rantala M.H., Peltoniemi O.A.T., Katila T., Taponen J., 2009a.
Effect of GnRH dose on occurrence of short estrous cycles and LH response in cyclic dairy heifers.
Reproduction in Domestic Animals 44, 647-652.

III Rantala M.H., Katila T., Taponen J., 2009b.
Effect of time interval between prostaglandin $F_{2\alpha}$ and GnRH treatments on occurrence of short estrous cycles in cyclic heifers and cows.
Theriogenology 71, 930-938. Erratum Theriogenology 72, 590.

IV Rantala M.H., Mutikainen M., Schuler G., Katila T., Taponen J., 2014.
Endometrial expression of progesterone, estrogen and oxytocin receptors and of 20α-hydroxysteroid-dehydrogenase and cyclo-oxygenase II two and five days after ovulation in induced short and normal estrous cycles in dairy cows.
Theriogenology 81, 1181-1188.

ABBREVIATIONS

ANOVA	analysis of variance
AUC	area under the curve
C	control
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CIDR	controlled internal drug releasing device
CL	corpus luteum or corpora lutea
COX-II	cyclooxygenase-II
CT	cycle threshold
CV	coefficient of variation
D or d	day(s)
DNA	deoxyribonucleic acid
E_2	estradiol-17β
ER	estrogen receptor
Fig.	figure
FSH	follicle stimulating hormone
G	group
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin releasing hormone or its agonistic analogues
GPG	estrus synchronization protocol, also termed Ovsynch
h	hour(s)
hCG	human chorionic gonadotropin
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IHC	immunohistochemistry
im	intramuscular
LH	luteinizing hormone
min	minute(s)

mRNA	messenger ribonucleic acid
n	number
NC	normal length estrous cycle
OR	oxytocin receptor
P	probability
P_4	progesterone
PCR	polymerase chain reaction
PG	agonistic analogues of prostaglandin $F_{2\alpha}$
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFM	prostaglandin $F_{2\alpha}$ metabolite
PR	progesterone receptor
QPCR	real-time quantitative reverse transcriptase-polymerase chain reaction
RGE	relative gene expression
RIA	radioimmunoassay
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SC	short estrous cycle
SD	standard deviation
T	treatment
X_g	geometric mean
20α-HSD	20α-hydroxysteroid dehydrogenase
$\Delta\Delta$CT	comparative cycle threshold

1. INTRODUCTION

Dairy cows are the most important production animals in Finland (Vuorisalo 2014). At the end of 2013 there were approximately 8800 dairy farms in Finland (Vuorisalo 2014) and the number of dairy cows at the beginning of May 2014 was approximately 285 250 (Luke 2015). In 2013, these cows produced 2260 million liters of milk (Vuorisalo 2014). The most common dairy breeds in Finland were Ayrshire (59%) and Holstein (39.5%). There are also some dairy cows of Finnish Landrace breeds (1.2%) and the Jersey breed (n=237) (Faba 2015b). The average milk production of Finnish Ayrshire, Holstein, Landrace and Jersey breeds in 2013 was 8644, 9518, 6117 and 7872 liters, respectively.

In 2013, data from almost all dairy farms (89.2%) were gathered in a national dairy disease register (Faba 2015a). The most common reason for treatments of those dairy herds was various fertility disorders (18.4% of all animals). The second and third most common health problems were mastitis and other udder-related illness (14.5%) and milk fever (3.4%). Thus, approximately every fifth dairy cow on those farms was treated for fertility problems. The number of cows treated for fertility disorders increases with increasing milk production: when the level of milk production is high (exceeds 10500 kg per cow per year), the percentage of treated animals is also high (28.8%), and when the level of milk production is low (less than 7500 kg per cow per year), the percentage of treated animals is lower (13.1%). In 2013, at an annual average milk production level of 8845 kg per cow, the percentage of cows treated for fertility disorders was 16.2%. The average calving interval on those farms was 420 days in 2013.

Fertility in modern, highly-productive dairy cows in comparison with heifers has decreased worldwide (Wiltbank et al. 2011). This is due to increased milk production and hormonal imbalances, mainly progesterone, estradiol, luteinizing hormone (LH), follicle stimulating hormone (FSH) and gonadotropin releasing hormone (GnRH) around estrus. This results in decreased estradiol-17β secretion around estrus, ovulation of large and aged follicles, and/or increased frequency of anovulation or double ovulation (Wiltbank et al. 2011). In practice, decreased fertility warrants use of hormonal estrus synchronization protocols to control follicular waves and luteal regression to achieve acceptable pregnancy rates. The goal is to shorten the time of follicular dominance and to increase the length of

proestrus, without unwanted side effects such as short estrous cycles (Wiltbank et al. 2011). Several protocols for estrus and ovulation synchronization have been developed for dairy and beef cattle. These have used agonistic analogues of prostaglandin $F_{2\alpha}$ (PG) and GnRH in different combinations and doses, after different time intervals and/or with other hormones (estradiol, progesterone, eCG or FSH) or with calf removal, and were recently reviewed by Wiltbank and Pursley (2014). The duration of the luteal phase is most commonly reduced with PG causing luteal regression and increased with progesterone extending follicular dominance, or with GnRH or estradiol which cause changes in follicular wave dynamics (Macmillan et al. 2003). The length of the follicular phase is most often reduced via induction of ovulation with GnRH or estradiol (Macmillan et al. 2003). The goal of these sequential hormonal treatments is to allow timed artificial insemination after synchronization of ovulation, i.e. to regulate the CL, ovarian follicles and the whole periovulatory hormonal milieu correctly (Wiltbank et al. 2011, Wiltbank and Pursley 2014).

Aberrations in these hormonal estrus synchronization regimes can lead to unwantedly reduced fertility, as reported by Peters and Pursley (2003). They investigated the optimal time interval (0, 12, 24 and 36 h) between PG and GnRH, and short estrous cycles occurred more frequently as the time interval between treatments decreased. However, after numerous studies, according to Wiltbank and Pursley (2014), further research is needed to make these estrus synchronization protocols more effective, simple, practical and synchronious. The early diestrus, i.e. first five to eight days after synchronization protocols remains a grey zone, and therefore research should also be focused on that period (Skarzynski et al. 2013). More specifically, reasons behind the decreased progesterone secretion occurring after synchronization treatments as well as the causes of refractoriness of the newly developed CL to exogenous PG should be analysed further (Skarzynski et al. 2013). In a recent study, Sahu et al. (2014) investigated the GnRH - $PGF_{2\alpha}$ - GnRH (GPG) estrus synchronization protocol in dairy heifers with or without exogenous progesterone, and concluded that the positive effects of external progesterone administration are not mediated via changes in follicular dynamics. Further studies were warranted, and short estrous cycles occurring after synchronization of estrus were mentioned as a point of focus (Sahu et al. 2014).

Physiological short estrous cycles are common in postpartum cows and in pre-pubertal heifers, and lead to low pregnancy rates if animals are bred to such cycles (Garverick and Smith 1986, Lishman and Inskeep 1991, Hunter 1991, Garverick et al. 1992). Short estrous cycles can be induced in cyclic cattle when PG and GnRH are given in sufficiently close succession (Stevens et al. 1993). Similarly to postpartum short cycles, induced short estrous cycles clearly are of low fertility, despite a progestagen phase preceding them. In a study by Pursley et al. (1994), the pregnancy rate after simultaneous PG and GnRH administration was only 9%, compared with 55% when GnRH was given 48 h after PG and 46 % when the time interval between treatments was 24 h.

Earlier possible reasons behind short cycles were speculated to be "1) lack of sufficient luteotropin, 2) failure of the luteal tissue to recognize a luteotropin, and 3) presence of a luteolytic agent" (Odde et al. 1980). According to Garverick and Smith (1986), short cycles could be due to disturbances during both follicular and luteal phases. Events during follicular development and ovulation as well as during formation, support and regression of the CL could all lead to a shortened inter-estrous interval (Garverick and Smith 1986). Copelin et al. (1987) thought that mechanisms might be inadequate luteotropic stimuli, a premature release of or increased sensitivity to a luteolysin, or both, increased sensitivity of the CL to $PGF_{2\alpha}$, and an increased or premature release of $PGF_{2\alpha}$. Currently, the most accepted of these seems to be the presence of a prematurely released luteolytic agent, $PGF_{2\alpha}$.

In sheep, Brown et al. (2014) investigated endocrine and ovarian receptor changes during male-induced, physiological short estrous cycles in anestrous females. Before ovulation, a moderate loss of steroid acute regulatory protein (STAR) gene expression on thecal cells was detected, and at or following ovulation, significant changes in expression of genes involved in progesterone synthesis (STAR, CYP11A1, HAD3B1) and vascular development (VEGFA, VEGFR2) took place. No changes in expression of these genes on granulosal cells were detected. No changes in PGFM or in receptors for $PGF_{2\alpha}$ were recorded for short and normal length cycles. On Day 3 after the male-effect, the variation in the expression of the genes investigated was large, but two subpopulations could be differentiated; they were assumed to represent normal (high expression) and short (low expression) length cycles. The inadequate degree of STAR expression on thecal cells was

assumed to cause short cycles via follicle dysfunction, and the authors questioned the role of $PGF_{2\alpha}$ in causing physiological short cycles in ewes (Brown et al. 2014).

2. REVIEW OF THE LITERATURE

2.1. Events during follicular and luteal phase of the bovine estrous cycle

Cattle are polyestrous, puberty occurring at the age of 6 to 12 months when animals weigh about 200 to 250 kg (reviewed by Forde et al. 2011). The length of the normal estrous cycle is 21 ± 3 days, consisting of two phases separated by ovulation: follicular (proestrus and estrus, 4 to 6 days) and luteal (metestrus and diestrus, 14 to 18 days) (Forde et al. 2011). Follicular development starts as a response to FSH secreted from the hypophysis after hypothalamic release of GnRH (reviewed by Driancourt 2001). This causes 5 to 10 small (1 to 3 mm), gonadotropin-sensitive follicles to start growing. As these follicles reach about 4 mm in diameter, they become gonadotropin-dependent. This is termed follicular recruitment or wave emergence, and lasts for about two days. As the cohort of follicles matures, they become able to synthesize estradiol-17β from androgens (aromatase activity), insulin-like growth factor (IGF), activin, follistatin and inhibin, and their size dominance changes to functional dominance. During follicular growth, follistatin binds activin, causing the activin/inhibin-balance to change in favour of inhibin, and FSH decreases the production of IGF binding proteins (IGFBP) leading to increased concentration of unbound IGF. Aromatase activity and estradiol-17β production are increased by IGF and FSH, and increased concentrations of inhibin and estradiol-17β decrease FSH concentration, thus ending the period of follicular recruitment. Normally only one follicle (8 mm) is the first to be able to synthesize LH receptors on its granulosa cells (Driancourt 2001). This first follicle continues to develop with the aid of LH in the changing hormonal environment and becomes the dominant follicle. The subordinate follicles enter atresia due to decreased FSH. The level of androgens produced in thecal cells increase due to LH, inhibin and IGF. During the dominance phase, the dominant follicle continues to grow, and both nuclear and cytoplasmic maturation are needed for an ovum to become fertilized (Driancourt 2001).

Each follicular wave lasts about 7 to 10 days, and if the general hormonal milieu allows, the dominant follicle ovulates as a response to changes in LH secretion pattern (reviewed by Diskin et al. 2002). If ovulation does not take place, the dominant follicle enters atresia, and a new follicular wave can grow (Diskin et al. 2002). Most commonly two or three

follicular waves occur during the estrous cycle: one ovulatory and one or two non-ovulatory (Savio et al. 1988). According to Sirois and Fortune (1988), three follicular waves usually occur during an estrous cycle. In three-wave-cycles, follicular waves start approximately on Days 2, 9 and 16 (Sirois and Fortune 1988), and the dominant follicle is of maximum size on average on Days 6, 16 and 21 (Savio et al. 1988), and in two-waves-cycles, on average, on Days 6 and 15 (Savio et al. 1988). The dominant follicle of the second follicular wave is significantly smaller than that of the first wave or the third wave during the same cycle (Sirois and Fortune 1988). The diameter of the preovulatory follicle becomes larger than the diameter of other follicles during the same cycle (Savio et al. 1988). The maximum size of the dominant follicle in the first, second or third follicular wave in heifers, according to Sirois and Fortune (1988), ranged between 12 and 13 mm, 8 and 11.5 mm or 12 and 14 mm, respectively. Savio et al. (1988) reported that the maximum size of the dominant follicle in heifers was approximately 15 mm in the first and the second wave, and approximately 19 mm in the third wave.

As the LH peak induces the dominant follicle to ovulate, a dynamic transition from follicular to luteal phase starts (metestrus, 3 to 4 days) and the secretion of estradiol-17β from the ovulatory follicle ceases (reviewed by Diaz et al. 2002). The basement membrane breaks, small (~ 17 µm) and large (~ 38 µm) luteal cells develop from follicular thecal and granulosal cells of the follicle, respectively, and the CL thus formed begins to produce progesterone. Large luteal cells are independent of LH and secrete 80% of progesterone, but also have receptors for estradiol-17β and $PGF_{2\alpha}$. Small luteal cells have more LH receptors and fewer receptors for estradiol-17β and $PGF_{2\alpha}$ than the large luteal cells. Synthesis of progesterone in these cells is mainly constitutive, continuous and autonomous without acute stimulatory control (Diaz et al. 2002). LH causes blood-derived circulating lipoproteins to be converted to progesterone via pregnenolone (Diaz et al. 2002). The important capacity of the corpus luteum to regress at the appropriate time makes the CL a transient endocrine gland. Around Day 7, the capacity for luteal regression is gained and luteal cells are able to produce and release more $PGF_{2\alpha}$ in response to a small amount of uterine $PGF_{2\alpha}$ (Diaz et al. 2002). This creates a positive, auto-amplifying feedback loop leading to both functional and structural regression of the CL. After an adequate amount of $PGF_{2\alpha}$ is reached, peripheral blood progesterone is decreased within 12 h (Diaz et al. 2002). A schematic representation of reproductive hormones secreted from the

hypothalamus, anterior pituitary, ovaries and uterus, and their possible interactions is given in Figure 2.1.A.

In summary, the 21 ± 3 day long estrous cycles in cows consists most usually of two to three follicular waves, each lasting approximately 7 to 10 days. The estrous cycle is divided into the follicular and luteal phases, separated by LH-induced ovulation. After ovulation, secretion of estradiol-17β ceases, the CL is formed and the secretion of progesterone begins. At luteal regression, $PGF_{2\alpha}$ causes structural and functional regression of the CL.

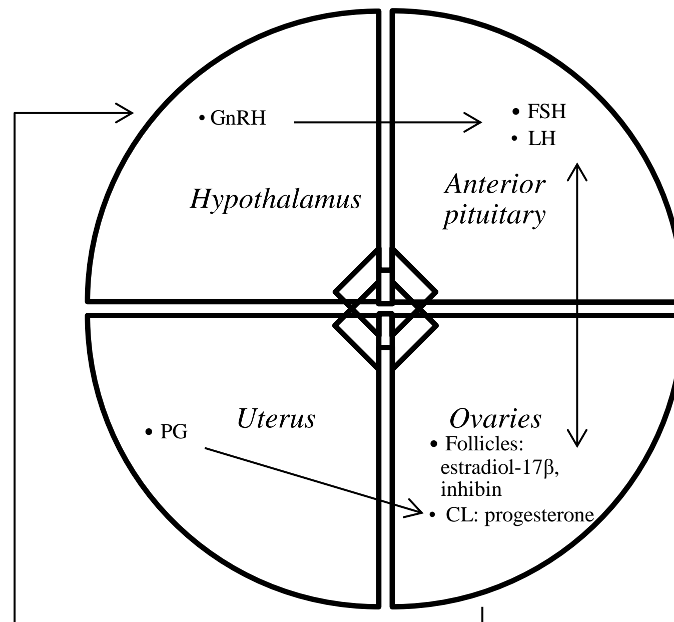


Figure 2.1.A. A schematic representation of reproductive hormones and their possible interactions - gonadotropin-releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol-17β, inhibin, progesterone and prostaglandin $F_{2\alpha}$ (PG) - secreted from the hypothalamus, anterior pituitary, ovaries and uterus.

2.2. Physiological short estrous cycles and their incidences

According to Odde et al. (1980), the most common length of physiological short estrous cycles is 8 days and the range is from 7 to 10 days. The incidence in their material of almost 3000 postpartum beef cows was 7% and 86% of short cycles were detected between the first and the second estrus postpartum. Hinshelwood et al. (1982) reported the incidence of short cycles (less than 17 days) in postpartum dairy cows to be 11%. The mean length of such short cycles was 11 days. Visual observation, rectal palpation and/or vasectomized bulls were used for heat detection in their study. According to Mackey et al. (2000), the length of postpartum short estrous cycles in suckling beef cows was 12.0 ± 1.5 days, and was almost unchanged if suckling was restricted to once daily (11.3 ± 1.2 days). Similarly, Stevens et al. (1993) reported the length of short cycles to vary between 7 to 13 days in diestrous dairy cows. In contrast, Schrick et al. (1993) reported a shorter luteal life span in postpartum beef cows exhibiting a short luteal phase, 6.9 ± 0.3 days. Standing estrus is less common during the follicular phase preceding these physiological short luteal phases in comparison with the follicular phase of the normal length cycles (Ramirez-Godinez et al. 1982b), and exogenous progesterone treatment significantly increases the number of beef cows exhibiting estrous signs at first estrus postpartum (Mackey et al. 2000). Cows inseminated or mated during the follicular phase of these short cycles do not conceive (Odde et al. 1980, Breuel et al. 1993).

Time from the ultrasonographical detection of a ≥ 5 mm follicle to ovulation in postpartum beef cows is significantly longer in normal cycles than in physiological short cycles (Schrick et al. 1993). Peaks of FSH and LH precede progesterone elevation during physiological short estrous cycles (Manns et al. 1983). The CL during these short luteal phases is morphologically normal, containing both large and small luteal cells (Manns et al. 1983). Fertilization, transportation from the oviduct to the uterus and early development of the embryo have been evaluated with the aid of embryo flushing (Ramirez-Godinez et al. 1982a, Breuel et al. 1993); both ovulation and fertilization are normal during the estrus preceding the short luteal phase (Ramirez-Godinez et al. 1982a, Breuel et al. 1993), and fertility at mating or insemination prior to the short luteal phase is not affected (Breuel et al. 1993, Schrick et al. 1993). The ova are normally transported from the oviduct to the uterus after fertilization, and also develop to the 4- or 8-cell stage similarly in untreated post-weaning beef cows exhibiting short cycles in comparison with norgestomet-treated

controls with normal length estrous cycles (Breuel et al. 1993). The rate of recovery, quality and developmental stage of embryos flushed from the uterus on Day 6 after estrus in postpartum beef cows does not differ between physiological short and normal length cycles (Schrick et al. 1993). Recovery rates of embryos in oviductal flushing (Day 3 after estrus) or uterine flushing (Day 6 after estrus) of beef cows are similar in post-weaning short estrous cycles and in norgestomet-treated controls (Breuel et al. 1993). In contrast, for normal, cyclic recipients Schrick et al. (1993) reported a tendency towards lower embryonic survival for donors exhibiting a short cycle than for donors inseminated in a normal cycle (23% vs. 47%, respectively, p = 0.08). The overall pregnancy rate of normal, cyclic recipients tended to be less for embryos from short cycle animals than for embryos from normal cycle animals (13% vs. 32%, respectively, p = 0.06), but statistical significance was not reached (Schrick et al. 1993).

Most studies concerning physiological short estrous cycles and their prevention have been done in beef cows and with exogenous progestagen supplementation prior to post-weaning estrus. In post-weaning beef cows the incidence of 8 to 12 day long physiological short cycles was 83% (Ramirez-Godinez et al. 1981). This incidence was reduced to zero when animals were pre-treated for nine days with norgestomet implanted at weaning (Ramirez-Godinez et al. 1981). If animals were implanted nine days before weaning, the incidence of short cycles was reduced to 30%, and also the conception rate increased from 0 to 33% (implant before weaning) or to 80% (implant at weaning). Troxel and Kesler (1984) used 0.25 mg of GnRH 24 h after progestagen implant for eight days in suckling, postpartum beef cows. This protocol induced ovulation in all cows, in comparison to significantly reduced ovulation rate in those given only GnRH (83%) or no treatment (0%). Animals given only GnRH had significantly more 8 to 12 days cycles than those treated with progestin and GnRH (80% vs. 33%, respectively). In a similar study by Schrick et al. (1993), the incidence of postpartum short cycles in beef cows was reduced from 74% in untreated controls to 21% in norgestomet-treated animals (implant for nine days, weaning seven days later). Also Breuel et al. (1993) noted the positive effect of exogenous progesterone as the pregnancy rate increased from nil (postweaning, untreated beef cow exhibiting short estrous cycle) to 50% (exogenous norgestomet for nine days, weaning seven days later, normal length cycles). Similarly, Mackey et al. (2000) significantly reduced the frequency of physiological short estrous cycles in postpartum beef cows with exogenous progesterone. Sheffel et al. (1982) reported that beef cows pre-treated with

norgestomet had a normal length cycle (19.6 d) compared with a short cycle length (13.4 d) in non-treated controls. Rutter et al. (1985) demonstrated a 53% incidence of short cycles when ovulation in beef cows was induced with 0.2 mg of GnRH 30 days postpartum. This incidence was effectively reduced with progesterone pre-treatment for four days prior to GnRH. Garverick and Smith (1986) reported similar resuts: the estrous cycle length was significantly shorter in untreated postpartum beef cows in comparison with norgestomet-treated ones. If daily peroral or injectable progestagen supplementation was started later (on Day 4 after mating or insemination), early luteal regression was not prevented (Breuel et al. 1993).

In conclusion, a preceding progestagen phase is essential in reducing the incidence of physiological, postpartum short cycles, and significantly improves fertility. Usually physiological short estrous cycles are exhibited between the first and the second ovulation postpartum. The length of physiological short cycles is less than two weeks. Signs of estrus are less visible, but ovulation, fertilization and transport of the ovum to the uterus occur normally. If animals are inseminated or mated during the follicular phase preceding these short luteal phases, fertility is nil.

2.3. Induced short estrous cycles and their incidences

Short estrous cycles also result when cyclic cattle are treated with PG and GnRH in sufficiently close succession (Stevens et al. 1993) when a preceding progestagen phase does not inhibit the occurrence of the short cycles. Schmitt et al. (1996) gave PG and 8 µg of buserelin 24 h apart to cyclic cows and heifers, and the incidence of induced short estrous cycles was about 35%. Cruz et al. (1997) reported short estrous cycles in 23% of cyclic, suckling beef cows treated with PG and 0.1 mg of gonadorelin 30 h apart - the incidence of short estrous cycles was lowest as the time interval between PG and GnRH administration was the longest. In a study of Taponen et al. (1999), a short estrous cycle of 9 to 10 days was recorded in 1/6 heifers and 1/3 cows that ovulated after PG and 0.1 mg of gonadorelin were given 24 h apart during early diestrus to cyclic animals. In subsequent similar studies of Taponen et al. (2002, 2003), the incidence was about 33% in cows (Taponen et al. 2002) and about 58% in heifers (Taponen et al. 2003). When the time interval between administration PG and 0.1 mg of gonadorelin is decreased from 2.2 to 1.2 days, the number of induced short estrous cycles in cyclic beef cows is increased from

12.5% to 50% (p = 0.1; Bridges et al. 2010). Similarly, when the time interval decreased from 2.25 days to 1.25 days, the proportion of short cycles significantly increased from 35% to 82% (Bridges et al. 2010). The effect of simultaneous treatment with PG and 0.1 mg of gonadorelin on Day 8 or Day 10 after standing estrus was noted by Stevens et al. (1993): all five cows that had an induced ovulation in 48 h after GnRH was given exhibited a short cycle (7 to 13 days). The authors concluded that such a hormonal protocol did not improve estrous synchrony compared with giving PG alone because it did not allow normal follicular development. When PG and 10 μg of buserelin were given to cyclic dairy cows either 40 h or 60 h apart, the percentages of animals without a functional CL on Day 7 after ovulation were 25% and 6%, respectively (Bollwein et al. 2010). In comparison, after spontaneous ovulation, 12% of animals had no functional CL on that day.

In a recent study by Núñez-Olivera et al. (2014), postpartum, anestrous beef cows (n = 46) received an intravaginal progesterone-releasing device combined with 2 mg of estradiol benzoate. At removal of the device eight days later, they were given 0.5 mg of estradiol cypionate and 0.5 mg of cloprostenol, and half of the animals also 400 IU of eCG. Ovulation rate was significantly increased with eCG, but despite pre-treatment with progesterone, eight short estrous cycles occurred (8/22; 36%). These short cycles were assumed to be caused by inadequate luteotropic support via decreased secretion of LH due to low body condition score. The length of these short cycles was either seven days (three cases after eCG and one case without eCG) or twelve days (three cases without eCG and one case after eCG). However, the authors did not specify whether the short luteal phases were physiological or induced, or both.

Similarly to physiological short estrous cycles, induced short luteal phases clearly decrease fertility if animals are bred to those cycles. The conception rate after simultaneous PG and GnRH administration was only 9% in comparison with 55% when GnRH was given 48 h after PG (Pursley et al. 1994). A significant reduction in pregnancy rates on Day 30 was also reported by Bridges et al. (2010): PG and 0.1 mg of gonadorelin given 1.25 days or 2.25 days apart resulted in pregnancy rates of 2.6% and 50.0%, respectively ($P < 0.01$). In another study with heifers, the pregnancy rates were 26% and 46% when GnRH was administered 24 or 48 h after PG, respectively (Schmitt et al. 1996). In comparison, the

pregnancy rate after artificial insemination at behavioural estrus in the same study was 48%. The reduced pregnancy rate was thought to be caused by short estrous cycles that were due to a GnRH-induced surge of LH prior to adequate development of the preovulatory follicle. When GnRH was given 24 or 48 h after PG, approximately 35% and 16% of heifers exhibited a shortened inter-estrus interval (≤ 16 days), respectively (Schmitt et al. 1996). Changing GnRH to hCG did not prevent the reduction in conception rate (Schmitt et al. 1996).

In conclusion, induced short estrous cycles are due to PG and GnRH given to cyclic animals in sufficiently close succession. The occurrence of these short luteal phases is increased when the time interval between PG and GnRH treatments is decreased. Induced short cycles are not prevented by a preceding progestagen phase. Fertility is also reduced when the time interval between PG and GnRH administration is decreased.

2.4. Hypothalamus-hypophysis-gonadal-axis

2.4.1. Gonadotropin releasing hormone

The hypothalamus synthesizes and releases gonadotropin releasing hormone, a decapeptide hormone GnRH. Its primary target organ is the pituitary, where, with high affinity, it binds to its receptors (GnRH-R) on the gonadotropic cell membranes, and releases LH and FSH. The amount of GnRH-R determines the effect of GnRH, and is regulated by GnRH itself, progesterone, estrogen, inhibin and activin (reviewed by Rispoli and Nett 2005). Normal pulsatory secretion of GnRH is necessary to maintain GnRH-R on the cell membranes. Continuous secretion of GnRH suppresses GnRH-R. During estrus (i.e. phase of low progesterone) the secretory pattern of GnRH changes allowing the up-regulation of GnRH-R. Thus, progesterone seems to be a very important regulator for GnRH-R. When progesterone level is high, the level of GnRH-R is down-regulated, and during periods of low progesterone, it is up-regulated, i.e. progesterone has a negative effect on the level of GnRH-R (Rispoli and Nett 2005). Estrogen and activin in turn increase, and inhibin decreases the level of GnRH-R up-regulation. The highest level of GnRH-R is reached just before ovulation, and is essential for the preovulatory release of LH (Rispoli and Nett 2005).

2.4.2. LH release in response to exogenous gonadotropin releasing hormone

Analysis and description of the LH secretion from the pituitary can be done using several parameters. The magnitude of secretion is described by the amplitude of the LH peak and/or with total secretion evaluated as the area under the LH curve (AUC). Other parameters used are the time interval between GnRH administration and LH peak and the duration of the LH surge. According to Mikél Jensen et al. (1983) and Chenault et al. (1990), AUC is the best tool to estimate the GnRH-induced LH release quantitatively. After PG and 0.1 mg of gonadorelin were given 48 h apart to dairy cows, the peak release of LH was reached 1.5 h later (Bas et al. 2014). After PG and 0.1 mg of gonadorelin administration 72 h apart, the duration of LH secretion in heifers was 4.0 to 6.8 h (Lucy and Stevenson 1986). Around luteolysis, a natural-like LH peak in heifers may be induced with as little as 5 µg of gonadorelin (Ginther and Beg 2012).

The individual variation in LH responses is considerable (Yamada et al. 2002). In many studies, and especially with higher GnRH doses, no dose effect of GnRH on LH secretion has been demonstrated (Kaltenbach et al. 1974, Fonseca et al. 1980, Wettemann et al. 1982, Yamada et al. 2002). Zolman et al. (1984) found a significant effect of gonadorelin dose on LH concentrations, but only with the lowest doses, when 0.005, 0.04 or 0.32 mg of gonadorelin was given during late diestrus and proestrus to dairy heifers. Schams et al. (1974) observed a linear dose dependency of the LH release from 0.0625 mg up to 1.5 mg of gonadorelin. The differences in responses between doses of 0.25 mg and 0.5 mg appeared non-significant. Some studies in cyclic or anestrous beef or dairy cows demonstrated that by using such GnRH levels as used in practice, or even lower, a positive linear effect on LH secretion was achieved (Echternkamp et al. 1978, Mikél Jensen et al. 1983, Chenault et al. 1990, Mee et al. 1993). Chenault et al. (1990) used various doses of GnRH products at their labelled dosages in nine replicates for each treatment: saline; 0.025, 0.05, 0.1 or 0.2 mg of fertirelin acetate; 0.1, 0.25 or 0.5 mg of gonadorelin; and 0.01 or 0.02 mg of buserelin, all given to dairy heifers during diestrus. A classical dose response, measured in terms of both peak concentration and AUC, was observed in LH concentration following injection of fertirelin acetate. However, the increase was not significantly different between doses of 0.1 mg and 0.2 mg. Gonadorelin and buserelin treatments gave similar dose responses, but results were not tested statistically. Mikél Jensen et al. (1983) studied the dose response for gonadorelin in dairy heifers treated at

five dose levels (0, 0.01, 0.05, 0.1, and 0.25 mg) and on five treatment days during proestrus and early diestrus. The LH response increased with increasing doses of gonadorelin, and the largest increase was recorded when the dose was raised from 0.05 to 0.1 mg. However, individual variation seemed to be wide, and one heifer did not respond to any of the doses. In addition, some evidence for the existence of an individually variable threshold dose was detected, which may explain the very inconsistent results obtained in different studies.

Moreover, the preparation of the GnRH product used affects the LH release, probably via differences in absorption and product qualities (Martinez et al. 2003). Souza et al. (2009) investigated the LH response after administration of 0.05 mg and 0.1 mg of four different gonadorelin products (Cystorelin®, Ovacyst®, Factrel® and Fertagyl®) given on Day 7 after the last GnRH of the Ovsynch programme to dairy cows. No difference in AUC, time to LH peak and LH peak concentration among the products was noticed, and the pooled data showed that doubling the dose of GnRH doubled the peak release of LH, and AUC was increased by about 80%. The ovulatory response after Factrel® given during the luteal phase was significantly less in comparison with the response to other products. Similarly, Martinez et al. (2003) compared 0.1 mg of three different gonadorelin formulations (Cystorelin®, Fertagyl® and Factrel®) administered intramuscularly to dairy cows and beef heifers on Day 6 or 7 after ovulation. Cystorelin® released significantly more LH than the other two products did. The mean and mean peak LH values increased as compared with the others, as did the ovulation rate in dairy cows, but not in beef heifers. Furthermore, Palasz et al. (1989) reported differences in LH release following a 0.1 mg dose of two different gonadorelin products (Cystorelin® and Factrel®). The LH peak values were not different, but the total secretion was increased in animals administered with Cystorelin® in comparison with Factrel®. In an estrus synchronization protocol, where PG was given seven days after the first GnRH, and followed 48 h later with another GnRH treatment, synchronization rate, double-ovulation rate, conception rate and pregnancy loss were similar for 0.05 and 0.1 mg doses of gonadorelin, but the cost of treatment was significantly altered (Fricke et al. 1998).

All the studies described above investigated effects of an intramuscular administration of gonadorelin. In a recent study, intrauterine administration of 0.2 mg of gonadorelin was compared with an intramuscular administration of 0.1 mg of gonadorelin in dairy cows

(Bas et al. 2014). Induction of ovulation in response to gonadorelin given 48 h after PG occurred in all animals after intramuscular injection (8/8), but not in every animal after intrauterine administration (6/9). In comparison, only two untreated control cows (2/8) ovulated within 48 h.

In conclusion, increasing the intramuscular dose of GnRH above 0.1 mg of gonadorelin in an attempt to release more LH during any stage of the estrous cycle does not increase the benefit of the GnRH treatment, but does increase the cost of the treatment. The release of LH after GnRH is best evaluated using AUC (area under the LH curve). The preparation of the GnRH product may affect LH release.

2.4.3. LH

Several reports have been published concerning the connection between LH and induced or physiological short estrous cycles, but in almost all of them no differences between short and normal length estrous cycles were reported. Mean peak serum LH levels and AUC before, during or after estrus were similar when comparing physiological short estrous cycles and subsequent normal estrous cycles (Ramirez-Godinez et al. 1982b). Differences in LH secretion pattern were not studied, but LH deficiency was concluded not to be a cause of physiological short estrous cycles. According to Garverick et al. (1988), inadequate LH secretion was not a cause of physiological short estrous cycles, as mean concentration, amplitude and duration of LH secretion were similar for physiological short cycles and normal length cycles. In a more recent study by Bridges et al. (2010), the mean LH secretion, AUC and LH peak concentration after 0.1 mg of gonadorelin given 2.25 days or 1.25 days after PG were similar for induced short and normal length cycles. The amount of follicular LH receptors on thecal and granulosal cells in postpartum beef cows anticipated to exhibit a short cycle was less than in animals having a normal length cycle (Braden et al. 1989). Inskeep et al. (1988) detected a significant increase in the amount of LH receptors both on thecal and granulosal cells after progestagen treatment of postpartum beef cows in comparison with untreated controls exhibiting physiological short estrous cycles. In contrast to those studies, Rutter et al. (1985) reported that changes in the concentration of LH receptors in the CL or in large/small luteal cell ratio do not cause postpartum short estrous cycles.

Regarding attempts to decrease the incidence of physiological short estrous cycles, several reports have been published on LH secretion following administration of different combinations of progestagens, GnRH and/or PG treatments. In beef cows during post-weaning period, pre-treatment with norgestomet increased the mean secretion of LH and the frequency of LH pulses in comparison with non-treated cows, which mainly exhibited physiological short cycles (Garcia-Winder 1986). Cruz et al. (1997) gave PG and 0.1 mg of gonadorelin 30 h apart to investigate the LH secretion in both cyclic and anestrous postpartum suckling beef cows. Short cycles were more common in anestrous cows at resumption of ovarian cyclicity (85%) than in cyclic (23%) cows, and cows with a short luteal phase had a significantly lower peak amplitude (98.0 vs. 142.5 ng/ml) and smaller AUC (19.0 vs. 28.8) during 4 h in comparison with animals with normal length cycles. Troxel and Kesler (1984) used 0.25 mg of GnRH 24 h after progestagen treatment for eight days in suckled, postpartum beef cows, or only 0.25 mg of GnRH. As a result, the total secretion of LH and LH peak concentration were both significantly less for the GnRH group in comparison with the progestagen + GnRH treated group. In non-treated controls LH remained low during the experimental period (Troxel and Kesler 1984). Similarly, the number of LH pulses released did not decrease with exogenous progesterone given for six days to postpartum beef cows exposed to restricted suckling once a day (Mackey et al. 2000).

In conclusion, preovulatory release of LH did not differ between induced or physiological short and normal length estrous cycles.

2.4.4. Basal LH secretion

Basal LH secretion during bovine estrous cycles seems to vary among different studies, possibly due to differences in experimental settings, LH analysis methods and/or LH pulsatility detection methods (Swanson and Hafs 1971, Zolman et al. 1974, Schallenberger et al. 1984, Peters et al. 1994, Cupp et al. 1995, Ginther et al. 1998 and Hannan et al. 2010). In experiments of Ginther et al. (1998) and Hannan et al. (2010) mean basal LH secretion (approximately 0.3 ng/ml) was much less than in the earlier studies mentioned above. Ginther et al. (1998) investigated early luteal phase and Hannan et al. (2010) the entire estrous cycle from one ovulation to another. Significant variation in the baseline LH values (between 0.9 and 2.0 ng/ml) among animals has been reported (Swanson and Hafs

1971). Although basal LH secretion was not affected by the stage of the luteal phase, the number of LH peaks or pulses was. During early luteal phase, fewer LH peaks occurred in comparison with the phase of luteal regression and proestrus, and during mid and late luteal phases even fewer LH peaks occurred in comparison with during early luteal phase (Schallenberger et al. 1985). The number of LH peaks during early luteal phase was reported to be 7 per 12 h (Schallenberger et al. 1985), 13 per 24 h on Day 2 after behavioural estrus (Peters et al. 1994), and 4.9 per 12 h on Day 5 after behavioural estrus (Cupp et al. 1995). Another parameter used for LH peak analysis is the mean inter-pulse or inter-peak interval (Schallenberger et al. 1984, Walters and Schallenberger 1984). Additional discrepancies in results might be caused by diurnal variation in basal LH secretion reported in some studies (Swanson and Hafs 1971, Hannan et al. 2010). Swanson and Hafs (1971) reported higher basal LH values in the morning and in the afternoon and Hannan et al. (2010) in the morning on Days 5 to 9 after ovulation, but not on Days 10 to 14.

In conclusion, the number of LH peaks or pulses is affected by the stage of the luteal phase. In addition to differences in LH analysis methods, experimental settings and LH peak detection methods, basal LH secretion varied diurnally in some studies.

2.4.5. FSH

Garverick et al. (1988) and Schrick et al. (1993), for beef cattle, reported no difference in FSH secretion for physiological short estrous cycles and norgestomet-treated normal length cycles. Similarly, Garcia-Winder et al. (1986) report no difference in FSH concentration in postpartum beef cows treated or not treated with norgestomet. They concluded that a threshold secretion of FSH is needed for follicular development. Only according to Ramirez-Godinez et al. (1982b) were serum FSH levels for four days before ovulation lower in physiological short cycles than in the subsequent, second postpartum cycle. The authors suggested further studies to establish whether FSH has a role in initiating physiological short estrous cycles. The amount of FSH receptors on granulosal cells was similar for physiological short estrous cycles and norgestomet-treated controls (Inskeep et al. 1988). In conclusion, there is no evidence that changes in FSH secretion cause physiological short cycles.

2.5. Uterus and short estrous cycles

2.5.1. Endometrial expression of progesterone, estrogen-α and oxytocin receptors and cyclo-oxygenase-II

Endometrial hormone receptors can be investigated in several ways. Before the advent of the polymerase chain reaction (PCR), methods included were radio-receptor analysis of tissue homogenates (Zelinski et al. 1982, Zollers et al. 1993, Mann and Lamming 1994, Leung and Wathes 2000) and immunohistochemistry (IHC) of tissue slices (Boos et al. 1996, Boos 1998, Dall`Aglio et al. 1999, Kimmins and MacLaren 2001). Samples for analysis were collected at slaughter, or taken as biopsies from live animals. Particularly when repeated samples are needed, transcervical biopsy is a good option; even repeated biopsies taken at the end of the estrous cycle do not shorten the luteal phase via premature release of $PGF_{2\alpha}$ (Mann and Lamming 1994).

Steroid receptors are nuclear receptors, and their action is mediated via slow-acting (i.e. hours) genomic responses, but fast-acting (i.e. seconds or minutes) non-genomic responses have been suggested to exist (reviewed by Stormshak and Bishop 2008). Estrogen receptor (ER) is present in two different forms, ERα and ERβ, which differ in their DNA binding affinity (Stormshak and Bishop 2008). They are found in different tissues and cells (Stormshak and Bishop 2008). Also progesterone receptor (PR) occurs in two different forms, PR-A and PR-B, in different tissues and in different ratios: in cattle, PR-A is dominant in the ovarian and uterine tissue and PR-B in the mammary gland tissue (Stormshak and Bishop 2008). In contrast, only one oxytocin receptor (OR) gene has been found (reviewed by Ivell et al. 2000). Before puberty, the expression of uterine OR is constitutive, low to moderate, and OR also exists in the foetal uterus during the late third trimester prior to birth, if not earlier (Fuchs et al. 1998). Before puberty there is no circulating progesterone present, but small concentrations of estrogen (2 to 3 pg/ml) occur, allowing the expression of OR (Fuchs et al. 1998). In these pre-pubertal animals the endometrium was though not capable to release $PGF_{2\alpha}$ in response to oxytocin, most probably due to lack of COX-II expression (Fuchs et al. 1988). At puberty OR was suppressed due to the changing hormonal milieu (Fuchs et al. 1998). In ovariectomized cows without effects of steroids, OR concentration was relatively high, and unable to release $PGF_{2\alpha}$ as a response to oxytocin (Mann et al. 2001). Similarly, *in vitro* OR was

spontaneously up-regulated in the absence of hormonal stimulus, suggesting that *in vivo* regulation of OR is mainly inhibitory (Leung and Wathes 2000).

2.5.1.1. Progesterone, estrogen and their endometrial receptors

The uterus is a target organ for the ovarian secretion of progesterone and estrogen, and many studies have been conducted to investigate the cyclical relationship and uterine dependency on steroids. In ovariectomized cattle, the expression of ER and PR was constitutive, and treatment with exogenous estrogen or progesterone respectively increased and decreased the expression of their own receptors (Kimmins and MacLaren 2001). Boos et al. (1996), Dall`Aglio et al. (1999) and Robinson et al. (2001) among others, reported that the follicular phase (estrogen) promoted endometrial PR and ER synthesis, and luteal phase (progesterone) down-regulated them. Also in the oviduct the progesterone phase inhibited both ER and PR (Valle et al. 2007). On the other hand, during superovulation treatment the concentration of blood estrogen and progesterone were clearly higher than during a natural estrous cycle, but no effect on the expression of PR and ER in oviducts of heifers was noticed (Valle et al. 2007). Both endometrial ER and PR were maximally present during or immediately after estrus, and concentrations declined during the subsequent luteal phase (Kimmins and Maclaren 2001). ER concentration was maximal at metestrus, i.e. Days 1 to 3 after ovulation, and was down-regulated between Days 7 to 17 after ovulation (Kimmins and MacLaren 2001). Concentration of PR was maximal during metestrus and early diestrus, i.e. Days 1 to 6 after ovulation, and down-regulated as the diestrus proceeded (Kimmins and MacLaren 2001). Moreover, Okumu et al. (2010) reported significantly higher ERα and PR expression on Days 5 and 7 after estrus in comparison with Days 13 and 16.

In addition to the above-mentioned time-specific changes, ER and PR undergo spatial changes in the bovine uterus, which can further complicate the interpretation of results. ER was expressed in all layers of the endometrium during estrus, in deep glands during the whole estrous cycle, and in increased amounts in the luminal epithelium during mid-luteal phase (Robinson et al. 2001). PR was expressed mostly in the stroma, and the expression was maximal during estrus and early luteal phase (Robinson et al. 2001). According to Boos et al. (1996), surface epithelial cells exhibited at least low staining for ER through the whole cycle, but increased intensity was recorded between Days 8 and 15 after

behavioural estrus (= Day 1). For glands and stroma, maximal staining intensity for ER occurred at estrus, and minimal staining on Day 15. At estrus PR was still low on the endometrial surface and in glandular epithelium, reaching its maximum on Day 8 after behavioural estrus (Day 1), and starting to decline subsequently (Boos et al. 1996). Similar results were obtained by Meikle et al. (2001): maximal mRNA results for ER and PR occurred around estrus, and both started to decrease on Day 5 after the standing estrus. According to a review by Robinson et al. (2008), this timing of maximal expression of PR in the endometrium might be related to the relationship between adequate amounts of progesterone secretion and embryonic development at the early luteal phase. In early embryonic deaths of cattle, inadequate progesterone secretion changed endometrial hormone receptor concentrations, and thus indirectly affected the uterine secretion of embryo-supporting histotroph, embryo development and maternal recognition of pregnancy (Lonergan 2011). During late diestrus an up-regulation of ER in the endometrial surface or glandular epithelial cells was noted (Boos et al. 1996). This was thought to be important for the initiation of luteal regression via the OR because almost no PR in the endometrial surface or glandular epithelial cells was present on Days 15 and 19 after behavioural estrus (Day 1) in non-pregnant cows, but the amount of ER was highest between Days 8 and 15 (Boos et al. 1996).

In pregnant animals on Day 16 after ovulation, ER was present in shallow endometrial glands, and absent in non-pregnant animals on that day, which was suggested to be associated with interferon-τ (INF-τ) induced support for the embryo via effects on histotroph secretion (Kimmins and MacLaren 2001). In another study on Day 16 there was no difference between pregnant and cyclic cows: ER was equally present in luminal epithelium and glands (Robinson et al. 1999). According to Okumu et al. (2010), ERα was detected in all layers of endometrium, myometrium and stroma. ERα was unaffected by the pregnancy status, but ERβ was up-regulated between Days 5 and 7, and remained high until Day 16 (Okumu et al. 2010). Exogenous progesterone significantly shortened this up-regulation (high until Day 13). The expression of PR in luminal epithelium and superficial glands decreased to a low level between Days 7 and 13 both in pregnant and non-pregnant heifers, and was still low on Day 16 (Okumu et al. 2010). This decrease in expression was significantly more pronounced in pregnant heifers than in cyclic ones, and was hastened by exogenous progestagen. According to Robinson et al. (1999), PR was equally present in the endometrial stroma of non-pregnant and pregnant cows on Day 16. In contrast, when

PG and GnRH were given either 60 h or 36 h apart to early diestrous beef cows, on Day 15.5 after treatment with GnRH (= Day 0) a reduction in the staining intensity of endometrial PR in the deep glandular epithelium was detected (Bridges et al. 2012). On Day 15.5, the amount of IFN-τ from embryos transferred on Day 7 was similar between groups, as was also the mRNA concentration of OR and PR (Bridges et al. 2012).

In conclusion, the cyclical expression of endometrial ER and PR is cell-specific and temporal, regulated by progesterone and estrogen, and possibly connected to OR up-regulation at luteal regression. During the early luteal phase, i.e. prior to Day 16, the uterus seems to prepare for pregnancy. The actual differentiation between non-pregnancy and pregnancy takes place subsequently.

2.5.1.2. Up-regulation of endometrial oxytocin receptor

In early pregnant cattle at the time of maternal recognition of pregnancy, luteal regression needs to be prevented via release of foetal IFN-τ, which has a direct suppressive effect on the translation of OR and ER (Robinson et al. 2001). Endometrial OR was clearly down-regulated during the first week after ovulation (Ivell et al. 2000), both in luminal epithelium and superficial glands, and stayed low, probably due to progesterone acting directly on PR and indirectly on OR (Robinson et al. 2001). According to Mann et al. (2001), only progesterone, not estrogen, was needed in ovariectomized cows to induce oxytocin responsiveness, i.e. release of $PGF_{2\alpha}$ measured as PGFM. Without release of IFN-τ, endometrial OR concentration in dairy cows started to rise from low concentrations prior to first $PGF_{2\alpha}$ release, to reach five-fold peak concentration only after luteal regression was complete (Mann and Lamming 2006).

The exact factors behind OR up-regulation are still not completely understood. Robinson et al. (2001) proposed two possible causes. First, PR during the luteal phase lost its dominance via down-regulation due to progesterone acting on its receptors (Robinson et al. 2001). Exogenous progesterone supplementation during early luteal phase hastened the down-regulation of PR in superficial glands and luminal epithelium (Okumu et al. 2010). Inhibition of OR by progesterone (progesterone block) was suggested to occur in sheep by McCracken et al. (1984). The down-regulation of PR was not a sufficient stimulus alone, and secondly, but less probably according to Robinson et al. (2001), estrogen might have

acted via ER to up-regulate OR. In the luminal epithelium ER significantly increased in non-pregnant cows on Days 16 to 18 after estrus (Robinson et al. 2001) or on Days 14 or 16 after ovulation depending on the estrous cycle length (Kimmins and MacLaren 2001). In non-pregnant cows ER were not present on Day 16 (Robinson et al. 1999) or Day 18 after estrus (Kimmins and MacLaren 2001, Robinson et al. 2001).

On the other hand, according to Robinson et al. (2001), the initial up-regulation of OR on the luminal epithelium on Days 16 to 17 (in some animals as early as on Day 14) was not preceded by changes in ER or PR expression, and the up-regulation of ER between Days 16 and 18 in non-pregnant cows occurred after up-regulation of OR. There was no difference in ER or PR between pregnant and non-pregnant cows on Day 16, and inhibition of OR up-regulation in pregnant animals was probably unrelated to the expression of ER or to the maintenance of PR (Robinson et al. 1999). The item of evidence against the role of ER in priming the luteal regression was that in cattle the OR promoter region has an interferon response element (IRE), but no estrogen response element (ERE), suggesting that estrogen may have used steroid receptor cofactors (SRC), such as SRC1e, and ERE half sites to achieve the estrogenic effect on OR (Telgmann et al. 2003).

Several other theories for causes of OR up-regulation have been suggested. Leung and Wathes (2000) concluded that both positive and negative modulators for OR expression exist, but the primary regulator was not estrogen, which if present, could speed up the up-regulation via ER. Leung and Wathes (2000) also concluded, “local factors from the endometrium are required to regulate oxytocin receptor expression in the endometrium via interaction with the oestrogen receptor”. In a review by Stormshak and Bishop (2008) it was assumed that estrogen up-regulated and progesterone down-regulated the uterine OR, but the overall situation might have been more complex. Goff (2004) stated that ovarian steroids were needed for luteal regression, but their role was still somewhat unclear. A review by Ivell et al. (2000) concluded that steroids have not been proved to have any direct effect on the OR gene or OR protein, but progesterone seemed to have an indirect, paracrine and inhibitory effect on the OR gene. Another review suggested that the effect of estrogen is modulatory, and progesterone has a direct effect (Oruda et al. 2002). This effect of progesterone on oxytocin responsiveness could be mediated via post-receptor signalling pathways and/or enzymes involved in the prostaglandin synthesis (Mann et al. 2001).

Morover, during short estrous cycles, the premature loss of endometrial progesterone dominance and/or increased concentration of endometrial OR were assumed to cause premature $PGF_{2\alpha}$ release (Zollers et al. 1993). On Day 5 after ovulation, endometrial PR concentration in short cycle animals was significantly less than in normal cycle animals, and endometrial OR concentration in short cycle animals was significantly higher than in normal cycle animals (Zollers et al. 1993). Estrogen levels prior to ovulation were assumed to determine the length of the subsequent progesterone dominance, i.e. luteal phase, through altered levels of PR expression (Zollers et al. 1993). At the time of luteal regression during short cycles, high concentrations of endometrial OR were present, and peaks of oxytocin and PGFM coincided, allowing the luteal regression to be initiated (Hunter 1991).

In conclusion, for up-regulation of OR, the suppressive role of progesterone and PR is acknowledged, but the roles of estrogen and ER remain unclear. Ovarian steroids are needed and both positive and negative modulators of OR up-regulation are assumed to exist. Differences in results of all studies using IHC, radioactive competitive binding assay, and/or QPCR when analysing endometrial receptors, may be due to different sensitivities of the methods, or existing or lack of true differences between induced and physiological short estrous cycles.

2.5.2. Prostaglandin $F_{2\alpha}$

In the initiation of luteal regression, the most important event is the up-regulation of OR, which in turn allows the endogenous release of $PGF_{2\alpha}$ (mini-review by Goff 2004). Arachidonic acid, derived from cell membrane phospholipids, is enzymatically converted to prostaglandins. First prostaglandin G/H synthase (PGHS), known as cyclooxygenase (COX), produces PGH_2. Two different COX enzymes exist: COX-1 (PGHS1, constitutive) and COX-2 (PGHS2, inducible), and PGH_2 is further converted to PGE_2 or $PGF_{2\alpha}$ (Goff 2004). It seems that most prostaglandin synthesis in the bovine endometrium is mediated by COX-II (Parent et al. 2003) because no expression of mRNA for COX-I was present during the bovine estrous cycle (Arosh et al. 2002).

COX-II was expressed at low and high levels between Days 1 to 12 and 13 to 21, respectively (Arosh et al. 2002). To produce $PGF_{2\alpha}$, PGH_2 is further converted to

prostanoids by three possible synthase-enzymes: PGD 11-ketoreductase (from PGD_2), PGH 9-11-endoperoxidase (from PGH_2) and PGE 9-ketoreductase (from PGE_2). The latter is identical to 20α-HSD, which belongs to an aldo-ketoreductase family (AKR1C; Goff 2004). Also another aldose-reductase enzyme, AKR1B5, was strongly expressed in the bovine endometrium at the time of luteal regression (Madore et al. 2003). Its peak expression occurred around peak progesterone concentrations, i.e. Days 12 to 18 (Madore et al. 2003). AKR1B5 synthesized $PGF_{2\alpha}$ from PGH_2, and locally lowered progesterone concentrations via degradation (Madore et al. 2003). PGE-synthase (PGES) produced PGE_2, and in comparison with $PGF_{2\alpha}$, showed a different endometrial expression pattern: Days 1 to 3 moderate, Days 4 to 12 low and Days 13 to 21 high (Arosh et al. 2002). During the high expression period PGES was significantly correlated with COX-II mRNA expression (Arosh et al. 2002). During luteal regression the pulsatile release pattern of $PGF_{2\alpha}$ made the CL sensitive to $PGF_{2\alpha}$, and prevented desensitization (reviewed by Okuda et al. 2002). In pregnant animals the basal levels of $PGF_{2\alpha}$ were higher than in non-pregnant ones. The maximum concentration of $PGF_{2\alpha}$ occurred for 2 to 3 days during luteal regression and after it, i.e. during follicular phase and estrus (Okuda et al. 2002). In six dairy cows during a 10 h sampling period, 2.2 ± 0.5 episodes of $PGF_{2\alpha}$ were released on average prior to luteal regression, and each episode took 4.0 ± 0.4 h on average (Mann and Lamming 2006).

Another theory concerning the connection between the endometrium and $PGF_{2\alpha}$ or PGE_2 exists: during the luteal phase, i.e. during high progesterone, prostaglandins significantly reduce the arterial blood flow to the endometrium, leading to local hypoxia and remodelling of the endometrium (Krzymowski and Stefánczyk-Krzymowska 2008). The increased mass of the uterus during the luteal phase is due to water retention via increased oncotic pressure and increased albumin retention. Albumin may bind $PGF_{2\alpha}$ and its metabolites. Remodelling releases $PGF_{2\alpha}$ from endometrial cells to the lymphatics. $PGF_{2\alpha}$ is further transferred via a retrograde countercurrent system from the venous blood to the arterial blood, and in the ovary $PGF_{2\alpha}$ may induce luteal regression. During early luteal phase this system is supposed to prevent premature luteal regression and to protect early pregnancy. Pulsatile elevations of $PGF_{2\alpha}$ measured in the peripheral blood are only a reflection of remodelling events occurring in the endometrium, and only very small amounts of $PGF_{2\alpha}$ are needed for luteal regression. When estrogen levels are high PGE_2 is secreted and arteries are relaxed. Oxytocin pulses secreted by the ovary or the

hypothalamus induce varying amounts of uterine contractility depending on the amount of endometrial OR, and the contractions put pressure on uterine tissues. Increased pressure then causes blood and lymph to flow more (Krzymowski and Stefánczyk-Krzymowska 2008).

Copelin et al. (1987) proved that an intact uterus was needed for $PGF_{2α}$-induced luteal regression, and short estrous cycles could be prevented with hysterectomy. As a response to oxytocin injection, $PGF_{2α}$ was released as early as on Day 5 in postpartum beef animals expected to have a short cycle, but not in animals having a normal length cycle (Zollers et al. 1989). *In vitro* on Day 5, $PGF_{2α}$ was secreted from endometrium in animals expected to have short estrous cycles (Zollers et al. 1991). The CL during short cycles was not more sensitive to $PGF_{2α}$ than during normal length cycles (Copelin et al. 1986, Garverick et al. 1988). Basal serum oxytocin and PGFM concentrations were significantly elevated throughout the cycle in postpartum animals having cycle lengths less than 17 days in comparison with animals with normal length cycles (Peter et al. 1989). The release of oxytocin and PGFM were related (Peter et al. 1989). Embryo flushing medium on Day 6 in short cycle beef animals contained significantly more $PGF_{2α}$ in comparison to normal length cycles (Schrick et al. 1993), and this difference tended to be correlated with embryo quality on that day. The $PGF_{2α}$ secretion on Day 5 in postpartum short cycle animals was similar to secretion during luteal regression at the end of normal length cycles on Day 16 (Zollers et al. 1989). Taponen et al. (2003) showed that luteal regression in dairy heifers during induced short estrous cycles was caused by a premature release of $PGF_{2α}$, which resembled the release during normal, spontaneous luteal regression.

In conclusion, endometrial up-regulation of OR is followed by release of $PGF_{2α}$ and leads to luteal regression. According to another theory, this release of $PGF_{2α}$ may only be a reflection of endometrial remodelling events. The release of $PGF_{2α}$ prior to luteal regression occurs both during physiological and induced short estrous cycles.

2.6. Estradiol concentration in peripheral blood

The estradiol secretion at estrus in postpartum short estrous cycles was significantly less than in progesterone-treated controls (Garcia-Winder et al. 1986, Garverick et al. 1988). In beef cows, estradiol secretion significantly increased three days before estrus in

postpartum normally cyclic animals in comparison with animals exhibiting short cycles (Schrick et al. 1993). The number of cows in standing estrus was increased with norgestomet treatment (Sheffel et al. 1982). In postpartum beef cows anticipated to exhibit a short cycle, the estradiol concentration of the follicular fluid was four times lower than in animals having a normal length cycle (Braden et al. 1989). The significantly increased concentration of estradiol in follicular fluid of norgestomet-treated beef cows in comparison with untreated animals exhibiting physiological short estrous cycles was reported by Inskeep et al. (1988). In a study with beef cattle by Bridges et al. (2010) the peak concentration of estradiol during proestrus was significantly lower in animals having a shorter interval between PG and GnRH treatments (1.2 d vs. 2.2 d), as was also the estradiol concentration around ovulation (Days -1.9 to 0). The peak ovulatory concentration of estradiol in most short cycle cases (4/5) was less than 10 pg/ml, and if the concentration of estradiol was over 10 pg/ml, most cows (10/11) had a cycle of normal length (Bridges et al. 2010).

On the other hand, the size of the preovulatory follicle, above or below 10 mm, at induced luteal regression did not lead to differences in estrogen secretion, because follicles were allowed to grow and ovulate spontaneously (Robinson et al. 2005). In another study, the increasing follicular size was associated with increasing blood estradiol concentration (Atkins et al. 2008). Secretion of estradiol could be an important determinant of physiological maturation of follicles and initiation of estrus, but the absolute diameter of the follicle or the magnitude of GnRH-induced LH secretion was thought to be less important (Atkins et al. 2008). Mann and Lamming (2000) postulated that low preovulatory levels of estradiol could be the cause of postpartum short estrous cycles via impaired OR inhibition. Thus premature luteal regression during postpartum short cycles was not due to lack of progesterone priming. Endometrial OR levels could be decreased with exogenous estradiol in the absence of progesterone, and the degree of OR expression was related to the amount of estradiol secreted during estrus (Mann and Lamming 2000).

In conclusion, the amount of estradiol secreted is not related to the size of the preovulatory follicle if follicles can ovulate spontaneously. When ovulation is induced with GnRH, decreasing the length of proestrus also decreases the secretion of estradiol around estrus. Secretion of estradiol is also decreased during physiological short cycles, and may be increased with exogenous progesterone.

2.7. Ovulation induction with gonadotropin releasing hormone and the size of the ovulatory follicle

During Ovsynch or GPG protocol, two doses of GnRH are separated by a single administration of PG 6 to 7 days after the first GnRH injection (Wolfenson et al. 1994, Twagiramungu et al. 1995). The first GnRH treatment caused follicular ovulation or atresia, thus allowing growth of a new follicular wave within two days. The GnRH treatment may be repeated 24 h (heifers) or 48 h (cows) after PG administration to induce ovulation of the dominant follicle in 24 to 32 h (Pursley et al. 1994). According to Silcox et al. (1995), a combination of PG and 0.1 mg of gonadorelin given 48 h apart was ineffective because ovulation in heifers was induced too late (30 to 31 h after GnRH), similarly to controls treated with saline (30 ± 6 h). This time interval in cows did not affect fertility as ovulation was induced earlier, i.e. 26 ± 1 h after GnRH (Silcox et al. 1995). In a study by Martinez et al. (2003), dairy cows ovulated 35.0 ± 2.5 h after 0.1 mg of gonadorelin, i.e. somewhat later.

The maximal diameter of the ovulating follicle is influenced by the stage of the estrous cycle when synchronizing estrus with GPG (Vasconcelos et al. 1999). During mid-cycle (Days 5 to 13) ovulating follicles were smallest. Also the time interval between PG and GnRH affected the size of the ovulatory follicle: ovulatory follicles were smaller on the day of GnRH administration in dairy cows treated simultaneously with PG and GnRH, in comparison with animals treated 24 h apart (Peters and Pursley 2003). The size of the ovulatory follicle was reduced 30 % when the time interval between PG and 10 µg of buserelin was 40 h rather than of 60 h (Bollwein et al. 2010). In contrast, no difference was detected in the ovulatory follicular diameter in beef cattle when the time interval between administration of PG and 0.1 mg of gonadorelin was decreased from 2.2 d to 1.2 d or from 2.25 d to 1.25 d (Bridges et al. 2010).

Follicle size at spontaneous ovulation had no effect on fertility, but small follicles induced to ovulate with GnRH reduced blood estradiol on insemination day and decreased the rise in and concentration of blood progesterone (Perry et al. 2005). This led to decreased pregnancy rate and increased embryonic mortality (Perry et al. 2005). A modified Ovsynch programme, where the second GnRH was given 40 h after PG, diminished the size of the preovulatory follicles compared with the case for spontaneously ovulating dairy cows

(Bollwein et al. 2010). The shortened preovulatory phase could exert a negative effect on fertility via a decreased ovulatory follicle size and inadequate follicle development, and also decreased luteal blood flow (Bollwein et al. 2010). However, the size of the ovulating follicle was not correlated with follicular blood flow (Bollwein et al. 2010). Bridges et al. (2010) suggested that follicular characteristics other than size at ovulation were more likely to explain the decreased fertility induced by a shortened preovulatory period. They thought the most likely cause to be the altered preovulatory concentrations of estradiol and progesterone due to a shortened proestrus phase.

In conclusion, the size of the preovulatory follicle is affected by the stage of the estrous cycle when inducing luteal regression and ovulation with PG and GnRH. Also decreasing the time interval between PG and GnRH administration has an effect on the size of the preovulatory follicle, and may also have negative effects on fertility, possibly via changes in estradiol and progesterone concentrations around estrus.

2.8. Peripheral blood progesterone concentration and corpus luteum

Lucy and Stevenson (1986) investigated serum progesterone concentrations following PG and 0.1 mg of GnRH or saline given 72 h apart to cyclic dairy cows and heifers. During 21 days following estrus the progesterone secretion was lower in GnRH-treated animals in comparison with saline-treated ones. Progesterone rose more quickly during the first week following estrus in animals ovulating spontaneously, compared with animals that ovulated after treatment with GnRH (Lucy and Stevenson 1986). Several possible explanations for the reduced luteal function were reviewed: a short term depletion of pituitary LH stores (of less duration than 12 h) due to extra LH release, an LH surge of shorter duration, fewer mitotic divisions in thecal and granulosal cells before LH release and following inadequate luteinization, down-regulation of luteal LH receptors, an asynchronious hormonal environment at induced ovulation, or a direct GnRH-induced suppression on luteal cells (Lucy and Stevenson 1986). However, conception rate was higher in animals receiving GnRH compared with animals given only saline. More slowly rising progesterone levels were assumed to improve fertility via unknown effects on embryonic survival (Lucy and Stevenson 1986).

If GnRH was given during proestrus, a less mature follicle ovulated and formed a CL that secreted less progesterone at the beginning of the cycle and during mid-luteal phase, thus lowering conception rates (Macmillan et al. 2003). According to Macmillan et al. (2003), the occurrence of short cycles following hormonal synchronization treatments should be taken as a sign of impaired effectiveness, and low doses of GnRH may be a cause of short cycles through an abnormal corpus luteum formation. Rutter et al. (1985) compared corpora lutea from postpartum cows with a normal cycle with cows having a short cycle: CL were heavier in cows having a normal cycle, but LH receptor concentration, progesterone production, and the small/large luteal cell ratio were similar on Day 6.5 after GnRH treatment. Luteal adenylate cyclase activity, phosphodiesterase activity, weight, number of LH receptors, and luteal or plasma progesterone concentrations were reported to be similar on Day 5 after estrus in postpartum beef animals having a short or normal cycle (Smith et al. 1986). Adenylate cyclase and phosphodiesterase were studied as authors hypothesized LH-induced progesterone secretion to be mediated via these enzymes, and changes in their activity might have caused short estrous cycles. In studies by Ramirez-Godinez et al. (1981, 1982b) serum progesterone concentrations declined after Day 6 following estrus in postpartum beef cows exhibiting a short cycle.

Bridges et al. (2010) also reported a decrease in progesterone concentration during the mid-luteal phase of short cycles in comparison with normal length cycles. In a study by Bollwein et al. (2010), luteal blood flow was dependent on the time interval between PG and GnRH. The highest values were recorded in animals after spontaneous ovulation, and significantly lower values occurred when ovulation was induced with 10 μg of buserelin given 40 h after PG. However, no correlation either between follicular size and follicular blood flow or between CL size and CL blood flow existed, but the size of the ovulating follicle and the size of the CL on Day 7 after ovulation were significantly, positively correlated. The follicular blood flow was increased in animals ovulating spontaneously, as compared with hormone treated animals induced to ovulate with 10 μg of buserelin given 40 h after PG. If ovulation was induced 60 h after PG with 10 μg of buserelin, there was no significant difference between the groups (Bollwein et al. 2010). The authors suggested that the optimal time interval between PG and GnRH administration might be cow-specific, and a shortened time prior to ovulation decreased both follicular and luteal blood flow (Bollwein et al. 2010).

In conclusion, induction of ovulation with GnRH decreases progesterone secretion and leads to a slower rise in progesterone level during early and midluteal phase. Shortened proestrus also affects follicular and luteal blood flow.

2.9. Peripheral blood progesterone, basal LH secretion and follicle size

The size of the ovulating follicle is linked to other hormonal changes around estrus. In dairy cows, the dominant follicle before ovulation was significantly bigger when luteal regression and ovulation were induced during early diestrus than during late diestrus (Vasconcelos et al. 1999). The average progesterone concentration at the time of treatments was significantly lower during early diestrus than late diestrus (Vasconcelos et al. 1999). In a study by Lüttgenau et al. (2011), lowered progesterone concentration in dairy cows during the luteal phase was linked to the increased size of the first wave dominant follicle, reported earlier in dairy heifers by Adams et al. (1992), and in beef cows by Pfeifer et al. (2009). Lüttgenau et al. (2011) concluded this to be caused by an increase in LH pulse frequency during Days 9 to 15 after ovulation, but LH concentrations were not analyzed. Follicular dynamics were suggested to be the cause behind increased LH pulse amplitude around Days 7 to 12 after behavioral estrus, because this could not be explained by changes in progesterone or estradiol levels (Cupp et al. 1995). Increased LH pulse frequency and mean of all LH concentrations coincided with follicular wave deviation at around Days 2 and 12 after ovulation (Ginther et al. 1998). Pfeifer et al. (2009) reported a significant increase in basal and mean LH concentrations in animals with lowered progesterone concentration during dominant follicle growth. Decreased or increased progesterone concentration, respectively, increased or decreased the LH pulse frequency within 6 h (Bergfeld et al. 1996). A larger ovulating follicle resulted in a larger CL, with a subsequent increase in progesterone secretion (Pfeifer et al. 2009). This increase in luteal size and progesterone concentration was, however, not reported by Lüttgenau et al. (2011). The cause for the reduction in the size of the dominant follicle is a progesterone-induced decrease in LH concentration (Ginther et al. 2001a, 2001b), and LH and progesterone oscillations are positively and temporally related (Hannan et al. 2010).

In conclusion, mean progesterone concentration affects follicle size via differences in LH pulse frequency and basal LH secretion.

3. AIMS OF THIS THESIS

The research for this thesis was carried out to elucidate possible mechanisms behind PG and GnRH induced short estrus cycles in cyclic dairy cows and heifers. The specific aims of different projects were as follows:

I To investigate whether the cycle day (early vs. late diestrus) affects the incidence of induced short estrus cycles or the size of the preovulatory follicle. Also to analyze the preovulatory peak of LH and postovulatory, basal LH release during induced short estrus cycles in comparison with spontaneous ovulation and normal length estrus cycles in cyclic dairy heifers.

II To investigate whether the dose of gonadorelin (low vs. high) affects the preovulatory release of LH, the size of the ovulating follicle or the incidence of short estrous cycles in cyclic dairy heifers.

III To investigate whether the time interval (0 vs. 24 h) between PG and GnRH administration affects ovulation rate, follicle size at ovulation and the incidence of induced short estrus cycles in cyclic dairy heifers and cows.

IV To investigate whether endometrial expression of the receptors estrogen-α, progesterone, oxytocin and the enzymes cyclo-oxygenase-II and 20α-hydroxysteroid-dehydrogenase on Days 2 and 5 after ovulation differ between normal length estrous cycles and induced short estrous cycles in cyclic dairy cows. Also to investigate whether follicle size and estradiol secretion at estrus differ between normal and induced short estrus cycles.

4. MATERIALS AND METHODS

An overview of materials and methods is presented in this section, and detailed information can be found in the original publications (I-IV).

4.1. Animals

Healthy, normally cyclic dairy heifers and highly productive dairy cows were used. All experiments took place at Viikki Research Farm, University of Helsinki, Finland, between years 2000 and 2007. Heifers (Experiments I to III) were loose housed, and cows (Experiments III and IV) were kept stanchioned. Animals were fed grass silage, concentrate and straw (heifers) or hay (cows) according to Finnish standards. Most animals were Finnish Ayrshires, only two heifers (Experiment III) and one cow (Experiment IV) were Holstein-Friesians. Number of animals in each experiment, age of heifers at the beginning of the experiment, and the experimental period are shown in Table 4.1.A.

Table 4.1.A. Number of dairy heifers and cows, heifer age at the beginning of each experiment, and the experimental period (month/year) in Experiments I to IV.

Experiment number	*Number of cases*	*Heifer age at the beginning of experiment*	*Experimental period (month/year)*
I heifers	19	12 to 21 months	9/2000 - 2/2001
II heifers	25	11 to 14 months	12/2002 - 3/2003
IIIa heifers	21	13 to 18 months	1 - 3/2004, 2 - 6/2005
IIIb cows	26	-	11/2000 - 6/2001
IV cows	14	-	4 - 6/2005, 1 - 5/2006

4.2. Experimental designs

Animal welfare was taken into account when planning the experimental settings, and all experiments were approved by the Ethics Committee of the University of Helsinki or by the Animal Experiment Board at the University of Helsinki. Animals were first assigned randomly into the treatment groups. After at least one unmanipulated estrous cycle animals could be subjected to another treatment. In all experiments the intramuscularly administered GnRH was 0.1 mg of gonadorelin (Fertagyl® 0.1 mg/ml, Intervet International, Boxmeer, The Netherlands), with the exception that a 0.5 mg dose was used also in Experiment II. Luteal regression was induced with an intramuscular administration of an agonistic analogue of prostaglandin $F_{2\alpha}$: 0.15 mg of dexcloprostenol (Genestran® 75 µg/ml, Vetcare Ltd, Salo, Finland) was used in all experiments except in IIIb, where 0.5 mg of cloprostenol (Estrumat® 0.25 mg/ml, Mallinckrodt Veterinary Ltd., Harefield, Uxbridge, UK) was used instead. All treatments and samplings were performed by the same operator at the same time of day.

In all experiments, the estrus synchronization procedure was the same: the cyclic status of each animal was determined using a transrectal ultrasound examination, and estrus was induced with a single dose of PG. Starting from the first (Experiments I, III, IV) or second (Experiment II) day after PG administration, the animals were examined daily using transrectal ultrasonography to monitor the occurrence of ovulation. The second luteal regression was thereafter induced with PG on the following days after ovulation (= Day 0): Experiment I - Day 7 or Day 14; Experiments II and III - Day 7; Experiment IIIb - Day 8, 9 or 10; and Experiment IV - Day 8. Subsequently, the protocol continued differently in each experiment.

4.2.1. Treatment groups

Experiments I, II and IIIa included two different treatment groups, Experiment IIIb three different groups, and Experiment IV only one treatment group. In Experiment I, an unmanipulated control group was included. Numbers of animals in different subgroups in Experiment I to IV are presented in Table. 4.2.1.A.

Table 4.2.1.A. Number (n) of animals in different subgroups (D7, D14, C, T500, T100, T0, T24, D8, D9, D10) in Experiments I to IV.

	Experiment I	*Experiment II*	*Experiment IIIa*	*Experiment IIIb*	*Experiment IV*
Treatment group	D7: n = 6 D14: n = 6	T500: n = 15	T0: n = 23	D8: n = 18 D9: n = 5 D10: n = 3	n = 11
Control group	C: n = 7	T100: n = 10	T24: n = 23		

- ✓ Experiment I: the 2nd PG was administered either on Day 7 (D7) or on Day 14 (D14) after ovulation, and GnRH was given 24 h after PG. An unmanipulated control group (C) was included, and synchronized with CIDR (CIDR+®, Vetcare, Salo, Finland) inserted for nine days.
- ✓ Experiment II: two different doses of gonadorelin were administered 24 h after PG, either 0.5 mg (T500) or 0.1 mg (T100). Group T100 served as a control group.
- ✓ Experiment IIIa: PG and GnRH were given at different time intervals, simultaneously (T0) or 24 h apart (T24). Group T24 served as a control group.
- ✓ Experiment IIIb: the animals were treated simultaneously with PG and GnRH either on Day 8 (D8), Day 9 (D9) or Day 10 (D10) after ovulation.
- ✓ Experiment IV: PG and GnRH were given 24 h apart to all animals.

4.2.2. Blood sampling and treatment manipulations

In Experiments I, II, IIIa and IV blood sampling for plasma progesterone (P_4) determination began immediately before the 2nd PG administration and continued once daily until the 2nd ovulation after the GnRH treatment. The samples were collected into heparinized blood tubes (Vacutainer®, Becton Dickinson Vacutainer Systems, Plymouth, UK) by vacuum puncture of a tail blood vessel. After immediate centrifugation (Experiment I 2200 x *g*, 10 min; Experiment II 1500 x *g*, 10 min; and Experiments IIIa and

IV 1400 x *g*, 10 min), the plasma was harvested, frozen, and stored in plastic tubes at –20 °C until analyzed. Blood samples for LH analysis (Experiment I and II) were treated similarly.

In Experiment I, the heifers were treated during early (D7) or late (D14) diestrus with PG and GnRH 24 h apart. Follicles thus induced to ovulate were the first and second wave dominant follicles, respectively. Blood sampling for hormone determinations began immediately before the 2nd PG, and continued once daily during the entire following estrous cycle until the next ovulation. In Groups D7 and D14, an indwelling catheter was inserted into the jugular vein for LH analysis some hours before the 2nd PG. Frequent sampling periods were as follows: beginning immediately before GnRH administration and continuing every 30 min for 6 h and on Days 1, 3, and 5 every 10 minutes for 3 h. After the last frequent sampling period, the catheters were removed and sampling continued once daily from a tail blood vessel. In the control Group C, blood was collected once daily for 16 days starting from the day of the CIDR removal. These heifers were catheterized for frequent blood sampling as in other groups. Blood sampling started 36 h after the removal of the CIDR device, and samples were collected every 30 min for 31 h. On Days 1, 3, and 5 after the ovulation, blood samples were taken every 10 min for 3 h as in other groups.

In Experiment II, the heifers were administered either 0.5 mg (Group T500) or 0.1 mg (Group T100) of gonadorelin 24 h after PG to induce ovulation. Five heifers in both groups were catheterized with an indwelling catheter some hours before GnRH administration, and blood samples for LH response analysis were collected every 30 min beginning one hour before GnRH administration and continued for 6 h after it.

In Experiment IIIa, all heifers were given GnRH either 0 (Group T0) or 24 hours (Group T24) after the PG administration. In Experiment IIIb all animals received simultaneous PG and GnRH injections on Day 8 (n = 18), Day 9 (n = 5) or Day 10 (n = 3) after ovulation.

In Experiment IV, the cows were given GnRH 24 h after PG. After ovulation, transcervical endometrial biopsies were taken on Days 2 and 5.

4.2.3. Milk sampling

Whole milk samples for P_4 determinations were collected daily in Experiment IIIb beginning immediately before the PG and GnRH treatment and continued until signs of next estrus were detected. Samples were collected immediately after the morning milking into plastic tubes containing a tablet of bronopol, after which they were frozen and stored in the original tubes at –20 °C until analyzed.

4.3. Ovarian examinations

In all experiments, ovarian examinations were performed with a real-time B-mode ultrasound scanner (Aloka SSD-210DXII, Aloka, Japan) equipped with a 7.5-MHz rectal linear array transducer. The ovaries were scanned several times to determine the largest cross-section of follicles and/or a CL. By freezing the image, the largest and smallest diameters were measured and recorded, and the average diameter was calculated later. The central cavities of CLs were measured and recorded in the same way. All follicles equal to or larger than 5 to 6 mm were measured. Locations of follicles larger than that were coded to follow their growth. Occurrence of ovulation was defined as a sudden disappearance of a large follicle between two consecutive ultrasound scans. Day of ovulation (Day 0) was the last day when the follicle was intact prior to the subsequent examination showing that the follicle had disappeared.

In Experiments I, II, IIIa and IV, transrectal ultrasonographic examinations of the ovaries were started 24 h after GnRH administration, and repeatedly performed once hourly (Experiment II), every 6 h (Experiments I and IIIa) or every 12 h (Experiment IV) until detection of ovulation, and thereafter once daily starting immediately (Experiments I, IIIa and IV), or on Day 4 (Experiment II), and continuing until the next ovulation or for at least 9 d (Experiment I). Possible signs of estrus and metestrous bleeding were recorded daily. In Experiment I, daily scanning was continued in all animals in Groups D7 and D14 until next ovulation occurred in short cycles, i.e. for at least 9 d, and in normal cycles again when signs of estrus were noticed. In Group C ultrasound examinations were started on the day of CIDR removal, and continued as in normal cycle animals. In Experiment IIIb, ultrasound examinations were performed daily beginning from the day of PG and GnRH treatment until the appearance of a new CL, thus confirming luteal regression, ovulation

and development of a new CL. During expected occurrence of short cycles, ultrasound examinations were performed daily, and after that normal length cycles were intermittently followed up until the next estrus.

4.4. Hormone analyses

4.4.1. Progesterone

The concentration of plasma P_4 was measured in one sample per day throughout the sampling period in Experiments I, II, IIIa and IV. The measurements were performed by radioimmunoassay (RIA) using commercial kits (Coat-A-Count® Progesterone, Diagnostic Products Corporation, Los Angeles, USA; or Spectria®, Orion Diagnostica, Orion Corporation, Espoo, Finland). The detection limit of both assays was 0.3 nmol/l. In Experiment IIIb, the whole milk P_4 concentration was measured with RIA using a commercial kit (Spectria®, Orion Diagnostica, Orion Corporation, Espoo, Finland) in single tubes. Immediately before analysis, the samples were allowed to thaw at room temperature. After thawing, they were warmed in +45 °C for 15 minutes and then carefully shaken with a single tube vortex for 30 seconds in order to redisperse their fat content. The detection limit of the assay was 1.0 nmol/l. Sample type and intra- and inter-assay coefficients of variation (CV) are summarized in Table 4.4.1.A.

4.4.2. LH

In Experiment I, peripheral plasma LH concentration was measured once in each sample using a RIA method described earlier by Forsberg et al. (1993). The intra- and inter-assay CVs for LH, as well as detection limits of the assay, are shown in Table 4.4.2.A. Pulses of LH on Days 1, 3 and 5 after ovulation were defined as values above individual basal LH values, i.e. peaks, detected according to a skewedness method described earlier by Zarco et al. (1984). In summary, mean and standard deviations (SD) of all samples were calculated, and samples greater than two SD above the mean excluded. This procedure was continued until no further peaks were detected. The mean of remaining values represented the average basal secretion of LH in each animal, and values above that were considered to represent a significant secretion of LH, i.e. pulses of LH. The LH wave after CIDR

Table 4.4.1.A. A summary of progesterone (P_4) sample type (blood or milk), intra- and inter-assay coefficients of variation (CV, %) and P_4 levels for CV calculations (nmol/l) in Experiments I to IV.

Experiment number	*Type of P_4 sample*	*P_4 level for CV calculations (nmol/l)*	*Intra-assay CV (%)*	*Inter-assay CV (%)*
I	Blood	4.6	6.1	7.4
		7.9	4.1	6.4
II	Blood	6.0	6.7	11.4
		10.8	8.3	10.8
IIIa	Blood	16.2	6.9	10.3
		33.1	15.0	12.3
IIIb	Milk	15.9	< 8.5	< 8.5
IV	Blood	8.0	12.3	One assay
		25.1	10.7	One assay

removal in Group C was detected similarly, i.e. several consecutive values above individual basal level.

In Experiment II, peripheral plasma LH concentrations were measured using a direct double-antibody RIA described and validated by Niswender et al. (1969) with the following minor modifications. Samples were first incubated with a buffer solution and bovine LH antibody (Tucker Endocrine Research Institute, the USA) for 24 h, after which a radioactively labelled LH (Bovine LH, Tucker Endocrine Research Institute, the USA) was added. Incubation was continued for 48 h, after which a solid-phase second antibody-coated cellulose suspension (SAC-CEL i.e. Solid Phase Second Antibody Coated Cellulose Suspension, IDS Ltd., Boldon, UK) was added to separate bound and unbound labels. Incubation was continued for 30 min. After centrifugation (3000 x *g*, +4 °C, 10 min) the radioactivity of the solid phase was measured using a gamma counter (MiniGamma 1275 Gamma Counter, Wallac). Controls and standards were included, as well as quality controls. A standard radioactivity curve for labeled LH was determined for concentrations ranging from 16 ng/ml to 0.125 ng/ml by serially diluting the original

sample with a buffer. The detection limit of the assay was defined as three SD for 0-binding values. The intra- and inter-assay coefficients for LH, as well as detection limits of the assay, are shown in Table 4.4.2.A.

4.4.3. Estradiol-17β

In Experiment IV, the concentration of peripheral blood estradiol-17β (E_2) was measured daily throughout the sampling period using a sequential RIA as described by Klein et al. (2003). Plasma (0.25 ml) was extracted with toluene; the antiserum used was directed against E_2-6-carboxymethyloxim (CMO)-BSA. The minimum detectable concentration was 2 pg/ml and quality controls were included in each assay.

4.5. Uterine biopsies

In Experiment IV, endometrial biopsy samples were obtained on Days 2 and 5 after ovulation. The cows were sedated and epidural anesthesia was induced. The vulva and perineum were washed, disinfected, and a 56 cm, sterile, guarded biopsy instrument (metallic, home-made with a mechanism corresponding to that of Tru-Cut® biopsy instrument,) was introduced via the cervix to either uterine horn, aided by manipulation per rectum. Four to five endometrial sections (about 20 mm x 5 mm) were cut on both days. One section was incubated in 10% phosphate buffered formalin solution for 24 h at +4 °C (tissue-formalin ratio 1:10), and three or four sections were immediately frozen with liquid nitrogen and stored at -80 °C until RNA extraction. The formalin-incubated biopsy sample was stored for one week in phosphate buffer at +4 °C, after which it was cut longitudinally into two sections, dehydrated, embedded in paraffin wax and kept refrigerated until immunohistochemistry analysis.

4.6. Immunohistochemistry

In Experiment IV, a microtome (Mikrom HM 400) was used to cut 4-µm sections of the biopsy samples, which were mounted on SuperFrost® Plus glass slides (Menzel Glaeser, D-38116, Braunschweig, Germany) and dried at +37 °C. Sections were deparaffinized with xylene (2 x 5 min) and rehydrated with a graded alcohol series (99%, 95% and 70%, 2 x 2 min each), and rinsed with running tap water for 5 min. For antigen retrieval,

Table 4.4.2.A. The intra- and inter-assay coefficients of variation (CV, %) for luteinizing hormone (LH) analysis, CV calculation levels (ng/ml) and the detection limit of the assay (ng/ml) in Experiments I and II.

Experiment number	*LH level for CV calculations (ng/ml)*	*Intra-assay CV (%)*	*Inter-assay CV (%)*	*Detection limit of the assay (ng/ml)*
I	three different	4.6 to 7.5	5.7 to 7.7	0.1
II	3.7	1.0 to 19.2	19.2	0.025

sections were pre-incubated in citrate buffer for 5 min at room temperature, heated in pre-heated citrate buffer in a microwave oven (560 W, 3 x 5 min), cooled for 20 min, and rinsed with running tap water for 5 min. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 30 min. Thereafter, samples were washed with immunohistochemistry (IHC) buffer (phosphate buffered saline and 0.3% Triton X, pH 7.2-7.4) for 5 min, drained and incubated with 10% blocking serum to block non-specific binding sites. For PR, ER and COX-II, 10% horse serum was used for blocking. After draining the serum, the samples were incubated with the respective primary antibody in a humid chamber at +4 ºC overnight. Primary antibodies for PR (1:500, mouse monoclonal IgG2a clone 10A9, dianova-immunotech, Hamburg, Germany), ER (1:200, mouse monoclonal IgG2a Ab-8, clone AER311, Lab Vision Corporation, Fremont CA 94539 USA) and COX-II (1:100, mouse monoclonal IgG clone 33, BD Biosciences Pharmingen, Becton, Dickinson and Company) were diluted in IHC buffer. All primary antibodies used were validated earlier for use in bovine uterine tissue (Schuler et al. 1999, 2002, 2006). Negative antibody control for PR, ER and COX-II was mouse monoclonal antibody (MsIgG2a, BeckmanCoultier) diluted in IHC buffer (1:100).

On the following day the sections were washed in IHC buffer for 20 min, drained and incubated at room temperature for 30 min with a secondary biotinylated antibody diluted in IHC buffer. The secondary antibody for PR, ER and COX-II was anti-mouse IgG (Ba-2000, Vector Laboratories, Burlingame, CA 94010 USA) diluted in IHC buffer (1:200). After draining, the samples were washed with IHC buffer and incubated at room

temperature for 30 min with streptavidin-peroxidase complex (ABC-system, Vector Laboratories, Burlingame, CA 94010 USA) diluted in IHC buffer according to the manufacturer´s instructions. After draining and washing in IHC buffer for 10 min, the sections were incubated with the substrate (Nova RED, Vector Laboratories, Burlingame, CA 94010 USA) diluted in distilled water according to the manufacturer´s instructions for an appropriate time for each receptor (COX-II and ER 10 min; PR 4 min). Thereafter, the sections were drained and washed under running tap water for 10 min. They were then counter-stained with haematoxylin and dried with a graded alcohol series (rapidly in 80%, 2 x 2 min changes in 96% and 99%) and xylol (2 x 10 min). Finally they were mounted in Histokit (Assistant, D-37520 Osteorode, Germany) and covered with a coverslip.

4.7. Semi-quantitative immunohistochemical evaluation

In Experiment IV, the staining intensity for all receptors and enzymes was evaluated semi-quantitatively from individual sections by the same person using a light microscope. For COX-II the cytoplasmic staining intensity was scored as no stain (0), weak (1), intermediate (2) or strong (3) stain. Surface epithelium, gland tubules (superficial and deep), gland openings and stroma (superficial or intermediate) were each evaluated separately. The amount of staining for ER and PR was evaluated in terms of immunoreactivity according to Boos et al. (1996). Nuclear staining was classified in random locations in at least 500 surface epithelium, endometrial gland, gland opening and stromal cells. If there were insufficient numbers of target cells, all possible cells were counted (in one sample the minimum number of gland opening cells was 40 and 66 for ER and PR, respectively).

4.8. Quantitative real-time polymerase chain reaction

In Experiment IV, relative mRNA concentrations for endometrial receptors ERα, PR, OR and enzymes 20α-HSD and COX-II, and for the house-keeping gene GAPDH, were analyzed using quantitative real-time RT-PCR (QPCR) from the endometrial biopsy samples stored at -80 °C. Two deep-frozen biopsy samples were homogenized with an ultra turrax (Ultra Turrax T8, IKA Werke GmBH&Co KG, Germany). For total RNA extraction, TRIzol® Reagent (Molecular Research Center Inc. Cat. No. 15596-026) was used according to the manufacturer’s instructions. The resulting RNA concentration was

determined with spectrophotometry in a Nano Drop ND-1000 (NanoDrop Technologies, Wilmington, DE), and samples were diluted in RNAase-free water to a concentration of 100 ng/µl. DNAase treatment (Sigma-Aldrich, St Louis, MO, USA, Cat. No. AMPD1) was applied to eliminate genomic DNA according to the manufacturer´s instructions. cDNA was prepared using a reverse transcription kit (Sensiscript® Reverse Transcriptase kit, Qiagen) in a total volume of 60 µl, according to the manufacturer´s instructions. All samples were run in duplicate. Programmable Peltier thermal cyclers (PTC-100®, MJ Research Inc., and DNA Engine®, Biorad) were used for all incubations. Random primers (3 µl, Promega, Madison, WI, USA) were first mixed with 900 ng of RNA and incubated at +70 °C for 10 min, after which they were cooled for 5 min. Subsequently, 45 µl of RT-PCR-mix, containing Sensiscript RT buffer, dNTPs (5 mM), RNAasin (40 IU/µl, Promega, Madison, WI, USA) and RNAase-free water, were added and samples were incubated at +37 °C for 2 min. Finally, 3 µl of the reverse transcriptase were added to treatment samples (RT+), and RNAase-free water was added to control samples (RT-) in place of reverse transcriptase. Incubation was continued at +37 °C for 1.5 h, and the reaction was stopped by heating to +94 °C for 5 min. The resulting cDNA was stored at -20 °C until analysis.

The Applied Biosystems Assays-by-design service was used to order all-in-one-tube TaqMan reagent-based assays for gene expression studies (forward and reverse primers and probes as listed in Table 4.8.A.). All QPCR analyses were run using the ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). For QPCR, 5 µl of diluted cDNA sample (100 ng/µl) was used in a 20 µl reaction mixture containing 10 µl TagMan Universal PCR Master Mix (Applied Biosystems), 1 µl 20X Assay Mix (Applied Biosystems) and 4 µl RNAase-free water. All the RT+ samples were run in triplicate for each gene and RT- samples once per gene. Two-fold serial dilution series were created from Day 17 endometrial cDNA samples in order to run standard curves for all genes. Three replicates for each ten dilution points were run in QPCR to create standard curves. Amplification conditions were the same for all targets assayed: one cycle at +50 °C for 2 min and one cycle at +95 °C for 10 min followed by 40 cycles at +95 °C for 15 s and at +60 °C for 1 min. Cycle threshold (CT) results in triplicate were screened for possible outliers ($SD < 0.5$), which were removed prior to further analysis (two in 20α-HSD, and six in COX-II), after which those samples were analyzed in duplicate only. Relative gene

Table 4.8.A. Forward and reverse primers and probes for genes of cyclo-oxygenase (COX-II), house-keeping gene GAPDH (GAPDH), estrogen receptor α (ERα), progesterone receptor (PR), 20α-hydroxysteroid-dehydrogenase (20α-HSD) and oxytocin receptor (OR) in Experiment IV.

Gene	*Forward primer*	*Reverse primer*	*Probe*
COX-II	CGAGGACCAGCTTTCACTAAGG	GCAGCTTATGCTGTCTCTCTAAAGA	AAGTCCACCCCATGGTTC
GAPDH	CCTCAACGACCACTTTGTCAAG	CTGTTGCTGTAGCCGAATTCATTG	TCGTACCAGGAAATGAG
ERα	GGAGAAGAGTTTGTGTGCCTCAA	AGAGTGCTGGACAGAAATGTGT	ACACTCCAGAATTAAGCAAGATG
PR	CCTGTGGAAGCTGTAAGGTCTT	CAATCGTTTCTTCCAGCACATAAGT	ATGCTGTCCTTCCATTGCC
20α-HSD	GACTACCTGGACCTCTACCTCATC	TGCCGTCCTCATCCAATGG	AAGTCCTTCCCAGGCTTG
OR	CGTGCAGATGTGGAGTGTCT	CCAGGAGCATGGCGATGAT	CAAGGAAGCCTCACCTTT

expression (RGE) was calculated using the comparative CT method (ΔΔCT method) and reported as n-fold differences in comparison with the sample of the lowest amount of the respective gene transcripts (calibrator) after normalizing the samples referring to the house-keeping gene GAPDH.

4.9. Statistical analysis

The data were analyzed using different versions of SPSS software for Windows. Differences in LH (Experiments I and II), E_2 (Experiment IV) and P_4 (all experiments) concentrations between different groups were analyzed using repeated measures analysis of variance (ANOVA) with group as the between-subject factor and time as the within-subject factor. The significances of time effects and time by group interaction effects were evaluated using Greenhouse-Geisser-adjusted P-values. The differences between groups in incidences of short cycles were evaluated with Fisher's exact test in all experiments. Also the differences between groups in incidences of anovulations after GnRH administration (Experiment III) were evaluated with Fisher´s exact test. The differences in the average diameter of the dominant follicle before ovulation between groups were analyzed using the

independent samples´ t-test in all experiments. Differences in time from GnRH administration to ovulation (Experiment II), in the size of the CL after ovulation (Experiment I), in the postovulatory, basal LH secretion (Experiment I) and in AUC (Experiments I and II) and LH peak values (Experiment I) were all analyzed with the independent samples t-test. In Experiment IV, the IHC results were analyzed using a Mann-Whitney U-test, and values for relative gene expression (RGE) from QPCR between groups were analyzed with one-way ANOVA. Due to the right-skewed distribution, these data were transformed logarithmically prior to statistical evaluation, and presented as geometric mean x deviation factor $^{\pm 1}$. In all experiments results were expressed as means or percentages (± SD). The differences were considered significant at $P < 0.05$.

5. RESULTS

5.1. Excluded cases

Over all experiments, only those cases in which ovulation occurred less than 48 h after GnRH, i.e. in a clear response to GnRH administration, were included in the analysis of data. Most excluded cases occurred in Experiment III, when GnRH was given to heifers and cows either 0 or 24 h after PG, and were due to ovulatory failure. In Experiment IIIb in cows, after simultaneous PG and GnRH administration, 8/26 cases (4/18 treated on Day 8, 3/5 on Day 9, and 1/3 on Day 10) were excluded. The average size of the dominant follicle at the time of treatment was 16.5 ± 2.2 mm and no further examinations or samplings were done in these cases. In Experiment IIIa, 12 heifers were excluded: ten belonged to Group T0, and two to Group T24. Thus significantly more cases failed to respond to GnRH in Group T0 in comparison with Group T24 ($P < 0.01$). In four of these excluded 12 cases the dominant follicle failed to ovulate as a response to GnRH, but continued to grow and ovulated 3 to 4 d after GnRH was given. At the time of GnRH treatment, the average size of these dominant follicles was 12.3 ± 2.3 mm (min. 11.0 mm, max. 15.0 mm). On ovulation day, the average size of the preovulatory follicles was 15.5 ± 2.4 mm. The additional eight cases led to atresia of the dominant follicle and to the emergence of a new dominant follicle and ovulation. At the time of GnRH treatment, the average size of these dominant follicles was 11.9 ± 1.5 mm (min. 10.0 mm, max. 14.5 mm). Ovulation of a new follicle occurred in three cases 5 to 6 d, and in one case 6 to 7 d, after GnRH administration. In four cases, the actual ovulation date remained undetermined. Two additional cases of heifers were also excluded due to incomplete luteal regression after administration of PG. In one of these cases, ovulation occurred as a response to GnRH, and an accessory CL developed.

Sporadic exclusions occurred also in other studies. In Experiment I, a short cycle in Group D7 was excluded from the exact calculation of the estrus cycle length and preovulatory follicle size due to an anovulatory estrus and cyst formation at the end of the experimental period. The length of estrous cycle in this case was approximately 9 d, based on the day of the luteal regression. In Group D14, the data from one animal were excluded due to anovulation after GnRH was given. In Group C, one animal was excluded from

preovulatory LH analysis due to secretion starting before the sampling period. Also all data from one animal were excluded due to the absence of an ovulatory LH release during the sampling period.

In Experiment II, one heifer was excluded from the analysis of LH results due to its biphasic LH release after 0.5 mg of gonadorelin was given: LH release started 30 min before GnRH administration, reached its peak value 30 min after GnRH administration, reverted to basal values, and rose again, reaching a new peak at 210 min after GnRH administration. This may have been due to spontaneous LH release at the time of GnRH administration.

In Experiment IV, one case was excluded due to incomplete luteal regression following administration of PG, and the accurate cycle length could not be determined in two cases because luteal regression was followed by an anovulatory estrus (a normal length cycle) or a follicular cyst formation (a short cycle). These two cases were not included in the cycle length calculations.

In conclusion, ovulatory failure was the major cause of exclusions, and occurred mainly when PG and GnRH were given simultaneously to cyclic, diestrous heifers or cows in Experiment III.

5.2. Lengths of the estrous cycles

The duration of estrous cycles was calculated from the day of induced ovulation (= Day 0) to the day of subsequent spontaneous ovulation monitored using daily ultrasound scanning in all experiments except IIIb. In Experiment IIIb, ultrasound examinations were performed daily from the day of simultaneous PG and GnRH treatment until the appearance of a new CL, thus confirming luteal regression, ovulation and the development of a new CL, and during the period of possible luteal regression of a short cycle. Thereafter, normal length cycles were followed up with daily milk samples for progesterone analysis until the next estrus occurred.

As the length of estrous cycles in all experiments was clearly bipartite, all cases were further classified into either short (SC) or normal (NC) cycle length groups. The mean

length (± SD) and minimum and maximum values of normal length cycles and short estrous cycles in all experiments are presented in Tables 5.2.A. and 5.2.B., respectively. The incidences of induced short cycles in different subgroups and their 95% confidence intervals (CI), as well as numbers of cases in short and normal cycle groups are presented in Table 5.2.C. In Experiment I, no short cycles occurred in the control group C, i.e. the length of all estrous cycles exceeded 16 days.

In Experiment I, the difference with regard to incidences of short estrous cycles between Groups D7 and D14 was not statistically significant. Also in Experiment II, the incidence of short cycles after either 0.1 mg (Group T100) or 0.5 mg (Group T500) of gonadorelin was similar. In Experiment IIIa, the incidence of short estrous cycles in Group T24 (PG and GnRH 24 h apart) was 47.1%, and in Group T0 (PG and GnRH simultaneously) 100%, the difference being statistically significant ($P < 0.01$).

In conclusion, the length of estrous cycles in all experiments was bipartite, i.e. short or normal. The difference in incidences of short estrous cycles was significantly different when PG and GnRH were given simultaneously on Day 7 after ovulation to cyclic, diestrus heifers in Experiment IIIa.

Table 5.2.A. Normal length estrous cycle, their mean length (± SD), and minimum (Min) and maximum (Max) values in days (d) in Experiments I to IV.

Experiment	*Mean (d)*	*SD (d)*	*Min (d)*	*Max (d)*
I heifers	18.8	1.0	18	20
II heifers	19.2	2.2	17	23
IIIa heifers	18.1	1.7	16	21
IIIb cows	23.0	1.0	22	24
IV cows	20.3	1.5	18	21

Table 5.2.B. Induced short estrous cycles and their mean length (± SD), and minimum (Min) and maximum (Max) value in days (d) in Experiments I to IV. D7 and D14: prostaglandin and gonadotropin releasing hormone given 24 h apart beginning on Day 7 or Day 14 after ovulation, respectively.

Experiment	*Mean (d)*	*SD (d)*	*Min (d)*	*Max (d)*
I heifers D7	8.0	1.0	7	9
I heifers D14	7.0	0	7	9
II heifers	7.9	1.1	6	11
IIIa heifers	7.5	0.5	7	8
IIIb cows	9.0	1.3	7	12
IV cows	8.7	0.6	8	10

5.3. Gonadotropin releasing hormone -induced ovulations and ovulatory follicles

The mean size of the dominant follicle (± SD, mm) at GnRH administration and at ovulation in all experiments is presented in Tables 5.3.A. and 5.3.B., respectively. In all included cases of heifers that were given PG and GnRH 24 h apart starting on Day 7 after ovulation, the average size of the preovulatory follicle at ovulation day was 14.1 ± 1.8 mm. Ovulations occurred in most heifers in 24 to 30 h after GnRH was given, and in most cows in 24 to 36 h (Experiment IV) or in 24 to 48 h (Experiment IIIb) after GnRH administration. In Experiment I, one animal in Group D7 ovulated later (32 to 43 h after GnRH administration), and one animal in Group D14 earlier (20 to 23 h after GnRH administration) than the others. In Experiment II, three follicles were ovulated some hours earlier (24 to 26 h after GnRH given, Group T500) or later (29 to 30 h, Group T500 and 30 to 40 h, Group T100) than other follicles. In Experiment IIIa, two follicles in Group T0 were ovulated later than others, i.e. 30 to 47 h after GnRH administration, and in Experiment IV, only one cow ovulated later than 36 h after GnRH administration (between 36 and 48 h). In Group C all ovulations occurred 60 to 84 h after CIDR removal and 22.0 ± 3.0 to 26.5 ± 3.0 h after the maximal LH value (Experiment I). No differences between

Groups T100 and T500 were detected in terms of time intervals between GnRH administration and ovulation (Experiment II).

In conclusion, the size of the preovulatory follicle is not related to the occurrence of short or normal length estrous cycles in cyclic dairy cows and heifers induced to ovulate with PG and GnRH either 0 h or 24 h apart. When estrus and ovulation are induced with PG and GnRH given 24 h apart during early (Group D7) or late (Group D14) diestrus, the mean preovulatory follicle diameter during all three days before ovulation is significantly different ($P < 0.05$). This difference in the preovulatory follicle size also occurred between the re-divided Groups SC and NC three days ($P < 0.05$) and one day ($P = 0.01$) prior to ovulation.

Table 5.2.C. The incidence of induced short cycles (SC, %) and number of short cycles (SC/all cases) in different subgroups in Experiments I to IV, and their 95% confidence intervals (CI). D7 and D14: prostaglandin (PG) and gonadotropin releasing hormone (GnRH) given 24 h apart beginning on Day 7 or Day 14 after ovulation, respectively. T100 and T500: PG and 0.1 mg or 0.5 mg of gonadorelin given 24 h apart, respectively. T0 and T24: PG and GnRH given 0 or 24 h apart, respectively. D8, D9 and D10: PG and GnRH given simultaneously on Day 8, Day 9 or Day 10 after ovulation, respectively.

Experiment	*Total incidence of SC (%)*	*95% CI*	*Incidence of SC (%) in subgroups (SC/all cases)*
I heifers	67	40 – 93	D7: 100 (6/6) D14: 33 (2/6)
II heifers	76	59 – 93	T100: 70 (7/10) T500: 80 (12/15)
IIIa heifers	68	51 – 85	T0: 100 (11/11) T24: 47 (8/17)
IIIb cows	78	73 – 83	D8: 71 (10/14) D9: 100 (2/2) D10: 100 (2/2)
IV cows	62	35 – 88	8/13

Table 5.3.A. The mean size of the dominant follicle (± SD, mm) at gonadotropin releasing hormone (GnRH) administration in different groups (D7 and D14, T100 and T500, T0 and T24, or SC and NC) in Experiments I to IV. D7 and D14: prostaglandin (PG) and GnRH given 24 h apart beginning on Day 7 or Day 14 after ovulation, respectively. T100 and T500: PG and 0.1 mg or 0.5 mg of gonadorelin given 24 h apart, respectively. T0 and T24: PG and GnRH given 0 or 24 h apart, respectively. SC and NC: short and normal length estrous cycle, respectively.

	Experiment I heifers		*Experiment II heifers*		*Experiment IIIa heifers*	*Experiment IV cows*
D7	15.2 ± 1.9	T100	13.8 ± 2.0	T0	13.6 ± 1.9	
D14	12.1 ± 0.9	T500	15.0 ± 2.1	T24	13.8 ± 2.2	
SC	14.3 ± 2.2		14.9 ± 2.1		13.4 ± 2.1	16.4 ± 2.4
NC	11.7 ± 0.8		14.3 ± 2.0		14.4 ± 2.3	17.5 ± 4.9

Table 5.3.B. The mean size of the ovulatory follicle (± SD, mm) at ovulation day in different groups (D7, D14 and C, T100 and T500, T0 and T24, or SC and NC) in Experiments I to IV. D7 and D14: prostaglandin (PG) and gonadotropin releasing hormone (GnRH) given 24 h apart beginning on Day 7 or Day 14 after ovulation, respectively. C: control group. T100 and T500: PG and 0.1 mg or 0.5 mg of gonadorelin given 24 h apart, respectively. T0 and T24: PG and GnRH given 0 or 24 h apart, respectively. SC and NC: short and normal length estrous cycle, respectively.

	Experiment I heifers		*Experiment II heifers*		*Experiment IIIa heifers*		*Experiment IV cows*
D7	14.8 ± 1.4	T100	14.0 ± 1.5	T0	13.6 ± 2.3		
D14	11.9 ± 0.5	T500	14.9 ± 1.8	T24	13.3 ± 1.9		
C	14.0 ± 2.3						
SC	14.1 ± 1.7	SC	14.7 ± 1.0	SC	13.9 ± 2.0	SC	17.6 ± 2.5
NC	11.7 ± 0.6	NC	14.5 ± 1.0	NC	13.6 ± 1.0	NC	18.9 ± 2.5

5.4. LH concentration in the peripheral blood

5.4.1. Preovulatory secretion of LH

Secretion of LH during 6 h following administration of GnRH, representing the LH surge, in Experiment I (Groups D7 and D14, and SC and NC), is presented in Fig. 5.4.1.A. The mean LH surge in the control group (C) is presented in the same figure, and thus adjusted to begin when LH exceeded 3 ng/ml and to end when LH fell below 3 ng/ml. The preovulatory basal LH value in Group C ranged between 1.6 and 1.8 ng/ml and an increase above that, i.e. the LH surge, began 36.0 to 53.5 h after the CIDR removal, and lasted for 7.5 to 10.5 h. After LH exceeded 3 ng/ml, it took 2.0 to 3.5 h to reach the maximal LH value in Group C.

The secretion of LH did not differ between early (Group D7) and late (Group D14) diestrus groups, or between short (SC) and normal (NC) cycle length groups. The mean peak LH concentration was reached either 1.5 h (Group D7) or 2.0 h (Group D14) after administration of GnRH (12.2 ng/ml and 9.8 ng/ml, respectively). In Group C the mean peak LH concentration was 10.7 ng/ml, and similar to Groups D7 and D14. In Groups D7 and D14, LH secretion was below 2 ng/ml 4.5 h after GnRH administration and until the end of sampling.

The total LH secretion during 6 h after GnRH administration in Groups D7 and D14, and during the first 6 h of the LH surge in Group C, was evaluated in terms of AUC (± SD), and was 1779 ± 660, 1674 ± 316 and 2834 ± 994 ng*min/ml in Groups D7, D14 and C, respectively. The AUC did not differ between Groups D7 and D14 or between Groups SC and NC, but there was a statistically significant difference between Groups D14 and C ($P < 0.01$). A similar difference, approaching statistical significance, was apparent between Groups D7 and C ($P = 0.06$). Also in Experiment II, total LH secretion was evaluated in terms of average AUC (± SD) during 6 h after either 0.1 or 0.5 mg of gonadorelin, and was 903 ± 140 and 845 ± 132 ng*min/ml, respectively. This difference between groups was not significant. In re-divided groups SC and NC the average AUC (± SD) was 833 ± 139 ng*min/ml and 966 ± 55 ng*min/ml, and did not differ between groups (Experiment II).

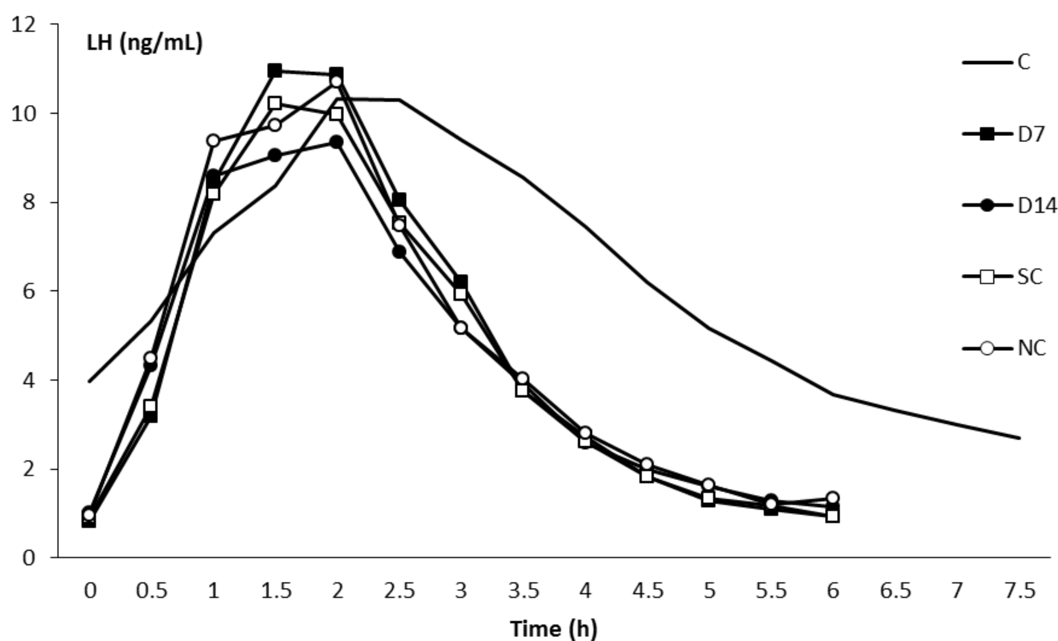
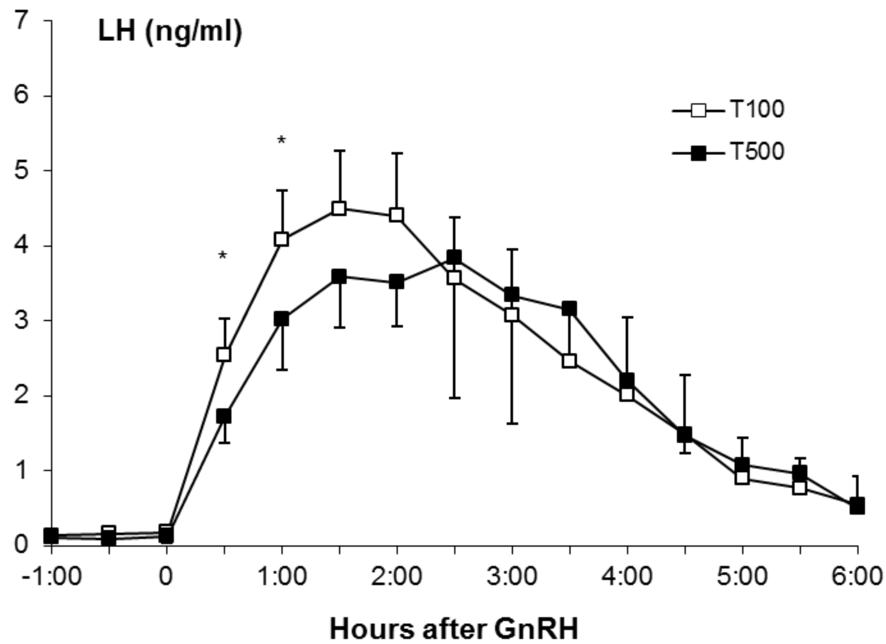


Fig. 5.4.1.A. LH secretion (ng/ml) in Experiment I during 6 h following the administration of GnRH, when PG and GnRH were given 24 h apart beginning on Day 7 (D7, n = 6) or on Day 14 (D14, n = 5) as well as in heifers showing a short (SC, n = 8) or normal (NC, n = 3) estrous cycle after the treatment. In the control group (C, n = 5), LH secretion was monitored as a spontaneous release after CIDR removal and it was adjusted chronologically to correspond with the secretion in other groups.

In Experiment II, when 0.1 mg or 0.5 mg of gonadorelin was given 24 h after PG, no significant differences were detected between the Groups T100 and T500 in the levels of LH curves. Average LH profiles from 1 h before to 6 h after administering 0.1 mg or 0.5 mg of gonadorelin, i.e. in Groups T100 (n = 5) and T500 (n = 4), are presented in Fig. 5.4.1.B. The individual LH curve rose more slowly in Group T500 than in Group T100: LH concentration was significantly lower ($P < 0.05$) in Group T500 than in T100 30 min and 60 min after GnRH administration. In addition to this, the peak values, 4.5 ± 0.8 ng/ml (T100) and 3.8 ± 1.9 ng/ml (T500), were reached somewhat later in Group T500 than in Group T100 (group averages 112 and 96 min, respectively). Of the nine heifers that were studied successfully for the LH response, 3/5 in Group T100 and 3/4 in Group T500 had a short estrous cycle. No significant differences between SC (n = 6) and NC (n = 3) were detected either in levels or profiles of LH curves.



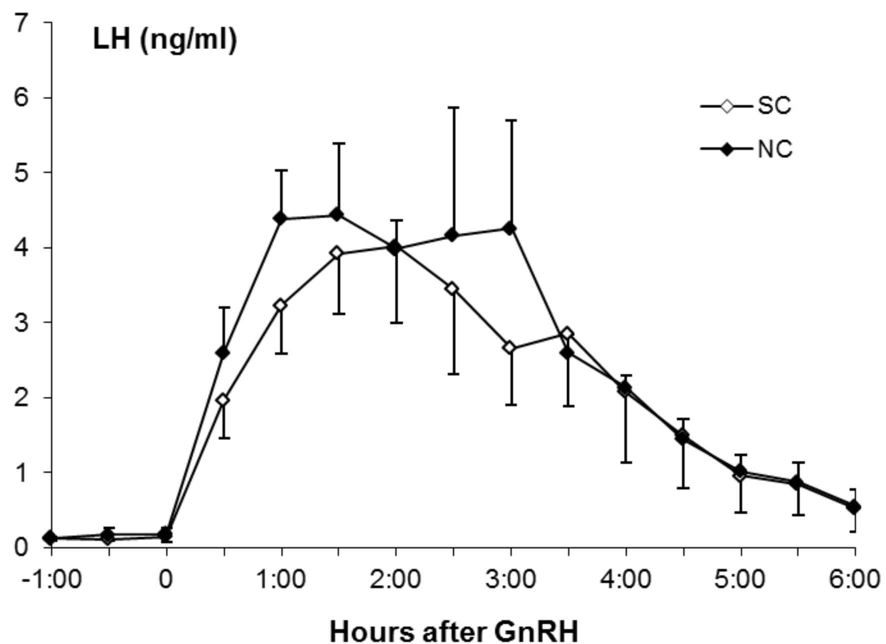


Fig. 5.4.1.B. LH concentration (mean ± SD, ng/ml) during a 7 -h period beginning 1 h before the GnRH administration in Groups T100 (n = 5) and T500 (n = 4) (upper panel) when either 0.1 mg (Group T100) or 0.5 mg (Group T500) of gonadorelin was administered 24 h after PG given on Day 7 after ovulation. The lower panel shows the LH concentration curves (mean ± SD, ng/ml) in re-divided groups of short estrous cycles (SC, n = 6) and normal length cycles (NC, n = 3). * $P < 0.05$.

In conclusion, when estrus and ovulation are induced with PG and GnRH given 24 apart to diestrus heifers starting on Day 7, the preovulatory secretion of LH preceding short and normal length estrous cycles is similar. The preovulatory LH secretion is also similar when estrus and ovulation are induced with PG and GnRH given 24 h apart during early (Day 7) and late (Day 14) diestrus. Increasing the dose of GnRH (0.1 mg vs. 0.5 mg of gonadorelin) had no significant effect on the preovulatory LH secretion when PG and GnRH were given 24 apart. Only the individual LH curve rose more slowly in Group T500 than in Group T100: LH concentration was significantly lower ($P < 0.05$) in Group T500 than in T100 30 min and 60 min after GnRH administration, and the peak values were reached somewhat later in Group T500 than in Group T100.

5.4.2. Basal secretion of LH

LH secretion parameters (mean number of pulses, mean inter-pulse interval, mean pulse duration, and mean basal secretion) during the 3 h sampling period on Days 1, 3 and 5 after ovulation in all animals (Groups D7, D14 and C), and in short and normal cycle length groups are presented in Table 5.4.2.A. (Experiment I). Between Groups SC and NC no difference in basal LH secretion occurred on Days 1, 3 and 5.

In conclusion, when estrus and ovulation are induced with PG and GnRH given 24 h apart to diestrous heifers, basal secretion of LH on Days 1, 3 and 5 after ovulation is similar between short and normal length estrous cycles.

5.5. Peripheral blood progesterone concentration

5.5.1. Progesterone concentration at PG administration and subsequent daily rise

Progesterone concentration shortly before PG administration on Day 7, i.e. during early diestrus, was 13.4 ± 5.3 nmol/l (Experiment I, Group D7), 15.3 ± 4.8 nmol/l (Experiment II, Group T100), 14.5 ± 2.7 nmol/l (Experiment II, Group T500), 13.9 ± 3.9 nmol/l (T0, Experiment IIIa) and 13.9 ± 3.5 nmol/l (Group T24, Experiment IIIa). In cows just prior to a simultaneous treatment with PG and GnRH either on Day 8, 9 or 10 after ovulation, milk P_4 concentration varied between 15.9 and 46.3 nmol/l, being on average 30.3 ± 8.9 nmol/l (Experiment IIIb). During late diestrus, i.e. on Day 14, P_4 concentration just before PG

administration was 24.4 ± 6.0 nmol/l (Experiment I). Just before the CIDR removal, the P_4 concentration in the control group C was 7.6 ± 1.9 nmol/l (Experiment I). At PG administration the concentration of P_4 was similar in Groups SC and NC (Experiment IIIa, IV).

In Experiments I and IIIa, P_4 concentration in all groups decreased to 1 nmol/l or less in 48 h after the administration of PG or CIDR removal (Group C, Experiment I). In Experiment IV, the lowest mean P_4 value (2.1 ± 1.3 nmol/l in Group SC and 0.4 ± 0.3 nmol/l in Group NC) was reached 48 h after PG administration. In Experiment IIIb with cows, milk P_4 concentration in all animals declined below 9 nmol/l in 48 h after PG treatment, but some exceptions occurred. In Experiment IIIa, in one heifer P_4 was still 2.8 nmol/l 48 h after PG, and in Experiment II, the P_4 concentration declined to below 1.0 nmol/l in 72 h after PG treatment in all heifers except two. In those two cases the lowest concentration (1.1 and 2.4 nmol/l) was reached on Day 3 after PG administration.

Table 5.4.2.A. Mean luteinizing hormone (LH) secretion parameters (number of LH pulses in 3 h, inter-pulse interval in minutes, basal LH secretion in ng/ml ± SD) during the 3 h sampling period on Days 1, 3 and 5 after ovulation (Day 0) in all (Groups D7, D14 and C) animals (ALL, n = 18) and in short (SC, n = 8) and normal (NC, n = 4) cycle length groups.

LH parameter		*Day 1*	*Day 3*	*Day 5*
Number of LH pulses in 3 h	ALL	1.4	1.4	1.8
	SC	1.5	2.0	2.5
	NC	1.9	1.5	1.8
Inter-pulse interval (min)		47	42	53
Basal LH secretion (ng/ml) ± SD	ALL	1.4 ± 0.4	1.4 ± 0.9	1.1 ± 0.7
	SC	1.1 ± 0.2	1.4 ± 1.2	0.8 ± 0.7
	NC	1.4 ± 0.2	0.9 ± 0.5	0.8 ± 0.4

In Experiment I in Groups SC and NC, the average blood P_4 rise between Days 3 and 7 after PG administration was 1.3 and 1.5 nmol/l/d, respectively. In Group C, P_4 rose until blood sampling was discontinued, i.e. Day 15 after treatment, and the average rise during that time was 1.4 nmol/l/d (Exp. I). In Experiment II, the average rise of P_4 between Days 1 to 4 was 2.4 and 2.0 nmol/l/d in Groups T100 and T500, respectively. In Experiment IV, the average rise between the lowest and highest P_4 value was 0.5 ng/ml/d in Group SC and 0.4 ng/ml/d in Group NC.

In conclusion, when estrus and ovulation are induced with PG and GnRH 24 h apart during early diestrus in cyclic heifers and cows, P_4 concentration before PG is similar between short and normal estrous cycles.

5.5.2. Maximum progesterone concentration during short and normal length cycles

In the groups of short cycles in Experiment IIIa, T0s and T24s, the maximum concentration of P_4 was reached similarly on Days 4.7 ± 0.7 and 4.6 ± 0.5 after ovulation, respectively (Experiment IIIa). Also in Experiment II, subgroups of short cycles, T100/SC and T500/SC, reached the highest P_4 concentration similarly, i.e. on Day 4.9 ± 1.1 and Day 4.8 ± 0.8 after ovulation, respectively. Individual cows exhibiting a short cycle in Experiment IIIb reached the maximum P_4 concentration on either Day 4 (n = 1), Day 5 (n = 9), Day 6 (n = 2) or Day 7 (n = 2) after ovulation. In Experiment IV with cows exhibiting short cycles, the peak value of P_4 was detected on Day 7 after ovulation. In Experiment I heifers exhibiting short cycles, the mean peak value of P_4 was 6 days after GnRH administration.

5.5.3. Difference in progesterone secretion during short and normal length estrous cycles

In Experiments I and IV differences in the levels and profiles of P_4 concentration between groups were analyzed from the ovulation day for 8 days. In Experiment II, differences in the levels and profiles of P_4 concentration between groups were analyzed from the day of ovulation for 5 days. In Experiment IIIb, differences in P_4 concentrations between groups were analyzed from Day 1 to Day 7 (Experiment IIIa) or Day 8 (Experiment IIIb) after ovulation. Between Groups SC and NC, a significant difference occurred in the level ($P < 0.05$) and in the profile ($P < 0.001$) of P_4 secretion in Experiment I (Figure 5.5.3.A). The

difference in the secretion profile emerged on Day 6 ($P < 0.05$) after ovulation and also occurred on Days 7 and 8 ($P < 0.001$). Due to variation in the occurrence of short and normal cycles between the groups, a corresponding significant difference in P_4 secretion was detected between Groups D7 and D14 ($P < 0.05$) and between Groups D7+D14 and C ($P < 0.01$).

In Experiment II, examination of the P_4 profiles in subgroups SC and NC of both Groups T100 and T500 (Figures 5.5.3.C and 5.5.3.B, respectively) revealed no differences. In Experiment IIIb, a significant difference in the profiles of P_4 curves emerged between groups SC and NC ($P < 0.05$), as Group SC attained the maximum P_4 concentration on Day 5 after ovulation, whereas in Group NC the concentration rose steadily. In Experiment IIIb, a significant difference in the profiles of P_4 curves existed between the groups SC and NC ($P < 0.05$). In group SC, the maximum P_4 concentration was reached on Day 5 after ovulation while in group NC the concentration increased steadily. A similar significant difference ($P < 0.001$) in the secretion profile of P_4 concentration during Days 1 to 7 after ovulation between Groups SC and NC occurred in Experiment IV. This profile difference emerged on Day 7 ($P < 0.01$) and occurred also on Day 8 ($P < 0.001$). The level of P_4 secretion during that time period did not differ between Groups SC and NC.

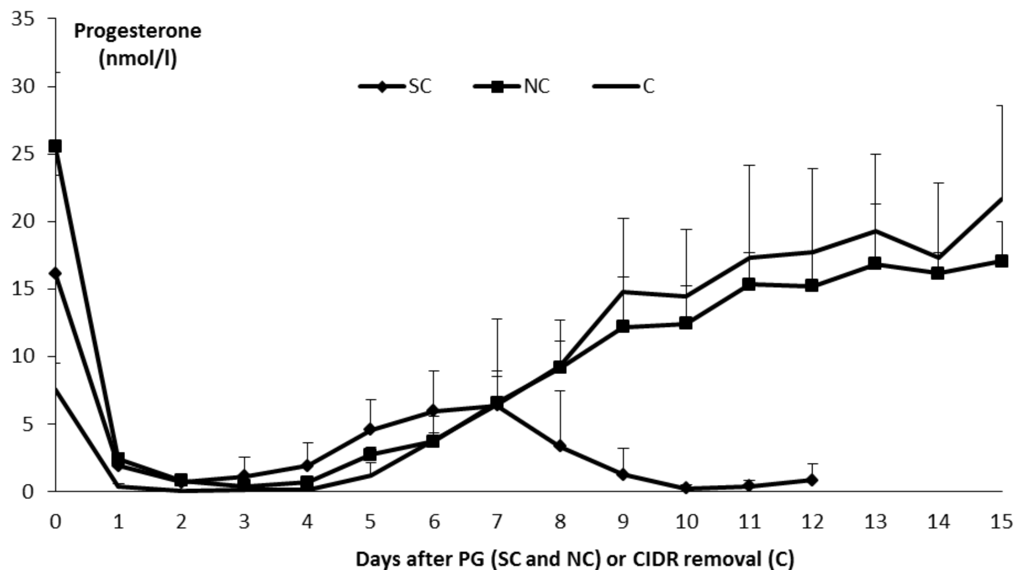


Figure 5.5.3.A. Progesterone profiles (mean ± SD, nmol/l) during the first 12 to 15 days of the estrous cycle in the short (SC) and normal cycle (NC) groups after PG (=Day 0) and GnRH administration 24 h apart, and in the control group (C) after CIDR removal (=Day 0) in Experiment I.

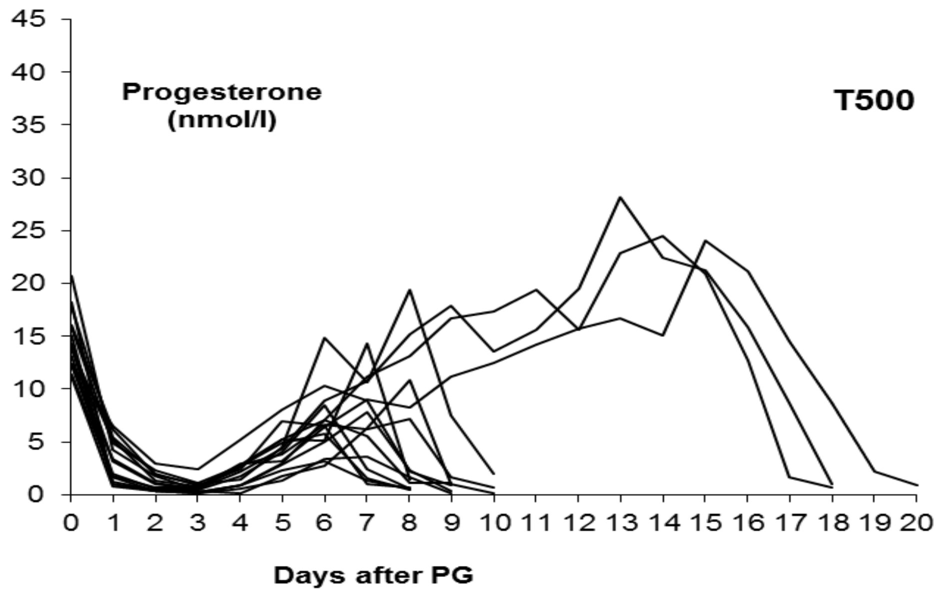


Figure 5.5.3.B. Individual progesterone profiles (nmol/l) from dexcloprostenol (PG) administration to the second estrus in Group T500, when 0.5 mg of gonadorelin was administered 24 h after the PG administered on Day 7 after ovulation.

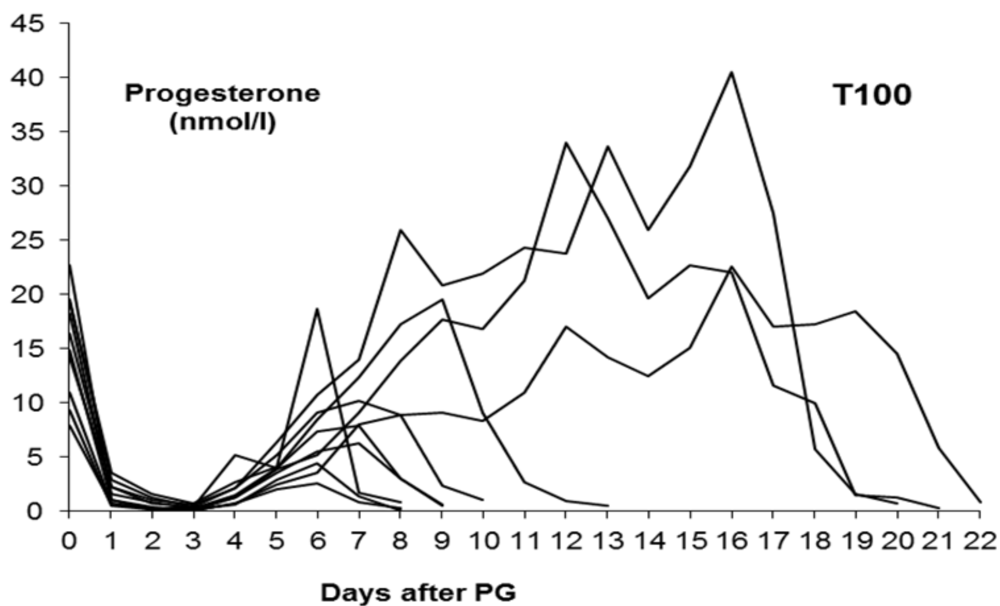


Figure 5.5.3.C. Individual progesterone profiles (nmol/l) from dexcloprostenol (PG) administration to the second estrus in Group T100, when 0.1 mg of gonadorelin was administered 24 h after the PG administered on Day 7 after ovulation.

In Experiment IIIa, a significant difference in levels ($P < 0.01$) and profiles ($P < 0.01$) of P_4 secretion between the groups was detected, and individual P_4 profiles showed a clear bipartite reaction in Group T24. As a result, the cases in Group T24 were re-divided for further analysis based on the length of the estrous cycle into groups of short (Group T24s) or normal (Group T24n) length cycle. Among Groups T0, T24s, and T24n, a significant difference in the levels ($P < 0.001$) and profiles ($P < 0.001$) of P_4 curves was detected (Figure 5.5.3.D). This difference appeared on Days 6 and 7, when the P_4 concentration in Group T24n was significantly higher. No difference between Groups T0 and T24s was observed.

The geometric mean of peripheral blood P_4 concentrations and their scatter ranges in Groups SC and NC in Experiment IV are presented in Figure 5.6.A.

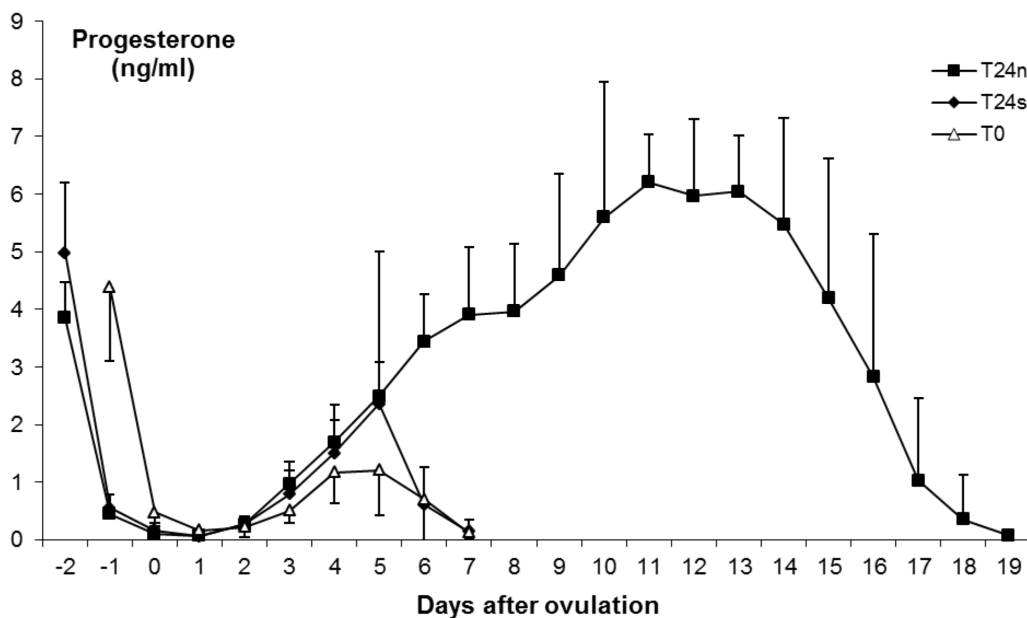


Figure 5.5.3.D. Progesterone concentrations (ng/ml, mean ± SD) in Groups T0 (PG and GnRH administrated simultaneously), T24s (PG and GnRH administered 24 h apart, animals with a short estrous cycle), and T24n (PG and GnRH administered 24 h apart, animals with a normal estrous cycle) during the subsequent estrous cycle. PG was given in Group T0 on Day -1 and in Groups T24 on Day -2. Among Groups T0, T24s, and T24n, a significant difference in the levels ($P < 0.001$) and profiles ($P < 0.001$) of P_4 curves was detected on Days 6 and 7, when the P_4 concentration in Group T24n was significantly higher. No difference between Groups T0 and T24s was observed.

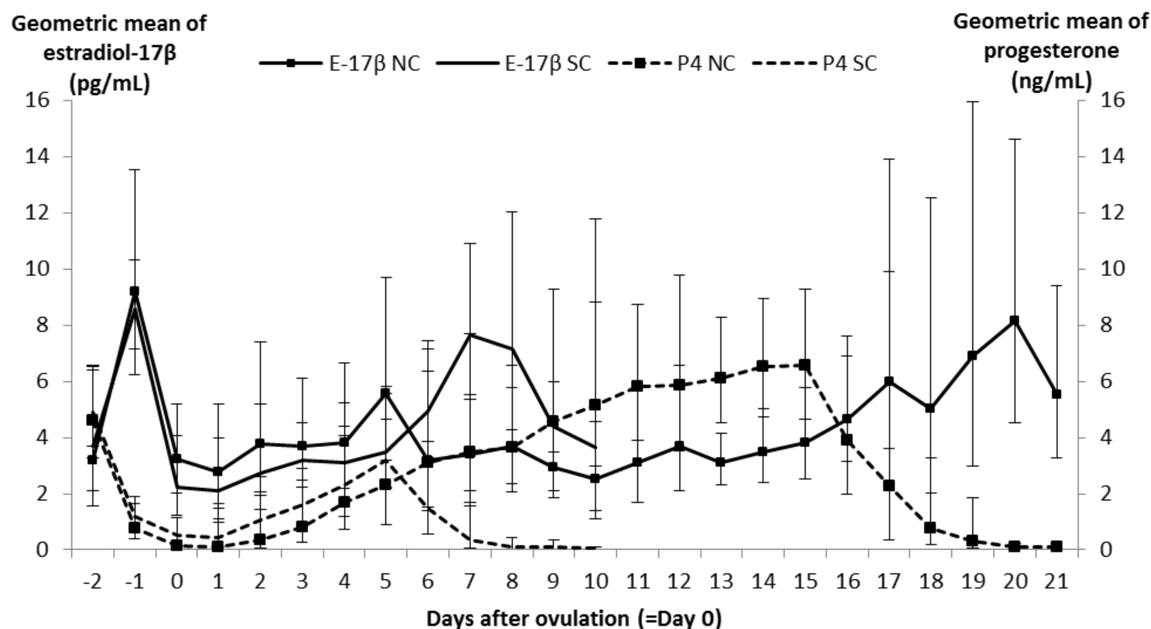


Figure 5.6.A. Geometric mean of peripheral blood estrogen-17β (E_2, pg/mL) and progesterone concentration (P_4, ng/mL) and their scatter ranges (Xg x deviation factor$^{\pm1}$) in induced short (SC, n=8) and normal (NC, n=5) length cycle groups after ovulation (=Day 0) in Experiment IV.

In conclusion, when estrus and ovulation are induced with PG and GnRH 24 apart in diestrus cows and heifers, a significant difference in P_4 profile between groups occurs during the first week after ovulation. This difference is due to occurrence of induced short estrous cycles.

5.6. Peripheral blood estradiol concentration

The geometric mean of peripheral blood estradiol-17β (E_2) and progesterone concentrations and their scatter ranges in Groups SC and NC in Experiment IV are presented in Figure 5.6.A. The mean peak of E_2 was reached one day before ovulation, and was similar in short and normal cycle length groups. Thereafter in the normal cycle length group, the average E_2 fluctuated below 4 pg/ml, except on Day 5 (5.6 x 1.8$^{\pm1}$ pg/ml) and on days after Day 20. A peak (8.2 x 1.8$^{\pm1}$ pg/ml) was reached on Day 20. In the short cycle length group, average E_2 after ovulation was below 4 pg/ml until Day 6 and beyond. Differences in E_2 concentration between Groups SC and NC were analyzed from two days

before ovulation until eight days after it. The secretion profile of blood E_2 was significantly different between Groups SC and NC ($P < 0.001$). This difference emerged on Day 7 ($P < 0.05$). Also on Day 5 there was a tendency towards significance ($P = 0.059$). The blood E_2 concentration 24 to 48 h before ovulation did not correlate with the size of the ovulatory follicle.

In conclusion, when estrus and ovulation are induced with PG and GnRH 24 h apart during early diestrus in dairy cows, the mean peak of E_2 is reached one day before ovulation, and is similar in induced short and normal cycle length groups. Blood E_2 concentration 24 to 48 h before ovulation does not correlate with the size of the preovulatory follicle. E_2 concentration is significantly different between short and normal length estrous cycles on Day 7 after ovulation.

5.7. Immunohistochemistry

Immunostaining for ER and PR is presented in Table 5.7.A. In Experiment IV, most immunostaining for COX-II was noted in the cytoplasm of surface epithelial, gland tubule and superficial stromal cells, and no staining was evident in deep gland tubule cells.

The range for COX-II immunostaining was from 0 (superficial gland tubule cells in Group NC on Days 2 and 5) to 1.5 ± 1.3 in gland opening cells on Day 5 in Group SC. For receptors ER and PR most immunostaining was noted in the nucleus of surface epithelial, gland tubule and gland opening cells. Significant non-specific staining was not observed. No statistically significant differences in any of the cell types mentioned above were detected in the average endometrial ER, PR or COX-II staining intensity between Groups SC and NC.

5.8. Endometrial receptor and enzyme expression

In Experiment IV, for two samples taken on Day 2 after ovulation from two animals (one in both short cycle and normal cycle length groups), no amplicon was evident in QPCR and those samples were thus excluded from further analysis. Geometric means of relative gene expression (RGE) for ERα, OR, PR, 20α-HSD and COX-II on Days 2 and 5 in short and normal cycle length groups are presented in Fig. 5.8.A. No statistically significant difference was detected in RGE between these groups.

Table 5.7.A. Immunoreactivity scores of endometrial surface epithelium, gland opening, gland tubule and stromal cells for estrogen (ER) and progesterone (PR) receptors in animals with short or normal length cycles (SC and NC, respectively) on Days 2 and 5 after ovulation (=Day 0) calculated according to Boos et al. (1996). No significant differences between Groups SC and NC were evident.

		Surface epithelium	*Gland opening*	*Gland tubules*	*Stroma*
ER					
Day 2	SC	92 ± 108	127 ± 76	108 ± 29	64 ± 25
	NC	241 ± 88	163 ± 0	127 ± 30	44 ± 25
Day 5	SC	99 ± 57	66 ± 52	97 ± 44	28 ± 14
	NC	129 ± 128	172 ± 0	125 ± 101	61 ± 57
PR					
Day 2	SC	156 ± 98	128 ± 43	132 ± 26	75 ± 29
	NC	153 ± 88	97 ± 0	132 ± 42	54 ± 30
Day 5	SC	124 ± 50	122 ± 22	134 ± 23	58 ± 22
	NC	156 ± 19	160 ± 39	111 ± 50	69 ± 14

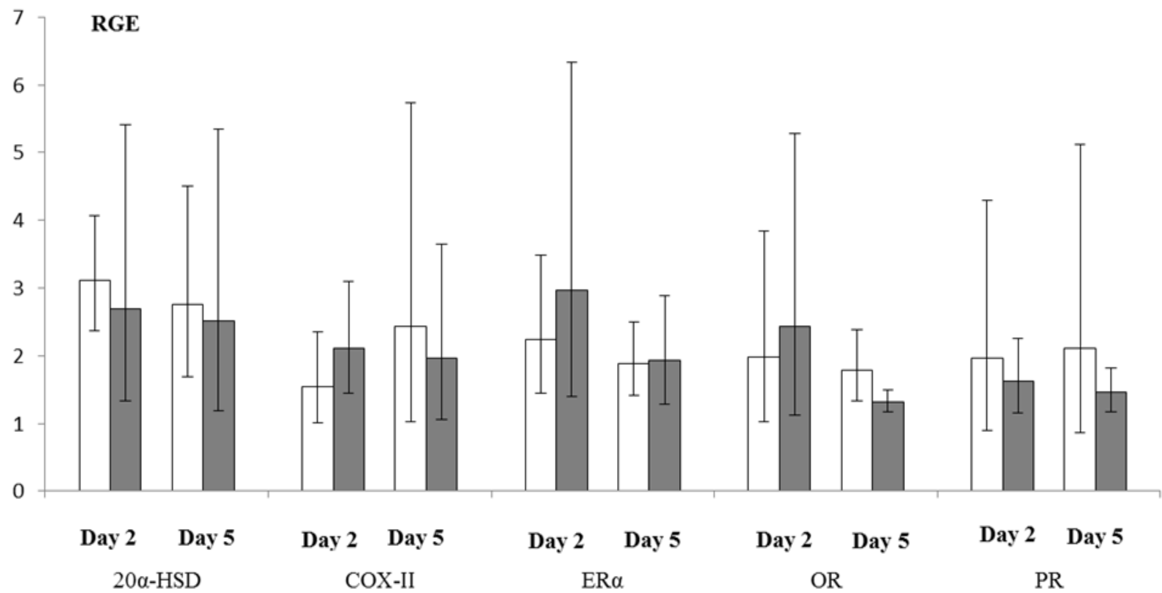


Fig. 5.8.A. Geometric mean of relative gene expression (RGE) for 20α-hydroxysteroid-dehydrogenase (20α-HSD), cyclo-oxygenase II (COX-II), estrogen receptor α (ERα), oxytocin receptor (OR) and progesterone receptor (PR) and their scatter range (Xg x deviation factor$^{\pm 1}$) in normal (white bars) and short (grey bars) cycle groups on Days 2 and 5 after ovulation (= Day 0) in Experiment IV. No statistically significant differences were evident among the groups ($P > 0.05$).

6. DISCUSSION

6.1. Exclusion of cases

In this thesis most excluded cases occurred due to ovulatory failure when PG and GnRH were given simultaneously to diestrous heifers or cows in Experiment III. Similarly disrupted follicular dynamics, premature ovulation and delayed return to normal estrus and ovulation were reported when Stevens et al. (1993) gave simultaneously PG and 0.1 mg of gonadorelin or saline to diestrous cows either on Day 8 or Day 10 after standing estrus. Significantly more saline-treated cows (14/16) exhibited a normal estrus within two to five days after treatment, in comparison with simultaneous treatment with cloprostenol and gonadorelin (6/16). Those treated with simultaneous cloprostenol and gonadorelin and not showing a normal estrus within 2 to 5 days, were further analyzed. Five cows exhibited no signs of estrus, ovulated within 48 h and developed an ultrasonographically detectable structure resembling CL, and returned to estrus 7 to 13 days later. Three showed signs of estrus but returned to estrus 6 to 11 days later, suggesting induced follicular atresia and development of a new dominant follicle, and two showed estrus on Day 4 after treatment, but did not ovulate. In contrast to other experiments reported in this thesis, in Experiment III PG and GnRH were given simultaneously, i.e. GnRH was given during high blood P_4 concentration. According to Giordano et al. (2012), the ovulatory response after 0.1 mg or 0.2 mg of gonadorelin given to diestrous animals either during high (over 3 ng/ml) or low (in average 0.2 ng/ml) blood P_4 concentration remained unaffected, even though the preovulatory release of LH was less during high blood P_4 concentration in comparison with low blood P_4 concentration. In Experiment III of this thesis, LH release after simultaneous treatment with PG and GnRH was not analyzed.

6.2. Length of estrous cycles and incidence of induced short estrous cycles

The length of estrous cycles following PG and GnRH given 0 or 24 h apart to diestrous dairy cows or heifers in this thesis was clearly bipartite due to occurrence of induced short estrous cycles in addition to normal length cycles. The maximum length of these short estrous cycles, 11 d in heifers and 12 d in cows, i.e. less than two weeks, and the minimum length of 6 d in heifers and 8 d in cows are in accordance with similar reports by Taponen

et al. (2002, 2003). In those studies, the approximated length of induced short cycles ranged between 8 to 10 d in cows (Taponen et al. 2002) and 8 to 12 d in heifers (Taponen et al. 2003).

Simultaneous treatment with PG and GnRH on Day 7 after ovulation caused the highest incidence of induced short estrus cycles (100%) in cyclic, diestrous dairy heifers. This was also significantly more in comparison with the incidence of short estrous cycles when PG and GnRH were given 24 h apart. Also Bridges et al. (2010) in their two experiments noted that decreasing the time interval between administration of PG and 0.1 mg of gonadorelin from 2.2 d to 1.2 d increased the incidence of short estrous cycles in cyclic beef cows. When the time interval was decreased from 2.25 d to 1.25 d, the proportion of short cycles was significantly increased (Bridges et al. 2010). Similarly when Schmitt et al. (1996) gave PG and 8 µg of buserelin 24 h apart, the incidence of induced short estrous cycles in cyclic cows and heifers was about 35%. This incidence was an estimate, as the occurrences of luteal regression and ovulation were not confirmed, and the inter-insemination interval was based on estrus detection. Similarly, Taponen et al. (2002, 2003) gave PG and 0.1 mg of gonadorelin 24 h apart to cyclic dairy heifers and cows. The incidence of short cycles was about 33% in cows (Taponen et al. 2002) and about 58% in heifers (Taponen et al. 2003). In those studies, the 95% confidence interval of incidences was wide due to small sample sizes: in cows 6% to 60%, and in heifers 30% to 86%. In studies included in this thesis, numbers of cows and heifers were somewhat higher and the total incidence of induced short estrous cycles was thus less variable than in earlier studies of Taponen et al. (2002, 2003).

6.3. Size of the ovulatory follicle

In the basic experimental setting of the work comprising this thesis, estrus in heifers was induced with PG during early diestrus, on Day 7 after ovulation, and 0.1 mg of gonadorelin for ovulation induction was given 24 h later. No difference in the size of the preovulatory follicle was recorded between induced short and normal length cycles. Similarly, no difference in the size of the preovulatory follicle between physiological short estrous cycles and norgestomet-treated controls was evident in a study with beef cows (Shrick et al. 1993). When PG and GnRH were given during early diestrus, the preovulatory follicle present in the ovary was the first wave dominant follicle, which in

two-wave cycles in heifers reached its maximum size on Day 6 (Savio et al. 1988). The maximal diameter of the ovulating follicle was influenced by the stage of the cycle at the initiation of the GPG estrus synchronization protocol (Vasconcelos et al. 1999). During our experiments, the average size of the preovulatory follicle at ovulation day (14.1 ± 1.8 mm) in all included cases of heifers that were given PG and GnRH 24 h apart starting on Day 7 after ovulation, was slightly larger than the mean size of the second or third wave preovulatory follicle in heifers (13.0 ± 0.3 mm) according to Wolfenson et al. (2004). When heifers in our experiments were treated with PG during late diestrus, on Day 14 after ovulation, and given GnRH 24 h later, the size of the preovulatory follicle was even smaller, 11.9 ± 0.5 mm. This size difference of the preovulatory follicles in animals treated with PG and followed by GnRH 24 h later either on Day 7 or on Day 14 after ovulation was statistically significant.

A possible explanation for changes in follicle size during the estrous cycle in this study is that the size of the ovulating follicle is linked to hormonal changes around estrus. During early diestrus (Day 7) the average blood P_4 concentration was significantly lower in comparison with late diestrus (Day 14). Vasconcelos et al. (1999) reported a connection between follicle size and P_4 concentration in dairy cows. The lowered P_4 concentration during the luteal phase in dairy cows is linked to the increased size of the first wave dominant follicle (Lüttgenau et al. 2011), reported earlier in dairy heifers by Adams et al. (1992) and in beef cows by Pfeifer et al. (2009). In contrast, Giordano et al. (2012) gave 0.1 mg of gonadorelin to cows either during low or high P_4 (approximately 0.2 ng/ml and over 3 ng/ml, respectively). As a result, the average size of the dominant follicle in both groups was quite similar, 17.7 mm (14 to 21 mm) and 16.9 mm (14 to 24 mm), respectively, but this was not statistically evaluated.

In cyclic beef cows, the length of proestrus was altered when PG was given twice at a 12 h interval and followed by 0.1 mg of gonadorelin either 60 h or 36 h later during early diestrus (Bridges et al. 2012). This caused a significant decrease in the preovulatory peak concentration of estradiol-17β but the size of the ovulatory follicle at GnRH administration remained unaffected (approximately 12 mm). In contrast, follicular aspiration and induction of luteal regression with PG, followed by 0.1 mg of gonadorelin given when the dominant follicle reached 10 mm, significantly decreased the size of the preovulatory follicle (about -1.3 mm), shortened the proestrus (-1.5 days), and ovulation occurred about

1.1 d earlier than the spontaneous ovulation (Mussard et al. 2007). After induced ovulation, the mid-luteal P_4 concentration and the conception rate were both less in comparison with spontaneous ovulation. Mussard et al. (2007) concluded that follicular maturation is probably affected by the interaction of several factors, not directly connected to the size of the ovulating follicle, and thus follicles need to be physiologically mature prior to induction of ovulation in order to avoid decreased fertility.

Lüttgenau et al. (2011) assumed that the above-mentioned connection between blood P_4 concentration and follicle size during diestrus, noticed also in our experiment, was caused by an increase in LH pulse frequency during Days 9 to 15 after ovulation, but LH concentrations were not analyzed in their study. In our study, basal secretion of LH on Days 1, 3 and 5 after ovulation was similar between short and normal length estrous cycles. Reduced LH release on those days coincided with higher progesterone concentration. This combination of low basal LH and high P_4 concentration (or the high basal LH and low P_4 concentration) has been analyzed also in other studies: a significant increase in basal and mean LH concentrations occurred in animals with lowered P_4 during the growth of the dominant follicle (Pfeifer et al. 2009). The decreased or increased P_4 concentration is respectively known to increase or decrease the LH pulse frequency (Bergfeldt et al. 1996). In conclusion, the decreased size of the dominant follicle was caused by a P_4-induced decrease in LH concentration (Ginther et al. 2001a, 2001b), and LH and P_4 oscillations were positively and temporally related (Hannan et al. 2010). On the other hand, ovulation of a larger follicle has been reported to create a larger CL, leading to increased P_4 secretion (Pfeifer et al. 2009). This increase in luteal size and P_4 concentration was, however, not reported in the study of Lüttgenau et al. (2011).

6.4. Secretion of the preovulatory LH

In the experiments reported here, a five-fold increase in the dose of gonadorelin (0.1 mg vs. 0.5 mg) given 24 h after PG to cyclic dairy heifers did not have a significant effect on the preovulatory release of LH. In heifers on Day 15 after previous ovulation, i.e. around luteal regression, the amount of GnRH needed to induce a natural-like LH peak 1 to 2 h after treatment was as low as 5 μg of gonadorelin (Ginther and Beg 2012). The effect of varying doses of GnRH on LH response has been investigated in several studies with inconsistent results (see section 2. Review of literature), and the variance between LH peak

values in different studies has been wide even when comparable GnRH doses and/or products have been used. This variability is probably explained by differences in experimental settings and analysis methods, and makes comparison among studies difficult. Differences in experimental settings can be responsible for conflicting results in the preovulatory release of LH because the quantity of LH released appears to be significantly affected by the endogenous milieu of steroid hormones, i.e. the stage of the estrous cycle (Kaltenbach et al. 1974, Mikél Jensen et al. 1983). When diestrous cows during high P_4 (exceeding 3 ng/ml) were treated with 0.1 mg or 0.2 mg of gonadorelin, the preovulatory release of LH, measured as AUC, was significantly decreased in comparison with the same treatment given during low P_4 (in average 0.2 ng/ml; Giordano et al. 2012). The LH peak value after administration of 0.1 mg or 0.2 mg of gonadorelin during high and low P_4 was significantly different (3.3 ± 0.3 ng/ml and 15.7 ± 2.2 ng/ml or 8.5 ± 1.7 ng/ml and 23.6 ± 1.6 ng/ml, respectively), but the ovulatory response after both doses was similar irrespective of the P_4 concentration. The preovulatory release of LH exceeded 10 ng/ml more often during low P_4 (86%) in comparison with high P_4 (13%), and the time to LH peak tended to be reached more slowly during high P_4 when 0.1 mg of gonadorelin was used (1.3 ± 0.2 h) in comparison with other groups (approximately 0.8 or 0.9 ± 0.1 h), (Giordano et al. 2012). In a similar study by Giordano et al. (2013), 0.1 mg or 0.2 mg of gonadorelin was again given to diestrous cows. As a result, the LH response was dose-dependent only when a functional CL was present in the ovary. Similarly in beef heifers more LH was released in response to 0.1 mg or 0.2 mg of gonadorelin in animals with low (3 ng/ml) in comparison to high (7 ng/ml) P_4 concentration; pre-treatment with 0.25 mg of estradiol benzoate 8 h earlier did not increase the ovulatory response (Dias et al. 2010). Similarly to the studies above, Colazo et al. (2010) used 0.1 mg of gonadorelin in beef heifers and cows with low (3.0 ± 0.4 ng/ml) and high (5.7 ± 0.4 ng/ml) P_4 concentration, and reported a smaller and shorter release of LH and fewer ovulations in animals with high P_4 concentration in comparison with low P_4.

Apart from our studies, very few results on the preovulatory LH secretion preceding short estrous cycles have been published. In a study of Bridges et al. (2010), mean LH secretion, AUC and LH peak concentration after PG and 0.1 mg of gonadorelin administration either 2.25 d or 1.25 d (54 h vs. 30 h) apart were similar for induced short and normal length cycles.

6.5. Basal secretion of LH

The results for basal LH secretion reported in this thesis are in accordance with those reported by Swanson and Hafs (1971), Zolman et al. (1974), Schallenberger et al. (1984), Peters et al. (1994) and Cupp et al. (1995). The basal LH secretion values during the estrous cycle vary among studies, possibly due to differences in experimental settings, LH analysis methods and/or LH pulsatility detection methods. A more detailed discussion of different studies is included in section 2, the review of the literature.

6.6. Blood estradiol concentration

When estrus and ovulation were induced with PG and GnRH 24 h apart in cyclic dairy heifers, no difference in the level of blood estradiol-17β (E_2) concentration during 48 h before ovulation between subsequent induced short and normal length cycles was detected. The size of the preovulatory follicle was unrelated to the E_2 concentration during that time. In contrast, in postpartum beef cows exhibiting physiological short estrous cycles, the E_2 secretion at estrus was reported to be significantly less than in P_4-treated controls (Garcia-Winder et al. 1986, Garverick et al. 1988). The secretion of E_2 during the three days before first postpartum estrus was significantly more preceding normal length cycles than short cycles (Schrick et al. 1993). Also the E_2 concentration in the follicular fluid of postpartum beef cows was four times less if anticipated to exhibit a short cycle than in animals having a normal length cycle (Braden et al. 1989). The low preovulatory level of E_2 and impaired OR inhibition were postulated to be behind postpartum short estrous cycles (Mann and Lamming 2000). In the absence of P_4, endometrial OR levels could be decreased with exogenous E_2 and the degree of OR expression was related to the amount of E_2 secreted during estrus (Mann and Lamming 2000).

In contrast to our studies, the time interval between PG and GnRH has been more than 24 h in other studies investigating the release of E_2 during the periestrous period preceding induced short estrous cycles. Similarly to physiological estrous cycles reported in the previous paragraph, in cyclic beef animals decreasing the time interval between administration PG and 0.1 mg of gonadorelin from 2.2 d to 1.2 d significantly decreased the E_2 concentration around ovulation (Bridges et al. 2010). The peak concentration of E_2 during proestrus was also significantly decreased (Bridges et al. 2010). In most short cycle

cases (4/5), the preceding preovulatory E_2 peak concentration was less than 10 pg/ml, and when the concentration of E_2 was over 10 pg/ml, most cows (10/11) had a cycle of normal length. In a similar study, where PG and 0.1 mg of gonadorelin were given to cyclic beef heifers either 60 h or 36 h apart, the length of proestrus was decreased (Bridges et al. 2012). The preovulatory peak concentration of E_2 (8.9 ± 0.4 pg/ml vs. 6.7 ± 0.8 pg/ml, respectively) was again decreased (Bridges et al. 2012). In that study, despite the shortened proestrus, the P_4 concentration between Days 2 and 15 after GnRH administration was similar between groups. In contrast to short cycle studies above, the size of the preovulatory follicle at induced luteal regression, above or below 10 mm, did not lead to differences in E_2 secretion because preovulatory follicles were allowed to ovulate spontaneously (Robinson et al. 2005). Increased follicular size was associated with increased blood E_2 concentration (Atkins et al. 2008). In beef cattle large follicles secreted more E_2 on the day of ovulation induction with GnRH, and more P_4 on Day 7 after ovulation (Mesquita et al. 2014). In that study, follicular growth was manipulated in physiological limits with sequential treatments of exogenous progesterone, estradiol benzoate, PG and GnRH, in order to create small and large preovulatory follicles (Mesquita et al. 2014). Thus, secretion of E_2 can be an important determinant of follicular physiological maturation and initiation of estrus, but the absolute diameter of the follicle or the magnitude of GnRH-induced LH secretion are thought to be less important (Atkins et al. 2008).

Effects of giving PG and estradiol benzoate 24 h apart are very different from the results obtained after PG and GnRH are given 24 h apart. When PG is given to cyclic heifers at the beginning of follicular dominance of the second follicular wave (i.e. Days 8 to 13 after estrus), and followed by estradiol benzoate 24 h later, the time to the preovulatory release of LH was significantly decreased in comparison with control animals, but no significant effect on size of the preovulatory follicle or on the time to ovulation was noticed (Evans et al. 2003). When PG was given at the emergence of the follicular wave to cyclic heifers, and followed by estradiol benzoate 24 h later, the size of the preovulatory follicle was significantly decreased (Evans et al. 2003). Also time to estrus and time to the preovulatory LH peak were both decreased in comparison with controls (Evans et al. 2003). Neither of these treatment protocols above affected the size of the CL or the length of the following luteal phase. In conclusion, both GnRH and estradiol benzoate given 24 h after PG shorten the proestrus, but the effect on the following luteal phase is very different.

6.7. Endometrial receptors and enzymes analyzed with immunohistochemistry and real-time quantitative reverse transcriptase-polymerase chain reaction and their association with induced short estrous cycles

Our working hypothesis, that endometrial expressions of enzymes 20α-HSD and COX-II or receptors OR, ER and PR during metestrus and early diestrus differ for induced short and normal estrous cycles, was not supported. QPCR did not detect any significant difference between short and normal length cycles on Days 2 and 5 after ovulation. Moreover, semi-quantitative IHC did not indicate significant differences in the endometrial staining for ER or PR between induced short and normal cycles on those days. Boos et al. (1996), Dall`Aglio et al. (1999) and Robinson et al. (2001) among others reported that the follicular phase promotes endometrial PR and ER synthesis, and the luteal phase down-regulates them. The exact causes of OR up-regulation are still not completely understood, but Robinson et al. (2001) assumed two possible causes. First, PR during luteal phase loses its dominance via down-regulation due to progesterone acting on its receptors (Robinson et al. 2001). Such inhibition of OR by progesterone, or progesterone block, was suggested by McCracken et al. (1984) in sheep. Secondly, but less probably according to Robinson et al. (2001), E_2 might act via ER to up-regulate OR. A more detailed discussion on different studies concerning endometrial receptors and their regulation is provided in section 2, the review of the literature.

Our hypothesis was based on earlier information about the cause of premature $PGF_{2\alpha}$ release during physiological short estrous cycles (i.e. first cycle after calving): endometrial PR concentration on Day 5 after ovulation in cows exhibiting a physiological short cycle was significantly lower and endometrial OR concentration significantly higher than in cows with normal length cycles (Zollers et al. 1993). According to those authors, E_2 levels prior to ovulation determined the length of the subsequent P_4 dominance, i.e. luteal phase, through altered degree of PR expression. At the time of luteal regression during short cycles, high concentrations of endometrial OR were present, and peaks of oxytocin and PGFM coincided, allowing the luteal regression to be initiated (Hunter 1991). At the time of maternal recognition of pregnancy, luteal regression needed to be prevented via release of foetal interferon-τ, which had a direct suppressive effect on the translation of OR and ER (Robinson et al. 2001).

In addition to such time-specific changes, ER and PR in cattle undergo spatial changes in the uterus, which further complicate the interpretation of study results: ER is expressed in all the layers of endometrium during estrus, in deep glands during the whole estrous cycle, and in increased amounts in the luminal epithelium during the mid-luteal phase, and PR is expressed mostly in the stroma, and the expression is maximal during estrus and the early luteal phase (Robinson et al. 2001). Okumu et al. (2010) prefer to use IHC when cell-specific changes are analysed. Cell-specific changes cannot be detected using QPCR because during the process of QPCR tissue samples are homogenized.

Prior to our study, to our knowledge, no PCR-studies were conducted to investigate endometrial receptor concentrations specifically during induced short cycles in cattle. When the length of proestrus was decreased (PG and 0.1 mg of GnRH given either 60 h or 36 h apart), and in response to that, estradiol secretion of the preovulatory follicle was significantly reduced, the concentration of mRNA for ESR1 during late diestrus (Day 15 after GnRH) fell, but the concentration of mRNA for OR remained unchanged (Bridges et al. 2012). On Day 15, the staining intensity in IHC for PR in deep glands was significantly more when the length of proestrus was 60 h than when 36 h. In a similar experimental setting as in Bridges et al. (2012), no difference in endometrial PR, OR and ERα expression was detected with QPCR one day before or on Day 7 after ovulation, when 10 µg of buserelin was given to cows either 40 or 60 h after 0.15 mg of cloprostenol, or not at all (Bollwein et al. 2010). When buserelin was given 40 h after cloprostenol, 25% of the cows had no CL on Day 7 after ovulation (6 % when the time interval between treatments was 60 h and 12 % when no GnRH was used). Bollwein et al. (2010) concluded that changes in the expression of endometrial receptors are not a cause of decreased fertility after the above-mentioned synchronization protocol where proestrus is decreased, but inadequate follicular and luteal development are. In a recent study by Mesquita et al. (2014), follicular growth was manipulated in physiological limits with sequential treatments of progesterone, estradiol, $PGF_{2\alpha}$ and GnRH to create small and large preovulatory follicles. On Day 7 after the last treatment with GnRH (= D0, used to induce ovulation), endometrial samples were collected at slaughter. Larger follicles had secreted significantly more estradiol on the day of ovulation induction with GnRH and the CL formed after them secreted significantly more progesterone on slaughter day. ERα on Day 7 was up-regulated, and OR down-regulated in animals with larger preovulatory follicles, and estradiol on Day 0 was positively correlated with ERα on Day 7. The authors

concluded that the size of the preovulatory follicle, through changes in the peri-ovulatory secretion of estradiol and luteal phase secretion of progesterone, affects the expression of important endometrial genes during diestrus, and might affect fertility.

7. CONCLUSIONS

When PG and GnRH are given 24 h apart to cyclic dairy heifers

- ✓ short estrous cycles may occur both during early and late diestrus, i.e. Day 7 or Day 14 after ovulation
- ✓ the dose of GnRH, 0.1 mg vs. 0.5 mg of gonadorelin, does not affect the occurrence of induced short estrous cycles
- ✓ the induced preovulatory release of LH is similar for short and normal length estrous cycles, and unaffected by the dose of GnRH (0.1 or 0.5 mg of gonadorelin)
- ✓ basal secretion of LH on Days 1, 3 and 5 after ovulation is similar for short and normal length estrous cycles, and lower LH release on those days coincides with higher progesterone concentration
- ✓ the size of the preovulatory follicle is unrelated to the occurrence of short or normal length estrous cycles
- ✓ early diestrous (Day 7 after ovulation) dominant follicles are larger than late diestrus dominant follicles (Day 14 after ovulation), probably due to lower preovulatory progesterone concentration and thus increased LH concentration during early diestrus in comparison with late diestrus
- ✓ the timing of GnRH administration (0 h or 24 h after PG) significantly affects the ovulatory response and the incidence of short estrous cycles

When PG and GnRH are given 24 h apart to cyclic dairy cows

- ✓ the size of the preovulatory follicle is unrelated to the occurrence of short or normal length estrous cycles
- ✓ the size of the preovulatory follicle is unrelated to the amount of estrogen secreted at estrus
- ✓ the timing of GnRH administration (0 h vs. 24 h after PG), significantly affects the ovulatory response and the incidence of short estrous cycles
- ✓ no difference in endometrial expression of receptors OR, ER and PR or enzymes 20α-HSD and COX-II occurs between short and normal length estrous cycles on Days 2 and 5 after ovulation

In practice, decreased fertility warrants use of hormonal estrus synchronization protocols to control follicular waves and luteal regression to achieve acceptable pregnancy rates. During protocols that use sequential treatments of PG and GnRH, our results above should be taken into account to avoid decreased fertility due to occurrence of induced short estrous cycles. PG and GnRH should not be given simultaneously, and when given 24 h apart, many animals will exhibit a short estrous cycle. Ovulation failure is common if PG and GnRH are given simultaneously.

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