Effects of CIDR and eCG with estradiol-17β or estradiol cypionate on the follicular wave emergence synchronization in seasonally anestrous ewes

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Table of content

iv

LIST OF TABLES

LIST OF FIGURES

Abstract

Estrous, hormonal, and ovarian responses in anestrous ewes treated with different synchronization treatments were evaluated. Fourteen Texel ewes received controlled-internal-drug-releasing devices (CIDR) on Day -12 and eCG on Day 0. Animals received 350 μg estradiol-17β (E_2; n=5), 350 μg estradiol cypionate (ECP350; n=4), or 70 μg ECP (ECP70; n=5) on Day -6. Mean E_2 and FSH concentrations fluctuated during 48 h after E_2/ECP ($P<0.05$). Only one ECP70 ewe did not exhibit estrus. Estrous and ovulation features, follicular wave emergence synchrony, number of CL, and LH, FSH, P_4 and most E_2 patterns, in E_2 and ECP70 ewes were similar ($P>0.05$). Follicular wave emerged earlier in E_2 than ECP70 ewes ($P<0.05$). No ECP350 ewes ovulated by 7 days after eCG. Mean P_4 concentrations increased until 7 days after ovulation ($P<0.001$). Three E_2 and two ECP70 ewes became pregnant. Perhaps E_2 can be replaced with ECP70 in the CIDR-E_2-eCG anestrous ewe synchronization protocol.

LIST OF ABBREVIATIONS USED

AE	After E_2/ECP
BCS	Body condition score
BS	Blood sampling
BW	Body weigh
CIDR	Controlled internal drug releasing device
CH	Corpus hemorrhagicum
CL	Corpus luteum
DBD	DNA-binding domain
DE	Digestible Energy
E_2	Estradiol-17β
EB	Estradiol benzoate
eCG	Equine chorionic gonadotropin
ECP	Estradiol cypionate
EIA	Enzyme immunoassay
ERE	Estrogen-responsive element
ER	Estradiol receptor
EV	Estradiol valerate
FGA	Fluorogestone acetate
FSH	Follicle stimulating hormone
GnIH	Gonadotropin-inhibitory hormone
GnRH	Gonadotropin-releasing hormone
GPER	G protein-coupled ER1

hCG	Human chorionic gonadotropin
LBD	Ligand-binding domain
LH	Luteinizing hormone
MAP	Medroxyprogesterone acetate
MFD	Maximum follicle diameter
MGA	Melengestrol acetate
NEG	Net Energy for Gain
NEL	Net Energy for Lactation
NEM	Net Energy for Maintenance
NO	Nitric oxide
NR	Nuclear receptor
P_4	Progesterone
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PMSG	Pregnant mare serum gonadotropin
PRIDs	Progesterone releasing intravaginal devices
RFRP	RF-amide related peptide
RIA	Radio immunoassay
SM	Second messenger
TAI	Timed artificial insemination
TF	Transcriptional factor
TUS	Transrectal ultrasonography

ACKNOWLEDGEMENTS

CHAPTER 1: INTRODUCTION

As the world population is growing rapidly, demand is expected to significantly rise for meat and dairy products [1,2]. It is also expected that immigrants who consume lamb and goat more than beef, poultry, and pork will double in number by 2031 in Canada [3]. Thus, scientists, veterinarians, and extension specialists should accelerate efforts to find effective ways to provide population demands. The sheep industry in Canada in comparison with cattle and beef industries is relatively small. There are 72,700 farms producing cattle, but only 9,390 sheep farms. In 2020, Canadian farmers had a total of 11.2 million cattle, while they held only 795,500 sheep and lambs on their farms [4]. Furthermore, consumption of beef per capita was about 27.8 kg in 2020, while this amount was about 32 kg in 2000. However, the evidence shows a slight increase in consumption of lamb per capita, as it was 0.94 kg in 2000 and reached 0.98 kg in 2019 [5]. Production of sheep is rather more complicated than the production of some other farm animal species such as cows and chickens, as sheep show seasonal reproductive cycles. Thus, pregnancy and lambing rates annually are low (once a year) since they cannot naturally breed out of the breeding season (spring and summer). To remove the seasonality of lamb production, developing successful out of breeding season techniques are needed. Hormonal treatment is a common and effective way for animal reproduction management. There are many studies in bovine reproductive synchronization in order to improve their output [6–15], but the lack of such investigations in the ovine encourages researchers to study it. Estrous synchronization is an important means to improve ewe reproductive performance. Follicular wave emergence and ovulation can be synchronized through modified estrous synchronization protocols. A predictable and synchronized ovulation can improve timed artificial insemination (TAI)

and embryo transfer programs. In addition to increasing lambing rates, breeding synchronization contributes to genetic improvement. It can also synchronize and shorten lambing, resulting in more efficient use of animal facilities and labor. Breeding synchronization also improves the management of pregnant ewes, resulting in an increase in lamb survival and more homogenous weights in newborn lambs. Thus, this protocol has positive effects on lamb marketing [16]. Also, estrous synchronization assists in fixing the time of breeding; therefore, the time of parturition can be scheduled for a favorable season when newborns can be grown in an appropriate environment with enough food [17]. In order to synchronize estrus, progesterone (P_4) priming treatment in different forms and time frames has been used during the breeding and non-breeding season [17–23]. There are also some studies that show administration of E_2 with a P_4 treatment not only improves the percentage of ewes exhibiting estrus, but also can synchronize follicular wave emergence in seasonally anestrous ewes [18,24]. While, a P_4 treatment without E_2 injection did not synchronize the follicular wave emergence [18]. However, in many countries E_2 is not allowed to be commercially used. Thus, researchers are studying different forms of estrogen to find a proper replacement for E_2. It has been reported that estrogen esters improved bovine reproductive performance [14,15,25,26]. However, there is a gap in knowledge in sheep studies which encourages researchers to evaluate effects of estrogen esters on ewe reproductive performance as well. Estradiol cypionate is the only available intramuscular injectable estrogen ester in North America, which is typically used for estrous induction in cows and heifers. It is believed that ECP is as effective as E_2 in ewe synchronization programs. The objective of this project was to investigate ECP as an

alternative to E_2 to synchronize follicular wave emergence along with an estrous synchronization protocol in seasonally anestrous ewes.

CHAPTER 2: LITERATURE REVIEW

Through the literature review, related topics to this project will be discussed in the following section. Seasonality of the reproductive activity in sheep, reproductive cycle and folliculogenesis in the ewe, follicular wave emergence and endocrine profiles of the ovine estrous cycle. In addition, common methods of estrous and follicular wave emergence synchronization, and their results were examined.

2.1 Sheep Reproductive Structures, Cycles, and Phases

2.1.1 Seasonal Reproduction in Sheep

Sheep are seasonal polyestrous breeders. Photoperiod is known as the major factor for regulating this, however, several other factors such as breed, environmental temperature, nutrition, and time of lambing affect this phenomenon as well [27]. Fall and winter in most breeds in the northern hemisphere are considered as the breeding season [28]. The retina transfers photoperiodic information to the pineal gland, where light signals affect melatonin secretion. Melatonin is secreted more at night and less in the day [29]. The amount of melatonin secretion affects the hypothalamus by regulating the pulsatile secretion of gonadotropin-releasing hormone (GnRH) [29] via control of secretion of RF-amide related peptide (RFRP)- also known as gonadotropin-inhibitory hormone (GnIH). This hormone has a key role in the regulation of GnRH secretion by season [30]. During the anestrous season, when the secretion of melatonin is decreased, the secretion of GnIH is increased [31], resulting in the reduction of kisspeptin levels. Kisspeptins are peptides that stimulate the secretion of GnRH, hence, during the non-breeding season when the levels of kisspeptin are decreased the secretion of GnRH is reduced as well [31]. Variations in GnRH secretion cause changes in luteinizing hormone (LH) release that has an important

role in ovulation [29,32]. With low LH secretion ovulation and sexual behavior are absent-clear features of seasonal anestrus [33].

2.1.2 Estrous Cycle

In sheep the estrous cycle regulates breeding, the cycle duration is about 16-17 days [28]. The estrous cycle includes two main phases: follicular and luteal. Each phase includes two stages: the follicular phase is divided into proestrus and estrus and luteal phase is divided into metestrus and diestrus [32].

2.1.2.1 Follicular Phase

Proestrus

Proestrus is initiated at the start of P_4 reduction as a result of corpus luteum (CL; ovarian structures after ovulation) regression, this phase ends at the onset of estrus. During proestrus, antral follicles (ovarian structures that contain eggs) mature and the reproductive tract readies for estrus [32]. Maturing antral follicles secrete E_2; thus, E_2 concentrations increase considerably in this stage [32].

Estrus

Estrus is the period when the ewe accepts the ram and stands for mating, and this stage ends at ovulation. By the increasing of E_2 from mature follicles, estrus starts and continues for about 18-48 h (average 30 h); E_2 is the dominant hormone in this stage. In ewes, ovulation occurs about 24-30 h after the start of estrous behaviors [32]. Also, during estrus, the surges of gonadotropins (LH and follicle stimulating hormone (FSH)) occur which cause ovulation [34].

2.1.2.2 Luteal Phase

Metestrus

Metestrus starts after ovulation, in this stage serum E_2 concentrations decrease and P_4 concentrations increase [32]. The structure of the ovulated follicle undergoes modification and forms a structure known as the corpus hemorrhagicum (CH)- its appearance is bloody clot-like on the ovary. The CH is formed instantly after ovulation and remains for about three days [35]. Then the CH loses its bloody appearance and begins to enlarge forming the CL [32].

Diestrus

Diestrus is the longest period of the estrous cycle, the CL has completely developed and matured. Thus, there is high secretion of P_4- the dominant hormone in this stage. There is no estrous behavior during diestrus and this stage ends at the time of CL regression [32]. After this stage, the regressed CL changes to an inactive structure known as the corpus albicans [36].

2.1.3 Anestrus

Anestrus is the time when females do not have estrous cycles. At this time there is no ovulation and active CL. A lack of GnRH secretion leads to the lack of LH and FSH secretion, resulting in anestrus [32]. Serum E_2 concentrations also remain basal during anestrus [27].

2.1.4 Folliculogenesis

Folliculogenesis is defined as the growth and development of follicles. As the antral follicles grow toward the ovulatory stage, three important events occur: 1) recruitment,

selection, and dominance; 2) gonadotropin requirements: FSH for recruitment and LH for growth, dominance, and ovulation; 3) variability in the number of waves in every cycle and number of follicles in every wave [37].

2.1.4.1 Follicle Classification

When a layer of squamous follicular cells surrounds an oocyte, the primordial follicle is created. A transitory or intermediate follicle is defined as an oocyte that is surrounded by squamous and cuboidal cells (Figure 1) [28]. When a layer of cuboidal granulosa cells surrounds the oocyte, they are called primary follicles. Follicles with two or more layers of cuboidal granulosa cells and oocytes encapsulated by the zona pellucida are known as secondary follicles (Figure 1). Pre-antral follicles contain one or more small fluid-filled cavities and layers of interna and externa thecal cells surrounding a basement membrane that encapsulates the granulosa cells. Antral follicles contain only one centrally located cavity that is filled with follicular fluid [28]. Antral follicles consist of three different cell layers: theca externa, theca interna, and granulosa cell layers. Granulosa cells have FSH receptors and produce E_2, inhibin, and follicular fluid [32]. Antral follicles in the ewe grow in a wave-like pattern from a small antral follicle pool with an interval of 4-5 days enlarging to $\geq$ 5 mm in diameter before ovulation or atresia [38].

2.1.5 Follicle Regression

Atresia, the process of follicle regression, is an important aspect of folliculogenesis. After selection, the ovulatory follicle(s) become(s) dominant and develop(s) to either ovulate or go through atresia, while the subordinate follicles undergo atresia [39]. In the ewe depending on the breed or estrous cycle stage 50-70% of large antral follicles become atretic [39].
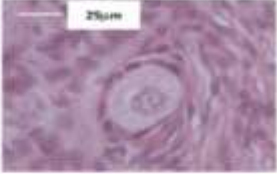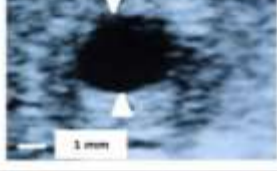
Follicle category and features	Ovine follicular images
Primordial An oocyte surrounded by a partial or complete layer of squamous follicular cells	
Intermediate/Transitory Follicles containing both squamous and cuboidal follicular cells	
Primary Follicles with a single layer (complete or partial) of cuboidal granulosa cells	
Secondary Follicles with multiple layers of granulosa cells and oocytes surrounded by the zona pellucida	
Pre-antral Follicles with one or more small areas filled with follicular fluid and distinctive layers of theca cells separated from granulosa cells by the basement membrane	
Antral (Graafian) Follicles with a single, centrally located fluid-filled cavity	

Figure 1. Ovarian follicles' categorization and characteristics in the ewe from the primordial follicle stage to the emergence of antral follicles during folliculogenesis. Scale bars on the histology images for microscopic follicles indicate 25 µm. The solitary scale bar on the ovarian ultrasound image (at the bottom) indicates 1 mm. Used by permission [28].

2.1.6 Follicular Wave

This ovarian follicular development process in the ewe is characterized by the frequent emergence of antral follicles which grow until they ovulate or become atretic. Generally, there are three or four follicular waves in every estrous cycle (Figure 2) [28,40]. The pattern of antral follicular growth is directly related to increasing daily serum FSH concentrations [28]; peaks of daily serum FSH concentrations occur before follicular wave emergence. In addition, the starting transient rise in serum E_2 concentrations is essential for follicular wave emergence [28,41]. In non-prolific ewes, the first follicular wave emerges around the day of ovulation (Day 0), while the second, third, and fourth waves emerge on Days 5, 9, and 12 after ovulation, respectively (Figure 2) [28]. However, in prolific ewes, the first wave emerges around Day 1, the second, third, and fourth waves emerge on Days 6, 10, and 13, respectively (Figure 2). Prolific breeds tend to have more and smaller preovulatory follicles than non-prolific breeds [28]. In some prolific breeds, the high ovulation rate is due to the ovulation of 50% of the ovulatory-sized follicles from the penultimate wave along with all follicles from the last wave of the estrous cycle [28]. While in non-prolific ewes, only 10% of the ovulatory-sized follicles from the penultimate wave and 77% of all follicles from the final wave ovulate [28].
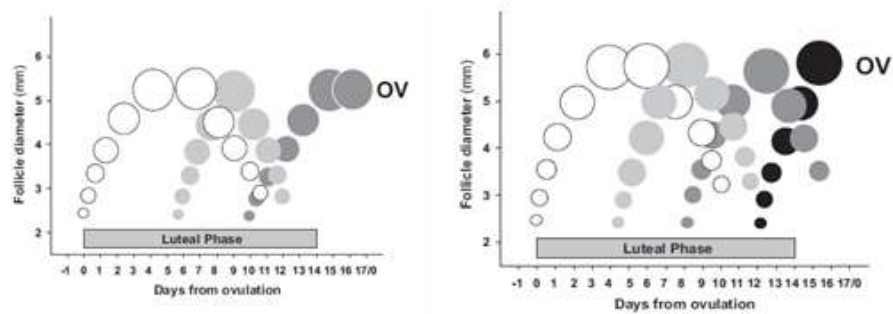
Figure 2. The pattern of the follicular wave emergence during the estrous cycle in ewes. Used by permission [28].

2.2 The Endocrine Profiles of the Ovine Estrous Cycle

2.2.1 Gonadotropin-Releasing Hormone

The most important phenomenon for normal reproductive function and fertility is the hormonal regulation of the hypothalamic-pituitary-gonadal axis. Hypothalamic GnRH neurons synthesize GnRH and release it into the hypophyseal portal circulation to act on the anterior pituitary [42]. The pulsatile discharge of GnRH regulates FSH and LH secretion [42].

2.2.2 Luteinizing Hormone and Follicle Stimulating Hormone

Gonadotropins enter the peripheral circulation, affecting ovarian control of folliculogenesis, ovulation, and steroidogenesis [42]. The decrease in P_4 and increase in E_2 secretion throughout the last phase of the estrous cycle (diestrus) cause the increase in GnRH concentrations [28]. In the late follicular phase when the P_4 concentrations are low and E_2 concentrations are at their maximum level, the LH surge begins about 30 h after P_4 starts to decline (Figure 3A,B) and lasts for approximately 10 h if GnRH concentrations remain elevated [32,43,44]. The LH secretion pattern in sheep is pulsatile (Figure 3A) [44]. The frequency of pulsatile LH release increases considerably during the follicular phase. The increased pulse frequency causes a sustained increase in circulating LH which is essential for the initiation of the preovulatory E_2 rise and for the successful completion of ovulation (Figure 3A,B) [45,46]. Increasing concentrations of E_2 produced by preovulatory follicles causes the preovulatory LH surge (Figure 3A,B), the surge is the most important factor for ovulation [47]. There is one pulse every 1-2 h before and one pulse every 20-30 min throughout the preovulatory LH surge [27]. During the estrous cycle, the negative feedback of ovarian steroids controls the secretion of GnRH and gonadotropins but at the

time of the preovulatory GnRH/LH surge E_2 has a positive feedback effect on the neuroendocrine axis. This results in a massive release of LH from the pituitary gland during the preovulatory period (Figure 3A) [47]. An increase in the amplitude and frequency (one pulse every 20-30 min) of LH pulses and basal LH concentrations brings about the preovulatory LH surge [28]. The preovulatory GnRH, LH, and FSH surges reach an apex about 14 h before ovulation. The frequency of LH pulses is reduced by the increase of P_4 after ovulation (Figure 3A,B) [48].
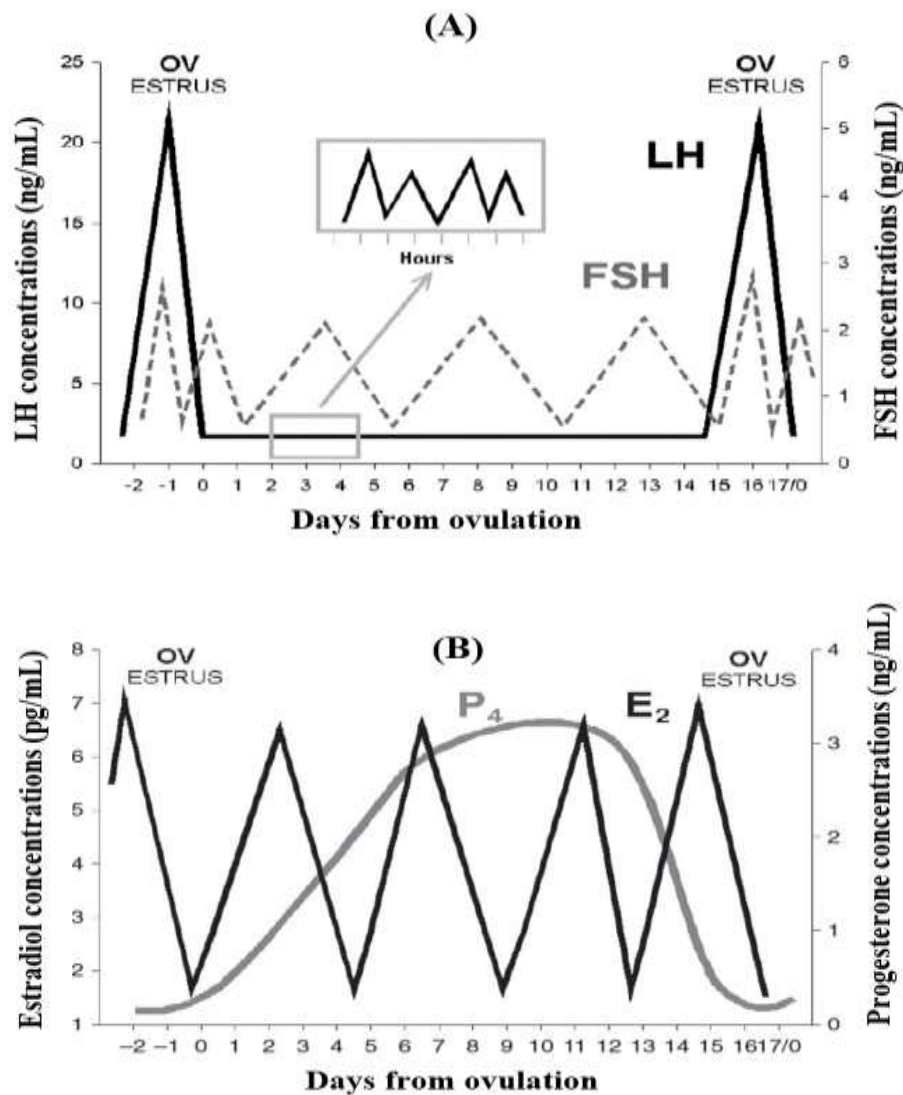
Figure 3. Changes in LH, FSH (Panel A), estradiol and progesterone (Panel B) concentrations throughout the ewe estrous cycle. Used by permission [49].

11

Follicle-stimulating hormone is the main hormone which controls follicle development; FSH secretion is stimulated by GnRH but is negatively regulated by E_2 and inhibin A [50]. An increase in FSH secretion causes several antral follicles to develop. However, the negative feedback of E_2 and inhibin from the recruited follicles causes a reduction in FSH (Figure 3A,B) to prevent more follicular selection when the dominant follicle is able to use LH, supporting its continued development [50]. In the ewe, regular fluctuations in daily serum FSH concentrations about every 4-5 days occur during the ovulatory cycle and anestrous season (Figure 3A) [28]. During the preovulatory period, FSH secretion occurs at two successive surges. The first one is concurrent with the preovulatory LH surge and the second one occurs between 20-36 h after it (Figure 3A). The amplitude of the second FSH release is lower, however, its duration is longer (20-24 h) than the preovulatory FSH surge (11-12 h; Figure 3A) [28].

2.2.3 Estradiol and Progesterone

In the ewe, E_2 secretion has a pulsatile pattern [44]. There are 3-4 rises in circulating E_2 concentrations throughout the estrous cycle (Figure 3B) [28]. Each coincides with the termination of the largest follicle development in the follicular wave [51]. A day after the beginning of CL regression an increase in E_2 concentrations is observed (Figure 3B); this increase is associated with the increase in LH pulse frequency (Figure 3A,B). Throughout the follicular phase the increase of E_2 secretion causes an increase in the density of LH receptors in preovulatory follicles. Then E_2 concentrations decrease to a minimum amount 16-24 h after the LH surge (Figure 3A,B) [28]. Estradiol is one of the most important factors for preparing animals to mate. It also causes increased blood flow in the genital

tract, increased mucosal secretion, the start of uterine gland growth, and increased myometrial tone [32].

In the ewe, P_4 secretion has a pulsatile pattern [52]. Following ovulation, the CL starts to produce increasing amounts of P_4 until Day 7 (Figure 3B). Afterward, P_4 concentrations in the peripheral plasma remain at a plateau until Day 12, then rapidly decrease and reach their lowest concentrations prior to estrus and ovulation (Figure 3B) [28]. Progesterone is needed for pregnancy establishment and maintenance [36]. This hormone controls the periodic increase of serum FSH concentrations and numbers of follicular waves through a negative feedback mechanism in cyclic ewes [28].

2.2.4 Inhibin and Prostaglandin $F_{2\alpha}$

Inhibin is secreted from ovarian granulosa cells, and regulates FSH secretion by a negative feedback mechanism [53]. During follicle development when the number of granulosa cells increases the production of inhibin increases as well. Thus, as the follicles enlarge FSH secretion is reduced [53]. In addition to its FSH secretion regulatory role, inhibin level is an indicator of the number of developing follicles and ovulation rates [54]. Prolific breeds produce low amounts of inhibin. The low concentrations of inhibin lead to an increase in FSH secretion and subsequently increased follicular development and ovulation rates [55].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a luteolytic hormone. This is secreted by uterine endometrial glands in ruminants [28]. During the late luteal phase, $PGF_{2\alpha}$ concentrations in the endometrium are higher than during the mid-luteal phase [56]. The reduction of circulating P_4 concentrations at the beginning of luteolysis increases pulsatile $PGF_{2\alpha}$ secretion. Throughout the luteal phase, $PGF_{2\alpha}$ is released into the uterine vein and by Day

13

13 sufficient amounts of $PGF_{2\alpha}$ reach the ovary inducing CL regression and subsequently causing a reduction in P_4 secretion [28].

2.3 Estradiol-17β, Estradiol Cypionate, Estrogen Receptors, and Ligand-Dependent Mechanisms of Action

Estradiol-17β is the major estrogen hormone, consists of 18 carbons with an aromatic ring and a hydroxyl group at position 17β (Figure 4A). The liver and many other tissues can convert E_2 to its metabolites (estrone, estriol, and their conjugates e.g., sulfates and glucuronides) through dehydrogenation, hydroxylation, and conjugation. Conjugation increases the water solubility of lipophilic steroids. Thus, they can better circulate and be excreted into the bile and urine. This is an important factor in the regulation of the serum concentrations of estradiol [57–60].

Estradiol cypionate is a fatty acid (cypionic acid) ester of E_2 (Figure 4B). Esterification of estrogens with fatty acids forms much more lipophilic estrogen metabolites with a longer half-life and more sustainable action. These esters mostly tend to be present in tissues than in the blood [60,61]. Intramuscular injection of estradiol fatty acid esters leads to the formation of a microcrystalline depot at the site of injection and sometimes in fat tissue, then the ester is slowly released and hydrolyzed in the liver and other organs. In comparison with shorter chain fatty acids, esters with a longer fatty acid chain have more lipophilicity but a lower rate of hydrolyzation. Esters with a longer fatty acid chain also have a more sustained action but lower serum concentrations [57,62]. Fatty acid esters of estradiol do not have a significant affinity to estradiol receptors (ERs). So, before imposing any estrogenic effects, estradiol has to be cleaved by enzymatic hydrolysis [57,60–63].
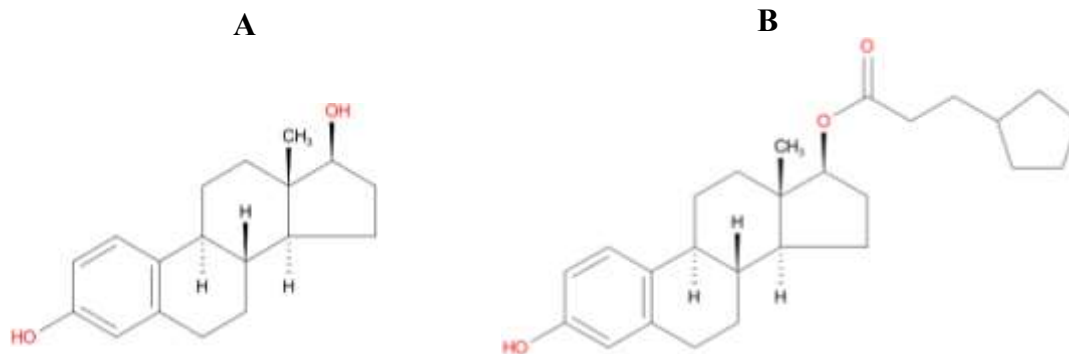
Figure 4. Molecular structures of estradiol-17β (A) and estradiol cypionate (B) [64]

Estradiol receptors act as mediators for the cellular signaling of estrogens. In mammals, ERs have two subunits, ERα and ERβ, whit their sequences being highly similar. Both these subunits are classified in the nuclear receptor (NR) family that represents a large group of transcriptional regulators [57,58,65,66]. Same as the other NRs, ERs contain some protein domains- different domains play various roles via binding to their hormonal activator and target gene (estrogen-responsive element (ERE)) [58,65].

Estrogen ligands such as E_2 trigger regulatory responses in the cells through two genomic mechanisms, direct and tethered pathways, and a non-genomic mechanism (Figure 5). In the direct pathway, E_2 binds to the ligand-binding domain (LBD), then the DNA-binding domain (DBD) directly interacts with ERE, and the structure of ER protein changes. This change increases the interconnection of the RNA polymerase II complex and its target genes and eventually changes the transcription rates [58,65].

In the tethered mechanism after E_2 attaches to LBD, E_2-ER interacts with DNA indirectly through other transcriptional factors (TF) [58,65].

In the non-genomic mechanism, E_2 activates either ER itself or a special receptor on the cell membrane. It also can activate ER by sending a signal in the cytoplasm. This interaction causes second messengers (SM) to affect ion channels or nitric oxide (NO) levels in the cytoplasm. This set of reactions triggers rapid physiological responses in the cell without any gene interference [58,65]. It has been shown that non-genomic mechanisms cannot direct main estrogen hormone responses. However, genomic mechanisms have a key role in the endocrine activity of estrogens. Studies on mice and rats have demonstrated that G protein-coupled ER1 (GPER) acts in nonreproductive systems such as cardiovascular and regulation of calcium in the kidney [58,65]. G protein-coupled ER1 belongs to the superfamily of 7-transmembrane receptors. These receptors share a basic structure containing seven highly conserved α-helical loops that can be situated on the cell membrane, although, GPER is mainly localized intracellularly. Despite structural similarity among this family of receptors, each subtype has a set of specific signal transduction activities, thus, estrogens binding to GPER is highly selective [67,68].

Studies on mice and rats have also indicated that life is possible without both or any ER subunits, but their elimination has a critical effect on reproductive functions [58]. The ERα has a dominant biological role among ERs, lack of ERα impacts the hypothalamic-pituitary-gonadal axis, uterine functions and generally disrupts the fertility of both sexes [65].

In comparison to ERα, eliminating ERβ in mice and rats causes reduced effects in reproductive performance. While males showed normal fertility without ERβ, in females the LH surge, ovulation, and eventually pregnancy rates were disrupted. There is also some evidence for the contribution of ERβ in the normal differentiation of granulosa cells [65].

In a study on seasonally anestrous ewes, it has been shown that ERβ-immunoreactive cells are detectable through the hypothalamus. Additionally, GnRH neurons can express ERβ [69].
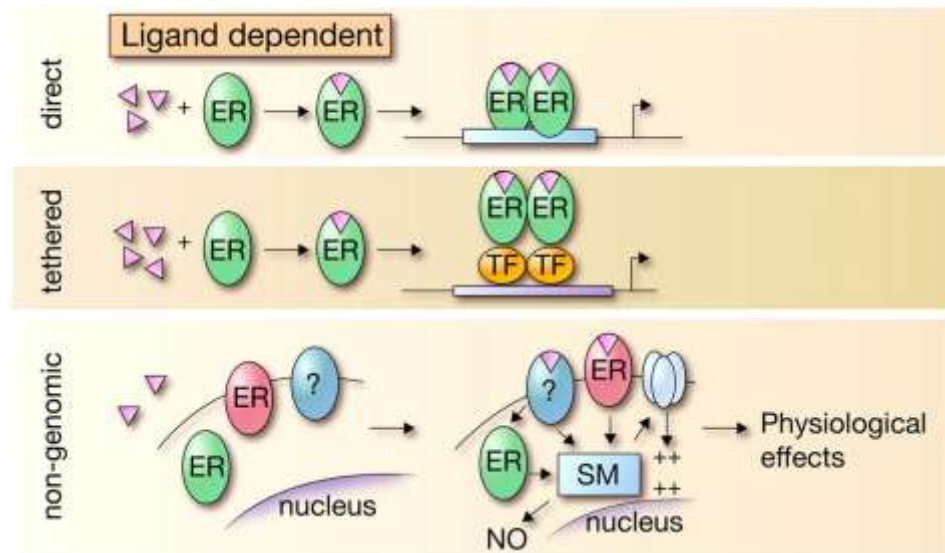
Figure 5. Different ligand-dependent regulatory mechanisms of ERs. In the direct pathway after the ligand binds to the ER, it directly binds to the ERE to modulate transcriptional activity. In the tethered pathway, the ER-ligand regulates the gene transcription indirectly via TF. In the non-genomic pathway, ligand activates ERs or a special receptor on the cell membrane or in the cytoplasm. This activation leads SM to affect some ion channels and NO concentrations in the cytoplasm. These reactions cause fast physiological responses [58]. Used by permission.

2.4 Ewe Fertility and Genetics, Nutrition, and the Ram Effect

The genotype is the most important intrinsic factor influencing the fertility of farm animals. Different genes control the diversity of ovulation rates in ewes, as a result, some breeds are prolific, nonprolific, or even infertile [70]. Nutrition is another important factor which affects the ovulation rate. Insufficient energy intake may have negative effects on ewe reproductive performance [70]. Flushing causes an increase in ovulation rate in animals receiving nutrient supplementation and a high energy diet before and during mating. A better body condition at mating and improved lambing rates are results of

flushing [70]. Introduction of a ram to a group of ewes increases the frequency of LH pulses and E_2 secretion causing an LH surge and ovulation, while no significant changes are observed in FSH secretion [71].

2.5 Follicle Size and Hormone Profiles During Seasonal Anestrus

During the non-breeding season follicles grow and undergo atresia, however, their size is similar to those detected during the luteal phase [27]. During the anestrous season there is follicular wave development and steroid secretion, however, follicles normally do not ovulate. Gonadotropins are released and there are many steroid negative and positive feedback mechanisms [27]. The pattern of E_2 secretion resembles the breeding season- an E_2 increase after every LH pulse. However, the frequency of GnRH and LH is very low- only one LH pulse every 8-12 h compared to one pulse every 3-4 h in the mid-luteal phase. There is no rise in tonic LH secretion and no increase in pulse frequency of GnRH and LH in seasonally anestrous ewes [27]. During the non-breeding season plasma P_4 concentrations remain at undetectable levels. Follicle stimulating hormone concentrations are the same in and out of the breeding season [27].

2.6 Estrous Synchronization

Estrous synchronization of cycling and anestrous animals involves mimicking the estrous cycle which causes a high percentage of a group of females to go into estrus in a short time (e.g., 24-48 h after treatment). It is especially helpful in sheep since they lack obvious heat signs. Feed additives, subcutaneous implants, intramuscular injections, and intravaginal devices are used for estrous synchronization protocols. Ewe reproductive performance after estrous synchronization is greater in the breeding season than in the

anestrous season [19,72]. The focus of the next sections is mainly on estrous synchronization during the non-breeding season.

2.6.1 Prostaglandin F$_{2\alpha}$

Administration of PGF$_{2\alpha}$ in animal reproductive management could be a preferred protocol since it is quickly metabolized and is not accumulated in tissues [17]. A PGF$_{2\alpha}$ treatment causes CL regression, although an active CL is needed to respond to exogenous PGF$_{2\alpha}$ [17]. The CL responds to PGF$_{2\alpha}$ from Day 4 of the estrous cycle to the day of natural luteolysis (12-15 days after ovulation) [28,73]. Thus, in anestrous animals or in the early/late luteal or follicular phase the CL does not respond to PGF$_{2\alpha}$ treatment. Observation of the estrous cycle stage in a group of females is difficult, thus it is necessary to apply two PGF$_{2\alpha}$ injections 9-10 days apart. With this procedure, almost all ewes will be in the mid-luteal phase at the second injection and will respond to the PGF$_{2\alpha}$ treatment during the breeding season. Although PGF$_{2\alpha}$ causes luteolysis and ovulation, significant lower fertility after this treatment has been reported [16,17]. Several studies investigated the administration of PGF$_{2\alpha}$ out of season. In seasonally anestrous ewes treated with two PGF$_{2\alpha}$ injections 11 days apart, the estrous onset, response, and pregnancy, and lambing rates were about 46 h after treatment, 57%, 70%, and 76%, respectively [74,75]. It has been reported that insertion of P$_4$ releasing intravaginal devices (PRIDs) for 11-12 days, a PGF$_{2\alpha}$ injection nine days after device insertion, an and eCG injection 48 h before or at device removal is required during the anestrous season [16,76].

2.6.2 Progesterone and eCG Based Methods

A common estrous synchronization procedure which mimics part or all of the luteal phase uses a P$_4$ treatment with an injection of eCG at P$_4$ removal. This protocol is applied

in and out of the breeding season in sheep [17–23,77]. Progesterone stimulates the brain priming areas which are involved with cyclicity [78]. In small ruminants, different forms of P_4 are used in estrous synchronization protocols: feed additives such as melengestrol acetate (MGA) [79], subcutaneous implants like the norgestomet ear implant [80], and intravaginal devices such as medroxyprogesterone acetate (MAP; 60 mg/sponge) and fluorogestone acetate (FGA; 20 mg/sponge) that all contain synthetic P_4, and the CIDR device that contains natural P_4 [16,81]. Because P_4 treatment controls GnRH, LH, and FSH secretion, it can be used to synchronize estrus by imitating the role of natural P_4 secreted by the CL [17]. Equine chorionic gonadotropin is a glycoprotein hormone extracted from the pregnant mare's serum - also known as pregnant mare serum gonadotropin (PMSG) [17,77]. Administration of eCG stimulates the last steps of follicular development [2,78]. Treatment with eCG at the time of P_4 removal stimulates follicle development, synchronizes estrus and ovulation, and increases the ovulation and lambing rates in seasonally anestrous ewes [2,16,18,20,76,77,80–86].

Tables 1-4 indicate that different estrous synchronization procedures give different results. Variations may be due to multiple factors such as animal breed, age, management, body weight, body condition score, hormone doses, and breeding systems. For example, different breeds were used in these studies. In some experiments, animals were kept outdoors only grazing native pastures, while in others, animals were kept indoors receiving a combination of forages and concentrates. In these studies, animal age ranged from seven months to eight years with body weight and body condition scores 40-60 kg and 2-4, respectively. Furthermore, the doses of applied hormones in all studies were not the same. For instance, eCG was used at 300-500 IU in different studies. Although all experiments

were done during the anestrous season, the ratio of natural light to dark varied in different latitudes. Various breeding methods (natural/artificial insemination), as well as the ratio of rams to ewes, could also affect outputs.

In ewes, the CIDR should be kept in the vagina for 5-7 or 12-14 days [19]. Seasonally anestrous ewes have been treated with CIDRs for 5-8 days or 11-14 days and an eCG injection at device removal (Table 1) [19,22,24,72,75,78,81,83,87–99]. However, in the 5-8-day treatment studies, the estrous duration and ovulation rates were not evaluated. Table 1 illustrates that the estrous onset and response, and pregnancy rates in 5–8-day treatment studies are similar to 11-14-day treatment studies. Lambing rates in 5-8-day treatments were higher than the 11-14-day treatments.

Table 1. Reproductive performance of seasonally anestrous ewes after 5-8/11-14 day CIDR-eCG synchronization protocols [19,22,24,72,75,78,81,83,87–99].

Parameters	5-8 days	11-14 days
Estrous onset (h after eCG injection)	40-48	30-48
Estrous response (%)	30-96	35-100
Estrous duration (h)	-	30-42
Ovulation rates	-	1.3
Pregnancy rates (%)	41-77	30-70
Lambing rates (%)	107-113	35-89

Seasonally anestrous ewes have been treated with MAP sponges for 6-7 days or 9-14 days and an eCG injection at sponge removal (Table 2) [19–21,40,72,75,81,83,94,98,100–113]. Table 2 shows that the estrous onset and response for both 6-7-day and 9-14-day MAP sponge treatments were similar. However, the range of estrous duration for 9-14-day

treatments is wider than 6-7-day treatments. Although, in 6-7-day treatment studies, follicle size and ovulation features were not evaluated, pregnancy rates were higher than in 9-14-day treatment studies. There is limited research on the 6-7-day MAP sponge treatment and impact on lambing rates. Available research indicates that lambing rates with the 6-7-day treatment is between the range of lambing rates in the 9-14-day treatment (Table 2). However, it is clear that more 6-7-day MAP treatment studies are needed to be compared with 9-14-day treatment studies.

Table 2. Reproductive performance of seasonally anestrous ewes after 6-7/9-14 day MAP-eCG synchronization protocols [19–21,40,72,75,81,83,94,98,100–113].

Parameters	6-7 days	9-14 days
Estrous onset (d after eCG injection)	1-4	1-4
Estrous response (%)	70-99	80-100
Estrous duration (h)	26-29	12-26
Follicle diameter (mm; 36-48 h after sponge removal)	-	5.5-6
Ovulation time (d after sponge removal)	-	2-3
Ovulation rates (%)	-	52-100
Pregnancy rates (%)	41-99	30-87
Lambing rates (%)	54	39-120

In seasonally anestrous ewes treated with a CIDR or MAP sponge for 9-12 days and an eCG injection one or two days before device removal, the interval from injection to estrous

onset, estrous response and duration, and ovulation, pregnancy, and lambing rates were about 21-47 h, 72%, 28 h, 1.2-1.9, 76%, and 52-74%, respectively [76,114,115].

Seasonally anestrous ewes have been treated with FGA sponges for 6-7 days or 11-14 days and an eCG injection at sponge removal (Table 3) [74,81,83–85,90,91,100,102,116–125]. Table 3 illustrates that with 6-7-day treatments, estrus started later than with the 11-14-day treatments, while the estrous duration for both treatments was similar. The wide range of estrous responses with 11-14-day treatments is probably due to different study conditions. In 6-7-day treatment studies, ovulation rates were not evaluated, however, pregnancy and lambing rates in these studies were between the range of pregnancy and lambing rates for the 11-14-day treatments (Table 3). In seasonally anestrous ewes treated with FGA sponges for 12 days and eCG at sponge removal with or without a GnRH injection 36 h after eCG treatment results showed that GnRH improved the synchrony of ovulation (about 3 days vs. 6 days) [126].

Table 3. Reproductive performance of seasonally anestrous ewes after 6-7/11-14 day FGA-eCG synchronization protocols [74,81,83–85,90,91,100,102,116–125].

Parameters	6-7 days	11-14 days
Estrous onset (h after eCG injection)	38-82	24-55
Estrous response (%)	84-92	17-100
Estrous duration (h)	29-45	29-35
Ovulation rates (%)	-	29
Pregnancy rates (%)	67-75	42-100
Lambing rates (%)	75-83	42-157

Seasonally anestrous ewes have been treated with norgestomet ear implants for 9-14 days and an eCG injection at device removal (Table 4) [80,83,102,105,127–129]. Table 4 shows that the estrous response and lambing rates seem to be lower with MGA treatments than norgestomet ear implant treatments [79,83,108,130,131], while pregnancy rates with norgestomet ear implant treatments are between the range of pregnancy rates with MGA treatments. Differences might be due to the kind of P_4 treatments as MGA is a feed additive. Thus, it is possible that animals did not receive sufficient amounts of MGA because of factors such as animal dominance, illness, stress, management, etc.

It has been reported that treating seasonally anestrous ewes with MGA for 7-13 days and an injection of eCG after the last feeding of MGA resulted in more luteal structures per ewe than only MGA treatment (2.3 vs. 1.8) [108]. In addition, in seasonally anestrous ewes treated with MGA for eight days and an E_2 injection 36-54 h after the last feeding, the estrous response and lambing rates were about 54-76% and 57-76%, respectively [132].

Table 4. Reproductive performance of seasonally anestrous ewes after 9-14 day norgestomet ear implant/7-13 d MGA-eCG synchronization protocols [79,80,83,102,105,108,127–131].

Parameters	Norgestomet ear implant-eCG	MGA with or without eCG
	9-14 days	7-13 days
Estrous onset (h after eCG injection)	24-52	-
Estrous response (%)	88-100	14-93
Estrous duration (h)	29	-
Ovulation time (h after P_4 removal)	65-69	-
Pregnancy rates (%)	50-72	44-93
Lambing rates (%)	60-100	10-73

Tables 1-4 illustrate that different P_4 treatments may affect seasonally anestrous ewe reproductive performance differently. For example, with intravaginal P_4 devices the range of estrous onset was wider than the other P_4 treatments (1-4 d vs. 1-2 d after device removal), but the estrous response with different P_4 treatments was similar. The estrous duration with norgestomet ear implants and MGA was between the range of estrous duration with intravaginal P_4 devices. Ovulation time also seems to be similar with different P_4 treatments. Pregnancy and lambing rates with intravaginal P_4 devices were lower and higher than with the other P_4 treatments, respectively. This information shows that the type of P_4 treatment, route of administration, kinetics of P_4 release, and metabolism rate and amounts of P_4 can impact fertility.

Although 9-14-day treatments with P_4 intravaginal devices (long-term P_4 treatment) have been approved in sheep and goat estrous synchronization programs, there are some studies that showed that 5-8-day P_4 treatments (short-term P_4 treatment) can also synchronize estrus in small ruminants. It has been reported that the estrous onset, response, and duration, and pregnancy and lambing rates in short-term P_4 treatments are similar to or higher than long-term P_4 treatments (Table 1, 2, 3). Researchers who agree with the short-term P_4 treatments believe that circulating P_4 concentrations go above 5 ng/mL about 4-5 days after P_4 device insertion. These concentrations are similar to P_4 concentrations observed through the mid-luteal phase (around seven days after ovulation). However, after 6-7 days after device insertion, circulating P_4 concentrations are reduced to less than 2 ng/mL (subluteal levels) [19]. In sheep and goats, subluteal P_4 concentrations increase LH pulse frequency (with no preovulatory LH surge occurrence), enlarging dominant follicle size and turning them into persistent follicles. Their oocytes have lower fertility, as oocyte quality decreases due to aging. To prevent these events, causing young follicles to ovulate, exposure to low P_4 concentrations for a long period should be avoided [19]. It has also been reported that ovulation of aged follicles does not have adverse impacts on the quality of embryos and fertility in ewes. The proportion of good quality embryos from ovulated aged follicles was the same as those from ovulated younger follicles. Lambing rates (mean 84%) were also similar among ovulated aged and younger follicles [133]. Thus, according to this study, ovulation of aged follicles is unlikely to result in a significant decrease in fertility. Furthermore, the number of short-term treatment studies is considerably less than the number of long-term treatment studies, therefore, the long-term treatment may be more acceptable in estrous synchronization programs. In contrast, some researchers disagree

26

with the short-term P_4 treatments and believe that 12-14-day P_4 treatments resemble the cyclic CL lifespan in sheep and goats. Thus, using a treatment based on the natural physiology of these animals may give more reasonable results.

Overall, in seasonally anestrous ewes, estrous synchronization does not result in follicular wave emergence synchronization. Maybe follicular wave emergence could be synchronized by additional administration of exogenous hormones, such as E_2, through an estrous synchronization protocol to improve seasonally anestrous ewe fertility.

2.7 Follicular Wave Emergence Synchronization

Follicular wave emergence can be manipulated either mechanically via follicle ablation or hormonally in estrous synchronization protocols [6–10,82]. Administration of GnRH at random stages of the estrous cycle causes ovulation in bovines but the interval from GnRH treatment to follicular wave emergence is inconsistent [10]. To remove the inconsistency a GnRH-PGF$_{2\alpha}$-GnRH (Day 0, 7, and 9, respectively) protocol known as the Ovsynch method was developed to control the CL lifespan, follicular wave, and ovulation in bovines. The intent of the first GnRH injection is to induce ovulation, resulting in a new follicular wave with a dominant follicle [6,7]. However, the addition of a P_4 treatment to a GnRH-based procedure improves ovulation and pregnancy rates in beef heifers and cows, and it is the most commonly used synchronization protocol in North America [6,7]. Several studies have confirmed the efficiency of P_4-E_2 treatment for follicular wave synchronization in bovines as well [6–8]. Although, E_2 is not allowed for commercial use in many countries. Using other kinds of E_2 such as its esters, estradiol benzoate (EB), valerate (EV), and cypionate (ECP), has been investigated. All E_2 esters caused follicle regression and a new follicular wave to emerge within 2-7 days after treatment when given

concurrently with a high level of plasma P_4 concentrations in cattle [6–10,134]. In a study on beef heifers which compared the effects of GnRH-PRIDs for five days -$PGF_{2\alpha}$-GnRH (Day 0, 0, 5, and 8, respectively; known as the Co-Synch protocol) with the same procedure but EB instead of GnRH injections, the interval from treatment to new follicular wave emergence was shorter with GnRH than EB treatment (2 vs. 4 days); ovulation and pregnancy rate did not differ between groups [6]. In beef heifers treated with a PRID for seven days -EB-$PGF_{2\alpha}$-ECP (Day 0, 0, 7, and 7, respectively) or a PRID for six days -EB-$PGF_{2\alpha}$-GnRH (Day 0, 0, 6, and 9, respectively) ovulation occurred about 65 h and 94 h after device removal, respectively [6].

Several studies have examined follicular wave synchronization techniques in ewes. Using $PGF_{2\alpha}$ based protocols, in seasonally anestrous ewes treated with FGA sponges for 6-14 days and a $PGF_{2\alpha}$ injection 1-2 days before sponge removal and an eCG injection at sponge removal, the estrous onset, response, and pregnancy and lambing rates were about 28-64 h after sponge removal, 86-100%, 33-100%, and 33-100%, respectively [86,121]. In seasonally anestrous ewes treated with FGA sponges for six days and $PGF_{2\alpha}$ at sponge insertion and eCG at sponge removal, the estrous onset, response, and duration were about 51 h after eCG injection, 92%, and 27 h, respectively [85]. In seasonally anestrous ewes treated with FGA sponges for seven days and a $PGF_{2\alpha}$ injection at sponge insertion or removal and eCG at sponge removal, the estrous onset, response, and ovulation rates were about 43 h vs. 48 h after sponge removal, 64% vs. 91%, and 20% vs. 24%, respectively. The number of total and growing medium follicles (3.5-5 mm) was higher in that group which received $PGF_{2\alpha}$ injection at sponge removal (2.5 and 1.5 vs. 1 and 0.3, respectively) at 24 h after sponge removal. The number of total and growing large follicles ($\geq$ 5.5 mm)

at 48 h after sponge removal was also greater in the group which received $PGF_{2\alpha}$ injection at sponge removal than those who received $PGF_{2\alpha}$ injection at sponge insertion (about 3 and 2 vs. 2 and 1, respectively) [84]. With an FGA sponge and $PGF_{2\alpha}$ injection at sponge removal treatment without eCG, estrous onset, response and pregnancy and lambing rates were about 60 h after sponge removal, 73%, 67%, and 47%, respectively [135]. In seasonally anestrous ewes treated with MAP sponges for 12 days and eCG-$PGF_{2\alpha}$ injections 48 h before sponge removal, the interval from sponge removal to estrous onset was about 32 h [76]. In seasonally anestrous ewes treated with MAP sponges for 6-9 days and an eCG-$PGF_{2\alpha}$ injection at 24 h before sponge withdrawal, the estrous onset, response, duration, timing of follicular wave emergence, number of 4-5 mm follicles, percentage of ovulated ewes, interval from sponge removal to ovulation, and number of ovulations were about 37 h after sponge withdrawal, 86%, 21-25 h, 3-4 days after sponge insertion, 0.8-1.4, 93-100%, 55-57 h, and 2-3, respectively [136].

In seasonally anestrous ewes treated with CIDRs for 14 days, and $PGF_{2\alpha}$ at CIDR insertion, a follicular wave emerged two days after CIDR removal [137]. In seasonally anestrous ewe treated with CIDRs for 5-7 days and E_2 at CIDR insertion and eCG or $PGF_{2\alpha}$-eCG at CIDR removal, estrous response, ovulation time, and pregnancy rates were about 32-66%, 60 h after eCG injection, and 17-35%, respectively. Also, results showed that the same CIDR-E_2-eCG treatment enhanced the frequency of estrous response [19,24,138].

Several studies have evaluated the effects of combinations of GnRH and $PGF_{2\alpha}$ as well. In a study on sheep using Ovsynch or Pre-synch protocols, in which seasonally anestrous ewes treated with GnRH-$PGF_{2\alpha}$-GnRH (Day 0, 7, and 9, respectively) or $PGF_{2\alpha}$-GnRH-$PGF_{2\alpha}$-GnRH (Day -7, 0, 7, and 9, respectively) pregnancy rates were 85.7% and 88%,

respectively [139]. In seasonally anestrous ewes treated with GnRH-PGF$_{2\alpha}$-PGF$_{2\alpha}$ (Day -15, -10, and 0, respectively) P$_4$ concentrations were higher than untreated ewes on Day -10 (about 3.50 vs. 2.70 ng/mL). Based on plasma P$_4$ concentrations (>1 ng/mL) on Day 70, about 98% of treated ewes had active CLs. Lambing and twin lambing rates were about 90% and 22%, respectively [140]. In a study using a similar Co-synch protocol called the U-synch protocol, seasonally anestrous ewes treated with PRIDs for seven days, a GnRH injection at device insertion and a dose of eCG and PGF$_{2\alpha}$ at PRID removal, the estrous, ovulation, and pregnancy rates were about 78%, 89%, and 69%, respectively [78]. However, in seasonally anestrous ewes treated with GnRH-FGA sponges for 14 days - GnRH-PGF$_{2\alpha}$ (Day -7, 0, 7, and 14, respectively), the estrous onset, response, and pregnancy and lambing rates were about 55 h after treatment, 67%, 67%, and 67%, respectively [135]. The estrous onset, response, and lambing rates in seasonally anestrous ewes treated with a GnRH-PGF$_{2\alpha}$ treatment (Day 0 and 5, respectively) were about 29-41 h after treatment, 47-86%, and 71%, respectively. Although, with the same procedure plus a second GnRH injection on Day 7, the estrous onset, response, and lambing rates were about 14-59 h after treatment, 29-33%, and 80%, respectively. Furthermore, with a GnRH-PGF$_{2\alpha}$-EB treatment (Day 0, 5, and 6, respectively) the estrous onset, response, and lambing rates were about 62 h after treatment, 63%, and 20%, respectively. In seasonally anestrous ewes treated with an EB-PGF$_{2\alpha}$-EB treatment (Day 0, 5, and 6, respectively) the estrous onset and response were about 49 h after treatment and 100%, respectively [122,141]. In seasonally anestrous ewes treated with CIDRs for 12 days and an eCG injection, or two FSH injections 12 h apart at CIDR removal, number of follicles and CLs on two and six days after treatment in eCG group were higher than FSH group [142].

Follicular wave emergence synchronization has also been investigated using E_2 based protocols in ewes. In seasonally anestrous ewes treated with only one E_2 injection or two E_2 injections 24 h apart no estrus was detected. The ovulation time and rate in the first group was about five days after treatment and 0-57%, respectively, while no ovulation was observed in the second group. Although a new follicular wave emerged about 2-3 days after treatment in both groups [143,144]. In seasonally anestrous ewes treated with norgestomet ear implants for 10 days and an injection of EV at implant insertion and an eCG injection at implant removal, the estrous onset, response, and pregnancy rates were about five days after eCG injection, 72%, and 51%, respectively [145]. In seasonally anestrous ewes treated with MAP sponges for 12 days, E_2 six days after sponge insertion, with or without eCG at sponge removal, the interval from E_2 injection to follicular wave emergence was 4-5 days. All ewes given eCG ovulated 3-4 days after eCG injection and all ovulated follicles formed CL [18]. In this study, ewes treated with MAP-E_2 with or without eCG showed more synchronized follicular wave emergence than ewes only treated with E_2 or MAP. The interval from E_2 injection to follicular wave emergence for the two last treatments was 0-5 and 0-4 days, respectively [18]. This study showed that when ewes were treated with P_4 and E_2 all ewes missed a follicular wave just after E_2 injection, while 50% of the ewes given only E_2 missed the next follicular wave [18]. Seasonally anestrous ewes treated with MAP sponges or CIDRs for 12 days, E_2 six days after device insertion, and eCG at device withdrawal ovulated about four days after eCG injection, while the number of ovulated follicles and CL was about 1.5 and one, respectively [146]. With the same procedure (CIDR-E_2-eCG) in seasonally anestrous ewes, estrous onset and duration, and pregnancy and lambing rates were about 1.5-2 days after eCG injection, 40 h, 33-62%,

and 30-61%, respectively [87–89]. This treatment resulted in a rise in LH concentrations after E_2 injection and caused a more synchronized preovulatory LH surge [89]. Thus, a P_4-E_2-eCG treatment may be an effective method for seasonally anestrous ewe synchronization protocols.

As it was previously mentioned, studies on the ewe follicular wave emergence are not as much as cattle studies, also estrous synchronization protocols do not synchronize the wave emergence, while the predictable ovulation is noteworthy for TAI and embryo transfer programs. Therefore, these reasons highlight the importance of the present study.

2.8 Hypotheses and Objectives

As ECP has been successfully used in synchronization programs in cattle, it is believed that it can also induce estrus, and synchronize follicular wave emergence and ovulation in seasonally anestrous ewes. It was hypothesized that ECP is as effective as E_2 in ewe synchronization programs. Thus, perhaps E_2 can be replaced with ECP in the CIDR-E_2-eCG seasonally anestrous ewe synchronization protocol.

The objective of this project was to compare the effects of ECP against E_2 on the synchronization of follicular wave emergence, estrus, and ovulation in an estrous synchronization protocol in seasonally anestrous ewes. Furthermore, the goal was to evaluate effects of E_2 and two different doses (70 and 350 µg) of ECP on reproductive hormone profiles, ovarian responses, follicular wave emergence, estrus, ovulation, and luteal development in CIDR-eCG treated seasonally anestrous ewes.

CHAPTER 3: Follicular dynamics and hormonal response to CIDR-eCG and estradiol-17β or estradiol cypionate in seasonally anestrous ewes

3.1 Abstract

Reproductive performance is low in seasonally anestrous ewes even with controlled breeding strategies. The P_4-E_2-eCG protocol has improved seasonally anestrous ewe reproductive output. This study compared the effects of E_2 with two different doses of ECP on the synchronization of follicular wave emergence, estrus, and ovulation in a seasonally anestrous ewe synchronization protocol. Fourteen Texel ewes received CIDRs on Day -12 and an eCG injection at CIDR removal (Day 0). The ewes were randomly divided into three groups based on the treatments on Day -6: ECP350 (350 μg of ECP; n=4), ECP70 (70 μg of ECP; n=5), and E_2 (350 μg of E_2; n=5). Ovarian ultrasonography and blood sampling were done to monitor follicular/luteal dynamics and pregnancy and evaluate hormonal changes. A haltered ram was introduced to the ewes to detect estrus. Mean serum E_2 concentrations peaked at 6 h after E_2/ECP injection (P<0.001). Mean serum FSH concentrations decreased from 0-6 h and increased from 12-42 h after E_2/ECP injection (P<0.05). Mean serum LH concentrations did not change (P>0.05). Mean serum E_2 concentrations at 6 h after E_2/ECP injection were higher in the E_2 group (127.9±8.1 pg/mL) than the ECP350 (25.2±10.7 pg/mL) and ECP70 (8.3±8.1 pg/mL) groups (P<0.001). However, mean E_2 concentrations at 18 and 24 h after E_2/ECP injection were higher in the ECP350 group (18 h: 48.5±10.7 pg/mL; 24 h: 39.6±9.0 pg/mL) than the ECP70 (18 h: 8.0±8.1 pg/mL; 24 h: 6.4±8.1 pg/mL) and E_2 (18 h: 4.7±8.1 pg/mL; 24 h: 2.8±8.1 pg/mL) groups (P<0.05). Mean FSH and LH concentrations at 0 and 24 h after E_2/ECP injection were respectively higher in the ECP350 group (FSH: 2.07±0.14 ng/mL; LH: 4.21±0.58 ng/mL) than the ECP70 (FSH: 1.24±0.12 ng/mL; LH: 0.13±0.52 ng/mL) and E_2 (FSH: 1.26±0.12 ng/mL; LH: 0.12±0.52 ng/mL) groups (P<0.001). Mean FSH concentrations at 48 h after E_2/ECP injection were lower in the ECP350 (0.70±0.14 ng/mL) than E_2 (1.52±0.12 ng/mL; P<0.01) group. Mean serum E_2 concentrations associated with estrous onset were higher in the ECP350 (7.5±1.7 pg/mL) than E_2 (2.6±0.1 pg/mL) group. Estrus started earlier in the ECP350 (30.0±0.0 h) than ECP70 (55.5±9.0 h) group after eCG treatment (P<0.05). Estrus was longer than 60 h in one ECP70 and two ECP350 ewes. Only

one ECP70 ewe did not express estrus. The interval from E_2/ECP injection to peak E_2 concentrations was shorter in the E_2 (6.0±0.0 h) than ECP350 (19.5±2.9 h; $P<0.05$) group. The interval from eCG to the preovulatory LH surge peak and first FSH rise was shorter in the ECP350 than ECP70 group (32.0±2.6 vs. 49.0±6.1 h and 32.0±2.0 vs. 46.5±5.1 h, respectively; $P<0.05$). Estrous and ovulation features, follicular wave emergence synchrony, number of CL, and LH, FSH, P_4 and most E_2 patterns in E_2 and ECP70 ewes were similar ($P>0.05$). Daily maximum follicle diameter (MFD) in the E_2 (3.7±0.3 mm) and ECP70 (3.1±0.3 mm) groups was larger than the ECP350 group (1.8±0.3 mm; $P<0.05$). Daily MFD decreased from Days -5 to -3 and increased from Days 0 to 2 relative to eCG injection ($P<0.05$). In the ECP350 group follicles were equal to or less than 2 mm in diameter from Days -3 to 3 and on Day 7 relative to eCG injection. Daily MFD was greater in both the E_2 and ECP70 groups than ECP350 group on Days 2 and 3 after eCG injection ($P<0.05$). Follicular wave emergence occurred earlier in E_2 than ECP70 ewes (3.6±0.5 vs. 6.0±0.8 days after E_2/ECP injection; $P<0.05$). Four E_2, three ECP70, and no ECP350 ewes ovulated by 7 days after eCG injection. Mean serum P_4 concentrations increased until 7 days after ovulation ($P<0.001$). For mean maximum serum P_4 concentrations in all ewes, the E_2 (7.48±0.65 ng/mL) was higher than ECP350 (0.54±0.02 ng/mL) group ($P<0.05$). Mean P_4 concentrations on Day 12 after ovulation were lower in the ECP350 (0.40±0.72 ng/mL) group than both the E_2 (5.78±0.64 ng/mL) and ECP70 (5.22±0.64 ng/mL) groups ($P<0.05$). Mean P_4 concentrations on Day 15 after ovulation were lower in the ECP350 (0.39±0.72 ng/mL) than the E_2 (4.89±0.64 ng/mL) group ($P<0.05$). Pregnancy was observed in three E_2 and two ECP70 ewes. In conclusion, perhaps ECP70 can be a replacement for E_2 in the CIDR-E_2-eCG seasonally anestrous ewe synchronization protocol.

Keywords: Anestrous ewes, Follicular wave emergence, Synchronization, Estradiol-17β, Estradiol cypionate.

3.2 Introduction

The world population is growing rapidly, so, demand is expected to significantly rise for meat and dairy products [1,2]. Sheep production is rather more complicated than the production of some other farm animal species such as cows and chickens, as sheep show seasonal reproductive cycles. Thus, pregnancy and lambing rates annually are low (once a year), since they cannot naturally breed during the non-breeding season (spring and summer). To tackle the seasonality of lamb production, developing successful out of breeding season techniques are required. Hormonal treatment is a common and effective way for animal reproduction management. There are many studies in bovine reproductive synchronization in order to improve their output [6–15], but the lack of such investigations in the ovine, inspires researchers to study it. Estrous synchronization is an important means to improve ewe reproductive performance. Follicular wave emergence and ovulation can be synchronized through modified estrous synchronization protocols. A predictable and synchronized ovulation can improve TAI and embryo transfer programs. In addition to increasing lambing rates, breeding synchronization contributes to genetic improvement. It can also control and shorten lambing, resulting in more efficient use of animal facilities and labor [16]. In ewes and cattle, administration of E_2 synchronized follicular wave emergence [11,18,59,134,144]. However, E_2 treatment is not licensed for commercial use in many countries. Thus, different forms of estrogen have been investigated to find a proper replacement for E_2. It has been reported that estrogen esters improved bovine reproductive performance [14,15,25,26]. Thus, it is hypothesized that ECP may also be a suitable alternative to E_2 in seasonally anestrous ewe synchronization programs. The objective of this study was to compare the effects of E_2 and two different doses (70 and 350 µg) of ECP

on reproductive hormone profiles, ovarian response, follicular wave emergence, estrus, ovulation, and luteal development in CIDR-eCG treated seasonally anestrous ewes.

3.3 Materials and Methods

3.3.1 Experimental Design, Animals, and Treatments

In this study, data/samples from fourteen Texel ewes were used. Clinically healthy sexually mature ewes aged between 4-8 years were weighed and body condition scored at the start of the experiment out of the breeding season (May-June) at the Dalhousie University Agricultural Campus Ruminant Animal Centre. Body condition was evaluated on a linear scale of one to five (1=very thin, 5=very obese) and was followed through by the same person on each occasion. Ewes were fed alfalfa hay and water ad libitum. Each animal was also given 150 g barley twice a day (Appendix Table A1). Treatments were balanced for age, body weight, body condition, and parity. Each ewe was given a CIDR (0.33 g of progesterone, Zoetis, Kirkland, QC, Canada) on Day -12 (Day 0=CIDR removal). Animals were randomly divided into three groups- treated with an intramuscular injection of ECP (Estrus®; DIN# 02299283, RAFTER8, Calgary, AB, Canada; 350 µg (ECP350; n=4) or 70 µg (ECP70; n=5)) or E_2 (Millipore Sigma, Oakville, ON, Canada; 350 µg; n=5) in sesame oil (Chemical grade, PCCA, London, ON, Canada) six days after CIDR insertion. On Day 0, CIDRs were removed and all ewes simultaneously received an eCG (Folligon®;500 IU; i.m.; Merck, Kirkland, QC) injection. A haltered ram was introduced every 6 h to the ewes on Days 1, 2, and 3 to detect estrous onset and duration. All ewes were weighed and body condition scored on the last day (Day 20) of the experiment (Figure 6). The experiment was done according to the standards of the Canadian Council on Animal Care published guidelines.

Figure 6. The project timeline (days of body weighing (BW), body condition scoring (BCS), blood sampling (BS), CIDR insertion and removal, transrectal ultrasonography (TUS), E_2, ECP, eCG injections, and detection of estrus in 14 seasonally anestrous Texel ewes).

| CIDR (n=14) | | | | | | | | | | | | | |

| Day -12 | -7 | -6 | -5 | -4 | -3 | -2 | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 7 | 20 |

CIDR in
BW
BCS
BS

BS
1x

BS
4x

BS
2x

BS
1x

BS
1x

BS
1x

BS 8x
Ram estrus check
started
2x TUS started

BS
8x

BS
8x

BS
2x

TUS
1x

BW
BCS

E_2 (n=5)
ECP350 (n=4)
ECP70 (n=5)
injection
Daily TUS started
BS 3x

BS 1x
CIDR out
eCG injection

Daily BS started
(ended on Day 20)
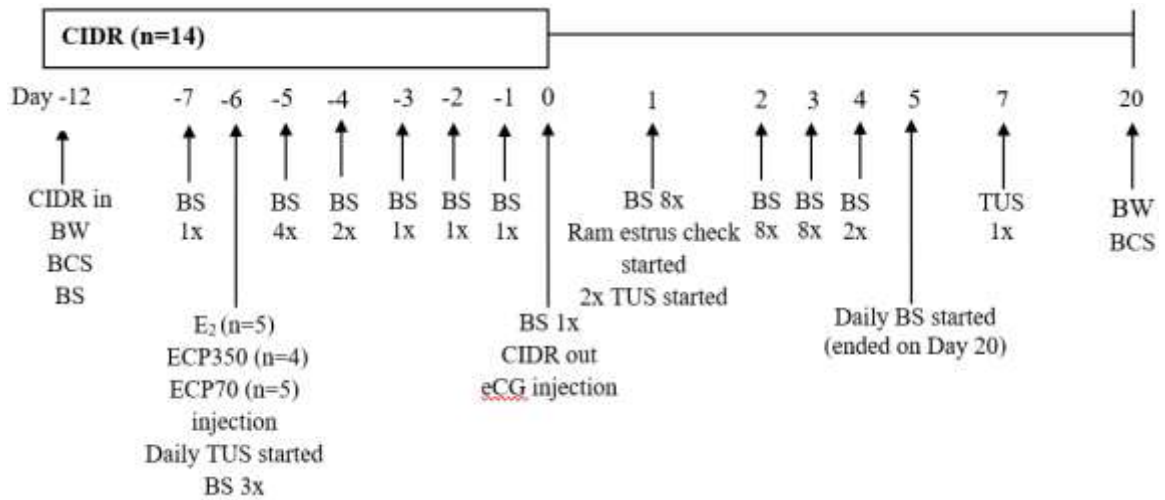
3.3.2 Transrectal Ultrasonography

Transrectal ovarian ultrasonography was done in all animals daily from Day -6 to 0, twice daily from Day 1 to 6, and one time on Day 7 (Figure 6). Ovulation was detected by ultrasonography when the largest follicle(s) ($\geq$ 5 mm in diameter) suddenly disappeared. Data for follicular waves were combined for the left and right ovaries of each animal. Ovarian ultrasonography was conducted using an ALOKA Prosound 2 B-mode, real-time echo camera (Imago Medical Inc. Vaudreuil-Dorion, QC, Canada). This technique has been previously validated for the observation of ovarian structures in ewes [147–149]. Ultrasonography was done to understand the effects of E_2/ECP treatment on follicular dynamics, ovulation, and CL development. Images were viewed at a magnification of 1.5X with constant gain and focal point settings. Ovarian images were recorded on a Super Multi DVD Recorder + HDD (LG RH398H-M, East Mississauga, ON, Canada) for later examination. Changes in the size and position of follicles and luteal structures on the ovaries were drawn on ovarian maps during ultrasonography for later assessment.

3.3.3 Blood sampling

Blood samples (3 mL) were taken from all animals by jugular venipuncture into vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) on Days -12, -7, and from Day -3 to 0, and also from Days 4 to 20. Blood sampling every 3-6 h was done using jugular catheters. Blood sampling was done on Days -6, -5, and -4 every 6 h (where time 0=i.m. E_2/ECP; 10 mL). Blood sampling was also done starting at 18 h after eCG injections every 3 h for 72 h (where time 0=i.m. eCG; 5 or 10 mL; Figure 6). All blood samples were kept at room temperature for 18-24 h to clot; then the samples were centrifuged (Thermo Fisher Scientific ST40R, Frankfurt, Hesse, Germany) for 10 min at 1500 × g at 20 °C. After that serum was removed and stored at -20 °C until analyzed.

3.3.4 Hormone analysis

The collected blood samples on Day -7 and from Days -3 to Day -1 were used for radioimmunoassay (RIA) of FSH concentrations [150,151]. The blood samples from Day 0 were RIA for E_2 [152] and FSH [150] concentrations. The blood samples from Days -6, -5, -4, and also from Days 1 to 4 were used to observe the effects of E_2/ECP treatment on circulating E_2, FSH, and LH [153] concentrations. The assay sensitivity (described as the lowest hormone concentration which is significantly able to dislocate radio/enzyme-labeled hormone from the antibody) for E_2 was 1.0 pg/mL and the ranges of standards were 1.0-100 pg/mL. The intra-assay and inter-assay coefficients of variations (CVs) were 12.1 % and 9.5% or 14.6% and 13.5% for reference sera with mean estradiol concentrations of 9.0 and 17.5 pg/mL, respectively. The assay sensitivity for FSH was 0.1 ng/mL and the ranges of standards were 0.12–16.0 ng/mL. All samples for FSH measurement were done in one assay; the intra-assay CVs were 5.6% and 6.8% for reference sera with mean FSH

concentrations of 0.86 and 1.10 ng/mL, respectively. The assay sensitivity for LH was 0.1 ng/mL and the ranges of standards were 0.06–8.0 ng/mL. All samples for LH measurement were done in one assay; the intra-assay CVs were 7.7 and 10.2% for reference sera with mean LH concentrations of 0.42 and 3.45 ng/mL, respectively. The collected blood samples on Day -12 and from Days 2 to 20 were used for enzyme immunoassay (EIA) of P_4 concentrations [154], to see if the ewes were cycling on Day -12 and the development of CL after ovulation. The assay sensitivity for P_4 was 0.03 ng/mL and the ranges of standards were 0.03–16.0 ng/mL. The intra-assay and inter-assay CVs were 8.9% and 9.2% or 4.0% and 13.6% for reference sera with mean P_4 concentrations of 2.34 or 5.58 ng/mL, respectively. Cycle-detection software was used to analyze FSH peak patterns in the collected blood samples [151].

3.3.5 Statistical Analyses

The statistical model included time, treatment (350 µg E_2, 350 µg ECP, or 70 µg ECP), and the time by treatment interaction. The data were normalized to the time of E_2/ECP and eCG injections. To analyze the effect of treatments, time, and treatment-time interactions on follicle size and serum hormone concentrations, the data were subjected to two-way repeated measures ANOVA (SigmaPlot for Windows Version 13.0, 2014, Systat Software, Inc., San Jose, CA, USA). One-way ANOVA was also used to analyze the effects of treatments on ovarian dynamics, such as follicular wave emergence and ovulation patterns, and hormone response, such as gonadotropin and steroid hormone patterns. Differences among means were tested by the Tukey test if the effects of time, treatment, or treatment-time interactions were significant ($P<0.05$). Data are expressed as mean ± S.E.M.

3.4 Results

3.4.1 Estrous Features

All the animals except one ECP70 ewe showed estrus after eCG injection. Estrus started earlier for ECP350 (30.0±0.0 h) than ECP70 (55.5±9.0 h) group (P<0.05); E_2 (36.0±3.3 h) group was in-between (P>0.05). Estrus ended at the same time for E_2 (72.0 ± 4.2 h) and ECP70 groups (76.0 ± 4.0 h; P>0.05; Table 5). Although estrus was longer than 60 h in one ECP70 and two ECP350 ewes.

Table 5. Mean (±S.E.M.) characteristics of estrus in seasonally anestrous Texel ewes.

Variables	E_2	ECP350	ECP70
Estrous response (number of ewes)	5/5	4/4	4/5
Estrous onset (h after eCG)	36.0±3.3[ab]	30.0±0.0[a]	55.5±9.0[b]
Estrous end (h after eCG)	72.0±4.2	---	76.0±4.0
Estrous duration (h)	36.0±6.3	---	22.0±8.7
Estrous range (h)	12-48	---	6-36

Ewes were treated with CIDRs for 12 days then assigned to the following groups: 350 µg E_2 (E_2; n=5), or 350 µg ECP (ECP350; n=4); or 70 µg ECP (ECP70; n=5) 6 days after CIDR insertion. At CIDR removal all ewes received an eCG injection.
--- Estrus was longer than 60 h in two ECP350 ewes.
a,b Indicate differences among groups within a row (P<0.05).

3.4.2 Ovarian Structures, Reproductive Hormone Profiles, and Pregnancy

3.4.2.1 Ovarian Structures and Pregnancy

There was a day, treatment, and interaction effect on daily MFD (P<0.005; Figure 7). Daily MFD in E_2 (3.7±0.3 mm) and ECP70 (3.1±0.3 mm) was larger than ECP350 (1.8±0.3 mm) group, (P<0.05; Figure 7). Daily MFD decreased from Day -5 to -3 and increased from Day 0 to 2 after eCG injection (P<0.05). Daily MFD was greater in E_2 than ECP350 and ECP70 groups from Day -1 to 1 (P<0.05; Figure 7). Although, daily MFD in ECP70

on Day 1 was smaller than E$_2$, it was greater than ECP350 group (P<0.05). Daily MFD was greater in E$_2$ and ECP70 than ECP350 group on Days 2 and 3 (P<0.05). In ECP350 group follicles were equal to or less than 2 mm in size from Day -3 on (Figure 7).
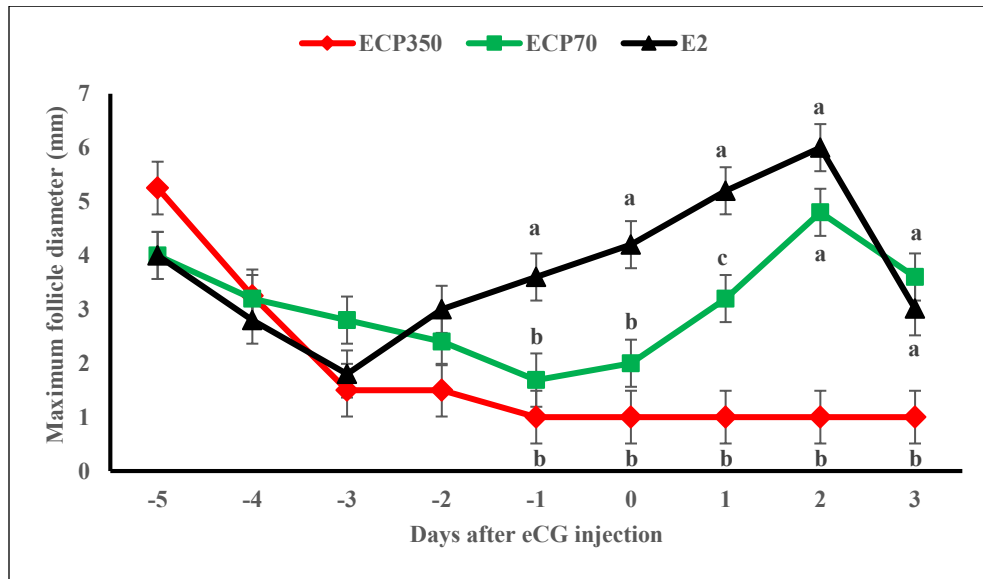
Figure 7. Follicular dynamics in seasonally anestrous ewes treated with CIDRs for 12 days then assigned to the following groups: 350 μg E$_2$ (E$_2$; n=5), or 350 μg ECP (ECP350; n=4); or 70 μg ECP (ECP70; n=5) 6 days after CIDR insertion. At CIDR removal (Day 0) all ewes received an eCG injection.
a,b,c Indicate differences among groups within day (P<0.005).

The follicular wave emerged earlier in E$_2$ (3.6±0.5 d) than ECP70 (6.0±0.8 d) group after E$_2$/ECP injection (P<0.05; Table 6). There was no significant difference between E$_2$ and ECP70 group follicular wave emergence synchrony. Four E$_2$ ewes, no ECP350 ewes, and three ECP70 ewes ovulated by no later than Day 7 after eCG injection (Table 6). Ovulation tended to be earlier in E$_2$ (2.5±0.0 d) than ECP70 (3.3±0.8 d) group after eCG injection (P=0.073). However, the synchrony of ovulation in E$_2$ and ECP70 groups was similar (Table 6). The number of ovulations and CLs per ewe in E$_2$ and ECP70 groups were also similar. Pregnancy was detected in three E$_2$ and two ECP70 ewes (Table 6).

Table 6. Mean (±S.E.M.) characteristics of ovarian responses in seasonally anestrous Texel ewes.

Variables	E_2	ECP350	ECP70
Time of follicular wave emergence (d after E_2/ECP)	3.6 ± 0.5^a	---	6.0 ± 0.8^b
Synchrony of wave emergence (d from mean)	0.9 ± 0.3	---	1.2 ± 0.5
Number of ewes ovulating*	4/5	0/4	3/5
Time of ovulation (d after eCG)*	2.5 ± 0.0	---	3.3 ± 0.8
Synchrony of ovulation (d from mean)	0.0 ± 0.0	---	0.3 ± 0.2
Number of ovulations per ewe	0.8 ± 0.2	---	1.0 ± 0.5
Number of CLs per ewe	0.8 ± 0.2	---	0.8 ± 0.6
Number of pregnant ewes	3/5	0/4	2/5

Ewes were treated with CIDRs for 12 days then assigned to the following groups: 350 μg E_2 (E_2; n=5), or 350 μg ECP (ECP350; n=4); or 70 μg ECP (ECP70; n=5) 6 days after CIDR insertion. At CIDR removal (Day 0) all ewes received an eCG injection.
* Ewes that ovulated by no later than Day 7 after eCG injection.
a,b Indicate differences between groups within a row ($P<0.05$).

3.4.2.2 Serum E_2 Concentrations

For mean serum E_2 concentrations, there was a time, treatment, and interaction effect ($P<0.05$) during 48 h after E_2/ECP injection. Mean E_2 concentrations peaked at 6 h after E_2/ECP injection ($P<0.001$) and were only different between ECP350 (24.5 ± 4.3 pg/mL) and ECP70 (5.6 ± 3.4 pg/mL) groups ($P<0.05$); E_2 (17.8 ± 3.4 pg/mL) was in-between ($P>0.05$). Mean E_2 concentrations at 6 h after E_2/ECP injection were higher in E_2 (127.9 ± 8.1 pg/mL) than ECP350 (25.2 ± 10.7 pg/mL) and ECP70 (8.3 ± 8.1 pg/mL) groups ($P<0.001$; Figure 8A). Mean E_2 concentrations at 18 and 24 h after E_2/ECP injection were higher in ECP350 (18 h: 48.5 ± 10.7; 24 h: 39.6 ± 9.0 pg/mL) than ECP70 (18 h: 8.0 ± 8.1;

24 h: 6.4±8.1 pg/mL) and E_2 (18 h: 4.7±8.1; 24 h: 2.8±8.1 pg/mL) groups (P<0.05), respectively (Figure 8A).
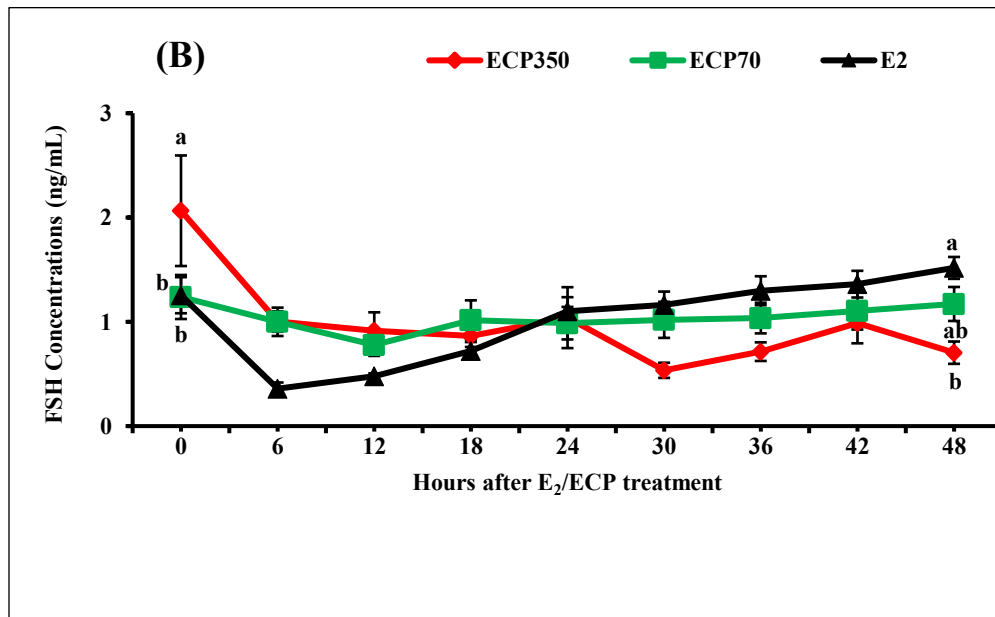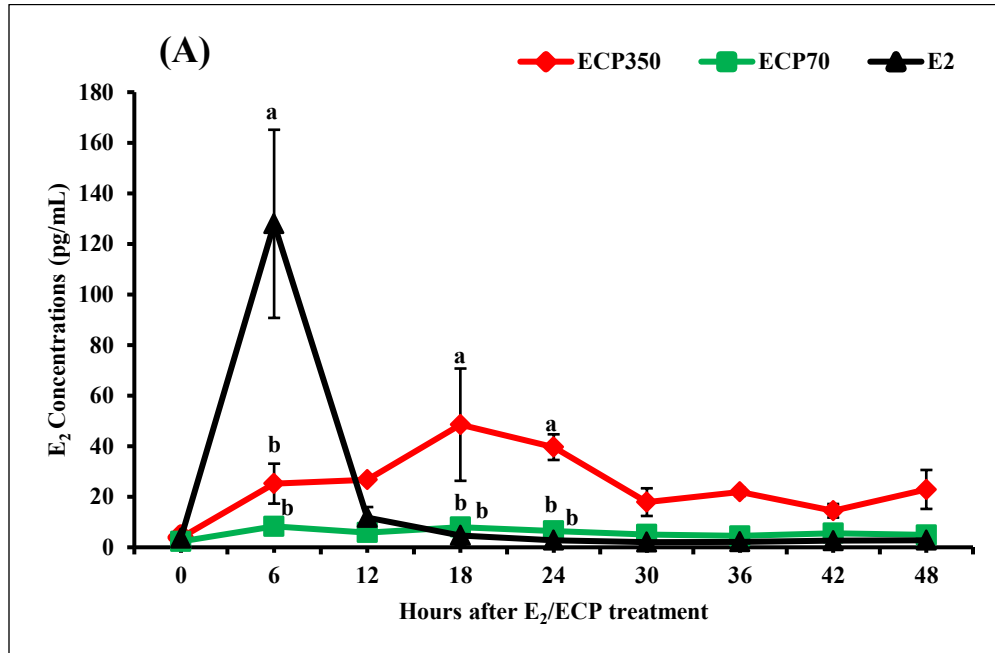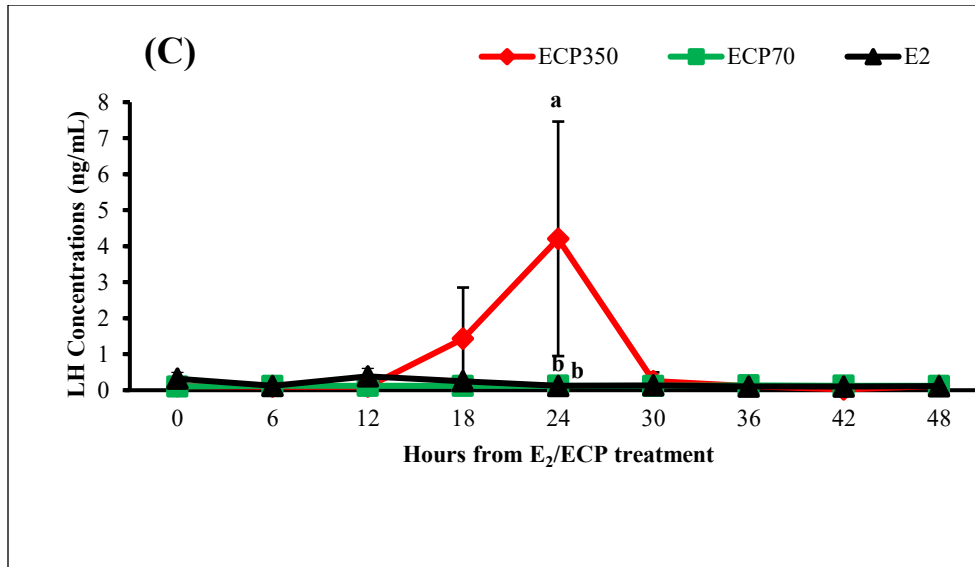
Figure 8. Mean (± S.E.M.) serum estradiol (panel A), FSH (panel B), and LH (panel C) concentrations for a 48 h interval after E_2/ECP treatment in seasonally anestrous Texel ewes treated with CIDRs for 12 days then assigned to the following groups: 350 μg E_2 (E_2; n=5), or 350 μg ECP (ECP350; n=4); or 70 μg ECP (ECP70; n=5) 6 days after CIDR insertion. At CIDR removal all ewes received an eCG injection.
a,b Indicate differences among groups within hour ($P<0.05$).

3.4.2.3 Serum FSH and LH Concentrations

For mean serum FSH and LH concentrations, there was a time and interaction effect ($P<0.05$), but no significant treatment effect during 48 h after E_2/ECP injection. Mean FSH concentrations decreased from 0 to 6 h and increased from 12 to 42 h after E_2/ECP injection ($P<0.05$); LH concentrations did not change. Mean FSH concentrations at 0 h after E_2/ECP injection was higher in ECP350 (2.07 ± 0.14 ng/mL) than ECP70 (1.24 ± 0.12 ng/mL) and E_2 (1.26 ± 0.12 ng/mL) groups ($P<0.001$; Figure 8B). Mean FSH concentrations at 48 h after E_2/ECP injection were lower in ECP350 (0.70 ± 0.14 ng/mL) than E_2 (1.52 ± 0.12 ng/mL) group ($P<0.01$); ECP70 (1.17 ± 0.12 ng/mL) was in-between ($P>0.05$; Figure 8B). Mean LH

concentrations at 24 h after E_2/ECP injection were higher in ECP350 (4.21±0.58 ng/mL) than ECP70 (0.13±0.52 ng/mL) and E_2 (0.12±0.52 ng/mL) groups (P<0.001; Figure 8C).

The interval from E_2/ECP injection to peak E_2 concentrations in E_2 (6.0±0.0 h) was shorter than ECP350 (19.5±2.9 h) group (P<0.05); ECP70 (12.0±3.8 h) was intermediate (P>0.05; Table 7). The interval from eCG injection to E_2 rise in E_2 (28.8 ± 3.5 h), ECP350 (30.0±4.9 h), and ECP70 (43.2±9.0 h) was similar (P>0.05). Mean E_2 concentrations associated with the estrous onset in ECP350 (7.5±1.7 pg/mL) was higher than E_2 (2.6±0.1 pg/mL) and ECP70 (3.3±0.8 pg/mL) groups (P<0.05; Table 7). The interval from eCG to the first FSH rise was shorter in ECP350 (32.0±2.0 h) than ECP70 (46.5±5.1 h) group (P<0.05); E_2 (38.4±1.5 h) group was intermediate (P>0.05; Table 7). The interval from eCG to the preovulatory LH surge peak was shorter in ECP350 (32.0±2.6 h) than ECP70 group (49.0±6.1 h; P<0.05); E_2 group (37.2±1.5 h) was intermediate (P>0.05). Preovulatory LH surge synchrony in ECP350 (3.3±1.2), ECP70 (7.3±3.2), and E_2 (2.6±0.8) groups were similar (P>0.05; Table 7).

Table 7. Mean (±SEM) hormone response timing or concentration after CIDR-E_2/ECP-eCG treatment in seasonally anestrous Texel ewes.

Variables	E_2	ECP350	ECP70
Interval from E_2/ECP injection to peak E_2 concentrations (h)	6.0 ± 0.0^a	19.5 ± 2.9^b	12.0 ± 3.8^{ab}
Interval from eCG injection to E_2 increase (h)	28.8 ± 3.5	30.0 ± 4.9	43.2 ± 9.0
E_2 concentrations associated with estrous onset (pg/mL)	2.6 ± 0.1^a	7.5 ± 1.7^b	3.3 ± 0.8^a
Interval from eCG injection to first FSH increase (h)	38.4 ± 1.5^{ab}	32.0 ± 2.0^a	46.5 ± 5.1^b
Interval from eCG injection to peak of the preovulatory LH surge (h)	37.2 ± 1.5^{ab}	32.0 ± 2.6^a	49.0 ± 6.1^b
Preovulatory LH surge synchrony (h from mean)	2.6 ± 0.8	3.3 ± 1.2	7.3 ± 3.2

Ewes were treated with CIDRs for 12 days then assigned to the following groups: 350 μg E_2 (E_2; n=5), or 350 μg ECP (ECP350; n=4); or 70 μg ECP (ECP70; n=5) 6 days after CIDR insertion. At CIDR removal all ewes received an eCG injection.
a,b Indicate differences among groups within row (P<0.05).

3.4.2.3.1 Characteristics of Peaks in Serum FSH Concentrations

The number of FSH peaks in E_2 (1.2±0.2), ECP70 (1.0±0.3), and ECP350 (1.0±0.6) groups was similar (P>0.05; Table 8). The daily FSH peak was detected only in two ECP350 ewes four days after E_2/ECP treatment. There was only one ewe in E_2 and one ewe in ECP70 groups that had two FSH peaks after E_2/ECP treatment. The peak concentration and amplitude of the FSH peak in E_2 group (2.10±0.22 ng/mL and 1.12±0.28 ng/mL) tended to be higher than ECP70 group (1.42±0.25 ng/mL and 0.47±0.09 ng/mL), (P=0.079 and P=0.086, respectively; Table 8). The duration of the FSH peak in E_2 (2.8±0.4 d) and ECP70 (2.8±0.5 d) groups was similar (P>0.05). The interval from E_2/ECP injection to the first FSH peak within 48 h, in E_2 group (30.0±6.0 h) was similar to ECP350 group

(34.0±5.3 h P>0.05; Table 8). The FSH peak was detected only in two ECP70 ewes at 18

h and 24 h after E_2/ECP treatment. The interval from E_2/ECP injection to the first FSH

peak within five days in E_2 group (2.6±6.0 d) was similar to ECP70 group (4.0±0.9 d

P>0.05; Table 8). The FSH peak associated with follicular wave emergence in E_2 group

(3.2±0.5 d) tended to be shorter than ECP70 group (5.0±0.8 d) after E_2/ECP treatment

(P=0.085; Table 8).

Table 8. Mean (±S.E.M.) characteristics of peaks in serum FSH concentrations in seasonally anestrous Texel ewes.

FSH peaks	E_2 n=5	ECP350 n=4	ECP70 n=4 or 5	P-value
Number of peaks	1.2±0.2	1.0±0.6	1.0±0.3 (n=5)	0.902
Peak concentration (ng/mL)	2.10±0.22	---	1.42±0.25 (n=4)	0.079
Peak amplitude (ng/mL)	1.12±0.28	---	0.47±0.09 (n=4)	0.086
Peak duration (d)	2.8±0.4	---	2.8±0.5 (n=4)	0.936
Interval from E_2/ECP injection to the first peak within 48 h (h)	30.0±6.0	34.0±5.3	---	0.643
Interval from E_2/ECP injection to the first daily peak within 5 d (d)	2.6±6.0	---	4.0±0.9 (n=5)	0.230
Interval from E_2/ECP injection to the peak associated with follicular wave emergence (d)	3.2±0.5	---	5.0±0.8 (n=5)	0.085

Ewes were treated with CIDRs for 12 days then assigned to the following groups: 350 µg E_2 (E_2; n=5), or 350 µg ECP (ECP350; n=4); or 70 µg ECP (ECP70; n=5) 6 days after CIDR insertion. At CIDR removal all ewes received an eCG injection.
--- FSH peaks occurred only in two ECP350 ewes within 5 d, and two ECP70 ewes within 48 h after E_2/ECP injection.

3.4.2.4 Serum P_4 Concentrations

Mean serum P_4 concentrations on Day -12 in ECP350 (0.69±0.08 ng/mL), ECP70

(0.64±0.06 ng/mL), and E_2 (0.63±0.06 ng/mL) groups was similar (Table 9). In

ovulated ewes (no later than Day 7 after eCG injection), for mean P_4 concentrations there was a day effect ($P<0.001$), but no significant treatment or interaction effect during the 17 days after ovulation. Mean P_4 concentrations increased from Day 0 to 7 after ovulation ($P<0.001$).

The average of the ovulation times in the ovulated ewes (3 days after eCG injection) was considered as the ovulation time for those ewes that did not ovulate by Day 7 after eCG injection. One E_2 ewe did not ovulate, however, she formed two luteinized follicles. Progesterone concentrations in this animal increased from Day 3 to 12 after eCG injection. In one ovulated ECP70 ewe, P_4 concentrations were approximately two times higher than the other ovulated ewes from Day 7 to 17 after ovulation. In ECP350 ewes P_4 concentrations increased slightly during the 20 days after eCG injection.

For mean maximum P_4 concentrations in all ewes, ECP350 (0.54 ± 0.02 ng/mL) was lower than E_2 (7.48 ± 0.65 ng/mL) group ($P<0.05$); ECP70 ewes were intermediate (5.93 ± 2.60 ng/mL; $P>0.05$; Table 9). In the ewes that ovulated by Day 7 after eCG injection, there was no significant difference in the mean maximum P_4 concentrations between E_2 (8.07 ± 0.34 ng/mL) and ECP70 groups (8.17 ± 3.84 ng/mL; Table 9).

Table 9. Mean (±SEM) serum P_4 concentrations (ng/mL) on the day of CIDR insertion (D -12) and during 17 days after ovulation in seasonally anestrous Texel ewes.

Serum P_4 concentrations (ng/mL)	E_2	ECP350	ECP70
Day -12	0.63±0.06	0.69±0.08	0.64±0.06
Maximum concentrations in all ewes	7.48±0.65[b]	0.54±0.02[a]	5.93±2.60[ab]
Maximum concentrations in ovulated ewes*	8.07±0.34	---	8.17±3.84

Ewes were treated with CIDRs for 12 days then assigned to the following groups: 350 μg E_2 (E_2; n=5), or 350 μg ECP (ECP350; n=4); or 70 μg ECP (ECP70; n=5) 6 days after CIDR insertion. At CIDR removal (Day 0) all ewes received an eCG injection.
* Ewes that ovulated by no later than Day 7 after eCG injection.
a,b Indicate differences between groups within a row (P<0.05).

Mean P_4 concentrations for all ewes on Days 0, 7, 12, 15, and 17 after ovulation were analyzed. The average of the ovulation times in the ovulated ewes (3 days after eCG injection) was considered as the ovulation time for those ewes that did not ovulate by Day 7 after eCG injection. For this analysis there was a day and interaction effect (P<0.05) and a tendency for a treatment effect (P=0.075). Mean P_4 concentrations increased from Day 0 to 7 after ovulation (P<0.001; Figure 9). Mean P_4 concentrations in the ECP350 group (0.43±1.12 ng/mL) tended to be lower than the E_2 group (3.94±1.00 ng/mL; P=0.092), although, the ECP70 group (3.74±1.03 ng/mL) was similar to both the E_2 and ECP350 groups. Mean P_4 concentrations on Day 12 after ovulation were lower in the ECP350 group (0.40±0.72 ng/mL) than both the E_2 (5.78±0.64 ng/mL) and ECP70 (5.22±0.64 ng/mL) groups (P<0.05; Figure 9). Mean P_4 concentrations on Day 15 after ovulation were lower in the ECP350 group (0.39±0.72 ng/mL) than the E_2 group (4.89±0.64 ng/mL; P<0.05); the ECP70 group (4.49±0.64 ng/mL) was intermediate (P>0.05; Figure 9).
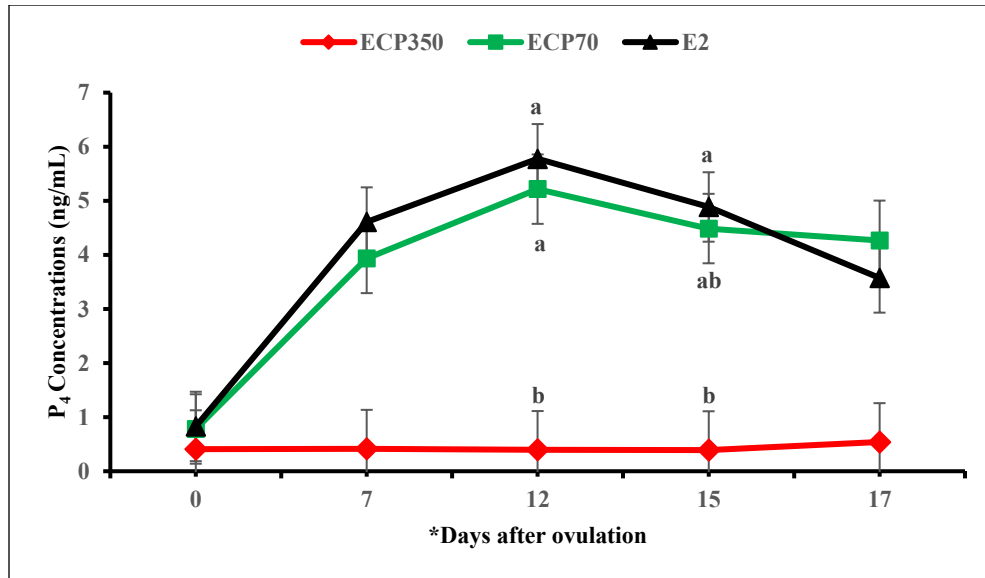
Figure 9. Mean (± S.E.M.) serum P_4 concentrations on Days 0, 7, 12, 15, and 17 after ovulation in seasonally anestrous Texel ewes treated with CIDRs for 12 days then assigned to the following groups: 350 µg E_2 (E_2; n=5), or 350 µg ECP (ECP350; n=4); or 70 µg ECP (ECP70; n=5) 6 days after CIDR insertion. At CIDR removal all ewes received an eCG injection.
*No ECP350 ewes ovulated by Day 7 after eCG, so, the average ovulation time in E_2 and ECP70 ewes (Day 3 after eCG) was considered as the ovulation time for ECP350 ewes.
a,b Indicate differences among groups within day ($P<0.05$).

3.5 Discussion

In modern agricultural systems, assisted reproductive technologies are used for reproductive performance and genetic improvement [155]. To our knowledge, the present study was the first study that compared the effects of CIDR-eCG plus different doses of ECP with E_2 on the hormonal profiles and ovarian responses in seasonally anestrous ewes. In this study, seasonally anestrous ewes received CIDRs for 12 days and an injection of E_2/ECP six days after CIDR insertion, and an eCG injection at CIDR removal. Before treatment initiation, all ewes were anestrus as indicated by serum P_4 concentrations being less than 1 ng/mL on Day -12. It has been documented that administration of estrogen or its esters can improve estrous response in sheep and cows [14,15,138,156,157]. The high

estrous response in the present study (only one ECP70 ewe did not exhibit estrus) agrees with this theory. The high estrous expression could be a result of estrogen treatment which increases circulating E_2 concentrations [14]. In our study, estrus in both the E_2 and ECP350 groups started around the same time (30-36 h after eCG), similar to the previous study [88]. Whereas in the ECP70 group the estrous onset was significantly delayed (~ 56 h after eCG) and it was close to another study (~ 53 h after eCG) on seasonally anestrous ewes [87]. In the ECP350 group, only small follicles were observed from Days 0 to 3 and on Day 7 after eCG injection. Although, ovaries were not observed from Days 4 to 6 after eCG injection, it is assumed based on known ovine reproductive biology that only small follicles and no ovulation would be observed during this time period. All ECP350 ewes showed estrus, it is obvious that the residual exogenous estradiol and not endogenous estradiol from preovulatory follicles induced estrous behaviors in ECP350 ewes. This idea is supported by a study on beef heifers, in which ECP treatment induced estrus in most heifers with small follicles. The author suggested that it seems to be as a result of prolonged elevated E_2 concentrations after ECP treatment [14], which is in agreement with the serum E_2 changes in ECP350 ewes in our study. In the present study, the interval from eCG injection to E_2 increase in E_2 and ECP groups was about 29-43 h. However, this interval in CIDR-E_2-eCG treated seasonally anestrous ewes was about 18 h in a previous study [89]. Differences could be due to animal breed, body weight/condition score, nutrition, and management practices. The estrous duration in two ECP350 ewes lasted more than 60 h. While in E_2 and ECP70 ewes the estrous length was similar (~ 30 h) and close to the estrous duration (37 h) in CIDR-E_2-eCG treated seasonally anestrous ewes in a previous study

[89]. We inferred that extended exposure to E_2 for a longer period caused a very long estrous duration in the ECP350 group.

It has been reported that, in sheep and cows, estrous exhibition results in higher pregnancy rates [13,15,122,135,158–160]. Although in these studies endogenous E_2 secreted from large follicles induced estrus and consequently resulted in higher pregnancy rates. These results are in agreement with the higher pregnancy rates in the E_2 group in our study. However, lack of large follicles by seven days after eCG injection resulted in no pregnancy in the ECP350 ewes. The relationship between estrous exhibition and pregnancy rates in the ECP350 group is in agreement with a study on seasonally anestrous ewes treated with a pharmacological dosage of EB (400 µg), in which, despite high estrous responses, fertility was low [122]. Also, 200 µg of EB in a P_4-EB treatment in goats induced estrus, a LH surge, and ovulation, but resulted in low fertility [72]. These results might be related to the animals' ovarian follicular dynamics after treatment. Thus, low pregnancy rates in ECP350 ewes in the current study and previous studies [72,161] seems to be due to prolonged high E_2 concentrations (as a result of a pharmacological dosage of estrogen ester treatments) and lack of mature dominant follicles.

In the present study mean serum E_2 concentrations in the E_2 group peaked 6 h after E_2 injection, which was in agreement with E_2 concentration changes in seasonally anestrous ewes and beef heifers in previous studies [18,59]. However, mean serum E_2 concentrations in seasonally anestrous ewes only treated with one or two E_2 injections, peaked 2 h [143] and 4 h [144] after injection. Differences may be due to administration of P_4, lack of exogenous P_4 priming, different E_2 doses, sampling methods, as well as nutrition and management systems. Similar to our study, E_2 concentrations increased faster and reached

a higher peak in beef heifers after E_2 injection than after treatment with estrogen esters [59,134]. A higher dose of ECP caused more prolonged elevated E_2 concentration than the other estrogen esters [59]. It seems that changes in serum E_2 concentrations depend on the formulation of the applied estrogen. Thus, the effects of various estrogen forms may vary on concentrations of gonadotropins and follicular wave emergence.

Serum FSH concentrations at 0 h after E_2/ECP injection in ECP350 ewes were higher than the other groups. This could not be a result of ECP treatment, but perhaps serum FSH concentrations in individual ewes in this group were higher than the other groups before treatment. Serum FSH concentrations decreased 6 h after E_2/ECP injection, resulting in blocked follicular wave emergence. This was in agreement with the effect of E_2 or its esters on seasonally anestrous ewes and beef heifers in other studies [18,59]. Serum FSH concentrations at 48 h after E_2/ECP injection in the ECP350 group were lower than the E_2 group. It seems to be related to the negative feedback of E_2 on FSH secretion [162] as ECP350 induced prolonged elevated serum E_2 concentrations, resulting in suppression of FSH concentrations for a longer period.

In our study, mean serum FSH concentration patterns after E_2 treatment were similar to previous studies on ewes [18,143,144,163,164]. Daily FSH peak concentrations and amplitudes induced by E_2 and ECP70 treatments were similar to previous studies in ewes treated with a P_4-E_2 treatment [18,163,164]. Also, the number of FSH peaks in the E_2, ECP70, and ECP350 groups in this study were close to those of previous studies [163,164]. Whereas the FSH peak duration in the E_2 and ECP70 groups were similar to [164], shorter [163], and longer [18] than the previous studies. Differences could be due to animal breed, management, and nutrition. The FSH peaks were detected only in two ECP350 ewes. The

occurrence of the FSH peak associated with follicular wave emergence in the ECP70 group was later than in the E_2 group, however, there was no significant difference between groups. But, the later FSH rise delayed follicular wave emergence in ECP70 ewes. These results are supported by studies on cows and ewes treated with E_2 or its esters in which estrogen ester treatments delayed follicular wave emergence [11,59,134,161,165,166]. It could be explained that as estrogen esters are metabolized and absorbed slower than E_2, they delay follicular wave emergence [11,59]. So, in comparison with E_2, ECP delays the FSH peak and follicle wave emergence. The ester might also cause more variable follicle wave emergence since the timing of metabolization can vary among different animals. This may support our results, if we suppose daily FSH peaks, follicular wave emergence, and ovulation in the ECP350 group occurred later than Day 7 after eCG injection (after metabolization of ECP350). However, it is possible that FSH peaks occurred between Day 5 and 7 after eCG injection because FSH peak analysis was not done over these days. These results seem to be related to the pharmacokinetics of ECP [57,60–62,167]; the production and release of FSH depends on the dosage and formulation of estrogen treatments.

In a previous study on seasonally anestrous ewes, supra-physiological concentrations of E_2 decreased FSH concentrations, but no follicular wave emerged during the 9 days after treatment [163]. These findings were close to the effect of ECP350 on the suppression of FSH and follicular wave emergence in our study. We inferred that since the endogenous FSH peaks after E_2/ECP injection were suppressed, perhaps the small follicles did not receive sufficient FSH signals to enter into a follicular wave. This idea is supported by studies on the FSH-dependency of the follicular wave in ewes [41,168]. According to Barrett et al. [41], follicles smaller than 2 mm cannot respond to the proper FSH signal to

enter a follicular wave. This can be a reason for no follicular wave emergence in ECP350 ewes who had follicles equal to or less than 2 mm after treatment. It means that there is a threshold FSH concentration that must be reached to cause follicular wave emergence [41].

Our findings show that E_2/ECP treatment reduced the diameter of the largest follicles and induced new follicular wave emergence about 3-6 days later, which agrees with other studies [18,138,144,169]. The time of follicular wave emergence in our ECP70 group is close to estrogen or estrogen ester treated seasonally anestrous ewes and cows in previous studies (~ 6 vs. 5-6 days after estrogen treatment) [18,59,134,138]. However, follicular wave emergence timing in our E_2 group is similar to [169] or different from [18] E_2 treated seasonally anestrous ewes in previous studies. The variation could be due to animal breed, (as the ovarian sensitivity/response can vary among animals) or the application of different kinds of P_4 devices. Furthermore, in our E_2 group time of follicular wave emergence agrees with the effect of reduced dosages of EV and EB on cows in previous studies (~ 3 vs. 3-4 days after estrogen treatment) [59,134]. In the current study, both the E_2 and ECP70 treatments synchronized follicular wave emergence; synchrony of wave emergence in the E_2 and ECP70 groups was similar. In the present study, the effect of E_2 and ECP70 treatments on follicular wave emergence synchrony agrees with the effect of E_2 and reduced dosages of estrogen esters in previous studies on cows and anestrous ewes [18,59,134,143,144].

In our study, E_2/ECP treatment brought about a rapid decrease in FSH concentrations, but not LH concentrations. This is in agreement with LH concentration changes in E_2 treated seasonally anestrous ewes in previous studies [143,144]. However, serum LH concentrations peaked at 24 h after E_2/ECP treatment in the ECP350 group. This increase

approximately coincided with the peak serum E_2 (18 h after E_2/ECP), and higher E_2 (at 18 and 24 h after treatment) concentrations in the ECP350 group than the other groups. These observations could be due to an increase of the pituitary gland sensitivity to GnRH after E_2/ECP treatment. The number of GnRH receptors on the pituitary gland regulates gonadotropin secretion [170,171]. It has been shown that both E_2 and EB increase the number of GnRH receptors and pituitary gland responsiveness in ewes and cattle, resulting in LH release [172,173]. Based on these studies it is suggested that elevated E_2 concentrations from 18-24 h after E_2/ECP injection induced the LH peak at 24 h after treatment in the ECP350 group.

The preovulatory LH surge is an important factor that can indicate if synchronous ovulation occurred [14,136,137]. In the present study the preovulatory LH surge occurred about 32-37 h after eCG injection in the E_2 and ECP350 groups. This was in agreement with the preovulatory LH surge timing (~ 37 h after eCG injection) in P_4-E_2-eCG treated seasonally anestrous ewes in an earlier study [89]. The LH surge timing in the ECP70 group (~ 49 h after eCG injection) in our study, was close to that of ECP treated beef heifers (~ 51 h after eCG injection) in a previous study [174]. Our findings showed that E_2 and ECP treatments synchronized the preovulatory LH surge, which is in agreement with the effects of E_2 in a CIDR- E_2-eCG treatment on seasonally anestrous ewes [89].

In our study E_2 and ECP70 treatments synchronized ovulation. This is in agreement with the effects of E_2 on seasonally anestrous ewes [18] and ECP and EB on beef cows [174]. It seems that exogenous estrogen or its esters synchronize the LH surge, resulting in synchronized ovulation. Although in our study ovulation rates in the ECP70 group were less than the ECP treated beef heifers (~ 60% vs. 80%) in a previous study [174].

Differences can be due to the animal species, which may differently respond to the hormonal treatments or dosages of ECP.

In the present study, ovulation did not occur in all ECP70 and E_2 ewes, similar to seasonally anestrous ewes treated with one/two E_2 injections or a MAP-E_2 treatment [18,143,144]. However, all seasonally anestrous ewes treated with a MAP-E_2-eCG treatment ovulated [18]. Based on a previous study on seasonally anestrous ewes [143], even if the follicles grow to a preovulatory size and LH surges are detected, no ovulation occurrence might be due to the immaturity of the largest follicle to respond to changes in LH concentrations [143]. Thus, it could be a reason for no ovulation occurrence in one E_2 and two ECP70 ewes in our study. However, there was no follicular development to the preovulatory size in the ECP350 group by Day 7 after eCG. The ovulation time in both E_2 and ECP70 groups (~ 3 days after eCG injection) in our study is close to the seasonally anestrous ewes treated with the same E_2 treatment [18,146], as well as ECP treated cows [174,175].

As was previously stated, follicular wave emergence and ovulation in the ECP350 group may have occurred later than Day 7 after eCG injection. This idea is supported by a study on cyclic ewes, treated with P_4 sponges and small or large E_2 implants [41]. In this study, follicular wave emergence and ovulation in the large E_2 implant group occurred later than the small E_2 implant group [41]. It has also been reported that a higher dosage of estrogen esters lengthened the interval from treatment to the FSH rise and follicular wave emergence in cows [59,134]. Thus, the delayed follicular wave emergence could be a result of an inadequate FSH signal until implant removal [41] or metabolization of estrogen esters in the cows [59,134] and the present study. However, these findings are not in agreement with

the effects of high and low doses of ECP on postpartum anestrous beef cows [176], in which the ovulation time was earlier in the higher ECP dose group than the lower ECP dose group [176].

Interestingly, similar to ECP350 ewes, the ovulatory follicle size did not improve or was smaller as the dose of ECP increased in cows [15,176]. These findings are somewhat in agreement with our findings. Although ovulatory follicles were not observed in the ECP350 group, daily MFD in this group was significantly smaller than the E_2 and ECP70 groups from Days -1 to 3 and Days 1 to 3 relative to eCG injection, respectively. Based on these observations, it seems that follicle growth depends on the dose of ECP treatment.

According to Bartlewski et al. [177], P_4 concentrations increase 3-7 days after ovulation and approximately peak at Day 12, then start to decrease if a ewe is not pregnant. The P_4 concentrations in the current study increased from Day 0-7 after ovulation, which is close to the findings of Bartlewski et al. [177]. There were no significant differences among P_4 concentrations on Days 7, 12, 15, and 17 after ovulation in our study. In addition, in seasonally anestrous ewes treated with a P_4-E_2-eCG treatment, P_4 concentrations increased from Days 1 to 11 after ovulation [146]. Many ewes were pregnant in our study. There was only one E_2 ewe that did not ovulate, but P_4 concentrations in this animal increased from Days 3 to 12 after eCG injection, probably because of the observed luteinizing follicles. In ECP350 ewes, ovulation likely occurred later on because P_4 concentrations increased slightly during the 20 days after eCG injection.

3.6 Conclusion

This study compared the effects of E_2, ECP70, and ECP350 treatments in a CIDR-eCG synchronization protocol on estrous, hormonal, and ovarian responses in seasonally

anestrous ewes. Mean E_2 and FSH concentrations fluctuated during the 48 h after E_2/ECP injection, while mean LH concentrations did not change during this time period. All ewes except one ECP70 ewe showed estrus. Estrous and ovulation features, follicular wave emergence synchrony, number of CL, and LH, FSH, P_4 and most E_2 patterns in E_2 and ECP70 ewes were similar. In the ECP350 group follicles were equal to or less than 2 mm in diameter from Days -3 to 3 and on Day 7 relative to eCG. Daily MFD was greater in both the E_2 and ECP70 groups than the ECP350 group on Days 2 and 3 after eCG. The follicular wave emerged later in the ECP70 group than the E_2 group. Four E_2, three ECP70, and no ECP350 ewes ovulated by 7 days after eCG. Mean P_4 concentrations increased until 7 days after ovulation. Pregnancy was observed in three E_2 and two ECP70 ewes. In conclusion, perhaps E_2 can be replaced with ECP70 in the CIDR-E_2-eCG synchronization protocol in seasonally anestrous ewes.

CHAPTER 4: CONCLUSION

4.1 Summary and Conclusion

Sheep are seasonal breeders; they cannot naturally breed during spring and summer. Thus, their annual pregnancy and lambing rates are low. To tackle the seasonality of lamb production, assisted reproductive technologies are required for out of season breeding. Ewes can be induced to breed during the anestrous season by the introduction of rams (ram effect) or using photoperiod regulation protocols, but hormonal treatments are necessary for tightly controlled synchronization programs. Synchronization protocols can be used with ram breeding and in TAI and embryo transfer programs, allowing producers to improve pregnancy and lambing rates and the genetics of their flocks.

In ewes, P_4-eCG treatments have successfully synchronized estrus and ovulation during the breeding and non-breeding seasons. However, estrous synchronization does not necessarily result in follicular wave emergence synchronization. With estrous synchronization protocols follicles either are not mature enough to ovulate, or the mature follicles ovulate at different time points of development. Administration of exogenous hormones such as E_2, in estrous synchronization protocols, can synchronize follicle wave emergence, estrus, and ovulation. However, E_2 is not licensed for commercial use in Canada, the USA, and many other countries, thus, estrogen esters have been investigated as alternatives to E_2. Estradiol cypionate is the only estrogen allowed for use in ruminant reproductive management in Canada. It has been shown that ECP can synchronize follicle wave emergence in cattle and, therefore, we believed that it could also synchronize the emergence of the follicle wave and ovulation in seasonally anestrous ewes.

Accordingly, this study compared the effects of two different doses of ECP with E_2 in a P_4-eCG protocol in seasonally anestrous ewes. In this study, 14 Texel ewes received CIDRs for 12 days and an eCG injection at CIDR removal. Animals were randomly divided into three groups, to receive one of the following treatments; 350 µg of E_2, or 350 µg or 70 µg of ECP six days after CIDR insertion. Mean E_2 and FSH concentrations changed during the 48 h after E_2/ECP injection, but mean LH concentrations were similar during this time period. Only one ECP70 ewe did not express estrus. Estrous and ovulation characteristics, synchrony of follicular wave emergence, number of CL, and LH, FSH, P_4 and most E_2 profiles in the E_2 and ECP70 groups were alike. In the ECP350 group, only small follicles (≤ 2 mm) were observed from Days -3 to 3 and on Day 7 relative to eCG injection. Daily MFD in both the E_2 and ECP70 groups was larger than the ECP350 group on Days 2 and 3 after eCG injection. Follicular wave emergence occurred earlier in the E_2 group than the ECP70 group. Ovulation occurred in four E_2, three ECP70, and no ECP350 ewes by Day 7 after eCG injection. Mean P_4 concentrations increased until 7 days after ovulation. Three E_2 and two ECP70 ewes became pregnant. Based on our results, ECP70 seems to be a proper replacement for E_2 in the CIDR-E_2-eCG seasonally anestrous ewe synchronization program.

If the P_4-ECP70-eCG treatment was successfully approved as a synchronization procedure in seasonally anestrous ewes, it could be applied in TAI and embryo transfer programs. Although small sheep producers tend to prefer natural mating rather than AI and embryo transfer (as assisted reproductive technologies are expensive), purebred sheep breeding in Canada and around the world does require TAI and embryo transfer programs. These programs improve flock pregnancy and lambing rates per year and prevent the

spread of infectious diseases. Furthermore, these programs remove the cost of ram maintenance, help in maintaining desired breeds, and result in genetic improvement. Synchronization programs are necessary for AI and embryo transfer, as they can provide predictable and synchronized estrus and ovulation.

Collectively, by comparing the effects of two different doses of ECP with E_2 treatment, the findings from this study suggest that perhaps ECP70 is a proper alternative to E_2 in the CIDR-E_2-eCG seasonally anestrous ewe synchronization protocol. However, further studies are still needed to fully ascertain the effects of ECP on seasonally anestrous ewe reproductive performance with synchronization programs.

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APPENDIX

Table A1. Nutritional value of alfalfa hay and barley.

Parameter	Alfalfa hay		Barley	
	As fed	Dry	As fed	Dry
Dry Matter (%)	90.69	---	89.24	---
Crude Protein (%)	12.38	13.65	18.70	20.95
ADF (%)	30.10	33.19	---	---
NDF (%)	49.20	54.25	---	---
TDN (%)	58.86	64.90	---	---
DE (Mcal/kg)	2.47	2.72	---	---
NEL (Mcal/kg)	1.26	1.39	---	---
NEG (Mcal/kg)	0.77	0.85	---	---
NEM (Mcal/kg)	1.41	1.55	---	---
Calcium (%)	0.254	0.280	1.05	1.17
Potassium (%)	2.238	2.468	0.84	0.94
Magnesium (%)	0.154	0.170	0.21	0.24
Phosphorus (%)	0.309	0.341	0.76	0.85
Sodium (%)	0.036	0.040	0.38	0.43
Copper (ppm)	6.84	7.54	6.26	7.01
Manganese (ppm)	107.62	118.67	190.83	213.84
Zinc (ppm)	27.66	30.50	187.04	209.59
Crude fat (%)	---	---	2.24	2.51

TDN=Total Digestible Nutrients, DE=Digestible Energy, NEL=Net Energy for Lactation, NEG=Net Energy for Gain, NEM=Net Energy for Maintenance.