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DEPARTMENT AND CLINIC OF REPRODUCTION

Stimulation of equine estrus early in the breeding season

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
BCS	body condition score
CL	corpus luteum
DYN	dynorphin
ELISA	enzyme-linked immunosorbent assay
E1	estrone
E2	estradiol
E3	estriol
GH	growth hormone
GnRH	gonadotropin releasing hormone
FSH	follicle stimulating hormone
LH	luteinizing hormone
HBCS	high body condition score
HPG	hypothalamo-pituitary-gonadal axis
IGF-1	Insulin like growth factor-1
IU	International unit
LBCS	low body condition score
NKB	neurokinin-B
ovPRL	ovine prolactin
PBS	phosphate buffered saline
PGs	prostaglandins
PRL	prolactin
P4	progesterone
reFSH	recombinant follicle stimulating hormone
reLH	recombinant luteinizing hormone
RHT	retino-hypothalamic tract
rpPRL	recombinant porcine prolactin
SCN	suprachiasmatic nucleus
TSH	thyroid stimulating hormone
V	volt
W	watt

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1. INTRODUCTION

Horse breeding has been practiced for centuries originating from the Bedouin people in the Middle East. The timing of the first breeding programme is blurry but first documents originate back to 1330 AD. The Bedouins reputation was to breed careful breeding of Arabian horses with extensive pedigrees of great bloodlines. Later on different cultures started breeding horses depending on their needs and different horse breeds were created.

Nowadays the demand for quality athletic horses has increased greatly and breeders and horse trainers troughout the world have worked hard to meet the markets consistent demands. Human directed selective breeding programs and better management techniques have improved the quality and consistency of the horses, whether it is for high quality sport horses or leisure horses. Successful reproductive performance is the main goal of every breeders regardless of what kind of horses they intend to breed for.

Most breed registries have imposed that all foals born in a partiular year have an industry imposed birthdate of 1st of January that year. Because of the fact that mares are seasonal polyestrous animals it is a great challenge for horse breeders and equine pratitioners to bring the time of parturition as close to 1st of January as possible. Breeding dates in the first 3 months of the year offer the greatest competitive edge but this is a great challenge as 75-80% of all mares in the Northern Hemisphere are still in deep winter anestrus or early transition at this time (Smith et al, 1989). Early foals are highly advantageous in breeds that compete in athletic events at young age because those born closest to 1st of January are more mature when coming to the track than those born at natural timing in April-June. Different methods to stimulate mares to come into estrus earlier in the breeding season have been examined for decades with significant progress. With more understanding of the neuro-endocrine system, more possibilities to control the seasonal changes are now available including environmental and/or hormonal factors.

This thesis will describe the horse reproductive physiology followed by different methods how to manipulate the mares‘ estrous cycle to hasten the onset of season based on researches performed.

2. REPRODUCTIVE PHYSIOLOGY OF THE MARE

2.1. Reproductive cycle of mares

Horse is a seasonal polyestrous species with a natural breeding season occuring from April to October in the Northern Hemisphere. The seasonal reproductive pattern is a result of hormonal changes associated with the increase in daylight, temperature and improved feeding. Those factors have made a favourable evolution for the combination of the 11 months long gestation and the optimal timing of parturition to occur when there is enough food and the conditions are optimal for the developing offspring (Hughes *et al*, 1975).

Winter anestrus

The winter anestrus is the period of the year characterized by prolonged ovarian inactivity when roughly 80% of mares will not show signs of estrus and do not ovulate. The hours of daylight is short which will cause high amount of melatonin to be produced, resulting in GnRH suppression. The length of the period depends on different factors:

- Breed: Pony mares tend to show absence of ovarian activity during winter and resume cyclicity later than larger breeds.
- Individuality: Mares which showed inactivity in the previous years are more likely to show ovarian inactivity in the following years
- Age: young mares do not show ovarian activity during the winter before their first reproductive season
- Physiological state: nursing and lactating mares will more likely be inactive in the coming winter
- Body condition: nutrition has shown to have a long term effect on initiation of cyclicity where mares in poor body condition will come later into season. (Ellendorff and Elsaesser, 1985).

To describe how to evaluate mares to be in deep anestrus, following criteria must be met:

- Circulating progesterone (P4) shall be <1 ng/ml in three consecutive samples taken with 10 day interval.
- Ovaries are small and firm on rectal palpation or examination with ultrasonography. Follicles shall be <20 mm in diameter, no corpus luteum (CL) for the last 30 days.
- Uterine and cervical tone examined by transrectal ultrasonography and palpation shall be in accordance with anestrus condition (Mari *et al*, 2009).

Spring transition

As daylight begins to get longer during springtime, hormones controlled by the brain act as chemical messengers in the mare‘s body to prepare her for the coming breeding season. As a result, the ovaries start to grow follicles and produce eggs further affecting the brain through feedback loops. Most influencing factor for this period is the light detected by the eye but other additional factors, such as temperature and body condition also play a big role. Green pastures are also thought to have an affect on making mares start cycling earlier, this is called ´Green grass effect´. Stress acts negatively on the mare by preventing her to come into cycle so travelling with her during this period should be avoided. This period is frustrating for many horse owners and veterinarians as the mares may show erratic signs of estrus but they are not able to conceive until after the first ovulation of the season which marks the end of spring transition period (Gunn *et al*, retrieved October 10th 2017) .

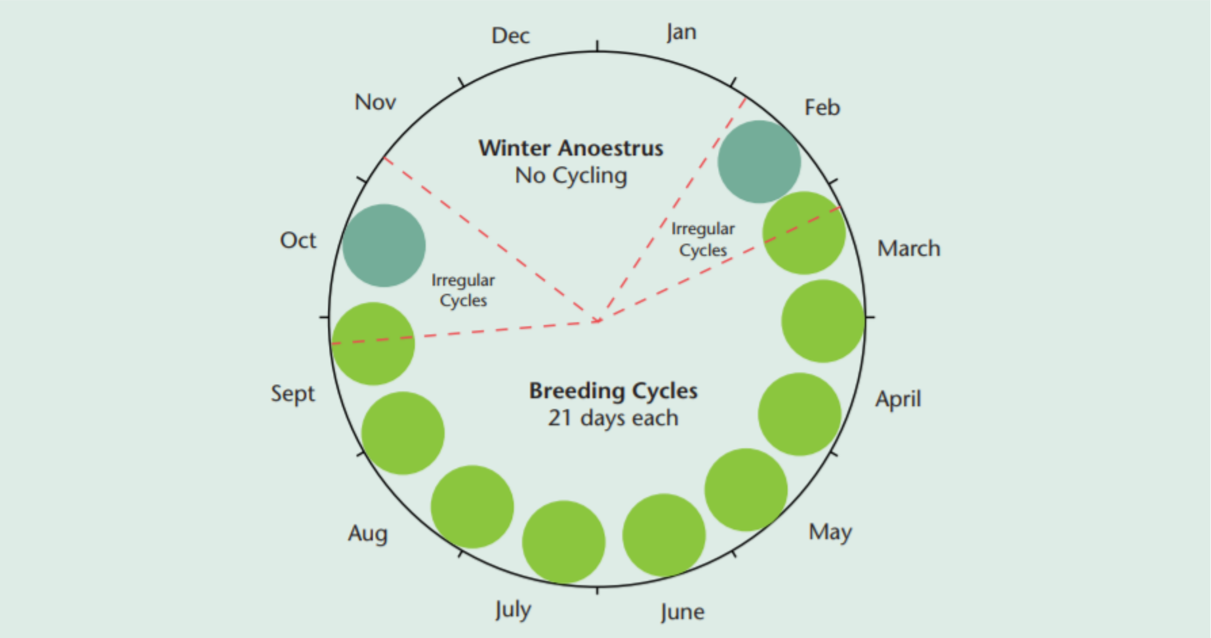


Figure 1: Mare‘s reproductive cycle (Adopted from Conlon and McArdle, 2016).

When follicles reach 20-25 mm in diameter it is marked as the onset of spring transition period which lasts for about 50-70 days on average. The number of antral follicles will increase during the period and will continue to grow and produce estrogen (McCue, 2006). The follicles however fail to ovulate because of insufficient production of LH (luteinising hormone) partly as a result of low concentration of estrogens, inhibin and other factors present in the follicular fluid such as IGF-1 (insulin-like growth factor) (Aurich, 2011). The low level of those hormones will simultaneously inhibit the negative feedback on FSH so GnRH level will remain high. The ovaries will show multiple follicular waves producing and secreting estrogen until one follicle eventually ovulates, marking the end of spring transition and the mare will generally continue to cycle at regular intervals (McCue, 2006).

Estrous cycle

The estrous cycle is the interval between two consecutive ovulations. The length of one estrous cycle in mares is approximately 21 days, but can vary between 18-24 days. Each cycle can be devided into stages based on behavior or gonadal events. The terminology is as follows:

- Proestrus: building up phase. Follicles are developing and produce estrogen. The mare may show signs of estrus but does not accept the stallion yet.
- Estrus: sexual receptivity. Elevation of estrogens from the mature follicles just prior to ovulation.
- Metestrus: sexual receptivity ends. After ovulation, granulosa cells form the developing corpus luteum and start to produce progesterone.
- Diestrus: Mature CL (corpus luteum) characterizes this phase with high concentration of progesterone and low levels of estrogen. Follicles may start to grow but are not able to ovulate (Klein, 2013).

Estrous cycle based on secretory patterns of the ovary:

a. Follicular phase: this phase includes proestrus and estrus. During this period, follicles at different stages are developing and there is an increase in E2 secretion. It has a duration of 5-7 days on average, but is shorter during spring and longer during autumn. The mare start to show estrus behaviour already early in the follicular phase and will within few days be sexually receptive to the stallion. Follicles are growing to provide optimal environment for oocyte maturation and during each follicular wave one or more follicles dominate while others undergo atresia. In the end, one dominant follicle to ovulate is ideal as multiple ovulation is not preferred in mares due to many complications. The follicles produce estrogen and as the follicles become larger, more estrogen is produced. The increase in estrogen and decrease in progesterone during this phase are responsible for the behavioural changes of the mare and 24-48 hours before the end of estrus the mare ovulates.

b. Luteal phase: this phase includes metestrus and diestrus. When the follicle ovulates it undergoes structural and functional changes, the granulosa cells transform into luteal cells and start to produce progesteone (P4) instead of estrogen (E2). Corpus luteum is now the primary ovarian structure and this process is called luteinization. Before the follicular cells tranform to luteal cells the follicular cavity fills with blood and fibroblasts, this intermediate structure is called corpus haemorrhagicum. It takes the corpus luteum about 5 days postovulation to reach high level of progesteron production which remains until the end of diestrus. Average length of the luteal phase is 14-15 days but may be longer during mid-summer and shorter during spring or autum. During this period of time the mare does not show sexual receptivity but follicular waves are already present to prepare the next estrus period.

2.2. Neuroendocrine control of the estrous cycle

2.2.1. The endocrine organs

Pineal gland

The pineal gland serves an important endocrine function in several body functions including the synchronization of the gonadal activity. The gland is a tubular evagination of the diencephalic roof and is composed of hormone producing pinealocytes, secreting melatonin. Even though the gland is derived from embryonic neural epithelium, the pinealocytes are not real nerve cells but are so-called paraneurons because of their ability to secrete neurotransmitter at a synaptic junction but without dentrites or axon. The pineal gland is able to convert a light signal input into a hormonal output and acts that way as neuro-chemical, neuro-endocrine transducer (Ueck and Wake, 1977).

Hypothalamus

The hypothalamus has many roles in the body and reproduction is one of its primary importance. It is a relatively small area of the brain at the ventral portion of the diencephalon and acts as the driving force of the hypothalamo-pituitary-gonadal axis (HPG). Hypothalamic nuclei are responsible for the production and secretion of several neuropeptides, GnRH being one of them. The GnRH secretion is regulated by more than fifty neurotransmitters, neurotrophic factors and other regulatory molecules forming feedback system.

- Positive feedback results in production and secretion of GnRH or other neuropeptides produced by the hypothalamus into the portal capillary system which further transports them to the anterior lobe of the pituitary gland.

- Negative feedback works the opposite way by inhibiting the secretion and release of neuropeptides into the portal capillary system

Pituitary gland

The pituitary gland consists of two lobes, the anterior and posterior pituitary gland. The gland is located just below the hypothalamus, separated by the pituitary stalk, also called infundibulum, and has both neural and endocrine function. The anterior pituitary gland, also known as adenohypophysis, is a neuron extension of the neurons of the paraventricular and supraoptic nuclei of the hypothalamus while the posterior pituitary gland, also known as neurohypophysis is a glandular tissue originating from the primitive digestive tract. When the pituitary gland is stimulated it releases its hormones into the hypophyseal portal system futher affecting other organs, including the gonads. The secreted hormones are follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), growth hormone (GH), prolactin (PRL) and adrenocorticotropic hormone (ACTH). The gonadotroph cells which produce FSH and LH are located in the pars distalis and pars tuberalis of the anterior pituitary gland (Carpe, retrieved September 16th 2017)

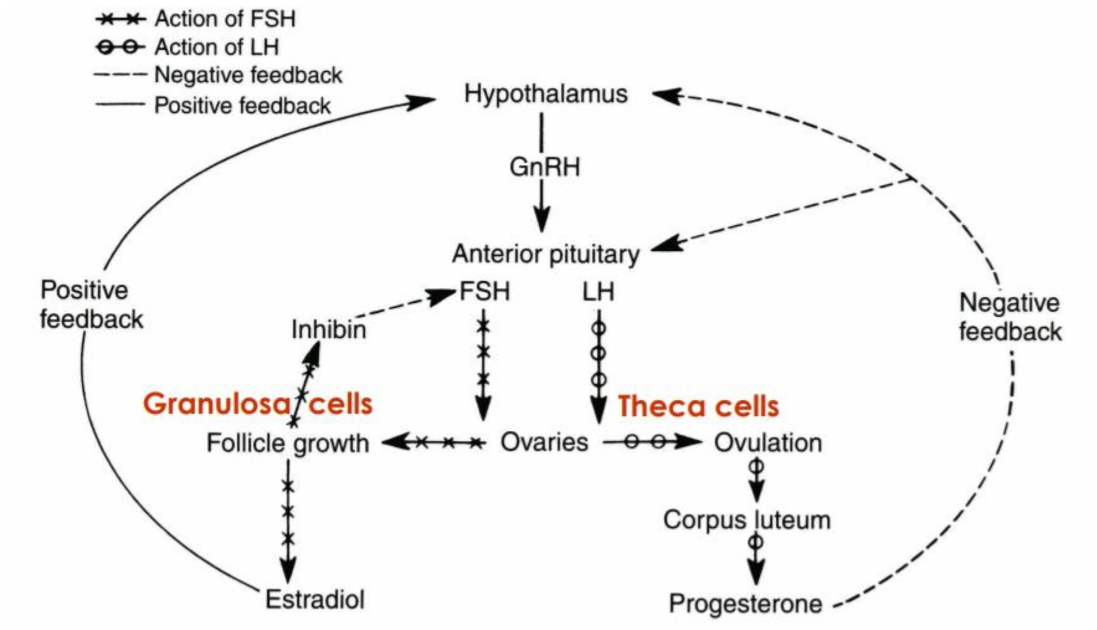


Figure 2: Hypothalamic-pituitary-gonadal-axis
(Adopted from Bearden *et al*, 2004).

Ovaries

The equine ovaries have unique architecture compared to ovaries of other species in that they appear to be inside-out, with the cortical zone located in the interior of the ovary while medullary zone is in the superficial region. Due to this different structure the follicles develop deep within the organ instead of in the surface region. The two main cell types in the developing follicles are the granulosa cells and thecal cells (McCue, 1998). Granulosa cells form the inner cell lining of the follicle and provide physical support and microenvironment required for the developing oocyte. As the follicles grows, granulosa cells are responsible for the production of estrogen which increases when the follicle grows larger (Skinner *et al*, 2010) but the cells also produce inhibin and activin (McCue, 1998). Thecal cells on the other hand surround the outside of the follicles, separated from the granulosa cells by the basal membrane. The main role of thecal cells is the production of androgens, in response to LH, which are converted by granulosa cells to estrogen (Skinner *et al,* 2010). After transforming to lutenizing cells they are responsible for the progesterone biosynthesis and release (Young and McNeilly, 2010).

2.2.2. The hormones

Melatonin

Melatonin is an ubiquitous molecule and is the so-called ´darkness hormone´. It is primarily produced and secreted at night by the arcuate nucleus of the pineal gland from serotonin, which is a neurotransmitter derived from the amino acid tryptophan. Melatonin has multiple functions throughout the body, including regulation of immune system, hormone production and secretion, reproductive rhythm, sleep and circadian cycles. Melatonin is produced during the dark period of the day independent of whether the animal is diurnally or nocturnally active. It further affects the synthesis and release of GnRH, gonadotrophins and gonadal hormones. For this reason, mares are long-day breeders because melatonin secretion is directly proportional to the length of darkness hours (Dubrovich, 2007).

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus, referred as the master circadian pacemaker controlling circadian rhythm of mammals, controls melatonin secretion in a multisynaptic pathway. Retinal ganglion cells in the eye express melanopsin which respond to light and travel a signal to the SCN‘s receptors in the hypothalamus stimulating the release of GnRH. The SCN contains two G-protein coupled receptors which participate in the regulation of sleep and circadian phase, MT1 and MT2. Those participate in implementing biological effects on the body originating from the RHT (Dubrovich, 2003).

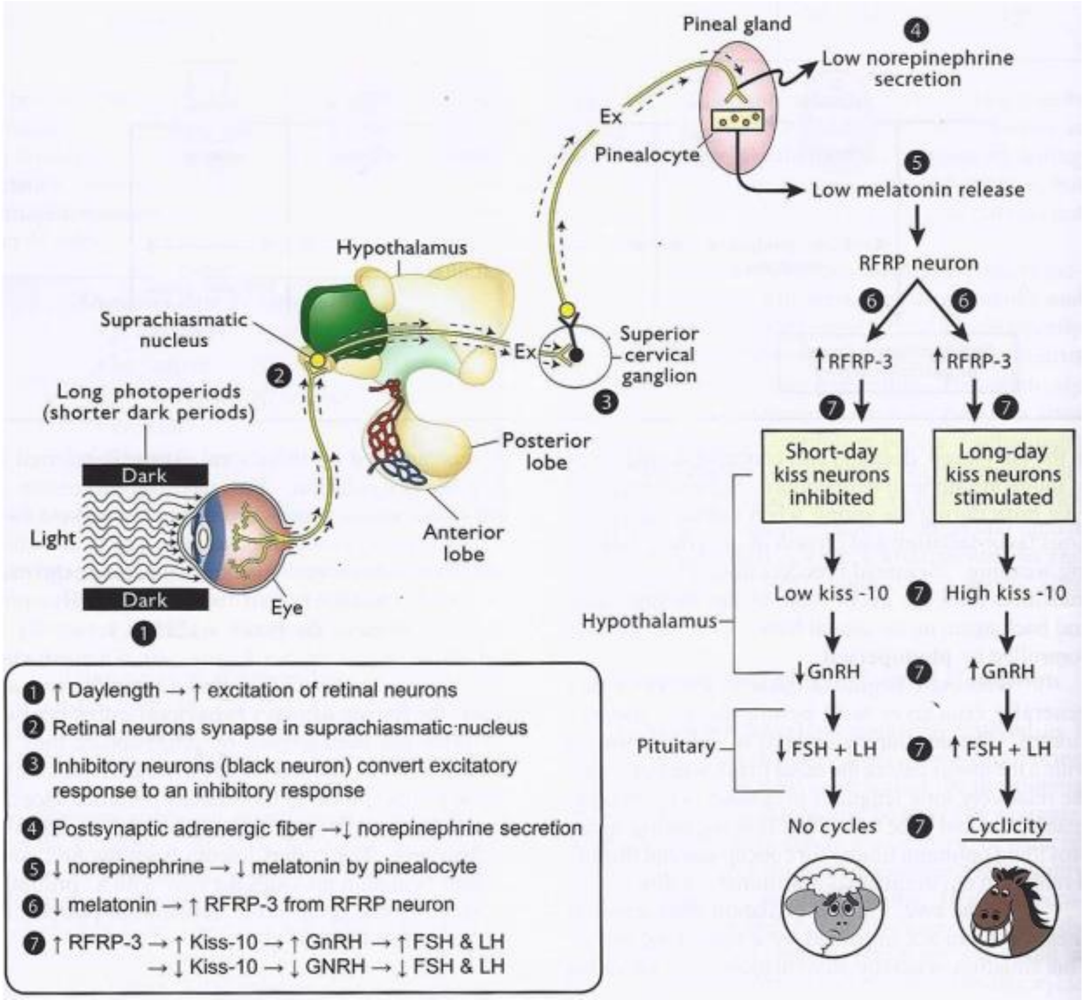


Figure 3: Retino-hypothalamic tract

(Adopted from Senger, 2012).

Kisspeptins

Kisspeptins are important neuromodulator proteins that take a major part in the regulation of the reproductive axis. They are produced an secreted by nerve cells together with two other neuropeptides, neurokinin B (NKB) and dynorphin (DYN). Kisspeptins were first discovered in 1996 and was originally called metastin due to its metastasis inhibitory effect in melanoma cell lines (Clarke et al, 2015). These small protein molecules act directly on hypothalamic neurons expressing the Kiss1 gene (Donato and Frazão, 2016). Kisspeptins stimulate these neurons resulting in GnRH release further stimulating the pituitary gland and gonads. Knowing how they stimulate the reproductive axis, experiments have shown their critical role in the onset of puberty, seasonal regulation on reproduction, adult fertility and the regulation of gonadal steroid feedback to the hypothalamus (Clarke *et al*, 2015).

GnRH

Gonadotropin releasing hormone (GnRH) is a decapeptide produced and secreted by the arcuate nucleus in the hypothalamus. It is a member of the hypothalamic-pituitary-gonadal axis by stimulating the anterior pituitary gland to produce and release the gonadotropins, lutenizing hormone (LH) and follicle-stimulating hormone (FSH) (Wheeler, 2013). After stimulation of GnRH neurons, the hormone is secreted from the median eminence into the fenestrated capillaries of portal circulation which carries it to the anterior pituitary gland. It serves a physiological signaling role by switching the gonads on and off with its pulsatile release modified by the feedback of steroids on the hypothalamus. The frequency and amplitude of those pulses of GnRH play a major role in initiating the gonadal activity at puberty as well as stimulating cyclicity in the seasonal breeders especially (Fink, 1997). The tonic center of the hypothalamus releases low frequency pulses of GnRH resulting in FSH secretion from the pituitary. When positive feedback of estrogen exceeds threshold level the surge center is activated resulting in preovulatory LH peak as a response to huge amout of GnRH released (England, 2005).

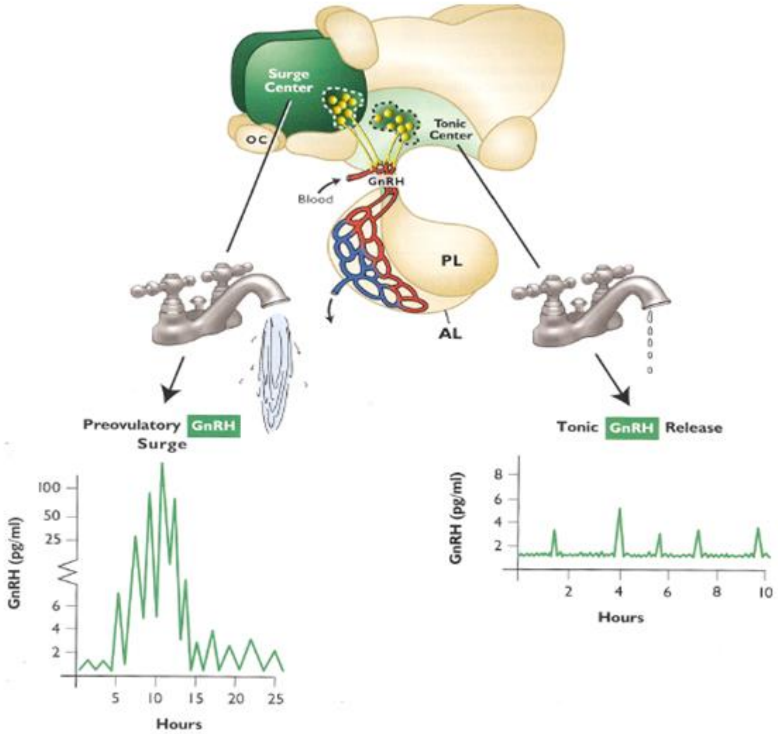


Figure 4: Surge- and tonic center of the hypothalamus (Adopted from Senger, 2012).

FSH and LH

Follicle stimulating hormone (FSH) and lutenizing hormone (LH) are glyoproteins produced and secreted by the anterior pituitary gland. Their production and secretion is regulated by a complex interaction between GnRH from the hypothalamus and estrogen and progesterone from the ovaries (Fischbach and Dunning, 2009). The secreted FSH is responsible for ovarian follicular development while LH is responsible for the ovulation.

Estrogen

Estrogens, estradiol-17β in particular, is the primary female sex hormone which promotes the development and maintainance of female characteristics among other roles in the body. It is a steroid hormone produced and secreted mainly by the ovaries and in a smaller amound by the adrenal cortex, testicles in males, fetoplacental unit and corpus luteum. They are shown to have negative and positive feedback effects on the hypothalamic-pituitary axis (Diczfalusy and Fraser, 1998). Estrogen is the common term for the compounds producing estrus . The three most common naturally occuring estrogens are estrone (E1), estradiol (E2) and estriol (E3). In the non-pregnant mare the main type and most active biologically produced estrogen is estradiol (E2) which is converted from androgens and testosterone by enzymatic processes of aromatase. The presence of E2 hormone has a negative impact on FSH but a positive impact on LH.

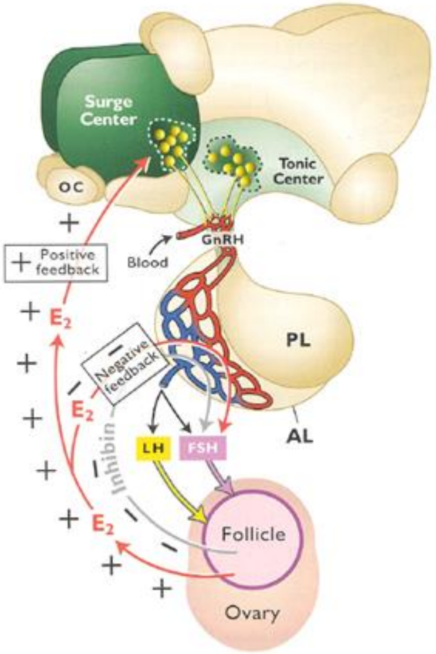


Figure 5: Feedback system of E2 (Adopted from Senger, 2012).

Progesterone

Progesterone is another steroidal hormone produced primarily by the ovary, more precisely by the granulosa-lutein cells of the corpus luteum. This hormone regulates a plethora of biologically distinct processes in different tissues throughout the body through the action of progesterone receptors (Al-Asmakh, 2007). Its role in reproduction include the prevention of estrus and exerting specific functions related to the preparation of the endometrium to accept and maintain pregnancy by helping in the development and inhibition of myometrial contractility (Younqquist and Threlfall, 2007). Progesterone has a positive impact on FSH and a negative impact on LH.

Leptin

Leptin is a polypeptide derived from adipose cells and was first disovered in the search for a satiety signal, hence it‘s called the ´satiety hormone´. Over the last decates the role of adipose tissue as an endocrine organ has become more evident and experiments have shown positive association between plasma concentration of leptin, insulin and carbohydrate content of feed (Fradinho *et al*, 2014). Leptin acts as a metabolic signal within the HPG-axis by providing a signaling link between nutritional status and reproduction. When leptin is secreted into the blood it either stimulates the secretion of GnRH indirectly through neuropeptide Y or by direct stimulation of GnRH neurons. However, if blood leptin concentrations are outside of the ideal range it will adversely influence reproductive capacity of the mare (Smith *et al*, 2002).

Prostaglandin

Prostaglandins (PGs) are a group of physiologiacally active lipids derived from arachidonic acid. The major prostaglandin involved in reproduction is $PGF2_{\alpha}$ with PGE playing a more limited role (Morel, 2015). Their effects are both positive and negative, including affecting ovulation, destruction of corpus luteum, implantation, and maintainance of pregnancy. Prostaglandins are frequently used in equine practice for the synchronization of estrus and may be used alone or in combination with other hormones (Weems *et al*, 2006). Prostaglandins have positive impact on GnRH release from the hypothalamus resulting in surge of LH to initiate ovulation (Randel *et al*, 1996).

Inhibin and activin

Inhibin and activing are glycoprotein hormones produced and secreted by the granulosa cells in the ovaries under the influence of FSH (De Jong, 1988). As inhibin‘s name indicates, its major action is the negative feedback control of FSH production by modulating the anterior pituitary response to GnRH. Activin on the other hand has the opposite effect, by increasing the secretion of FSH. These hormones therefore are involved in the development of the dominant growing follicle which will suppress the other follicles (Morel, 2015).

Dopamine

Dopamine is a neurotransmitter belonging to the group of catecholamines. It is mainly produced by the brain, primarily by substantia nigra and ventral tegmental area, and is released into the hypophyseal portal system. Dopamine affects the reproductive axis by activating D2 receptors in pituitary lactotrophs which will suppress the release of prolactin. It also acts on specific neurons in the hypothalamus expressing Kiss1 gene releasing the powerful activator kisspeptins (Donato and Frazão, 2016).

3. MANIPULATION OF THE MARE‘S ESTROUS CYCLE

3.1 Methods

This chapter will discuss the different methods and their efficacy in the stimulation of anestrous mares to come into estrus early in the breeding season. The methods discussed here are the following:

- Photostimulation
- Recombinant FSH and LH administration
- GnRH-agonist administration
- Progesterone and progestin administration
- Dopamin antagonist and prolactin administration
- Nutritional and environmental effects

3.1.1 Photostimulation

The idea of manipulating the mare‘s photoperiod originates all the way back to 1940‘s when Burkhardt suggested that increased light accelerated the first ovulation of the year to occur (Ellendorff and Elsaesser, 1984). He got the idea after Bissonnette‘s experiment in 1930 where he was able to bring anestrous ferrets into full estrus by exposing them to 200 W light bulb for 38-64 days (Burkhardt, 1947).

The first experiment to manipulate the photoperiod in mares was performed by himself and its results were reviewed in 1947. Increased lighting was applied to anestrous mares with the same idea as Bissonnette‘s. Twelve mares at the age of 6-14 years were used for the experiment and they were divided into four groups. From 13th of November their ovaries were examined twice weekly and at the onset of the experiment they were all in a state of deep anestrus. During the experiment the temperature in the boxes was checked daily and compared with the outside temperature. The four groups were as following:

> **Group A:** The group composed of 4 pony mares (n=4). 1000 W lamp hung from the roof in the box where they were kept during the experiment and they were fed 12 lb. of hay daily. The treatment varied from the 1st of January until 9th of March. The length of daylight was slowly increased during the period and the further in the period, the additional light exposure was shifted to later during the day because of naturally longer days.

Group B: This group also composed of 4 pony mares (n=4). The mares in this group were hooded to prevent light absorption through the retina because with this group Burkhardt wanted to examine if irradiation of the flank and belly with increased doses of ultraviolet light had an effect on the anestrous mares. The treatment varied for 4 week, from the 1st of January to 1st of February. After the treatment the mares were fed ad libitum and recieved additional protein sources.

Group C: This group composed of 2 pony mares (n=2) only and they represented a control group. They were kept inside the stable until 20th of February and fed ad libitum during the whole period.

Group D: This group was composed of 2 pony mares (n=2) like group C and also represented a control group. These mares on the other hand were kept outside in paddocks and fed once daily.

Mare	Stromal growth	1st appearance of follicles	1st oestrus
Group A			
1	1st February	20th February	23rd February
2	9th February	13th February	15th February
3	15th February	15th January	7th February
4	1st February	28 February	4th March
Group B			
1	24th February	24th February	27th February
2	4th March	14th March	18th April
3	19th February	28th March	21st April
4	28th February	5th March	19th March
Group C			
1	25th Marh	25th March	23rd April
2	14th March	3rd April	12th April
Group D			
1	25th March	25th March	10th April
2	25th March	3rd April	25th April

Figure 6: Reproductive activity of mares in the four groups (Burkhardt, 1947).

The results of Group A showed obvious changes in folliular growth after 2-3 weeks already and within 9 weeks all mares were in estrus. Mares in Group B did not show early follicular growth and did not come into estrus until after mid-March to April, exactly like in Marshall‘s ferret experiment, except for one mare. Follicular growth was observed on the 23rd of February and the mare came into estrus three days later. It should be noted that this mare did not have pigmented iris. As a result it can be interpreteted that more ultraviolet light has entered her retina than in the others in her group causing the ovarian stimulation. The control mares in Group C and Group D did not show follicular growth until March and did not come into estrus until mid and late April, just like the unaltered natural cycle of mares (Burkhardt, 1947).

Burkhardt‘s experiment made a history in equine reproduction and until now later experiments have verified that manipulation of the photoperiod is the most common and most effective method available to decrease the time to the first ovulation of the year in equine practice (Meyers-Brown *et al*, 2017). Experiments have shown that artificial photoperiod of 16 hours light and 8 hours dark starting on the 1st of December in the northern hemisphere hastens the first ovulation of the year. The extension of daylength can be achieved using 100-150 W light bulb in a stall of 3,6 x 3,6 m (Palmer and Driancourt, 1981). There is a lag period of 60-90 days mimicking the long summer days from the onset of artficial lighting to first ovulation (Slusher *et al*, 2014). This means that mares start cycling in February-March instead of March-April, resulting in parturition in January-February the year after as desired.

Recent studies have shown that the use of special masks with low intensity blue light with wavelength of 465-485 nm is most effective in advancing the breeding season in mares (Murphy *et al*, 2013). According to studies performed by Walsh *et al*, the threshold level of low wavelength light is 3-10 lux (Walsh *et al*, 2012). The mask mimics the light/darkness hours used in the above mentioned study of Burkhardt. The mask is placed on the mare‘s head and light of low wavelength is directed into a single eye. Compared to the in-stall experiment, the mare is able to stay on pasture under natural diurnal light pattern making the treatment period more comfortable for the mare.

University College of Dublin in Ireland performed a research reviewed in 2011 to examine the effect of use of light mask compared to mares housed under stall lighting and mares under natural photoperiod. Fifty nine mares were used for the experiment (n=59). The mares were divided into 3 groups with same average body condition and were at the age of 5-24 years. The groups were as following:

> **Group A:** The group consisted of 16 mares (n=16). They were kept on pasture during daytime and in stalls at night. Inside the stalls they were exposed by 250 Lux light from 16:30-23:00 hours daily. The mares were fed ad libitium and received 2,7 kg/day of low-starch diet.

Group B: The group consisted of 25 mares (n=25). They were further divided into two groups. Both groups were kept on pasture 24 hours each day and treated with bluelight masks. The special equipped mask exposed 50 Lux light into the right eye from 16:30-23.00 hours daily. The group was fed ad libitum and received 1,4 kg/day of mare nuts.

Group C: This group served as a control group and consisted of 19 mares (n=19). The mares were kept on pasture under natural photoperiod the whole experimental period.

Figure 7: Light mask (Picture adopted from equilume.com, visited 2nd October 2017).

The masks used were composed of a LED light using 4 Duracell Pro-cell AA sized 1,5 V batteries, powered circuit, on and off switch, clock and restrictors. The LED light used was Kingbright 7,6 x 7,6 mm Super Flux LED Lamp L-7676CQBC-D Blued with luminous intensity of 1300 mcd (millicandela). The light is placed in rubber eye cup, covered with aluminium on the inner surface to reflect light diffusely onto the eye. The experiment started on the 1st of December and varied until 10th of February. However, from 20th of November the mares were examined transrectally with ultrasound and venous blood collected to measure circulating progesterone concentration. During transrectal examination the ovarian activity, cervical tone and uterine edema was evaluated. The evaluation was registered as following:

- Oestrous cyclicity: follicles >20 mm, presence of CL or CH in conjunction with P4 level >1 ng/ml.
- Transitional phase: follicles >20 mm, typical physiological characteristics of oestrous activity but P4 level <1 ng/ml.
- Anoestrus: follicles >15 mm, no typical physiological characteristics for oestrous activity and P4 level <1 ng/ml.

In the beginning of the experiment when mares were first examined transrectally and circulating P4 levels were analysed it was surprising how large proportion of the mares were still reproductively active (Murphy *et al*, 2013). Previous studies indicated that about 20% of all mares cycle throughout the year and don‘t enter anoestrus phase (Ellendorff and Elsaesser, 1985). Even though the year is not over at the time of the beginning of this study, one would think that most of mares should have entered the anoestrus phase already. Those results therefore give us valuable information on the cyclicity because reproductive examinations are not commonly carried out at this time of the year.

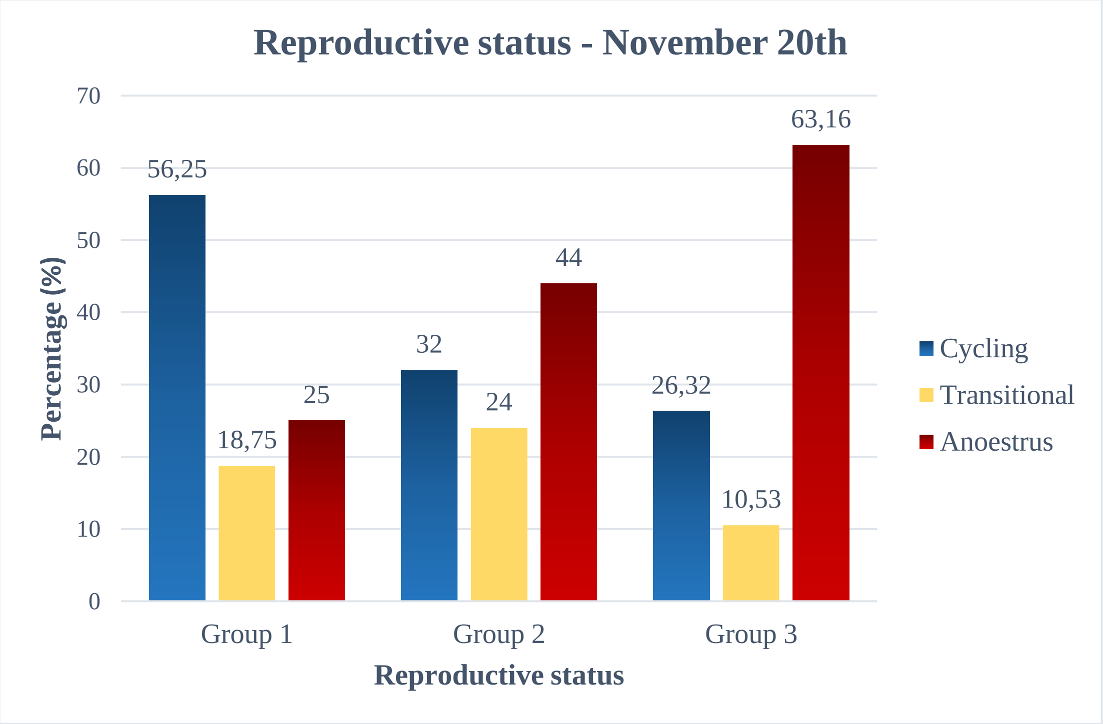


Figure 8: Reproductive status of mares before the onset of treatment (Murphy *et al*, 2013).

Based on results shown in Figure 8, 22 of the 59 mares used during the study were still cycling on 20th of November. The figure show the proportion of mares in each group which are still cycling, are in transitional period or have entered anoestrus.

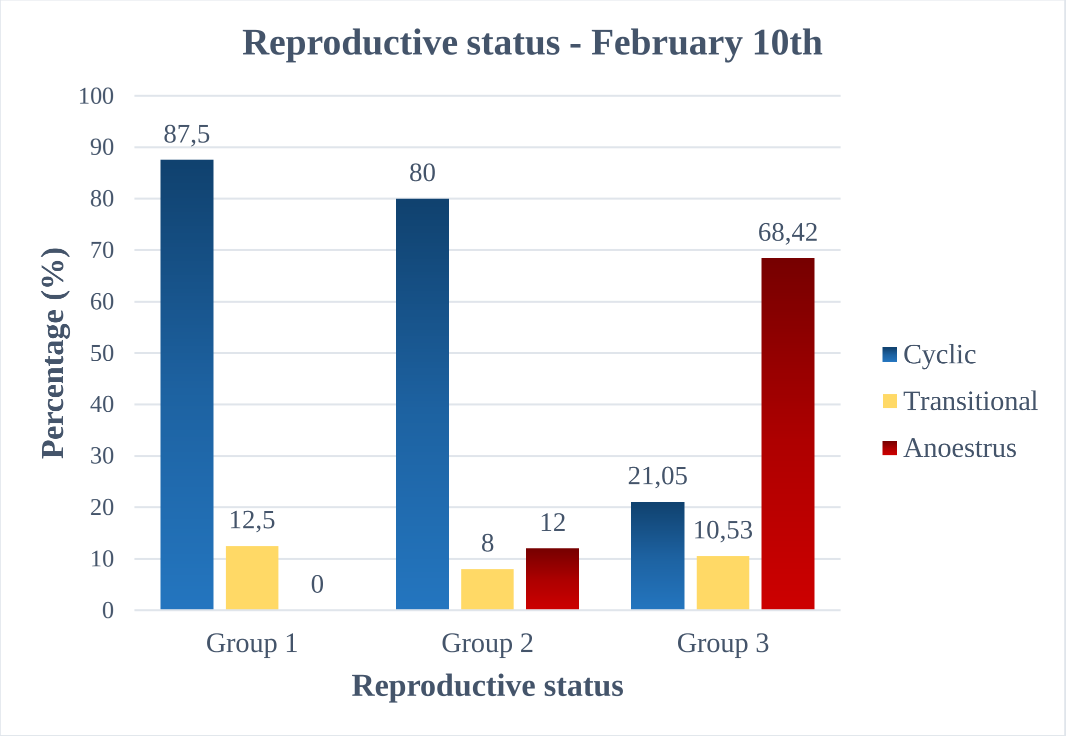


Figure 9: Reproductive status at the end of treatment (Murphy et al, 2013).

Results shown in Figure 9, show that >80% of the mares who were exposed by artificial light were already cycling on the 10th of February compared to 21% of the untreated control group (Murphy *et al*, 2013). Those results show that they were able to bring most transitional mares and mares in deep anoestrus into season within less than 10 weeks and that the use of bluelight mask is as effective as keeping mares indoor under artificial lighting.

At our university a group of Profs. Solti and Cseh with a former undergraduate student Theresa Schnitzler conducted a research to compare the efficiency of photostimulation and the long term gestagen treatment with the untreated controls. Fifteen mares of various age were used for the experiment and were allotted into three equal groups of five. The first group received light treatment (Equilume) from December 3rd until March 26th, the second group received 22 mg altrenogest (Regumate) daily for 15 days starting on February 5th whereas the last group served as untreated control. Three out of the five photostimulated mares came into oestrus in mid-March while the remaining two started cycling in April. These results are in good agreement with Burkhardt and Murphy *et al* confirming that the light stimulation is able to bring mares earlier into estrous. However, in the same experiment feeding with Regumate proved also to be effective in stimulating the mares to start cycling early in the breeding season (Solti *et al*, 2016).

3.1.2 Recombinant equine FSH (reFSH) and LH (reLH)

The use of recombinant equine FSH (reFSH) has been reported to induce follicular growth in cyclic mares (Jennings *et al* 2009; Meyers-Brown *et al*, 2010). Study reviewed in 2013 however determined the efficacy of it in deep anestrous mares to be very successful with ovulation rate of 76,7% in response to FSH treatment followed by hCG administration (Meyers-Brown *et al*, 2013).

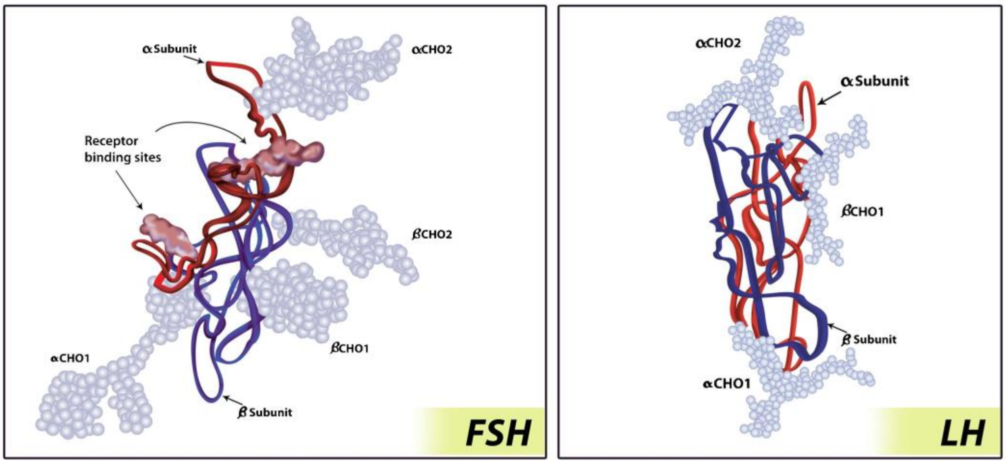


Figure 10: FSH and LH molecular structures (Leão and Esteves, 2014).

Most recent study on pharmaceutical compounds affecting deep anestrous mares reviewed in May this year was to determine the efficacy of recombinant equine (reFSH) and recombinant (reLH). 30 mares of 4-19 year of age used in the study were determined to be in deep anestrous by transrectal ultrasonography. The study did also take into account pregnancy rate and return to cyclicity after termination of pregnancy but their results will not be emphasized here. The mares were randomly divided into three equal groups of 10 mares and fed ad libitum.

Group A: examination of the effect of reFSH administration of 10 mares (n=10) under natural photoperiod. The mares were treated with 0,65 mg reFSH in 1,3 ml phosphate buffered saline (PBS) intramuscularly twice daily with an interval of 8 hours. When largest follicle reached 32 mm in diameter the administration of reFSH was ended but the mare still received PBS twice daily until the follicle reached 35 mm. During the next 36 hours no reFSH treatment was given but it was followed by iv administration of an ovulatory agent, 2500 IU human chorionic gonadotropins (hCG).

Group B: examination of the effect of reLH administration of 10 mares (n=10) under natural photoperiod. The mares were treated with 0,65 mg of reFSH in 1,3 ml PBS intramuscularly twice daily with an interval of 8 hour. When a follicle reached 29 mm, reFSH treatment was reduced to once daily but 1,5 mg reLH in 2 ml PBS intramuscular administration came in addition twice daily until the follicle continued to develop to 32 mm. At that stage reFSH administration ended and reLH continued to be administered three times daily with an interval of 8 hours until the follicle reached 35 mm. Then the mares received the same ovulatory treatment as group A received.

Group C: the group composed of 10 mares (n=10) kept under artificial lighting from 1st of December. The mares in this group (n=10) served as controls and received only 13 ml PBS intramuscularly twice a day.

The treatment started on the 6th of February. Mares in Group A and B were examined by transrectal ultrasonography every day during the treatment period while mares in Group C were examined once daily for 14 days. After that, mares were examined every other day until ovulation. In addition, jugular blood was collected for analysing of plasma LH, FSH, P4, E2 and immunoreactive-inhibin to evaluate the reproductive activity.

Ovarian parameters	**reFSH group**	**reFSH/reLH group**	**Control group**
Number of days to ≥ 35 mm follicle	5,9 ± 0,34	5,7 ± 0,37	15,8 ± 3,4
% of mares with ≥ 35 mm follicles at the end of reFSH, reFSH/reLH or at the end of 14 days treatment of control group	100%	100%	30%
No. of follicles ≥ 35 mm follicles at the end of reFSH, reFSH/reLH or at the end of 14 days treatment of control group	4,4 ± 1,3	3,5 ± 0,47	0,3 ± 0,17
% of mares receiving hCG during the 14 day treatment	100%	100%	30%
% of mares ovulating during the 14 day treatment	100%	100%	30%

Figure 11: Reproductive activity of mares treated with reFSH or reFSH/reLH (Meyers-Brown *et al*, 2017).

The results shown in figure 11 indicate that all deep anestrous mares treated with reFSH alone or reFSH and reLH in combination under natural photoperiod showed significant increase in follicular developement within 6 days on average and all of them ovulated within 10 days. In comparison the control group needed significantly longer time for follicular growth and only 30% of the conrol mares had ovulated at the end of the 14 days used for the experiment (Meyers-Brown *et al*, 2017).

3.1.3 GnRH agonists

GnRH is known to be responsible for the secretion of FSH and LH but studies performed to evaluate the efficacy of GnRH-agonists are conflicting. Previous studies indicate that administration of exogenous GnRH to acyclic mares has been shown to induce the release of both hormones (Ginther and Wentworth, 1974; Evans and Irvine, 1976; Alexander and Irvine, 1986) which induces follicular developement and ovulation, respectively. According to two studies in 1986, two injections of GnRH agonist each day or continous administation of GnRH agonist were able to induce follicular development and ovulation in acyclic mares (Fitzgerald *et al*, 1986; Sanderson *et al*, 1986). Another study, performed by Ginther and Bergfelt, demonstrates the same result where mares where about 60% of treated mares with GnRH-agonist ovulated within 21-day long treatment (Ginther and Bergfelt, 1990). The GnRH agonists available for mares include deslorelin, buserelin and historelin (Lindholm *et al*, 2011).

A study performed by McKinnon et al including two experiments evaluated the effiacy of deslorelin implants administered during early transition and late transition. The experiment was carried out as follows:

> **Experiment 1**: 21 mares in early transition divided in two groups were used. The treatment group consisted of 11 mares (n=11) which were treated every second day until either ovulation occured or 6 implants had been administered. The remaining 10 mares were used as control group (n=10). At the onset of treatment there was no difference in follicular size among the groups.
>
> **Experiment 2**: 20 mares in late transition were divided into two equal groups of 10 mares (n=10 x 2). The treatment group receiving deslorelin as described in Experiment 1. Those mares were in estrus, had a follicle ≥ 30 mm in diameter, and endometrial folds were demonstrated by ultrasonography. The remaining mares were used as control group.

Within 10 days after the onset of treatment in the first experiment, 6 out of 11 treated mares had ovulated whereas none of the control mares ovulated. The two groups represented the same interovulatory interval but one mare in the treatment group returned to transition for 60 days while another one had a persistent CL for about 37 days. In the second experiment, ovulation occured significantly earlier in the treated mares compared to the control group. The treated group ovulated within 11 days with an average of 3,7 ± 3,2 days compared to 21,9 ± 18,4 days in the control group. 8 out of 10 treated mares with a follicle ≥ 30 mm ovulated within 3 days while none of the control mares did. Mean number of implants needed for ovulation was 2,1. The treated mares showed prolonged interovulatory period of 25 ± 3,8 days compared to the control group with 19,7 ± 1,9 days. Similar to the first experiment, one of the treated mares returned to transition but in this case for 53 days (McKinnon *et al*, 1997).

As the studies mentioned above indicate, GnRH-agonist can be used to stimulate cyclicity. The response is in correlation with the follicular size at the beginning of the treatment and the depth of anestrus. This means that mares which are already in transition period are more likely to respond to the treatment than those who are in deep anestrus (Dascanio and McCue, 2014) due to insensitivity of GnRH. However, by using GnRH-agonists there is an incresed incidence that deep anestrous mares return to anestrous after the withdrawal of the treatment or the treatment may cause longer interovulatory interval. Another negative aspect of GnRH treatment in anestrous mares is the increased risk of early pregnancy losses due to inadequate luteal function (Bergfelt and Ginther, 1992).

3.1.4 Progesterone and progestins

Using progestins in equine reproduction is a common practice but their use in particular for seasonally anestrous mares is questionable. The use of progesterone in mares to hasten cyclicity has been examined in various studies showing conflicting results as in case of GnRH agonist researches. Majority of the studies performed indicate that mares in deep anestrus or early transition will not hasten first ovulation of the year with the treatment of progesterone (Squires *et al.*, 1979; Allen *et al.*, 1980; Turner *et al.*, 1981; Alexander and Irvine, 1991). However, studies have shown that mares which recieve progestins late in transition period when having at least one follicle > 20 mm in diameter at the onset of treatment exhibit regular post-treatment oestrous cycles earlier than control untreated mares (Webel and Squires, 1982). Therefore timing of the administration is a critical factor.

Progesterone administration suppresses LH release from the anterior pituitary and provides for storage and subsequent release of sufficient LH to induce follicular maturation and ovulation once progesterone supplementation ceases. This is called „rebound-effect“. The protocols available include orally or parenterally administered gestagens .

Intravaginal devices containing progesterne such as CIDR and PRID designed for cattle, administered off-label in mares, and progesterone-impregnated vaginal sponges have also been used in mares during transition period. Study performed by Hanlon and Firth during four consequitive breeding seasons, 2007-2010, examined the effect of intravaginal devices to reproductive performance of 227 transitional of Thoroughbred mares of 4-18 years of age. The number of mares used in the experiment varied between years. Only mares in transition phase of the anovulatory period were used in the experiment with largest follicle of 20-25 mm in diameter, did not have any CL, did not have any abnormalities of the reproductive tract and had shown signs of estrous behaviour for at lest 10 days. Intravaginal progesterone releasing device was administered in the treatment group and left in for 7-10 days. The mares were examined with transrectal ultrasonoprahy on Day 7 after inserting the device. In case of a follicle ≥ 35 mm in diameter the device were removed in those particular mares, but mares with follicles < 35 mm were examined again on Day 10. The control group receiving no treatment was examined by transrectal ultrasonography twice a week. After progesterone withdrawal of the treatment group, every mare was examined daily by ultrasonography and exposed to a stallion to determine behaviour of estrus. Mares with follicle ≥ 35 mm received 1,667 IU hCG intravenously to induce ovulation and were exposed to Thoroughbred stallion 24-36 hours later.

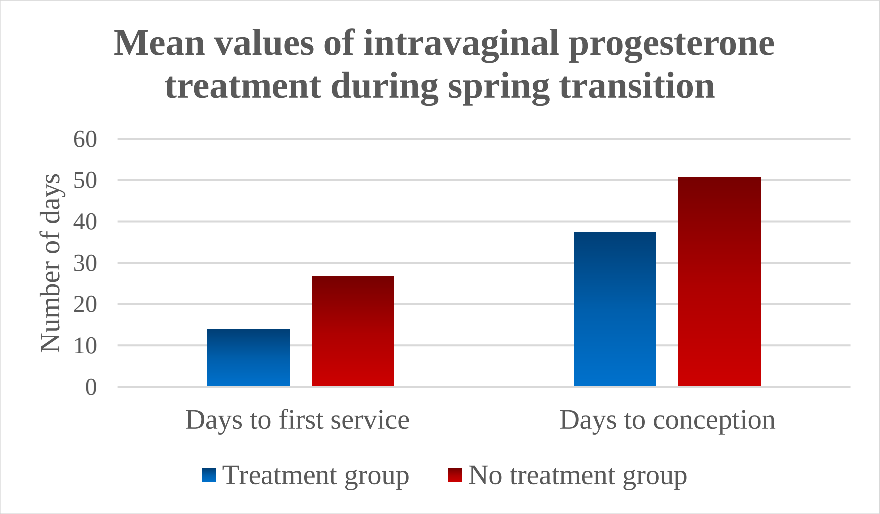


Figure 12: Mean number of days from onset of vaginal progesterone treatment until first service and conception (Hanlon and Firth, 2012)

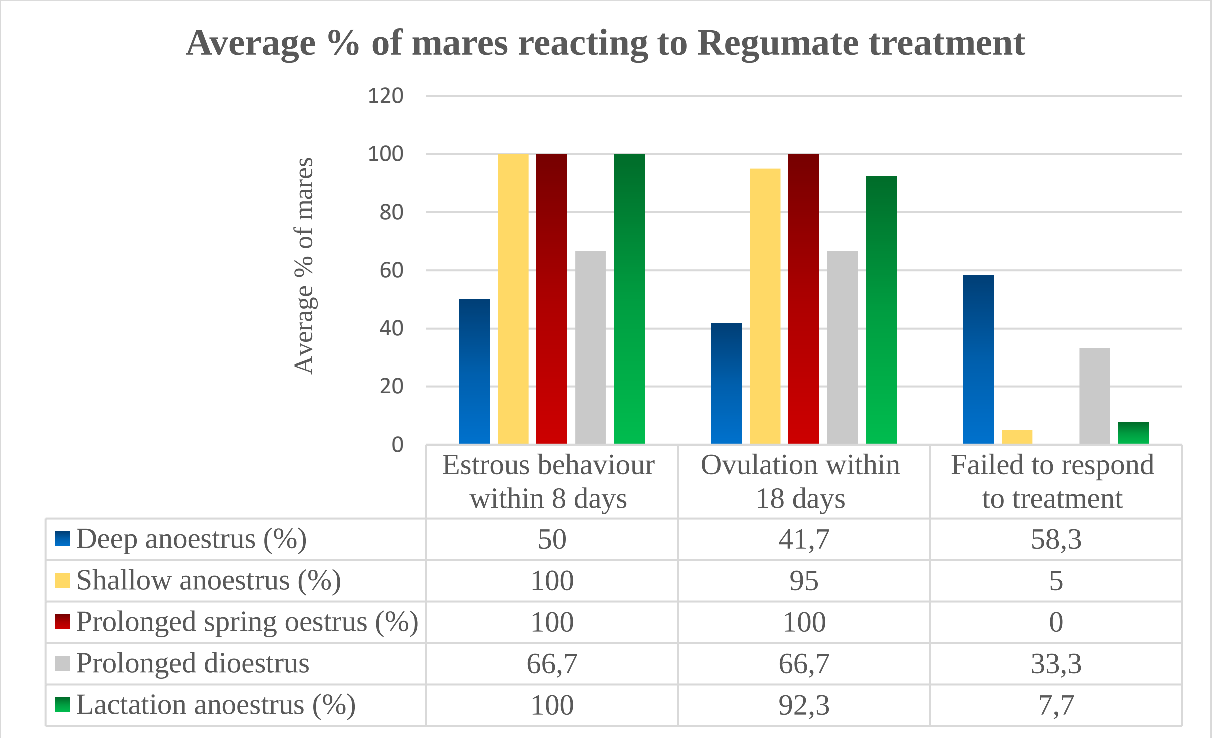
The treated group ovulated significantly earlier with an interval to first service of 13,9 ± 3,0 days compared to that of the control group of 26,7 ± 13,2 days. Mares treated for 7 days were served 2,5 days eariler than those treated for 10 days. Mares in treatment group conceived 37,5 ± 14,2 days compared to the control group conceiving at day 50,8 ± 21,3 (Hanlon and Firth, 2012). The results of the experiment showed that the use of progesterone have an advancing effect and hastenthe first estrous cycle of the year but the experiment does not take into account the depth of the anestrous.

Year	Number of mares	Mean interval to 1st service (days)	Mean interval to conception (days)
2007	38	21,2	49,9
2008	69	19,6	45,3
2009	68	17,5	41,7
2010	52	20,5	37,8

Figure 13: Mean interval from onset of vaginal progesterone treatment until first service and conception in the four consequitive breeding seasons (Hanlon and Firth, 2012).

Regumate is the most commonly used orally administered progestagen. Its active ingredient is allyl trenbolone, also called Altrenogest. Study performed by Allen *et al* evaluated the effect of oral progesterone treatment in seasonally anestrous mares. Eighty-four mares under natural photoperiod were used for the study and efficacy of Regumate was determined in mares at different stages of anestrus; deep anestrus, shallow anestrus, prolonged spring estrus and lactational anestrus.

The treatment group consisted of 61 mares while 23 mares were used as an untreated control group. The treated mares received 30 mg of allyl trenbolone (17α-allyl-trenbolone) in concentrates over a period of 10 days starting from the 15th of February. The 61 mares were divided into 4 groups according to their reproductive activity; deep anestrous, shallow anestrous, prolonged spring oestrus, prolonged dioestrus and lactation anestrous.



	Estrous behaviour within 8 days	Ovulation within 18 days	Failed to respond to treatment
🟦 Deep anoestrus (%)	50	41,7	58,3
🟨 Shallow anoestrus (%)	100	95	5
🟥 Prolonged spring oestrus (%)	100	100	0
⬜ Prolonged dioestrus	66,7	66,7	33,3
🟩 Lactation anoestrus (%)	100	92,3	7,7

Figure 14: Efficacy of Regumate treatment on mares in different phases of anestrous (Allen *et al*, 1980).

Signs of estrus was evaluated as well as analysis of plasma progesterone level performed. Within 8 days, 88% of the treated mares showed estrous behaviour and within 18 days after cessation of treatment 84% had ovulated. Only 16% of the mares failed to respond to the treatment. Deep anestrous mares showed significantly the lowest response but only 5 out of 12 mares ovulated (41,7%). Based on those figures the treatment gave a positive result in hastening cyclicity in mares but its response depends on the depth of the anestrous (Allen *et al*, 1980).

As previously indicated, Solti, Schnitzler and Cseh revealed that oral administration of Regumate was able to trigger cyclic ovarian funtion early in the transition period. Four out of 5 mares receiving long term gestagen treatment started cycling in early March, while the last mare showed cyclic ovarian activity by the end of April. Their results demonstrate and confirm that the use of long term gestagen treatment is also stimulating the mares to cycle in the transition period (Solti *et al*, 2016) which is in good accordance with the above mentioned results by Allen *et al*.

3.1.5 Dopamine antagonists and prolactin

Experiments have shown that inhibition of D2 dopamin receptors may hasten the onset of the ovulatory season in mares. The ones which have been studied are sulpiride, domperidone and perphenazine (Panzani *et al*, 2011). Studies performed on sheep observed that dopamin antagonists are effective in increasing LH secretion during anestrus by inhibiting the release of dopamine in the brain (Havern *et al*, 1991). Ideas of higher dopamine release during winter anestrus has been confirmed in studies measuring higher concentration of dopamine in cerebrospinal fluid in mares during deep anestrus compared to mares in season. The mechanism of change in dopamine concentration during transition period is due to reduction in negative feedback of estrogen on GnRH neurons (Havern *et al*, 1994).

Study reviewed in 2009, compared the efficacy of sulpiride and domperidone, two long acting dopamine antagonists, for the first time in the same study. The experiment was to evaluate the real efficacy of the pharmacological agents in inducing the ovarian activity in deep anestrous mares under the sme environmental conditions. 26 mares of 3-15 years of age were used for the experiment, were kept under natural photoperiod in stalls with access to paddocs during the day. The mares were divided into 3 groups, two groups of 10 (n=10) and one control group of 6 mares. One month before the onset of treatment, from the 3rd of February, all the mares were examined transrectally with ultrasound once every 10 days and plasma P4 concentration measured to make sure they all were in deep anestrus before the treatment started. After the treatment started the mares were examined every 3 days and largest follicle was registered each time. Once follicle of ≥25 mm was detected it was considered as the beginning of transition phase. Once a follicle became ≥40 mm, these particular mares were artificially inseminated.

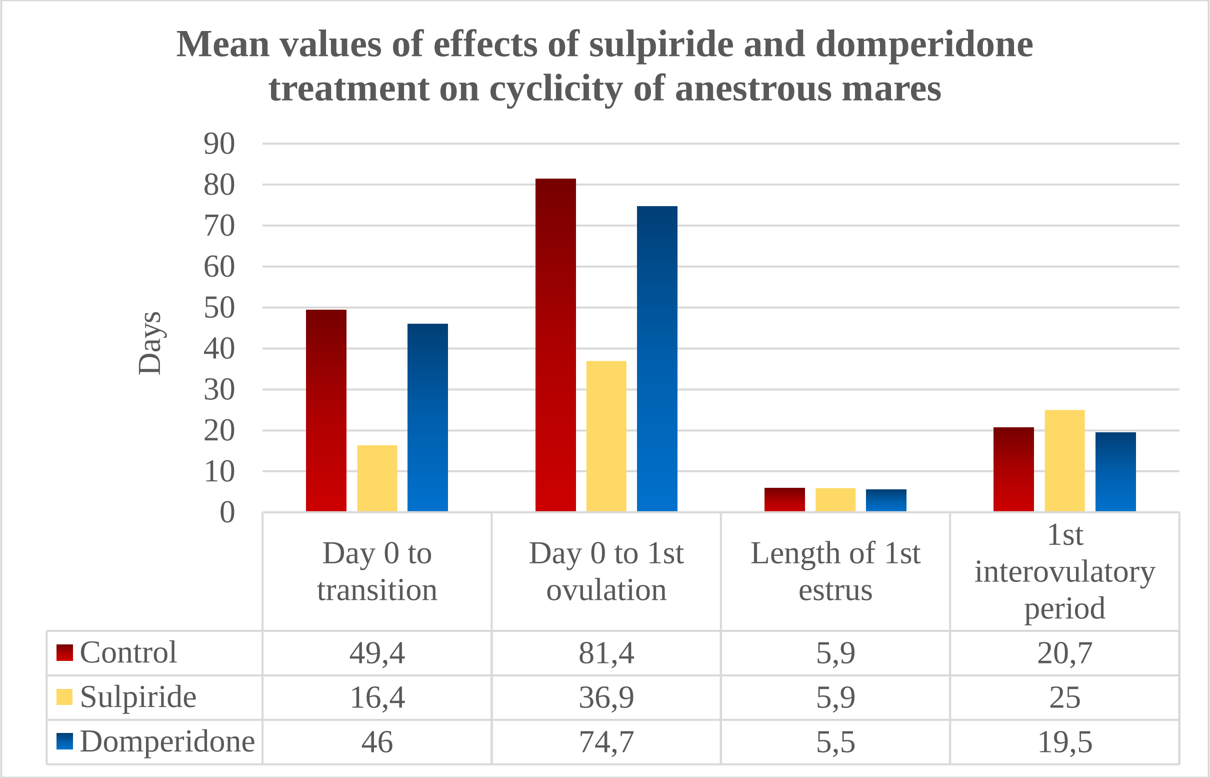
Group A: The group consisted of 10 mares (n=10) and was treated with sulpiride (1 mg/kg body weight).

Group B: The group consisted of 10 mares (n=10) and was treated with domperidone (1 mg/kg body weight).

Group C: The group consisted of 6 mares (n=6) and did not receive any treatment

The treatment of group A and B lasted for 25 days, between 3rd and 28th of February. In case of detected ovulation, the treatment of that particular mare was ended. The results indicated that 3 mares in Group A ovulated already during the treatment period, 3 mares ovulated within the first week after the end of treatment and 2 mares ovulated within two weeks. The last 2 mares of the group ovulated more than one month after the end of treatment. LH concentration of that group was significantly increased during the treatment period in comparison with Group B and

C. Follicular growth was already noticed on Day 3 after the onset of treatment. In comparison, 2 mares in Group B ovulated during the treatment period while the other 8 mares did not ovulate until at least one month after end of treatment. The LH concentration in Group B did increase, with a peak on Day 14, but never reached the level of Group A and at the end of the treatment the LH levels were equal to LH levels of the control group. Group B did not show difference in follicular growth compared to the control group until day 17. None of the mares in Group C ovulated during the treatment period.



	Day 0 to transition	Day 0 to 1st ovulation	Length of 1st estrus	1st interovulatory period
🟥 Control	49,4	81,4	5,9	20,7
🟨 Sulpiride	16,4	36,9	5,9	25
🟦 Domperidone	46	74,7	5,5	19,5

Figure 15: Comparison of efficacy of sulpiride and domperidone treatment (Mari *et al*, 2009).

Based on those results, sulpiride administration is the only one of the two drugs effective in hastening the transitional period and the first ovulation in deep anestrous mares (Mari *et al*, 2009).

As daylight increases, concentration of the hormone prolactin (PRL) increases as well. Dopamine is an inhibitor of PRL release and it has been suggested that prolactin adminstration may help in stimulating cyclicity in anestrous mares. Prolactin has a direct effect on the ovaries resulting in expression of GnRH receptors (McCue, 2006). Studies have confirmed that administration of recombinant prolactin has a stimulatory effect on anestrous mares. Porcine and equine prolactins have 92-95% similarity in primary structure. A study examined the effect of subcutaneous administration of recombinant porcine prolactin (rpPRL) on eight pony mares (n=8) for 45 days, starting on the 15th of January. Seventeen days after the onset of treatment, higher percentage of mares receiving treatment showed estrus signs compared to control mares and ovulation of the treated mares was hastened by more than a month, February 6th ± 3 days compared to March 14th ± 6 days (Thompson *et al*, 1997). Another study examined the effect of a large single dose of ovine recombinant prolactin (ovPRL), on the first week of January. In this study, significant stimulation of follicular development was noted but only one mare ovulated in comparison with the control group (Nequin *et al*, 1993).

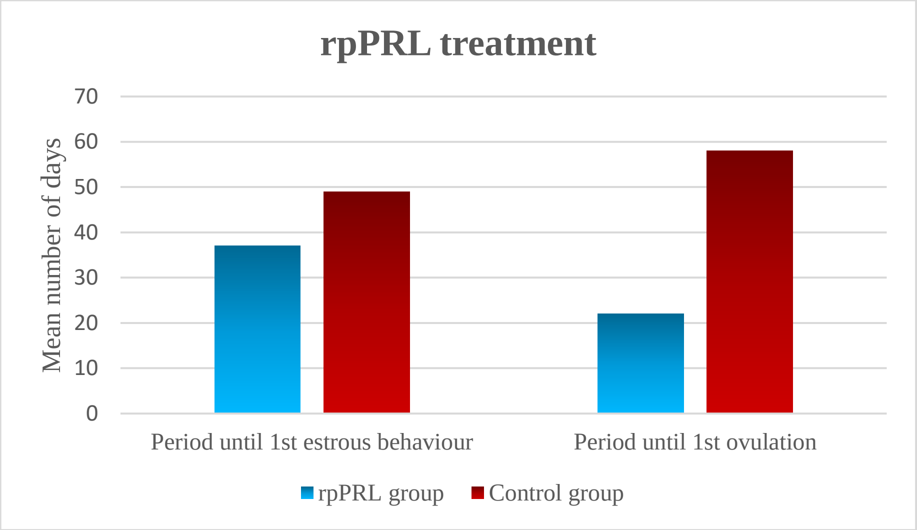


Figure 16: Mean number of days from onset of treatment until first behavioral estrous and first ovulation (Thompson *et al*, 1997).

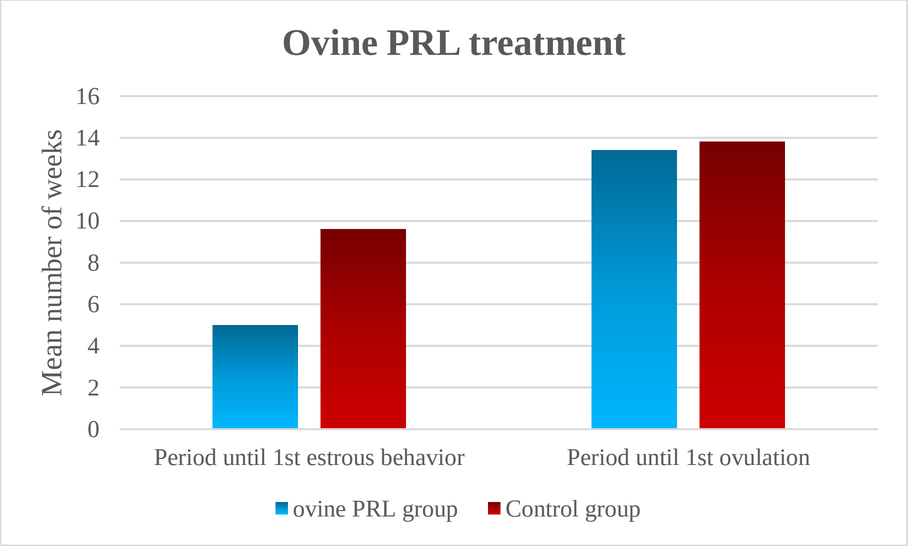


Figure 17: Mean number of weeks from onset of treatment until first behavioral estrous and first ovulation (Nequin *et al*, 1993).

Based on the above mentioned results the use rpPRL may hasten the onset of cyclicity while the use of ovPRL does not have a significant effect on ovulation despite its stimulatory effect on follicular developement.

3.1.6 Nutrition and environmental factors

Improving nutrition and increasing environmental temperature, both naturally associated with spring affect the reproductive cycle of the mare. Henneke et al reported that nutritional energy requirements for reproduction depend on the body condition score (BCS) of the mare (Henneke *et al*, 1983). Relationship between body condition score, leptin and reproduction has been examined and study of Gentry and Thompson reviewed in 2002 revealed results of their experiment on this relationship. They concluded that most mares with HBCS (high body condition score) continued to cycle throughout the winter compared to mares with LBCS (low body condition score) which exhibited a prolonged anovulatory season. Significantly lower levels of leptin were detected in LBCS mares than in HBCS mares even though their leptin ceased slightly during winter. However the follicular activity detected in HBCS mares was not associated with their leptin concentration and was therefore probably due to the high body condition itself (Gentry *et al*, 2002). Study performed by Waller *et al* examined the relationship between high body condition mares and leptin concentrations and they came to the same conclusion as Gentry *et al* that leptin and insulin concentration was not associated with alterations in cyclicity during winter and spring transition (Waller *et al*, 2006).

Study by Fitzgerald and McManus revealed that energy availability which may be linked to the circulating leptin concentration modified the inhibitory signal of melatonin. Their study verified that significanty greater proportion of mares with high concentration of circulating leptin remained cyclic during winther months (Fitzgerald and McManus, 2000). Another study, performed by Gastel *et al*, indicated that mares with low body condition needed longer time till first ovulation of the year in contrast with mares with high body condition. The delay of mares with low body condition was associated with reduced follicular development during transitional period and those mares had more multiple major anovulatory waves before their first ovulation (Gastal *et al*, 2004).

The so-called „green-grass effect“ or „pasture effect“ has been thought to stimulate anestrous mares to come into season and its stimulatory effect was evaluated by Carnevale *et al*. Treated mares showed increased reproductive activity but the stimulatory effect could not be distinguished from other factors, such as stress, exercise (Carnevale *et al*, 1997) and light exposure.

Environmental temperature has also been thought to play a role but the effect of temperature in particular on cyclicity is hard to evaluate. Study by Guerin and Wang evaluated the effect of environmental temperature over a 10-year period on first ovulation of the year. The mares used for the study were kept under natural condition. Difference in 20 days on average on first ovulation during this long period was noted but it was impossible to determine whether the changes were due to temperature or increase in daylength (Guerin and Wang, 1994).

4. CONCLUSION

By using the above mentioned methods foals get born earlier in the year thus meet the breeders‘ requirements of more mature individual of higher value. The effect of a horse‘s birth month on its future sport performance was examined by Langlois and Blouin. The result of their study demonstrated significant effect of birth month on the performance, especially on the early performances at 2-3 years of age (Langlois and Blouin, 1997). Subsequently, early foaling gives the mare more breeding oppurtunities during the season which may result in higher conception rate. This is for example in case of mares who have problems to get pregnant due to any reason.

Compared the different methods known today the most reliable one for early induction from anestrus or transition is the reFSH alone or combined reFSH and reLH treatment. All of the treated mares came into estrous within only 14 days from deep anestrous and within 6 days all mares showed already a significant follicular growth of ≥ 35 mm. The only problem with this method is that reFSH and reLH are not commercially available currently and therefore lighting protocols are the most reliable and widely used among the available methods.

The photostimulation has much longer treatment period, varying from about 1st of December till 10th of February compared to reFSH/reLH treatment of only 14 days. Despite the long treatment period, only 80% of mares treated in the study of Allen *et al* came into estrus and four out of five mares in the study of Solti *et al* started to cycle in the transition period. In comparison, 100% of the reFSH/reLH treated mares started cycling during the treatment period. However, the use of the photostimulation is easier to perform as no injection or feeding pharmaceuticals needs to be administered thus the mares can stay on pasture during the treatment period.

The other pharmaceutical methods did not show as strong evidence in deep anestrous mares compared to control groups and may be less practical due to frequent administration.

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