THESIS

Verena Flanter

2023

University of Veterinary Medicine of Budapest

Department of Animal Breeding, Nutrition and Laboratory Animal Science

Equine twinning - genetics

(Review of literature)

By:

Verena Flanter

Supervisor:

Dr. Zöldág László prof. emer

Budapest, Hungary

2023

Contents

Abstract

Equine twinning is undesirable due to the complications that are implied in its occurrence. Thus, it is from advantage to understand the background of this phenomenon for a proper approach. Multiple ovulations are the most frequent cause of twinning in horses. The factors contributing into it are various, but genetic traits are yet to be examined. Some horse breeds happen to be more affected than others by twinning, confirming that the reason of its occurrence is related to the genes. Furthermore, the statement is reinforced by the repeatability of twinning in family lines, proving that the related genes are inheritable.

Absztrakt

A lovak ikertenyészete nem kívánatos az előfordulásukkal járó komplikációk miatt. Ezért előnyös megérteni e jelenség hátterét a megfelelő megközelítés érdekében. A lovaknál a többszörös ovuláció a leggyakoribb oka az ikerterhességnek. Az ehhez hozzájáruló tényezők különbözőek, de a genetikai tulajdonságokat még nem vizsgálták. Egyes lófajták történetesen jobban érintettek az ikerterhességben, mint mások, ami megerősíti, hogy előfordulásának oka a génekkel függ össze. Továbbá az állítást erősíti az ikerszövetség megismétlődése a családi vonalakban, ami azt bizonyítja, hogy a kapcsolódó gének öröklődnek.

Abbreviations

BCS   Body Conditioning Scoring

MP   Multiple Pregnancy

MO	Multiple Ovulation
TB	Thoroughbred
BMP15	Bone morphogenic protein-15
GDF9	Growth differentiation factor-9
FST	Fixation index
SNP	Single Nucleotide Polymorphism
ROH	run of homozygosity
FSH	Follicle stimulating hormone
IGF	Insulin-like growth factor
hCG	human choriongonadotropin
LH	Luteinizing hormone
PGF2α	Prostaglandin F2 alpha
GC	granulosa cell
CC	cumulus cell
OSF	oocyte-secreted factor
TGF- β	Transforming growth factor- β
SMAD	SMA("small" worm phenotype) and MAD family ("Mothers Against Decapentaplegic")
BMPRII	Bone morphogenic protein receptor-II
STS	Steroid sulfatase
MAF	Minor allele frequency
eCG	equine choriongonadotropin
OPU	ovum pickup

Introduction

Throughout the past twin pregnancies in horses are an undesirable and therefore avoided phenomenon. The high risk of twinning impedes the pregnancy and birth process for the mare and the unborn (Crabtree, 2018). Even a successful twin pregnancy does not guarantee the foals survival, as the reported incidence of twin ovulations is high, with low incidence of abortions or foaling (Gintherayb, DouglasC, et al., n.d.-b). Usually, those animals are born weaker or not fully developed. Mummification, stillbirth and abortion of one or both foals are natural selection processes, as the equine reproductive organs are not constructed for such a matter (Ginther, n.d.-c). As consequence, high value breeding animals are usually excluded for a breeding season, resulting in immense economic losses (Crabtree, 2018). Thus, the aim is to prevent twin pregnancies from happening in first place. The most common approach is to abort the pregnancy, avoiding complications such as dystocia, abortion and fetal membrane retention difficulties. Many different methods are available for such an issue, depending on how advanced the pregnancy is or the timing of being noticed. On the other hand, most frequently, twinning is based on multiple ovulations (MO), which can be a favorable condition for embryo transfer techniques, gaining importance in nowadays horse breeding industry (Woods & Ginther, n.d.). Moreover, recent studies showed that the equine model is a plus in human medicine as well. There are many similarities in the follicular development, interovulatory interval and the ovulatory follicular waves between mare and women (Ginther et al., n.d.). The exemplification of the follicular dynamics will be explained in **Chapter 3, subchapter 3.1.**.

Furthermore, twin pregnancies are categorized into dizygotic or monozygotic twinning. Monozygotic twinning is extremely rare and occurs during the process of splitting of the morula or the blastocyst after fertilization. Monozygotic twinning was also described as the result of an embryo transfer (Morris & Allen, 2002). For dizygotic twinning, double ovulation (mostly referred as MO) is crucial to happen. If both oocytes are fertilized at the same time, a twin pregnancy occurs. Most of the twin pregnancies in horses are dizygotic.

In addition, the occurrence of twinning in Thoroughbreds (TBs) lead with 19% closely followed by other purebreds such as Quarter Horses with 9% and Appaloosas with 8%, (Davies Morel et al., 2005), leading to the estimation that there is a linkage between this phenomenon and certain horse breeds.

Aside from that, studies revealed a correlation between breeds and their genes, such as the Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) which have immense impact on the turn of a pregnancy. Their influence on the follicular development

in the ovaries is already proven and ongoing studies are still examining their full function related to this scenario (Stefaniuk-Szmukier et al., 2018).

GDF9 and BMP15 are growth factors, influencing cell proliferation. In certain breeds like the Thoroughbreds and Arabic horse those genes are very common, increasing the possibility for a twin pregnancy up to 30%.

The aim of this thesis is to focus on potential factors responsible for twinning in horses and the highlight the importance of their interaction.

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Chapter 1: The origin of twinning in horses, placental development.

Twins in horses are usually dizygotic and are therefore the result of multiple ovulation (MO). Monozygotic/identical twins in horses are extremely rare and polyovular follicles were never described in horses. Monozygotic twinning occurs during the process of splitting of the morula, four to six days after ovulation or splitting of the blastocyst, seven to eight days after ovulation, while leaving the zona pellucida. The rarity of this incidence can be explained to a unique feature. In comparison to other animals, the outside of the blastula in a horse access over an elastic capsule, that hinders a splitting of the embryo to a certain degree. Another cause for identical twins, is the result of an embryo transfer (Dijkstra et al., 2020). Dizygotic twins occur more frequently, as three different types of double ovulation are known. Synchronous, double

primary ovulation, where 2 ovulations occur randomly at the same time from both ovaries each, asynchronous double ovulations, with 2-10 days of time difference, and diestrus ovulations, parallelly to a single primary ovulation phase (Ginther, n.d.-c). Although, transrectal ultrasound allows a detection of the embryonic vesicle already on day 9 or 10 post ovulation – much sooner than in cattle, which can only be detected on day 20 (Ginther, 2021), it is not unusual for twin pregnancies to stay undetected for a certain time period. Factors known to influence the late detection, are embryo mobility and fixation (Miller & Woods, 1988).

In any equine pregnancy (independent if singleton or multiple), embryonic vesicles in the size of 3-9 mm preferred to stay in the uterine body till day 13, then moved to the uterine horns till fixation on day 16 to 18. For this reason, in disparate twins of an asynchronous ovulation, the smaller member can be found mostly in the uterine body, as they move most of the time independently from each other (Ginther, n.d.-a). Fixation wise, the smaller vesicle can maintain its' mobility, even after the larger vesicle is already fixed. In this cased the fixation will be unilateral, unlike in embryos with a similar diameter, which fixate bilaterally as shown in **Figure 1**. Furthermore, the reproductive status of a mare, is shown to be of great importance in the role of fixation as well. (Will be discussed in **chapter 2**).

During mobility and fixation, not only the presence of the vesicle allows us to make a conclusion about dizygotic or monozygotic twinning, but also the time between both ovulations can be estimated with help of the vesicles' diameter.
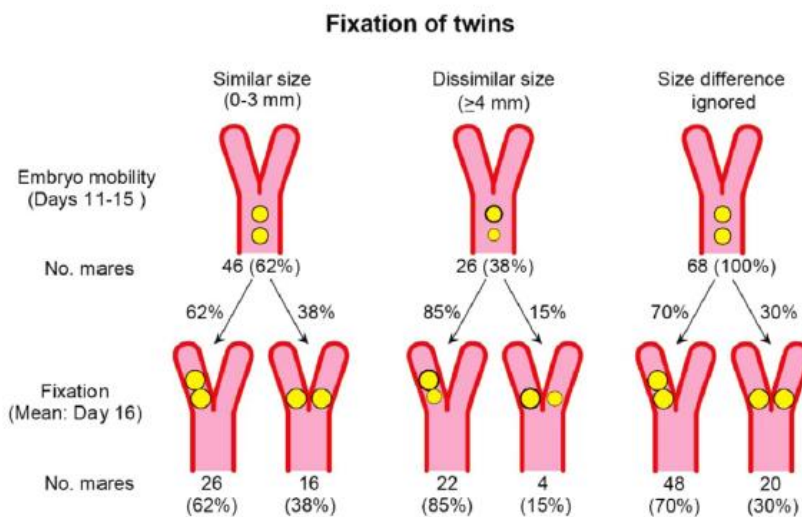
Figure 1: Effects of similarity versus dissimilarity in diameter of twin embryonic vesicles on unilateral vs bilateral fixation adapted from (GINTHER, 1989)*. Embryos of similar diameter (primarily from synchronous ovulations) occur more frequently than from dissimilar diameter (primarily from asynchronous ovulations). Embryos that come from asynchronous double ovulations are more likely to undergo unilateral fixation. The effect of ignoring diameter differences is also shown for comparison with reports that did not record embryo diameters.*

A study of the equine research station of 1973 in Suffolk examined the fetal membranes, to study the different placentation of dizygotic twins (Jeffcott & Lvhitwell, n.d.)

Each fetus possesses their own amnion and chorion, as demonstrated in **Figure 2**. Due to fixation, in most of twins (79%) one fetus occupied one uterus horn and nearly the whole uterus, while the second fetus occupied only a small part of the uterus body and the other uterus horn with its chorion invaginating the allantois of the bigger twin (**Type A**). In 10%, there was an even bigger distinction, as the larger twin occupied nearly all the surface area of the chorion, leaving the other twin mummified tightly enclosed in 25% of the leftover chorion (**Type C**). The different development can be explained through the competition between them for nutrients from the chorion, depending on the endometrial surface area. The chorion develops into the placenta together with the decidua basalis later. Another fact could be the separate ovulation timing, which can differ regarding the ovulatory pattern (Crabtree, 2018).

Ginther described that asynchronous ovulations can happen, if the circulating progesterone does not increase till the second ovulation, or in case of an ovulation during the diestrus. In such an incidence the corpus luteum regressed at the same time as the primary corpus luteum (Ginther, n.d.-c). The phenomenon of a diestrus, was never reported in other monovular species before and is highly paradox, as these ovulations occur during a high progesterone concentration, in an anovulatory follicular wave (Meintjes, 1995).

Only in 11% of a twin pregnancy, the surface area of the chorion is evenly divided, with both twins occupying the same space in the uterus and the horns, having equal access to the chorion (**Type B**).

Next to the decreased chorionic placental surface area, the contacting surface of the chorion is lacking on villi, leading to decreased nutrition of the embryos. This could explain the reason for twin foals being weaker than singleton foals. The outcome of the different placentation is demonstrated in **Figure 3**.
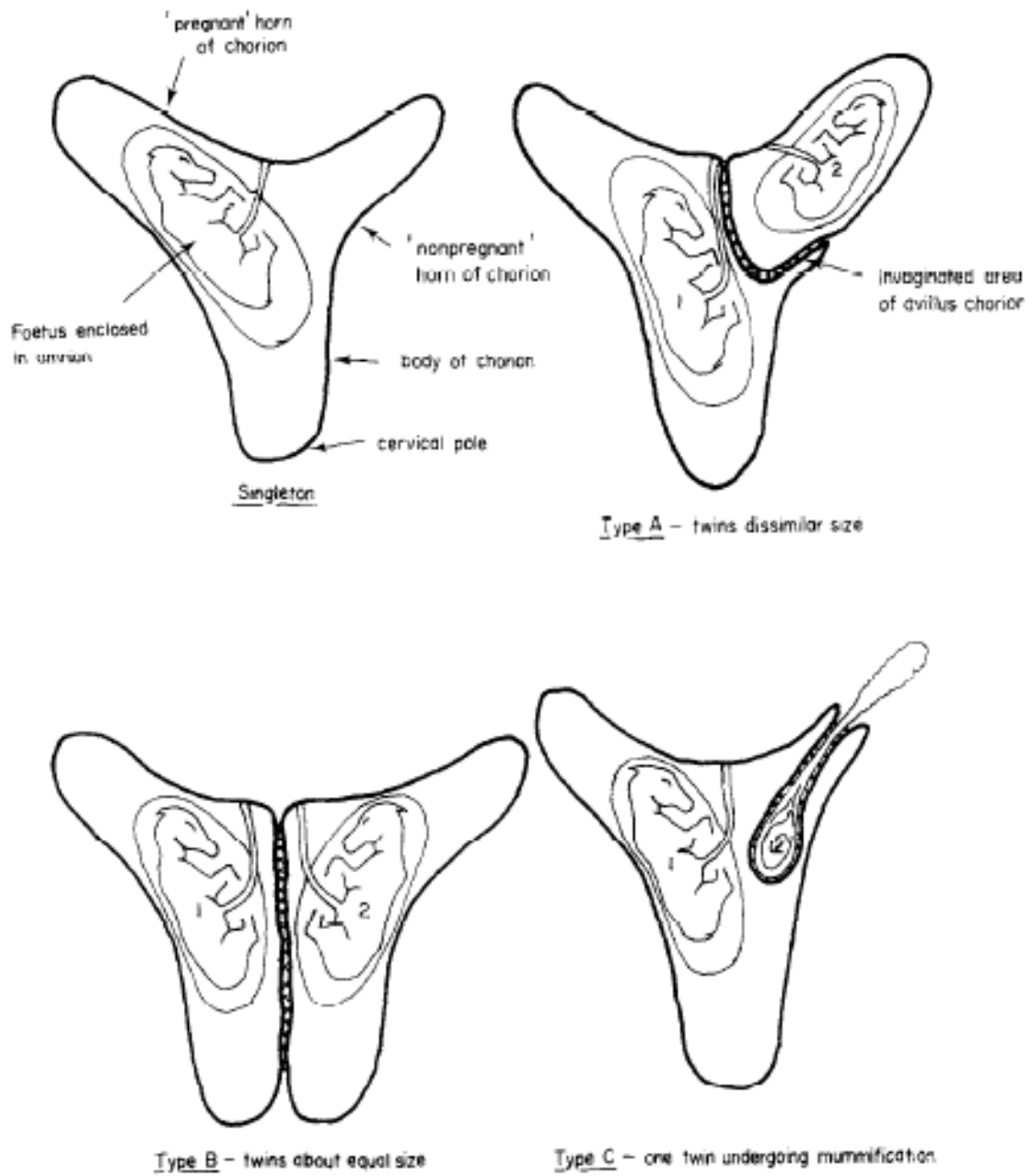
Figure 2: Diagrammatic representation of different twin placentation, adapted from (Jeffcott & Lvhitwell, n.d.).

	Type A		Type B		Type C (Term)	Total (3 mth– term)
	3–9 mth	10 mth–term	7 mth	Term		
No. of twin pregnancies	31	17	1	6	6	61
Total no. of foetuses	62	34	2	12	12	122
Foals born alive Twin 1	0	8	0	6	6	20
Twin 2	0	5	0	6	0	11
Total (%)†	0	13 (38%)	0	12 (100%)	6 (50%)	31 (25%)
Foals survived to 2 weeks of age Twin 1	0	5	0	5	3	13
Twin 2	0	1	0	4	0	5
Total (%)†	0	6 (18%)	0	9 (75%)	3 (25%)	18 (15%)

† (%) of total no. of foetuses.

Figure 3: Outcome of twin pregnancies according to the type of placentation, adapted by (Jeffcott & Lvhitwell, n.d.). *Due to the different positions in the uterus, the access of each twin to the chorion is connected to the different survival rates demonstrated here. (The Figure connects to **Figure 2**, regarding the different placenta types).*

Chapter 2: Factors influencing twinning.

2.1. Horse breeds

Besides the ovulation rate and the ovulatory patterns, there are other factors known to cause twinning in horses. The frequency of MO is shown to be positive in the thoroughbreds, quarter horses and Arabic horses, leading to the assumption of certain genes related to the occurrence of twinning in their reproductive performance. Moreover, this incidence proofed that those genes are breed related. In other animals the occurrence of twinning was also seen to be varying between different breeds. In addition, it was proven, that animals of different breeds, sharing a geographical location, show a huge variety in twinning rates, whereas the same breed in different geographical location, shows a similarity in the twinning rates (Johansson et al., 1974). This reinforces that twinning is inherited and of genetic origin.

2.2. Fertility

A base for twinning is fertility, which was defined as a proportion of reproductive length to the number of pregnancies (Asadollahpour Nanaei et al., 2020). Highly developed modern breeds are known to be affected the most regarding fertility, due to selective breeding of their desirable features. Therefore, it is not surprising that TBs are affected in a greater extent than other horse breeds, as they were bred for high performance in racing. The achievement of this trait is the final score of selection, narrowing their gene pool and neglecting other traits, such as reproductive performance, with MOs as the common cause for reproduction failure (Bower et al., 2011).

2.3. Candidate genes for twinning.

In cattle chromosomes 5, 7, 19 and 23 were found to be of great importance regarding the phenotype of twinning (Komisarek, 2002). With this information it is reasoned that in horses too, certain regions of a gene are responsible for twinning.

(Bower et al., 2011) described some candidate genes. He investigated the genetic resemblance between TBs and six other horse breeds (Akhal-Teke, Franches-Montages, Hanoverian, Holsteiner, Quarter Horse and Standardbred).

Therefore, he used the fixation index (FST). A measurement for population differentiation due to genetic structure. He demonstrated the variance in allele frequency of the six breeds, in resemblance to the allele frequency of the TBs.

To determine the FST, the genes were examined for SNPs (explained in **Chapter 5**) prior to the calculation. The value for TBs turned out to be significantly higher than in the other breeds and indicated, that individuals of this breed, were strongly separated from the other horse breeds, being genetically more distant due to selection.

Moreover, (Bjelland et al., 2013) pointed out the presence of homozygosity on many short genomic regions (ROH) and a low value for nucleotide diversity due to a relatively small long-term effective population size, indicating inbreeding in TBs. The outcome of the low genetic diversity is failure to adapt, which can be a reason of the low reproductive performance.

Also (Cunningham et al., n.d.) stated, that TBs are the horses with the highest level of inbreeding, as their alleles originate from 30 founders, with only one founder stallion being responsible for approximately 95% of paternal lineages.

The candidate genes identified by this research, were growth factors such as the Insulin-like growth factor and the Growth Differentiation Factor-9. All of them are known to regulate follicular development, implantation, pregnancy maintenance and fetal growth (Gobikrushanth

et al., 2018; Monget et al., 2002). Their function and relationship to twinning will be explained in **Chapter 3** and **Chapter 4**.

2.4. Repeatability and the Stallion effect

Another factor influencing twinning is repeatability. (Gintherayb, DouglasC, et al., n.d.-a) indicated a high degree of repeatability of MOs and twinning of certain family lines. A mare with a history of five ovulatory periods in four years, all containing double ovulations and two sets of twin fetuses was reported. Furthermore, two quarter horse mares – a dam and daughter from another holding were diagnosed twice with twin pregnancies and had at least three MOs each (Gintheralb & Douglas, n.d.). The recurrence is promoted to their breeds, known to be associated frequently with twinning. Moreover, the repeatability was linked to the stallion, due to reports of a stallion, having 24 daughters with all of them giving multiple births, although no occurrence of such a twinning was noted in their pedigree before (Hodder et al., 2008). In addition, sperm of highly fertile stallions, possess over a long survival time and hence access over the ability, to fertilize oocytes of asynchronous ovulations (Gintherayb, DouglasC, et al., n.d.-b) and are thus another possible factor known to cause twinning.

2.5. Age of the mare

A further environmental influence is the effect of age. (Davies Morel et al., 2005; Morris & Allen, 2002) examined the effect of age, related to MOs and multiple pregnancies (MP), as well as the hypothesis of the correlation between age and the vesicle diameter. The results were distinct, pointing out that mares of higher age, were associated with a decreasing pregnancy-and ovulation rate, but more likely to have MOs. The MPs were mostly observed in mares of medium age (6-8 years), due to the decreasing overall pregnancy and the increasing MO rate. Moreover, the hypothesis of the decreasing vesicle size due to age, was disproved. The age had nearly no impact on the size of a vesicle. (Jeffcott & Lvhitwell, n.d.) described the incidence of twinning seen even earlier (4-5 years old mares), pointing out that this is usually their first breeding season.

In addition, the decrease of the general pregnancy rate, can be explained due to endometrial cysts, which are more common findings with increasing age (Reproduction, 1995). Furthermore, a lack of nutrient supply of the endometrium for the embryo, is hypothesized by (Bracher et al., 1996). Caused by degenerative processes with increasing age (>11 years of age).

2.6. Breeding season

As for the age of a mare, fluctuations of the twinning trend were also observed in association with the breeding season (**Figure 4**). Temperature and the length of daylight have shown to play a crucial role on reproductive performance. With the breeding season starting from late February till the end of July, the pregnancy rates were reported to be steady until a rapid decrease in June, with a peak (35.5%) in April for singletons and a later peak (61.3%) of twinning in May-June (Jeffcott & Lvhitwell, n.d.). The late mating in June or July could be decisive for a mare to conceive during an asynchronous ovulation. In addition, MOs with a prolonged interovulatory interval of 5-6 days and a continuous estrus, were reported (Gintherayb, DouglasC, et al., n.d.-b). They tended to appear earlier during the breeding season. Furthermore, lightning programs were established to observe the effect of the month on MOs postpartum, showing lower results in the first postpartum period in January until March, then in April until May (Ginther, n.d.-d).
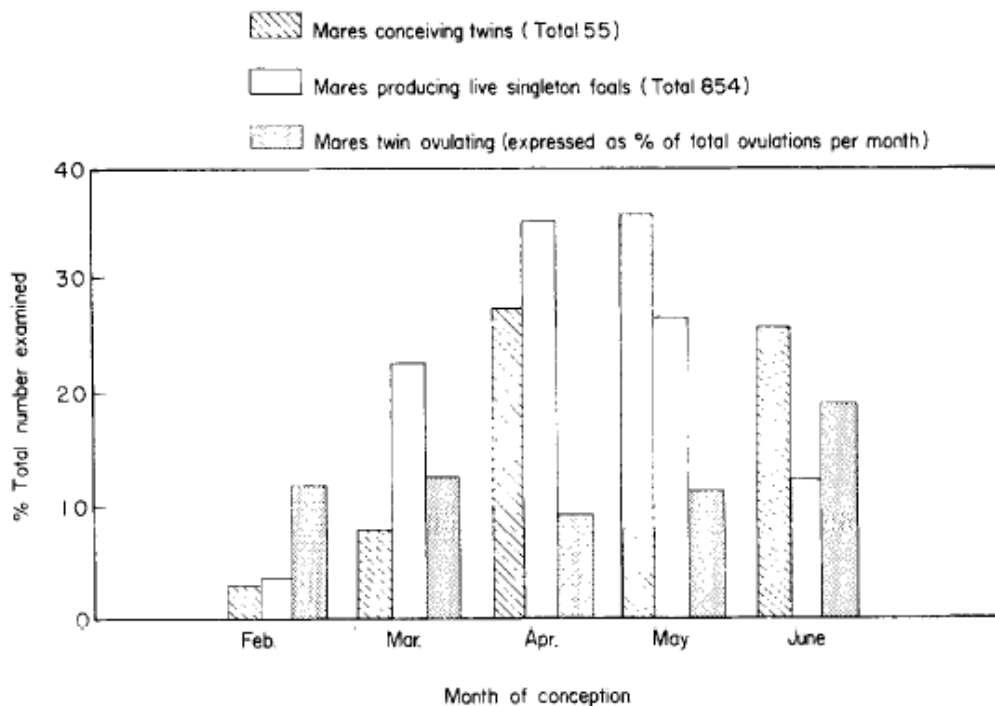
Figure 4: Demonstration of Breeding season and twinning, adapted from (Jeffcott & Lvhitwell, n.d.)**.**
Number of multiple pregnancies, multiple ovulations and number of singletons, referring to the different months

2.7. Reproductive status of a mare

During the postpartum period, mentioned in the previous chapter, foaling mares have the lowest incidence of MOs, compared to barren and maiden mares approximately 50% less. It is estimated that the reason for it might be the effect of nursing the newborn on the reproductive

function via the hypothalamic-pituitary-axis (Ginther, n.d.-d). In addition, twin pregnancies were observed in lactating mares, but less frequently then in non-lactating mares. They made up only 33% of all twin pregnancies (Merkt & Jöchle, 1993). The reasoning for this can be explained, by the high nutrient requirements needed in this period. Moreover, no correlation between the age and the mares' reproduction status could be made, regarding the occurrence of twinning (Gintherayb, DouglasC, et al., n.d.-b). Another study revealed also that the fixation of the embryo variates in mares with different reproductive status. In maiden and barren mares, its more common to fixate in the right horn, whereas in lactating mares, fixation mostly happens in the horn that was not used in the previous pregnancy (Miller & Woods, 1988). According to this fact, diagnosis of twinning is facilitated with the special focus to possible affected uterine horn.

2.8. Nutrition

As mentioned earlier, nutrition can be a determining factor for MOs as well. Usually, nutritional flushing is performed to enhance the reproduction performance to the maximum. MOs are more frequent to occur in a mare with a healthy body condition score (BCS). Therefore, a two to four weeklong dietary restriction, is a possible method for embryo reduction. In addition, natural embryo reduction, which is explained in **Chapter 6**, is hypothesized to be based on the shortened nutrient exchange over the placenta (Crabtree, 2018).

Chapter 3: Hormones influencing the follicular development and their use in regulation of a twin pregnancy.

3.1. Follicle selection and administration of FSH

Follicle selection, ovulation rate and luteinization are regulated by paracrine and endocrine factors. The interplay and feedback mechanism provides a basis for the regulation of folliculogenesis. Paracrine secretions are produced locally in the ovary during follicular development, leading to the regulation of ovarian functions, including follicular growth and differentiation, follicular selection, steroidogenesis, ovulation and luteogenesis (Haas et al., 2022). Based on rectal palpation, several large follicles (>20mm) are detected during the beginning of estrus, but the number is reduced 3-4 days before ovulation to just one follicle. That shows that horses are mono- ovulatory species, with usually just one dominant follicle

developing fully till ovulation, while the others regress. This action is based on an effective follicle selection mechanism (Ginther et al., n.d.) During follicular growth there are 2 different types of growth waves. Minor follicular waves, in which no follicle becomes dominant and major follicular waves, where the dominant follicle emerges. Depending on the stage of the reproduction cycle, major waves are either ovulatory or anovulatory. Both waves are stimulated by follicle stimulating hormone (FSH), which is secreted from the pituitary gland. FSH is known to peak twice during the different follicular waves. Therefore, many follicles undergo development in the common wave during diestrus. Based on that fact, the indication of FSH should lead to an increased number of follicles, like in cows and ewe. But, in a mare, the peak secretion of FSH is reached when the dominant follicle reaches 13mm in size, but the regression of the other follicles start only after dominant follicle reaches 22.5mm, showing the dominance of one follicle is associated with a decline of the FSH concentration. Based on this fact, it can be hypothesized, that the dominant follicle is inhibiting the further FSH secretion (Figure 5). If FSH is given onset of the estrus, atresia of the other follicles can be prevented, and MO can be induced (*Irvine1981*, n.d.).

In addition, with 2 days after the FSH peak, follicles respond by secreting estradiol, stimulating cell proliferation. The concentration of estradiol and other hormones such as Insulin-like growth factor 1 (IGF-1), inhibin-A and activin-A, within the follicles is shown to be varying according to the stage of development. The hypothesis, of them being responsible for the follicle selection mechanism was partly confirmed by (Ginther et al., n.d.), as he made the discovery of just IGF-1 to influence the microenvironment of an oocyte. The injection of an IGF binding protein was leading to regression of the dominant follicle. In case of natural twinning, two separate follicular waves must be considered. Furthermore, the incidence with double dominant follicles, does not automatically lead to double ovulation, as the chance that one follicle will regress is higher, as the incidence of MO (Ginther et al., 2004).
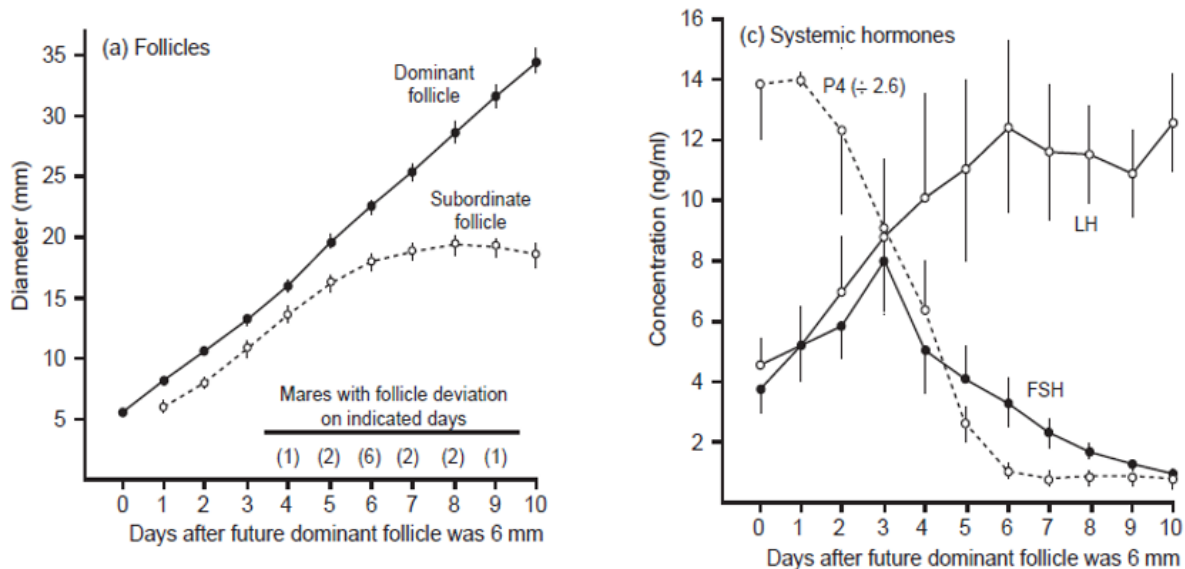
Figure 5: Mean ± SEM diameter of the dominant and subordinate follicles (two-follicle model) and hormone concentrations centralized to emergence adapted from (Gastal et al., 1997). *The numbers in parentheses for the follicle data centralized to emergence (a) are for number of mares with deviation on the indicated days. A demonstration of follicular dynamics related to the hormone concentration. The drop of FSH is said to start the deviation between the follicles.*

3.2. The influence of human chorionic gonadotropin

Originally human chorionic gonadotropin (hCG) was used to induce ovulation and shorten the period of the estrus to use the maximum reproductive performance in a mare, facilitating embryo transfers and artificial insemination, due to estrus synchronization. hCG, is a hormone, build in the placenta and chorion. It accesses over LH properties, stimulating luteal function to maintain pregnancies with help of progesterone.

Due to the influence hormones on the reproduction, the induction of estrus could be a possible cause leading to a twin pregnancy. The relationship between a pharmacological induction and MO was investigated.

(Woods & Ginther, n.d.) dissented by stating, that there was no significant difference between the number of MO of non-synchronized mares and the number of MO of synchronized mares with the help of hCG. Newer results on the other hand, rebutted previous studies, confirming that fact (Allen et al., 2007; Davies Morel & Newcombe, 2008; Perkins & Grimmett, 2001; Veronesi et al., 2003).

(Perkins & Grimmett, 2001) demonstrated a 3-fold increase in the likelihood of twinning, by administering hCG (1500 IU) intravenously 24 hours prior to breeding and 14 days after the onset of detection of diestrus followed by ovulation. (Davies Morel & Newcombe, 2008) took also the dosage of hCG into account (750 IU vs 1500 IU), resulting in 4% difference for MO and

only 1% difference for multiple pregnancies (MP). (Veronesi et al., 2003) aimed not only on the use of hCG but also on the use of prostaglandin F2alpha (PGF2α), which is used to induce estrus and cause abortion. This was the luteal phase can be shortened. The induction of any drug increased twinning from 6.5% to 16.6%. Whereas the use of 0.5mg of clorprostenol alone (n = 86; 17.4%), compared to the mares who received clorprostenol and hCG (n = 49; 30.6%), differed even more to the untreated control mares (n = 324; 6.5%) in twinning rate. In general, most follicles already regressed by more than 15 past ovulation and are therefore unresponsive the indication of any treatment. The induction of MO is for this reason discouraged.

Due to that fact, another drug tested regarding twinning in horses: Pituitary extract, widely used for its hormones due to their stimulating effect on ovulation.
(Woods & Ginther, n.d.) revealed the effect of influence of pituitary extract, used to induce multiple ovulations. One group of mares were treated 15-19 days post ovulation and the other on day 19-23 post ovulation. The mares receiving the extract earlier showed a MO rate of six out of seven, whereas the mares with later treatment only two out of seven had MOs. Aside from that, mares with pretreatment of PGF2α happened to have an increased ovulation rate and needed fever pituitary extract injections. Therefore, the distinct results of the experiments highlighted the importance of the indication timing and the addition of PGF2α prior to the indication of the pituitary extract. The dose applied was examined in a third trial showing no importance to MOs. All in all, the use of pituitary showed a possibility for a MO induced 15 days ovulation, contrary to the use of hCG. In addition, its effectiveness was proven to be of greater significance, than the use of FSH in super ovulating mares (Squires et al., n.d.). Unfortunately, pituitary extract is commercially not available, hence FSH can be still used as an alternative method to induce follicular activity and MOs. The FSH used for administration is usually porcine FSH (FSH-P), as the exploitation of FSH in horses, is nearly impossible due to a complex separation procedure from LH (*Irvine1981*, n.d.).

3.3. The influence of growth factors

The oocyte and the granulosa cells (GCs) are interacting with paracrine signaling mechanisms. This action is described as a functional two-way communication axis between the germ cells and its companion somatic cells (Gilchrist et al., 2006). At full maturity of a follicle, the oocyte is in a mass of cumulus cells (CCs), also known as oocyte-cumulus complex. The CCs originate from GCs and can therefore maintain on the communication by oocyte-secreted factors (OSFs).

GCs possess over receptors for the luteinizing hormone (LH). A study in mice showed, that the expression of the receptors can be suppressed by the oocyte when the oocyte-cumulus complex develops, as they were undetectable in cumulus cells (Eppig et al., 1997). Therefore, it has the be reasoned, that oocyte-derived growth factors (mentioned in **chapter 4**), promote the differentiation of granulosa cells as cumulus cells that can respond normally to events and processes downstream of the LH surge as well as enable the actual process of cumulus expansion after the LH surge (Su et al., 2004).

Both growth factors are expressed by the oocyte in different levels (explained in **Chapter 4**), confirming the study of (Crawford & McNatty, 2012), that a relationship between them and the species-specific ovulation patterns is existent.

In addition, a study tested the differences of the expression patterns of GDF9-and BMP15genes in Arabian and thoroughbred mares in different follicular stages, with the result that in both cases the maximal expression was reached in a follicle of 21–30 mm diameter and in ovarian parenchyma. For this reason, it can be inferenced, that they play and important role in the development of primordial follicles in horses (Stefaniuk-Szmukier et al., 2018).

Chapter 4: The function and importance of GDF9 (Growth Differentiation Factor-9) and BMP15 (Bone morphogenetic protein 15).

4.1. The signaling pathway of GDF9 and BMP15.

GDF9 and BMP15 are growth factors. A growth factor is a secreted molecule, that is affecting the growth of different cells, by promoting or inhibiting mitosis or cell differentiation. Growth factors can perform signal transduction, by acting on specific receptors on the surface of cells, transmitting growth signals to components within the cell. This triggers a cascade, resulting in a change of gene expression. A key role in this process is the phosphorylation of different proteins. When the receptor is activated by the binding of a growth factor, the intracellular proteins will be phosphorylated, resulting in transcription of a specific gene.

GDF9 and BMP15 are members of the transforming growth factor-β (TGF- β) superfamily and therefore cytokines. All cytokines, trigger signaling pathways, but only growth factors affect cellular differentiation, wound healing and cell proliferation (William L. Stone, 2022).

Both are excreted from the oocyte, during the growth of a follicle, playing a crucial role in follicular development. Their absence or deficiency had shown infertility in mice with deleted exons in the GDF9 gene (*Dong1996*, n.d.).

To understand the connection of GDF9 and BMP15 on the ovarian function, it is advised to review the role of the TGF-β pathway with all its key components, by looking at their detailed action on molecular level. Therefore, a brief summary of the signaling pathways is necessary.

Their signaling mechanism in the cells are very similar (visualized in **Figure 6**). Both bind to receptors, sitting in the cell wall, triggering the phosphorylation of so-called SMAD-proteins. For this action a TGF ligand like GDF9 or BMP15 is necessary to be binding to them. The receptor for BMP15 is known as the BMP receptor, whereas the receptor for GDF9 is the TGF-B receptor. Both are categorized into two different subunits, known as type I - and type II receptor. Those subunits are dimerized with disulfide bonds (Macias et al., 2015). For a functioning receptor complex, two type I receptors and two type II receptors in a heteromeric complex (Peng et al., 2013a), must be brought together via a ligand dimer docking on them. Usually, a ligand dimer consists or 2 GDFs or 2 BMPs, but in some cases GDF9 and BMP15 can link (**more explained in the subchapter 4.2.**). The type II receptor phosphorylates the type I receptor that contains kinase domains on the intracellular side. Phosphorylation of the type I receptor results in activation of its kinase activity. The kinase in turn, moves this type of action further by catalyzing the transfer of a phosphate to a SMAD protein, which is located in the cytoplasm of the cell.

SMAD-proteins act as transmitters of the signal to the nucleus, forming complexes which are either stimulating or inhibiting the transcription process of a gene. The minor difference is that Neogenin, a transmembrane protein, is necessary for BMP to bind to the receptor and activate the phosphorylation of the intracellular signaling proteins (SMADs), also known as transcription factors (Foti, 2017).

Furthermore, they bind to different SMAD-proteins. BMP signaling activates SMAD1, SMAD5 and SMAD8/9, whereas GDF signaling activates SMAD2 and SMAD3. Those SMADs are known as receptor-regulated SMADs or R-SMADs. Each of them is encoding a different gene (target gene) in a complex with the common partner SMAD (Co-SMAD), SMAD4.

The inhibition is regulated by the inhibitory SMADs (I-SMAD). For GDF, SMAD7 is degrading the TGF-receptor. For BMP signaling SMAD6 will competitively bind to SMAD4, inhibiting SMAD1,5,8/9 (Baba et al., 2022).
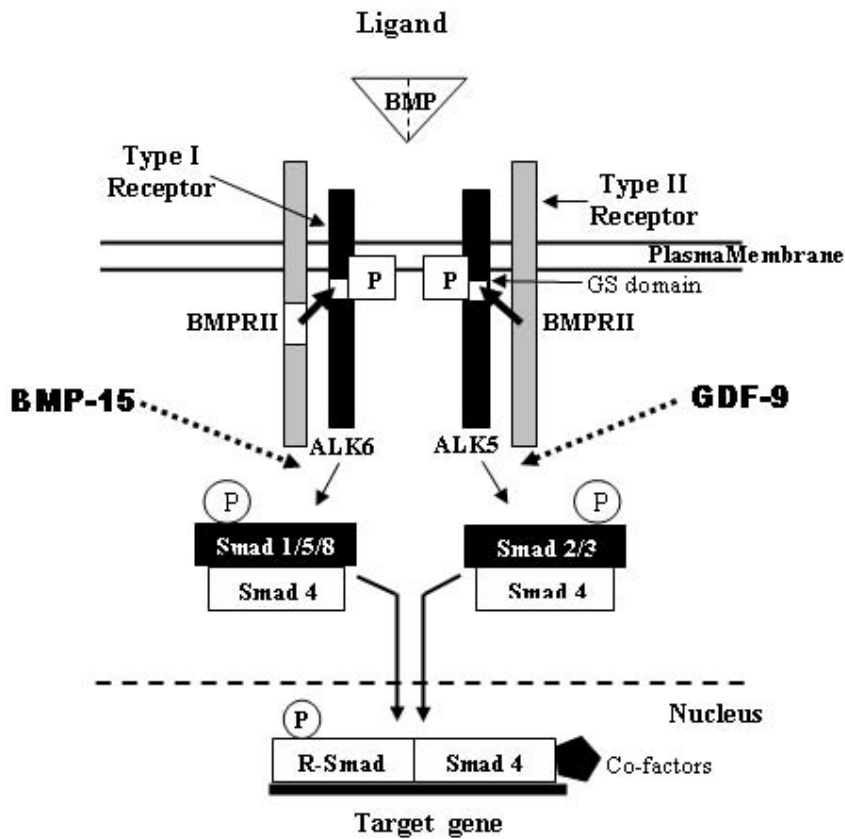

Figure 6: Receptors and signaling pathways of BMP-15 and GDF-9 adapted from(Vireque et al., 2008)**.** *The GDF-9 or BMP-15 dimers bind to specific type I and type II receptors and the formation of the tetramer (2 type 1 and 2 type II receptors for each ligand) permits the phosphorylation of the type I receptor by the type II receptor, resulting in the formation of the signal transduction complex. Type I ALK5 and ALK6 receptors respectively recognize and phosphorylate R-Smads 2/3 and 1/5/8. The phosphorylated R-Smads form complexes with Smad-4 (Co-Smads) and are translocated to the nucleus, where they interact with specific DNA motifs. The effective binding to the target gene is modulated by various nuclear cofactors that co-activate or co-repress transcription.*

If there is a change in the synthetized amount of the biomolecules, the ovulation process will drift from the norm due to a change in the differentiation and proliferation of the cells. Ovarian follicles and ovulation frequency can be inhibited leading to infertility or increased with the outcome of twinning (Cui et al., 2022).

4.2. Dimerization within the TGF-B transduction pathway

Taking focus on each step of the signal transduction pathway, certain possible changes from the norm can occur. First the TGF-B receptors can be altered by the dimerization process. Recent studies have shown in an experiment with human and mice, that the synergism of GDF9 and BMP15 and their dimerization, might influence the signal transduction. Interesting in this study was, that GDF9:GDF9 homodimers of mice have a significant activity, due to a species-specific bioactivity, compared to the ones of humans, whereas the BMP15:BMP15 homodimer is nearly inactive in mice. Therefore, it is estimated for each species to have a dominant dimer, being the active ligand in primary follicles (Peng et al., 2013a) . Aside from that, GDF9/BMP15 can form monomers, resulting in heterodimers, docking on the same cell surface receptors. This might have an influence on the different kinase domains, responsible for the phosphorylation. In addition, there is also an option for a dimer made of 2 homodimers or 2 heterodimers, with the ability to be biologically extremely potent (Mottershead et al., 2013) . The most bioactive ligands are GDF9:BMP15 heterodimers. Recent study showed the synergistic role of GDF9 and BMP15, regulating genes, necessary for cumulus cell expansion, as heterodimers in humans and mice. One example of such a heterodimer is cumulin, known to improve oocyte quality as a potent activator of the granulosa cells (Mottershead et al., 2015). In the granulosa cells SMAD2/3 is phosphorylated over a unique TGF-B receptor, the BMP-receptor type 2 (BMPR2)-ALK4/5/7-ALK6 receptor complex. ALKs (activin receptor-like kinases) are receptor specific kinases on the intracellular side. ALK4/5/7 needs ALK6 as an essential co-receptor to regulate the phosphorylation of SMAD2/3. Furthermore, as demonstrated in **Figure 7**, experiments on mice, humans and sheep, showed that the increased affinity of heterodimers is in vitro and in vivo (Peng et al., 2013a).
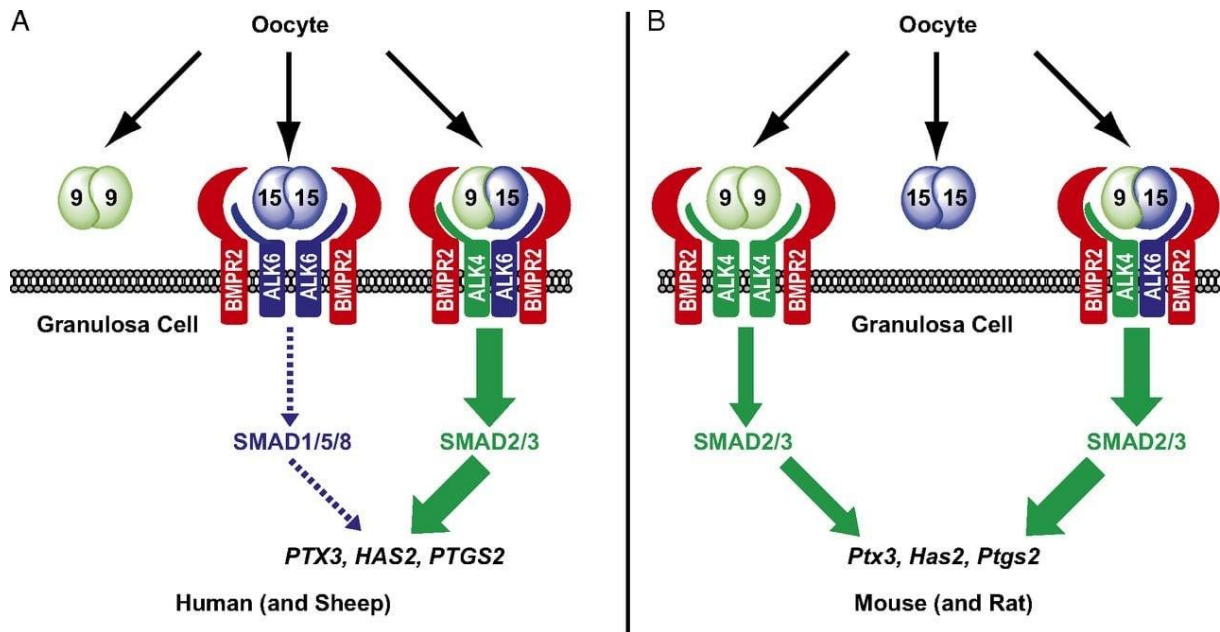
Figure 7: Models for BMP15 and GDF9 homodimers and GDF9:BMP15 heterodimers in regulating cumulus granulosa cell functions adapted from(Peng et al., 2013b)*. (A) In human (and sheep), the GDF9:GDF9 homodimer has extremely low activity. Active BMP15:BMP15 homodimer binds to BMPR2 and ALK6 to up-regulate genes minimally via a SMAD1/5/8 pathway, whereas the potent GDF9:BMP15 heterodimer likely binds to a BMPR2-ALK4-ALK6 receptor complex to transmit a signal through phosphorylation of SMAD2/3. (B) In mouse (and rat), BMP15:BMP15 is inactive, whereas the GDF9:GDF9 homodimer cooperates with the GDF9:BMP15 heterodimer to regulate granulosa cell function via a SMAD2/3 pathway starting from secondary follicles. The GDF9:GDF9 homodimer is likely the dominant (active) ligand in primary follicles.*

4.3. Specific membrane receptors of the TGF-β superfamily

As discussed in **subchapter 4.1.**, each of the receptors is consisting of type I - and type II receptors. The type I receptors, includes seven receptors (ALK 1 to 7) and type II receptors, comprise five receptors (Act RII, Act RIIB, AMHRII, TGF-βRII, and BMPRII) in mammals (Miyazawa et al., 2002).

The specific growth factors discussed here, bind to some of the receptors. GDF9 binds to the TGF-β receptor type I (ALK5) and BMPRII. BMP15 on the other hand, binds to BMP receptor type IB (ALK6) and to the BMPRII as well.

Due to the fact, that both ligands share the BMPRII receptor, which is expressed in the granulosa cells, there is a possibility for them to interact on each other's influence on cell proliferation. A study revealed that ectodomains (the contact surface of the receptor) of the BMP receptor, could partially block the GDF9 action as they differ in structure and receptor

type I. Furthermore, the expression level of the receptors can be modified due to a cellular response (Vitt et al., 2002).

Another study revealed that mutations in BMPRIB are capable to produce oocytes that can be fertilized in vitro, but not in vivo as defects in cumulus expansion prevent fertilization. In addition, the FecB allele is proven to be responsible for those mutations in sheep (Yi et al., 2001).

4.4. SNPs of BMP15 genes and STSs of GDF9

Another study, for variation screening of these growth factor genes, revealed the occurrence of 3 SNPs (single-nucleotide polymorphism) in BMP15 genes in Arabian and Thoroughbred mares with an increased ovulation rate (Stefaniuk-Szmukier et al., 2018). Single-nucleotide polymorphism is the change of a single base on a specific region of a genome of an individual. SNPs is a genetic variation, occurring within coding sequences (exons) of genes, intergenic regions between genes or non-coding regions (introns). They are basically point mutations. Single nucleotides can be changed via substitution, removed via deletion or added by insertion to a polynucleotide sequence during DNA replication. Twinning can occur as an outcome, if the new genetic variation results in an increased follicular growth. In humans two SNPs related to twinning were found in the SMAD3 gene and in the FSHB gene, known as the follicle stimulation hormone subunit beta (Mbarek et al., 2016). Another study presented the importance of a SNP in an exon of the FecX allele in the BMP15 gene in Rasa aragonesa sheep, resulting in increased prolificacy when heterozygous and sterility when homozygous in ewes(Calvo et al., 2020).

Furthermore, it is also revealed the occurrence of STS (Sequence-Tagged Site) in the GDF9 gene (Stefaniuk-Szmukier et al., 2018). STSs are unique sequence-based landmarks in a genome, with a sequence that cannot be found elsewhere in the genome. The unique sequences are usually located at the end of the genome and can be synthetized to unique DNA primers. For the detection of such a sequence PCR is used, specified with gel electrophoresis. Genetic diversity can be evaluated when the gene sequence is identified, helping a better understanding of its' link to certain traits, like the SNPs. Due to this ability, STSs are a useful tool for genetic analysis, known as molecular markers (Avinash Marwal, 2020).

The different genetic variant sequences are alleles, which can be on different positions on a chromosome. The position of a gene on a chromosome is known as locus.

During the study, genomes of five mares with reoccurring MO and no history of MO were analyzed with Sanger sequencing. The DTSC Sequencing Kit (Beckman Coulter, California, USA) and the CEQ8000 Genetic Analysis System (Beckman Coulter, California USA) were used for this action. The results showed 2 intronic SNPs in the BMP15 gene and in the intronic region of GDF9 locus, an STS sequence. Therefore, no relationship to MO can be made, as introns are spliced out after the transcription process. But the occurrence of this phenomenon, shows a possibility for such an incident.

Secondary to it, twinning also depends on the ovulation frequency, next to the development of ovarian follicles. Hence to this, the allele frequency of the already known SNPs and STSs were identified via PCR-RFLP and Fragment Analysis. To estimate and compare the genotype frequency, the MAF (minor allele frequencies) for each locus had to be carried out in both breeds. The MAF is the frequency of the second most frequent allele for a given SNP. This measure gives an idea about the variation of genotypes for a given SNP in a population, in other words it gives an idea about how common or rare that SNP is (Kim et al., 2011).

The results (**Figure 8**) showed that none of the genotype frequencies were significant regarding the ovulation, but still one locus was higher in TB mares then in Arabic mares, proving an increased tendency for twinning (Stefaniuk-Szmukier et al., 2018) .

Horse breed	n	BMP15			GDF9
		MAF g.40851607T>C	MAF g.40852502A>G	MAF g.40854998T>C	g.42750467GT [8]GA [12]
Arabians	73	0.19	0.32	0.49	0.34
Thoroughbreds	53	0.28	0.48	0.49	0.39

Figure 8: Minor allele frequencies for SNPs in BMP15 loci (g.40851607T>C, g.40852502A>G, g.40854998T>C) and STS (g.42750467GT [8]GA [11]) in GDF9 locus adapted from (Stefaniuk-Szmukier et al., 2018)**.** *The genotype frequencies comparison between investigated breeds showed similar distribution pattern. However, in BMP15 g.40851607T>C loci according to Chi2 test, significant differences (P-value 0.029) between Arabians and Thoroughbreds occurred with higher frequency in TB mares.*

Chapter 5: Demonstration of the occurrence of twinning in a model

Since twinning is an all-or-none-character it can be best demonstrated on threshold models. Threshold models function in assuming, that many factors with small individual effect, contribute together to a disorder. Hence, they can cover genetic, phenotypic and environmental results of research together and are therefore more applicable than linear models. Furthermore, they access over the capability to set a critical value, known as the threshold. In this case demonstrating that only an amount large enough – in interaction of genetic, phenotypic and environmental effect, will result in twinning. (Wolc et al., n.d.), demonstrated heritability and repeatability according to a threshold model, in correlation of the amount of mares/year of birth, twin frequency (= phenotype)/year of birth and the average genetic prediction/year of birth. The results were in all three cases declining. The declining phenotype in twinning, can be explained due to fewer mares born and genetic prediction with the advancement in ultrasound techniques. Whereas the genetic prediction, of a decline in twinning frequency, can also be made due to fewer mares. This demonstrates the correlation between all three factors to be necessary to trigger one event. The environmental factors, responsible for twinning, were described in **Chapter 2 and 3**.

Chapter 6: The natural reduction process of twins

The mare accesses over a natural mechanism of biological embryo reduction. This allows the mare to eliminate excess embryos, resulting from MOs to a singleton. A premise for such an activity is an age gap between the embryos of more than 2 days. In unilateral twins the reduction of the weak twin to a singleton is estimated to be 85% (Crabtree, 2018), whereas in bilateral twins it is 4% (Wolfsdorf & Macpherson, n.d.), as both members would have an equal chance of survival. As bilateral twins occur from synchronous ovulation, both ova have the same change for a fertilization, but surprisingly a disparity between the high incidence of double ovulation (of 4-43%) and the low incidence of a twin births or abortions of (1-2%) was noted (Gintherayb, Douglasc, et al., n.d.). This phenomenon was investigated by two different experiments. In the first one, double ovulation was stimulated with pituitary extract with the aim to produce twins. The experiment was unsuccessful, proving that such a mechanism must exist. In the second one, it was shown, that the pregnancy rate was higher in double ovulating mares than in single

ovulating mares, proving that the incidence of natural reduction must occur after fertilization. In addition, the process of embryo reduction only occurred after fixation at day 16 and before the end of the embryo stage at day 40 (Ginther, n.d.-b), but other studies disprove it, saying that this mechanism exerts its effect after the blastocysts enter the uterus by day 6. In this process, the nesting of the oocyte is impeded, based on the lack of nourishment of the reproductive system.(Gintherayb, DouglasC, et al., n.d.-b). Because of this, it can be hypothesized that the nature of this process requires further testing (Ginther, n.d.-c).

The elimination process of an embryo is not fully known yet, but it is hypothesized that in unilateral twins, the withdrawal of nutrients, by uterine massage will influence it (Gintheralb & Douglas, n.d.). In this event the larger vesicle will receive most of the access to the chorion, creating deprivation of nutrients for the smaller vesicle. A remnant of a twin pregnancy could only be the singletons vesicles orientation and later positioning of the fetus during the pregnancy.

The mechanism of natural reduction is complete by the 35th day of gestation. This is also the time when endometrial cups start to develop. They are a unique feature of equine pregnancies, responsible for secretion of the equine chorionic gonadotropin (eCG). The eCG concentration a crucial factor for future cyclicity. It is advised to not abort after their development, as the mare will not be able to reproduce again during the same breeding season (Estradé et al., 2016).

Chapter 7: hypothesis, ideas for further research

7.1. Reasoning for the lack of research on equine twinning

87% of twin pregnancies terminate with one or both fetuses aborted or result in stillbirth and neonatal mortality (Miller & Woods, 1988). If this is not the case, the management of equine twinning usually results in reduction of one embryo, by different techniques, such as manual crushing, dietary restriction, membrane slipping and prostaglandin induction, transvaginal ultrasound-guided puncture and craniocervical dislocation (Wolfsdorf & Macpherson, n.d.). Due to this issue, study on equine twins, is limited to the slaughterhouse specimen, abortions, stillbirths and fetal membranes. Moreover, many twin pregnancies are not recorded, due to failure of their identification, is not uncommon (Morris & Allen, 2002). The asynchronous ovulations in mares, differ in follicle size so that the smaller vesicle can be overlooked if the examination is done imprecisely or misdiagnosed as an endometrial cyst. Also, an early stop in

ultrasound monitoring of a pregnancy, can lead for the later ovulated vesicle, to go unnoticed. Diestrus ovulation are frequent affected by this incidence. A proper ultrasound guidance is therefore crucial in any pregnancy. Rectal palpation alone, is not efficient enough especially for unilateral fixation, as there is a higher change to misdiagnose two vesicles as one. Moreover, the vesicles mobility prior to the fixation impedes a diagnosis, if the whole uterus is not examined carefully (Hodder et al., 2008). Besides management issues, the research of reproductive genetics was out of focus for previous years, contributing to the lack of research. Horses were bred accordingly for favorable traits for human satisfaction, adapting their physiology and anatomy to it.

Furthermore, the research on twinning is impeded by the difficulty to obtain oocytes for study. The reason for it is that the adhesion of cumulus cells to the follicular wall is much stronger in horses, than in other species. Because of this instance, oocytes hardly recover post ovum pickup (OPU) from live donors. In addition, they also response poorly to gonadotropin preparation, used to mimic the physiological effect of gonadotropin hormones (Tharasanit & Thuwanut, 2021).

7.2. Grounds and goals for future experimentations and research.

7.2.1. Progesterone induction to prevent natural embryo reduction.

As embryo transfer, is gaining importance, it is advised to study factors increasing the rate of MOs. A convenient starting point would be to fully understand the effect of natural embryo reduction in a mare. Due to the discovery of (Gintheralb & Douglas, n.d.), that a possible factor for this phenomenon might be the effect of uterine massage, it can be inferenced, that the uterine contractions are leading to abortion. Therefore it might be of help to induce drugs, blocking the occurrence of contractions. An example for this, would be the indication of progesterone. Therefore, progesterone induction in the timeframe from the fertilization until the development of the endometrial cups can be tested, to maintain the pregnancy. A trial proved an increased chance of the smaller vesicles' survival demonstrated in a study from (Roberts & Myhre, 1983), who inducted progesterone after noticing, that in twinning the progesterone level happens to be often too low for both conceptions.

7.2.2. Gene profiling

Thus, to the stallion effect, it can be an advantage to make a gene profile of the stallion and mare to fully understand the gene expression of the different growth factors related to

homozygosity and heterozygosity, which might help to identify a gene locus responsible for twinning. Moreover, a genetic profiling in mares during follicle selection can be of big advantage to determine the genes responsible for follicle selection (Ginther et al., n.d.). In cattle such a gene profiling was already reported (Sisco et al., 2003). This can be made with help of sequencing technologies, as micro arrays are not available for the equine species.

Conclusion

Twinning is phenotypically an all-or-none occurrence but can be differentiated in its frequency. Therefore, it is important to understand factors that play a crucial role and trigger such an outcome. This gives us the ability to control twinning until a certain degree and meet the requirements. All those factors are briefly summarized in this thesis. In doing so, the importance of each factor is highlighted, but it is important to keep in mind that only an interplay between those factors will eventually result in the phenotype of twinning. Therefore, the phenomenon twinning can be best put for assumption by a threshold model.

Conspicuous is the clear distinction in the frequency of the occurrence of twinning in different horse breeds. For this reason, a genetic predisposition is assumed, leading to the statement that twinning is inheritable. The context of genes related to twinning, has been proven in other animals before. A gene for twinning was not identified, but certain SNPs in gene loci are related to such an occurrence can be found. Those genes are described as the candidate genes, which allow us to understand the background to this phenomenon and show us that it is not just influenced by the start of the pregnancy, but way before. Examples for those genes are growth factors, responsible for gene expression and cell proliferation. For this matter it is an advantage to understand correlations of molecular signaling such as the signaling pathway for growth factors. In addition, the physiology of endocrine and paracrine signaling pathway of hormones is necessary to be fully understood. The effect of FSH, hCG and many more hormones is proven to influence the fertility regarding the frequency of ovulations, creating ovulatory patterns and follicular waves. Moreover, the base for all pregnancies is fertility. Therefore, changes in fertility are the starting point to further investigate the phenomenon of twinning. These factors are either genetically predisposed or environmental. Examples of these, are age, season, the reproductive status and nutrition of the mare.

Due to the increasing importance of twinning in horses, further study is required. The understanding of potential factors is inevitable.

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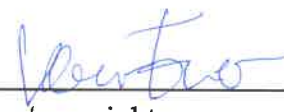
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Thesis progress report for veterinary students

Name of student: Verena Flanter

Neptun code of the student:

Name and title of the supervisor: Dr. Zöldág László prof. emer.

Department: Animal Breeding and Genetics

Thesis title: Twinning in horses - genetics (review of literature).

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	febr.	8	Discussion about the chosen thesis topic in general	
2.	2022	march	9	Rough content and design of the planned thesis	
3.	2022	april	18	Improvement of the planned thesis content	
4.	2022	may	5	Literature selection for the thesis	
5.	2022	june	8	Analysis and evaluation of the selected references	

Grade achieved at the end of the first semester: very good (5)

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	sept.	16	Discussion of the prepared rough manuscript	
2.	2022	oct.	20	Final talking over of the ready made manuscript	
3.	2022	nov.	10	Final glance onto the manuscript and approval	

| 4. | 2023 | jan | 18 | Discussion of the prepared rough manuscript | |
| 5. | 2023 | march | 10 | Final version | |

Grade achieved at the end of the second semester: very good (5)

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

....
signature of the supervisor

Signature of the student: ....

Signature of the secretary of the department: ....

Date of handing the thesis in............ ....

University of Veterinary Medicine

Name (student): **Verena Flanter**

Announcement of the chosen topic of the Thesis

I would like to ask for the permission of the Head of the Department of Animal Breeding, Nutrition and Laboratory Animal Science, to write my thesis in the following topic advertised and supervised by the Department.

Budapest, 2021-03-09

....
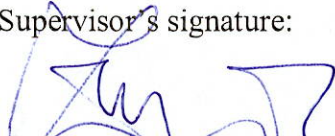Student's signature

Topic of the thesis:

This thesis work will be a review and analysis of the current research and genomic data about the twinning in horses. A special emphasis will be given to the current equine genomics and to the phenotype characteristics of the twinning in different horse breeds.

Title:

Equine twinning - genetics. (Review of literature)   TWINNING IN HORSES

Supervisor's signature:

....
Dr. Zöldág László prof. emer.

I approve:

....
Signature of the Head of the Department
Prof. Dr. András Gáspárdy