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# EPIGENETIC PROGRAMMING OF FOAL'S THROUGH NUTRITION, STRESS, UTERINE SPACE AND SPORT.

VETERINARY MEDICINE BACHELOR'S THESIS

Doble Grau en Veterinària i Ciència i Producció Animal

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## 1. Summary

Nowadays, to foresee probable phenotypical outcome as an adult of a foal, it is commonly done by checking their pedigree, their relatives' achievements and conditions as well as their temperament. However, noncommunicable diseases have actually been highly associated with in utero environment through epigenetics. This finding implies that common conditions that one would usually check before buying a horse, besides performance, won't necessarily be seen with the habitual methods used, as it mainly depends on the environment the individual had been exposed to throughout their life, even when in utero.

In the present study, four environmental factors, their phenotypical outcomes and the epigenetic marks involved have been researched and described. As available information in horses can be scarce, scientific evidence in other species has been researched and then extrapolated to horses.

The environmental factors (exposomes) researched are related to nutrition, stress, exercise and uterine space programming. The main epigenetic marks involved are: DNA methylation, miRNA and lncRNA, histone modifications and chromatin remodeling. As for the outcomes described, it involves some noncommunicable diseases like metabolic disorders (i.e., type 2 diabetes), cardiac and vascular conditions, obesity, osteocondrosis and neurodegenerative diseases. On top of that, increased fear and stress response, poor growth and immune function are some other potential outcomes of the exposomes above mentioned.

With all the collected information, a mare handling protocol is suggested based on up-to-date scientific evidence with the aim to contributing towards a healthy, sport-oriented foal.

**Resumen**

Hoy en día, para tratar de prever los probables fenotipos de un potro cuando sea adulto, es habitual verificar su pedigrí, los logros y enfermedades o lesiones de sus familiares, así como su temperamento. Sin embargo, las enfermedades no transmisibles se han asociado en gran medida con el ambiente intrauterino al que es expuesto el animal, explicado a través de la epigenética. ¿Qué significa eso? Implica que las enfermedades y lesiones comunes que uno suele revisar antes de comprar un caballo, además del rendimiento, no necesariamente se verán con los métodos habitualmente utilizados, ya que depende principalmente del entorno al que únicamente este individuo ha estado expuesto a lo largo de su vida, incluso cuando su madre se encontraba en su propia fase intrauterina.

En el presente estudio, se investigaron y describieron cuatro factores ambientales, sus resultados fenotípicos y las marcas epigenéticas involucradas. Como la información disponible en caballos puede ser escasa, se ha investigado la evidencia científica en otras especies y luego se ha extrapolado a los caballos.

Los factores ambientales (exposomas) investigados están relacionados con la nutrición, el estrés, el ejercicio y la programación del espacio uterino. Las principales marcas epigenéticas implicadas son: metilación del ADN, micro ARN y ARN no codificante de cadena larga, modificaciones de histonas y remodelado de la cromatina. En cuanto a los resultados descritos, algunos de ellos son: algunas enfermedades no transmisibles como trastornos metabólicos (como la diabetes tipo 2), afecciones cardíacas y vasculares, obesidad, osteocondrosis y enfermedades neurodegenerativas. Además de eso, el aumento del miedo y la respuesta al estrés, el crecimiento y la función inmunológica deficientes son otros posibles resultados de los exposomas mencionados anteriormente.

Con toda la información recopilada, se ha sugerido un protocolo de manejo de las yeguas basado en evidencia científica actualizada orientada a obtener potros sanos y con buenas capacidades hacia la práctica del deporte.

## Resum

Avui dia, per intentar preveure els probables fenotips d'un poltre quan sigui adult, és habitual verificar-ne el pedigrí, els èxits i les malalties o lesions dels familiars, així com el temperament. Tot i això, les malalties no transmissibles s'han associat en gran mesura amb l'ambient intrauterí al qual és exposat l'animal, explicat a través de l'epigenètica. Què vol dir això? Implica que les malalties i lesions comunes que un sol revisar abans de comprar un cavall, a més del rendiment, no necessàriament es veuran amb els mètodes habitualment utilitzats, ja que depenen principalment de l'entorn al que únicament aquest individu ha estat exposat al llarg de la seva vida, fins i tot quan la seva mare es trobava a la seva pròpia fase intrauterina.

En aquest estudi, s'han investigat i descrit quatre factors ambientals, els seus resultats fenotípics i les marques epigenètiques involucrades. Com que la informació disponible a cavalls pot ser escassa, s'ha investigat l'evidència científica en altres espècies i després s'ha extrapolat als cavalls.

Els factors ambientals (exposomes) investigats estan relacionats amb la nutrició, l'estrès, l'exercici i la programació de l'espai uterí. Les principals marques epigenètiques implicades són: metilació de l'ADN, micro ARN i ARN no codificant de cadena llarga, modificacions d'histones i remodelat de la cromatina. Pel que fa als resultats descrits, alguns són: algunes malalties no transmissibles com ara trastorns metabòlics (com la diabetis tipus 2), afeccions cardíaques i vasculars, obesitat, osteocondrosi i malalties neurodegeneratives. A més d'això, l'augment de la por i la resposta a l'estrès, el creixement i la funció immunitària deficients són altres conseqüències possibles dels exposomes esmentats anteriorment.

Amb la informació recopilada, s'ha suggerit un protocol de maneig de les eugues basat en evidència científica actualitzada que permeti la cria de poltres sans i amb bones capacitats per la pràctica de l'esport.

## 2. Motivation

Horses were first domesticated in around 3500 BC, probably on the steppes of southern Russia and Kazakhstan, and introduced to the ancient Near East in about 2300 BC. Before this time, people used donkeys as draught animals and beasts of burden. While in the past horses were used in war, in hunting and as a means of transport, nowadays, most horses are used for recreation, sport, entertainment, and competition.

**Horse performance** can be loosely defined as any form of work or forced physical activity carried out by a horse in contests. This can include walking, trotting, cantering, jumping and turning, depending on the competition. For instance, horses for racing require strong muscle tones and a fast metabolism to run short distances at full speed. Winning horses require great levels of stamina and alertness. Horses used for dressage need to be docile, agile, flexible and pay close attention to the rider. Endurance is a test of stamina for both the horse and the rider. It involves riding over long distances, typically between 25 to 500 miles.

To adjust to each of these needs, very **specific horse breeds** are used for each of these riding styles. Breeding selection has shaped the genetic content of each breed. Therefore, there is a genetic component in the traits that compose horse performance. Nowadays, several **DNA variants** have been identified in relation to body conformation, muscle power and muscle fibre composition (reviewed in Bailey et al., 2022[1] and Rivero et al., 2016[2]). Some of these variants, commonly known as the "speed gene" or the "gait gene", are being commercially exploited to raise the winning likelihood of horses (Rooney et al., 2018[3]). Although genetics might predispose a horse into racing, many **environmental factors** are essential to unlock the genetic potential of the horse. Diet, training routines, management practices have been adjusted to bring the best out of each horse.

However, little is known on how genetics and the environment interact. Recent evidence in other species highlight the relevance of **epigenetics** as a hinge between these two elements (summarised in Cavalli et al., 2019[4]). The conversation between genes and the environment work in both directions: cells use epigenetic labels to write cues from the environment into our genes, while our genes adjust to the intensity of the environmental signals also by using epigenetic tags.

Moreover, epigenetic signals can partially be **transmitted** to the next generation, resulting in the vertical transmission of acquired traits. This Lamarckian heritability raises many opportunities and concerns. For instance, can the performance of a young horse be enhanced by the diet of her pregnant mother? Can we adapt the activity of the mare so that foals have a better muscling capacity? How could we use the environmental factors to write the right epigenetic cues to the horse genome? What can we learn from other species?

# 1. Introduction

## 2.1. Epigenetics

The meaning of "epigenetics" has evolved with time and, especially, with scientific research. "Epigenetics" was first used to refer to the complex interactions between the genome and the environment that are involved in the development and differentiation in higher organisms. Nowadays it is used to refer to **heritable alterations** that are **not** due to **changes in DNA** sequence.

"Epigenetic tags" or "epigenetic labels" are marks in the DNA (such as: DNA methylation, histone modification, chromatin remodeling and non-coding RNA regulation), which allow or silence gene activity and the subsequent production of proteins that guide the growth and development of an organism.[5,6]

It's also been taken into consideration the fact that most non-infectious diseases might be explained thanks to epigenetic changes during an individual's life. Moreover, these changes might have happened during intrauterine development of this individual (i**ntergenerational inheritance**) or when this very same individual's mother was developing her germline cells in her own intrauterine period (**transgenerational inheritance**). In a practical sense, this means that the way an owner handles a mare during pregnancy might have an important effect on the offspring's health and performance (Figure 1).[7]

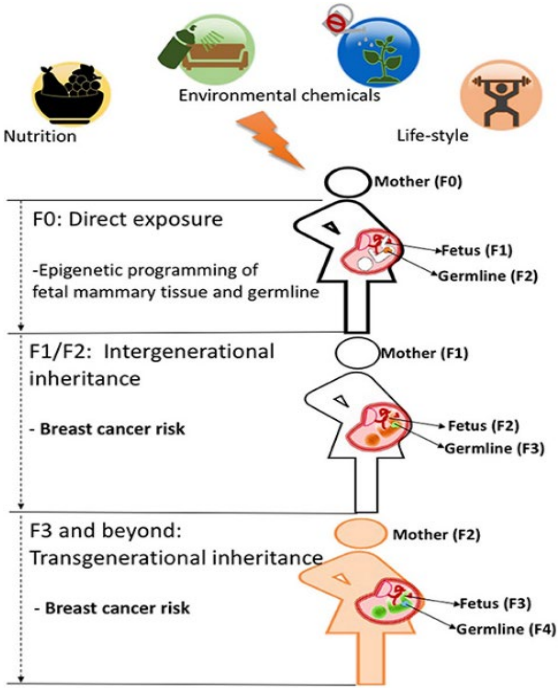



*Figure 1 - Intergenerational and transgenerational inheritance of epigenetic labels. The environment the F0 generation is exposed to during pregnancy can have an impact on the F2 generation phenotype through epigenetic labels on the germline cells. For example: nutrition during pregnancy, exercising rather than sedentary lifestyle. Image from Santana da Cruz et al., 2020*

To this day, owners wanting to buy a **racing horse** usually look into parental lines, genealogical books, number of wins in races of siblings and other relatives, conformation of the individual, temperament, offspring and their achievements, etc. but when diving into genetics, one huge field that should not go unnoticed is epigenetics. Good genetics are no good anymore if they can't be correctly expressed into the expected phenotype or if silent genes predisposing to diseases (and therefore never noticed before in other relatives) are enhanced by poor management.

Common practice in pregnant mares involves: feeding them concentrates, stopping them from exercising, stalling them and many others. These management practices are thought to be in the best interest of the mare and the unborn foal. While it is true that epigenetics in horses is something that still needs to be further explored, there is already strong evidence in other species which could be projected to horses. This may change through the years, as science is always evolving, but it certainly might do good to collect updated information in other species and extrapolating it to horses. That is what this present study aims for.

## 2.2. Known epigenetic tags

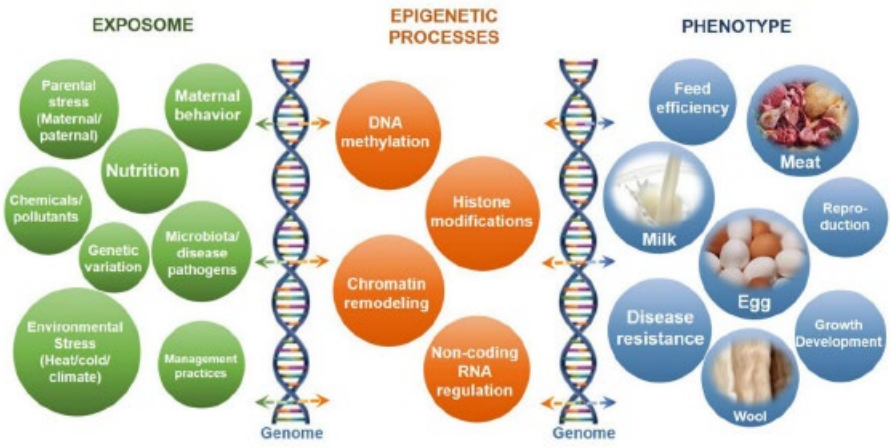



*Figure 2 - The impacts of epigenetic regulatory processes on livestock production in response to different impact factors or the exposome. In the image it's being showed common impact factors or exposome (left), their interact with epigenetic processes (center) and the genome (vertical DNA helix structure) to influence phenotypic outcome (right). Image from Wang M et al., 2020.*

Epigenetic signals can be driven by **four** different **processes** involving: noncoding RNA molecules, chemical modification of DNA, histone proteins and changes in the chromatin condensation. Figure 2 shows many exposomes which have been demonstrated to have an impact on an individual's phenotype through the studied epigenetic processes.

### *2.2.1. microRNA and long noncoding RNA*

Most of horse's genome is transcribed into RNAs with absent or limited protein-coding potential: mainly microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). They can regulate fundamental cellular processes via diverse mechanisms that involve gene expression modulation. Recent evidence indicates a cross talk between miRNAs and lncRNAs.

miRNAs are a large family of small noncoding RNAs, which can be as small as 18-25 nucleotides long, that can regulate translation of messenger RNAs (mRNAs); some of them can even prevent translation to happen by degradation. Cytoplasmic miRNAs are able to bind to 3' untranslated regions of target mRNA, therefore regulating what is being translated and what not as they need very small amount of nucleotide complementarity (6-8), a miRNA can potentially target hundreds of mRNAs.

In addition to this, **miRNAs** are expressed in abundance in the nucleus and regulate various nuclear events by binding directly to target genes. For instance, when **binding to promoter sequences**, they can control **transcription**, while by **binding to introns**, **RNA splicing** can be modulated. Through these mechanisms, the **translation** of a **gene into proteins is regulated**.[8]

Among several biological functions, miRNAs have been reported to regulate development, differentiation, cell cycle, cell proliferation and apoptosis. Some miRNAs are known to be regulators of important metabolic processes, including insulin signaling and glucose homeostasis. In fact, dysregulation of miRNAs has been associated with the development of chronic diseases such as cancer, cardiovascular diseases and many others.

Extracellular circulating miRNAs can resist adverse physiological conditions and can be found in body fluids such as blood, saliva, cerebrospinal fluid, urine and breast milk.[9] This represents an addition layer of cellular cross-talking.

Long non-coding RNAs (**lncRNA**) have also a very relevant role in epigenetic marking. lncRNA is a heterogenous family represented by long intergenic RNAs, circular RNAs, competing endogenous RNAs, antisense RNAs, etc. These are of absent or limited protein coding potential.

LncRNAs can act as **signal molecules**, they are transcribed and get to influence signaling pathways themselves or in combination with certain proteins like transcription factors. For example, p53-induced lncRNA-p21 has been shown to interact with nuclear heterogeneous ribonucleoprotein-K to inhibit expression of downstream genes in the p53 signaling pathway (implicated in the cell cycle control).

**LncRNAs** can also act as **guide molecules**, they aid specific proteins reaching their target location and, therefore, exerting their biological function. The mechanism by which they do this is regulating transcription of adjacent mRNAs by interacting with transcription factors in a specific DNA sequence. For example, Wang et al., 2011, found that lncTCF7 can recruit the

Switch/Sucrose-Non-Fermentable complex to the TCF7 promoter and so regulate its expression (this may lead to promote proliferation of liver cancer stem cells).[10]

Several **lncRNAs** can **recruit chromatin remodeling complexes** to a specific set of genes to activate or repress gene expression, for instance by its ability to regulate DNA methylation. A well-characterised example of lncRNA-mediated gene inactivation event is the inactivation of one X chromosome in females forming the bar corpuscle in somatic cells. This is a gene compensation event that is initiated by the activity of two noncoding, complementary RNAs—XIST and TSIX—. This phenomenon causes the calico phenotype in cats and horses (Figure 3), for instance.



*Figure 3. Calico phenotype in a horse.*

### *2.2.2. DNA methylation*

DNA methylation is the most stable and most studied type of epigenetic label[11–13]. This epigenetic mark involves the covalent transfer of a methyl group, usually towards a CpG dinucleotide. The family of enzymes responsible for this process is **DNA methyltransferases** (DNMTs; Figure 4).

The importance of DNA methylation on gene transcription varies depending on where it happens. It can happen in promoters, enhancers, transcription units, CpG rich-regions (CpG islands – CGIs), flanking CGIs or in CpG-poor regions. It has also been accepted that CGIs can (and often do) overlap with promoter regions and are **unmethylated** when the gene is actively **transcribed**. Conversely, when they do become **methylated**, transcription is strongly **repressed**.

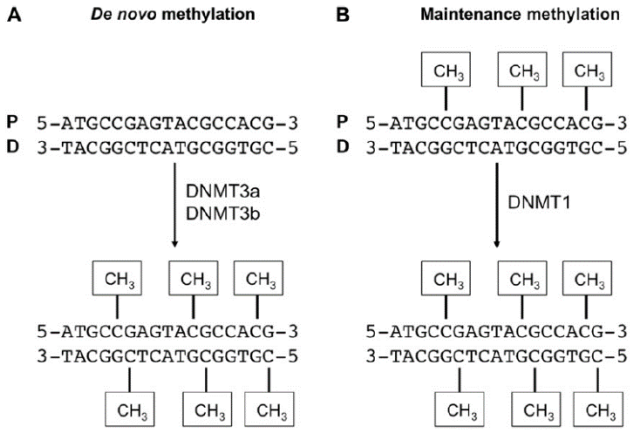



*Figure 4 - Schematic diagram of de novo and maintenance methylation. (A) The methyltransferase enzymes DNMT3a and DNMT3b are capable of adding methyl groups to CpG dinucleotides in the absence of a methylated template strand. (B) The methyltransferase enzyme DNMT1 uses the parental (P) template strand to stably propagate the methylation pattern on the newly synthesized daughter (D) strand. Image from Dindot et al., 2013.*

### 2.2.3. *Histone modifications*

DNA-histone association allows genomic information to be packed into a highly organized structures through the formation of nucleosomes.

Histone proteins have a protruding unstructured **N-terminal tail**. Some **amino acid** residues of this tail and a few others within the core of the nucleosome are potential **targets** for **post-translational modifications**: methylation (lysine and arginine), acetylation (lysine), phosphorylation (serine and threonine), ADP ribosylation, ubiquitylation (lysine), sumoylation (lysine), butyrylation, citrullination, crotonulation, formylation, proline isomerization and propionylation.

Combination of different histone modifications may regulate chromatin structure and transcriptional status at specific loci within given cell types. [6,11,13]

### 2.2.4. *Chromatin remodelling*

Together, the ncRNA, **DNA methylation** and **histone modification labels** strongly impact the **functional** and structural state of **chromatin**. Chromatin, that is, the nuclear complexes of DNA, RNA and histones, can be in an "open" or active state (referred as **Euchromatin**) or in a "closed" or inactive state (referred as **Heterochromatin**). Epigenetic labels are the key to set up these two states. Genes located in "open" chromatin are usually transcribed while genes in "closed" chromatin are usually silent (Figure 5).

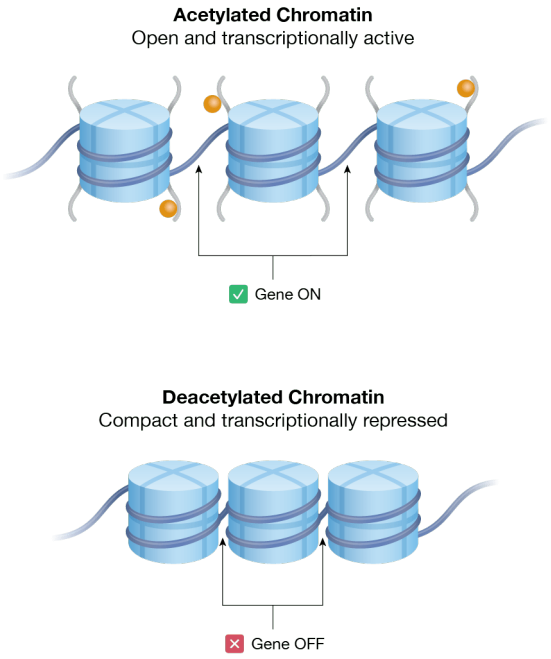



*Figure 5. Chromatin organization and epigenetic regulation. Relaxed chromatin puts the gene in a transcriptionally active state; densely packed chromatin makes the gene transcriptionally repressed. Picture by Bio Rad website publication: https://www.bio-rad.com/es-es/applications-technologies/epigenetics-chromatin-structure?ID=LUSNMFHYP*

### 2.3. Exposomes of special interest

Nutrition and exercise are the main exposomes researched in this Bachelor's Thesis, but intrauterine space and maternal stress during pregnancy are both of interest too when talking about horses bred for specific sport disciplines, like Thoroughbreds, which are specifically used in racing. It is a common discussion among breeders how to best feed a pregnant mare, whether to exercise them or not, stalling them or not, etc. That usually happens because a shiny coat in a mare might quickly catch the eye, just like a big newborn foal, but these outcomes don't necessarily mean the mare has gotten the best treatment in order to, for example, improve offspring's performance later in life. In other words, stalling a mare during pregnancy and feeding her concentrates might lead to a shiny coat and a big newborn foal, but it might also induce other long-term consequences, which take time to appear and might be undesirable. This project aims to study these consequences.

## 2. Objectives

Horse industry is moved mainly by pedigrees and, also, relatives and individual's performance. Handling protocols are usually designed according to short and mid-term consequences, like energy availability for a season's training and competition or to ensure shiny coats. But little is said about long-term consequences, like predisposing a horse to end up developing a metabolic condition by their *in-utero* environment or maybe leading their offspring to osteoarticular injuries. In other words, it might be interesting to identify which handling protocols are actually helpful for long-term benefit of horse's health and performance.

The aim of this project is to summarise published evidence on how nutrition, stress, uterine space and sport impact a pregnant mare's offspring through epigenetic changes. As studies in horses are scarce, information will also be extrapolated from other species' scientific studies.

Secondly, with this updated information, a handling protocol will be suggested on how to feed, treat and exercise pregnant mares in order to get a healthy foal who is set up for success given it is adequately handled in their training regime.

## 3. Methodology

### 3.1 Bibliographical research

This research project is mainly based on bibliographical research. In order to do so, scientific articles have been searched and extracted from reliable sources, such as: PubMed, Scopus, ScienceDirect, Wiley Online Library and Google Scholar.

Several key words have been used to extract as much useful information as possible. For introduction of epigenetics and epigenetic tags, the main key words used have been: epigenetics, epigenetic labels, DNA methylation, microRNA, noncoding RNA, histone modifications, chromatin acetylation. For epigenetic tags, some of the main key words have been: *in utero*, programming, foetal, nutritional programming, exercise during pregnancy, stress programming.

In order to get all bibliographical references correctly, it has also been used Zotero to save and import all articles researched and to properly reference them in every part of this paper.

With all information put together and with a reasonable amount of scientific evidence, a handling protocol is suggested for pregnant mares in order to get foals as healthy as efficient as runners as possible.

# 4. Results

The results of this study are presented according to four main factors: nutritional, stress and behavioural, intrauterine space and mother's exercise programming of the offspring. These are four of the most impactful exposomes generating epigenetic changes in horses, especially nutrition.

These exposomes impact most frequenctly on the offspring's **obesity, metabolic disorders** such as type 2 diabetes, developing **vascular diseases**, higher susceptibility to developing **OCD** (osteochondritis dissecans), enhanced or reduced **stress response**, etc.

In the following sections, scientific evidence on each one of these exposomes is described, to finalise with a **summary table** with the most **remarkable exposomes**, their **consequences** and the **epigenetic tags** involved**.**

## 4.1 Nutritional programming

### *4.1.1 General mechanisms*

There's two main ways by which the **supply of nutrients** and **oxygen** can be **altered** in the mammalian foetus:

1. There can be changes in the **availability** of these substances in the **maternal circulation** due to **alterations** in diet or maternal **metabolism** caused by **disease** or other environmental **stresses** and, therefore, impacting the **transplacental passage** of nutrients to the foetus.[14]

2. By changes in **placental function** influencing the foetal nutrient supply, impacting primarily the foetus but also producing alterations in placental hormone synthesis and metabolism, which may have effects on the mother and, therefore, impacting allocation of nutrients to the gravid uterus[14].

At a **cellular level**, nutrient availability can affect tissue development in several ways:

1. **Nutrients** themselves can **interact** with **molecules** within key intracellular signaling pathways, such as the **PI3K, MAPK and mTOR** ones, which control cell proliferation, protein synthesis and membrane abundance of specific nutrient transporters. These changes can be seen in the placenta, liver, skeletal muscle, and heart of offspring when manipulating diet, placental restriction or hypoxia during pregnancy.
2. Effects of nutritional state may be mediated by changes in the concentrations of **nutritionally sensitive hormones**, like insulin, insulin-like growth factors (IGF), leptin and glucocorticoids[15]. These hormones may affect foetal growth and development directly and indirectly by altering the transport phenotype of the placental. Several of them signal through cell surface receptors as mentioned in the previous point, but others act genomically via cytoplasmic receptors and response elements on gene promoters[15].
3. Another way is by the availability of **micronutrients** involved in one **carbon metabolism**: folate, glycine and methionine, which may alter the epigenome and, hence, expression of key metabolic and growth regulatory genes[16].

### *4.1.2 Nutrient variations during pregnancy and their impact on the offspring.*

It's hard to find experimental studies on this subject in **horses**, because they have a **long-cycle life**, with 11 months pregnancies and years before new-borns become adults. These have big impact on experimental studies, because it implies a large amount of time and economical expense. However, there is **evidence** in **other species** that can be studied.

This subsection of the study intends to sum up evidence found in other species, evidence in horses, and relate them altogether.

Fowden et al., 2013 summed up evidence found in mice, rat, guinea pig, sheep, baboon, human, pig and cow in epigenetic impact on offspring postnatal outcome of different nutrition treatments during pregnancy (table 1).

*Table 1 – Known phenotypic outcomes of experimental manipulation of maternal nutrition state during pregnancy on the postnatal phenotype of the offspring in different species. Fowden et al., 2013.*

| Treatment | Species | Δ Fetal wt | Age at Study | Postnatal Outcomes |
|---|---|---|---|---|
| Undernutrition | Mice | ↓ 23% | 6 months | Glucose intolerance, β cell dysfunction, Hypertension |
| | Rat | No Δ to↓ 15% | 11 weeks | Vascular dysfunction |
| | | | 12-14 weeks | HPA & sympatho-adrenal dysfunction |
| | | | 8 months | Altered adreno-medullary function |
| | Guinea pig | ↓ 15% | 3 months | Hypertension, Cardiac hypertrophy, Glucose intolerance, HPA dysfunction |
| | Sheep | No Δ to ↓ 20% | 19 weeks | Insulin deficiency |
| | | | 1 year | Insulin resistance, Renal & adipose tissue dysfunction |
| | | | 6 years | Cardiac remodelling, Altered insulin signalling |
| | Baboon | ↓ 8% | 3 years | Impaired cognitive performance |
| | Human | ↓ 10% | >50 years | Obesity, Cardiovascular dysfunction |
| Dietary composition | | | | |
| Low protein | Mice | No Δ to ↓10% | 8 weeks | Hypertension, Anxiety behaviours |
| | | | 10 weeks | Increased insulin sensitivity |
| | | | 16 weeks | Increased adiposity & leptin |
| | | | 6 months | Cardiac hypertrophy |
| | Rats | No Δ to ↓ 10% | 3 weeks | Hypertension |
| | | | 8 weeks | Impaired spatial memory |
| | | | 12 weeks | Increased insulin sensitivity, Altered hepatic glucogenesis |
| | | | 16 weeks | Altered food intake & glucose metabolism |
| | | | 20 weeks | Hypertension, Renal dysfunction, Impaired vascular function |
| | | | 6 months | Insulin deficiency, |
| | | | 21 months | Insulin resistance, Decreased insulin signalling |
| | Pig | ↓ 15% | 11 weeks | Impaired immunity, HPA dysfunction |
| | Cow | ↓ 10% | 2 years | Altered fat deposition & adipokines |
| High fat | Mouse | No Δ | 14 weeks | Glucose intolerance |
| | Rat | No Δ | 3 weeks | Increased obesity, Adrenal dysfunction |
| High fat-high sugar | Mouse | No Δ to ↓ 10% | 8 weeks | Cardiac hypertrophy |
| | | | 6 months | Hypertension, Glucose intolerance |
| Overnutrition | Rat | No Δ | 11 weeks | Altered hepatic glucose & lipid metabolism |
| | Sheep | No Δ | 2 years | Altered adiposity, & glucose tolerance |
| | | | 6 years | Altered Food intake & insulin secretion |
| Hypoxia | Rat | No Δ to ↓ 10% | 7 weeks | Decreased cardiac adrenoreceptors |
| | | | 12 weeks | Vascular & cardiac dysfunction, Altered insulin signalling |

May Table 1 be used as an example on how several alterations in maternal nutrition during pregnancy can impact birthweight and program the phenotype of the postnatal offspring. The main outcomes of this manipulation are changes in cardiovascular and metabolic functions, which are further explained in the following section.

When putting the focus in all the evidence regardless of the species of study, there is a lot of information available. In this section, some of this information is synthetized by putting the focus specifically in the three parts, those exposed in Figure 1: exposome, epigenetic process and phenotypic outcome.

### 1. Nutritional restriction/undernutrition

Prenatal malnutrition has been related as a risk factor for **type 2 diabetes**. This has been observed in rodents[17] and humans[18]. It has also been related to **obesity** in the adult offspring. Some examples and studies on nutritional restriction are as follows:

Malnutrition during pregnancy

Data on the **Dutch Famine** during the winter of 1944-1945 provided initial evidence on what severe malnutrition in utero may lead to, for example: **heart disease, type 2 diabetes, neurodegenerative diseases, obesity and cancer**.

The Dutch Famine refers to a short period of time during World War II where some areas of the Netherlands under German occupation had their ways of communication destroyed and so the supply of food stopped. However, birth and medical records of Dutch population at that time were impeccable and this provided some invaluable data, for example: malnutrition consequences[19,20].

Some of the findings were:

- Unborn males and females exposed at any stage in utero → Higher risk for type 2 diabetes and heart disease in adulthood.
- Female embryos exposed in early gestation → Increased prevalence of cardiovascular and cancer mortality.
- Males exposed to famine in early gestation → Higher risk for neurodegenerative diseases.
- Children whose mothers suffered from famine in utero → Heavier at birth.
- Children whose fathers suffered from famine in utero → Heavier in adult life.

As for the epigenetic changes involved, four CpG dinucleotides within the *IGF2* differentially methylated region (DMR) have been found to be less methylated in offspring who suffered from in utero famine[21].

Malnutrition in late pregnancy

This findings only partially overlap with those reported in **ewes**, where nutritional restriction during pregnancy has been found to produce in adult offspring: adrenocortical hypertrophy, disturbed regulation of the renin angiotensin system within the adrenal gland, effects on insulin signaling in foetal skeletal muscle, lipogenesis, heart, adipose tissue and liver metabolism and adrenocortical function[22].

Low protein diet

Maternal low-protein diet has been related to offspring developing **type 2 diabetes** as adults. It has been demonstrated in human[18] and some rodent species[17,23].

For example: in a study with rodent dams, half of them were fed an ad libitum diet containing 5-9% protein and the other half were fed a standard control diet containing 18-20% protein throughout the whole pregnancy. As a result, the first group had offspring with a

decreased transcriptional activity of *Hnf4β*, which is a key transcription factor for β-cell differentiation, and, therefore, being one cause of glucose intolerance in pancreatic islets. Expression of some key metabolic genes were affected too, including: *Igf2, GR, Nr3cl, Pparα* and *Cyp2c34*, as well as genes involved in amino acid response pathway in the liver of offspring rats. Furthermore, this study also concluded that protein-restricted diets increase expression of glucoregulatory genes like phosphoenolpyruvate carboxykinase (PEPCK) and reduce expression of nutrient transporters including glucose transporter (GLUT4), thereby predisposing to insulin resistance in adult life. As for the epigenetic labels involved in these outcomes, histone code modifications were found, such as: elevated activity of histone deacetylases HDAC1 and HDAC4, increased binding of DNMT3a and DNMT3b, deacetylation of histone 3 lysine 14, increased recruitment of heterochromatin protein 1α, and demethylation of H3K9 in adult life. As a result, the offspring had impaired glucose tolerance and decreased insulin sensitivity at weaning[17].

**2. Overnutrition**

Just like nutritional restriction, overnutrition can also cause epigenetic changes leading the adult offspring to **develop obesity, insulin resistance** and **alteration of body composition**.

Weight gain

In human, weight gain during pregnancy, independently from maternal obesity, has proven to cause obesity in children and adults[24]. In a systematic review[24], association between gestational weight gain and offspring's body weight were studied. Children and adolescents from ages 2 to almost 19 were included along with their mothers. Pregnancy data was provided, the stage of pregnancy was also included.

This review concludes that weight gain during pregnancy is a risk factor for **obesity** in children and adults, especially when this happens during early- and mid-pregnancy rather than late-pregnancy [24].

Another, and more recent, study[25] has been able to associate several offspring's epigenetic changes to maternal weight gain during pregnancy, leading to offspring's obesity as well. Three of these **changes** passed their validation analysis: **upregulation** of ***SNX5*** **methylation**, **downregulation** of ***FRAT1*** **methylation**, and ***KCNK3*** **underexpression**[25].

Overnutrition or obesity

Some studies have demonstrated that maternal overnutrition or **obesity** during pregnancy may **lead to** offspring's **obesity**. This has been observed in humans[26] and other species like mice[27], rats, sheep[28] and non-human primates[29].

For example, one of the studies performed in non-human primates hypothesized that maternal obesity would induce epigenetic changes in foetal baboon (*Papio hamadryas*) liver resulting in dysregulation of key metabolic pathways that impact lipid metabolism, therefore promoting obesity in offspring. In this study, they kept baboons in groups up to 15 in outdoor facilities where full socialization and free movement were allowed, females were non-pregnant nulliparous baboons of similar weight, body dimensions and age; they were assigned *ad libitum* regular/normal diet (n=6) or an *ad libitum* combination of normal diet and high-fat/high-fructose diet with free access to a sugar-containing drink (n=5) at least 9 months before pregnancy. The dietary regimens were maintained throughout pregnancy. Prior to pregnancy, weight gain of the second group was significantly higher ($P<0.001$); during pregnancy, analysis revealed five pathways in foetal livers (TCA cycle, proteasome, oxidative phosphorylation, glycolysis and Wnt/β-catenin signaling pathways) that were dysregulated when compared to the normal diet group; the study concluded that these characterized, interconnected pathways, which have shown to be important for liver metabolic function explain and evidence how maternal obesity may lead to offspring's obesity through foetal liver's dysregulation.

About epigenetic modifications in the offspring related to maternal obesity, both **DNA methylation** and **miRNAs** have been related to the expression of ***BDNF* gene** (which codes for a growth factor protein) and ***HIF3A* gene** (the dysregulation of which has been associated with obesity).[30,31] However, further study on more concrete epigenetic changes on the offspring associated with mother's body mass index needs to be done.

### 3. Feeding concentrates

Feeding the late-pregnant mare with concentrate feed is a frequent practice in the equine industry supplementation. However, there is some evidence that this might not be a good option for the foal once they are born and grown.

When comparing two groups of dams, one in a pasture-only diet through the whole pregnancy, and the other with supplementation of additional grain mix through the last 5 months of pregnancy (both of them with selenium supplement), the results were as follows:

- Pasture-only dams lost body condition and rump fat, showing greater mobilization of body fat stores in the absence of concentrate feed in the ration.
- Grain-fed dams were overall heavier (+10%) and fatter without achieving obesity. This group had elevated leptinemia (+116%) throughout the last 3 weeks of pregnancy; their

colostrum was significantly poorer in IgG and sugar; neonates from this group of mares, exhibited reduced leptinemia throughout the first 18 hours after birth, this implies less satiety of the foals, therefore having increased appetite and thus probably being more prone to metabolic disorders later in life as a result.

Importantly, dams fed from **concentrates** during pregnancy were more likely to produce foals with **osteochondrosis** lesions, with a 22% of probability difference among the two groups (4% vs. 36%). This finding is supported by other studies where foals whose dams were fed **cracked barley** in addition to hay and silage from the 7$^{th}$ month of pregnancy, had an increased **osteochondrosis** relative risk.[32,33]

**4. Vitamin B12 and folate deprivation**

In **ewes**, deprivation of vitamin B12 and folate during pregnancy, was found to produce overweight adult offspring with metabolic syndrome: high blood pressure, altered immune response and altered DNA methylation in the liver[22].

There isn't clear evidence on epigenetic tags explaining said exposome to its consequences, but it's enough evidence to keep food's vitamin availability in mind when designing a diet.

## 4.2 Stress and behavioural programming

### *4.2.1 General mechanisms*

Some welfare-related exposomes have been demonstrated to produce epigenetic changes that affect the **HPA axis** (hypothalamus, pituitary gland and adrenal gland) and, therefore, impact **endogenous glucocorticoid production** and **sensitivity**.

These exposomes include: licking and grooming from the mother to the newborn, maternal psychological stress during pregnancy

### *4.2.2 Behavioural programming of offspring*

Licking and grooming

Maternal licking and grooming (LG) of pups at birth have proved to produce epigenetic changes in the offspring resulting in less fear and stress responsive adults.

For example, in a study performed in rats[20] where pups at birth were cross-fostered to mother exhibiting different levels of licking and grooming, it was observed that high level of LG **reduced methylation** of the **gene** encoding the **glucocorticoid receptor** in the **hippocampus** and, therefore, pups of high LG mothers had higher numbers of glucocorticoid receptors in this area,

producing **enhanced glucocorticoid feedback sensitivity**. This resulted in a decreased release of adrenocorticotropic hormone, which leads to a **damped stress response**. In other words: pups of high LG mothers, showed reduced fear and lower stress responses as adults[20].

Maternal psychological stress

Human studies have associated maternal **stress** and maternal **depression** with offspring's **epigenetic changes** within the **glucocorticoid receptor (*NR3C1*) gene**, which is associated with the **HPA** (hypothalamus, pituitary and adrenal glands) axis and, therefore, HPA stress reactivity. In other words, maternal stress and maternal depression might influence offspring's response to stress factors.

For example, a study carried by Oberlander et al., 2008 studied prenatal exposure to increased third trimester maternal depressed/anxious mood in humans. Eighty-two women and their infants were studied during and after pregnancy: 33 of them were depressed and treated with serotonin reuptake inhibitor antidepressant, 13 were depressed but not medicated, and 36 were neither depressed nor treated. All of them were assessed during the second and third trimesters and three months postpartum. All mothers took a prenatal vitamin containing prenatal folic acid and all infants where full term. To study functional implications of the newborn methylation status of *NR3C1* in newborns (methylation of a CpG-rich region of the gene), HPA function was assessed at three months using salivary cortisol obtained before and following a non-noxious stressor and at a late afternoon basal time.

As a conclusion, methylation status of the human *NR3C1* gene in newborns was found to be sensitive to prenatal maternal mood. HPA reactivity, reflected by increasing cortisol stress change scores, was predicted by greater ***NR3C1*** methylation at CpG3 in newborns (infants who responded with increased cortisol levels had significantly higher *NR3C1* CpG3 methylation status compared with infants whose cortisol levels declined in response to three months stress challenge). On top of that, depressed maternal mood in the third trimester was associated with **increased** neonatal **methylation of CpG3**[34].

A systematic review performed by Nowak et al., 2020 reviewed the epigenetic changes produced by pregnant women's stress, anxiety or depression on offspring. They aimed to review studies that looked for DNA methylation near areas of interest: primarily genes linked to hypothalamic-pituitary-adrenal axis function **(*NR3C1*, *FKBP51***), growth/immune function (***IGF2***), and socioemotional regulation (***SLC6A4*, *OXTR***). As this article says, the majority of reports note associations among stress and offspring DNA methylation of said genes, and then it concludes that stress, anxiety and depression might work as predictors for epigenetic changes and,

therefore, **alteration** in expression of genes related to **endogenous cortisol secretion** and **sensitivity** to it, **insulin sensitivity, growth and immune function** and **socioemotional regulation**[35].

## 4.3 Uterine space restriction

### *4.3.1 Experimental evidence on intrauterine space programming*

Equine placentation occurs over the entire internal surface of the uterus. Therefore, **dam's size** determines the **nutritional supply** to the foetus. Increased or **restricted uterine capacity** represents both a **mechanical** and **nutritional constraint** for the developing foetus[32]. In consequence, inducing foetal growth, restriction or foetal growth excess through embryo transfer in a mare of a smaller or larger breed, respectively, than the embryo, results in foetal growth alterations that persist until adulthood.

The foetal growth excess group had altered blood pressure and adrenocortical response to ACTH as well as pancreatic β-cell sensitivity to glucose in the new-born foals.

In foetal growth restriction group, the sympathoadrenal responses to acute hypotension was augmented as neonates. Moreover, **restricted foals** appeared to be more prone to **osteochondrosis** than controls at six months of age[14,22].

Although embryo transfer is not currently a matter of interest in Thoroughbreds (as it's not an allowed breeding technique at the moment for this particular breed), uterine space restriction should be taken into account when considering the size of the mare and the size difference between the mare and the stallion.

Epigenetic changes which lead to offspring's increased osteochondrosis risk are not yet described, however, the relation between intrauterine environment and skeletal pathologies has been described for many years now. For example, Cooper et al., 1997 concluded so in human species.[36]

## 4.4 Exercise programming during pregnancy

### *4.4.1 Experimental evidence*

Interestingly, **epigenetic changes** produced by **adverse intrauterine environment** have shown to be **reversible** and **exercise** might have an important role on that. Moreover, exercise has shown to be **important for cognitive and muscle development** of the offspring.

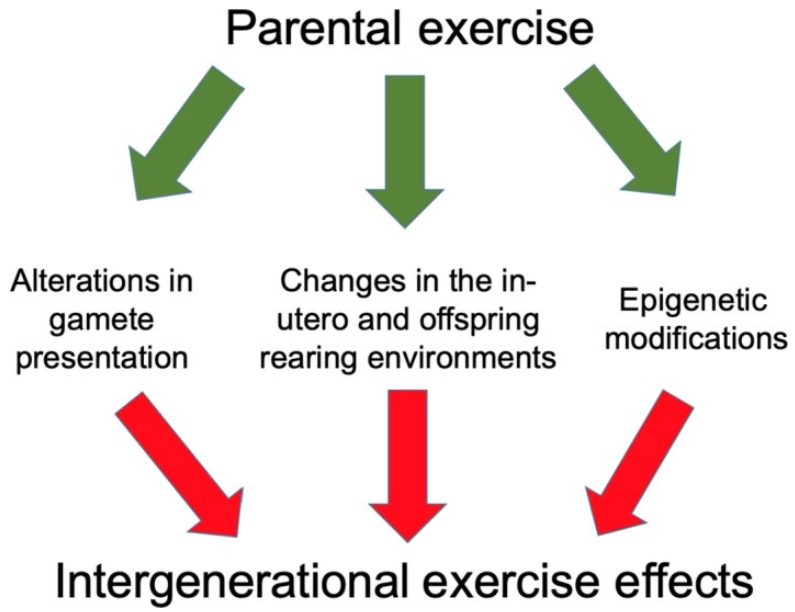



*Figure 6 – Mechanisms by which parental exercise impact offspring. The three main paths are through alterations in gamete presentation, changes in the in-utero and offspring rearing environment and epigenetic modifications Image from Axsom et al., 2019*

Exercise in females

There is evidence that exercise in pregnant females might **protect** offspring **from high-fat diet** induced consequences and it might also **promote muscle development**, which are both important when thinking of running Thoroughbreds.

For example, both high-fat diet and mother's obesity during pregnancy appear to induce **hypermethylation of *PGC-1α* promoter region**, which negatively correlates with **mRNA expression in skeletal muscle** of individuals with type 2 diabetes. Laker et al., 2014 were able to prove that maternal exercise protects offspring from maternal high-fat diet induced metabolic dysfunction at 0 months of age in mice[37].

Briefly, a group of female mice were tested by giving them high-fat diet and separating two groups: exercise and no exercise. Four individuals of 8 weeks of age were subjected to high-fat diet and sedentary activity, another group of the same characteristics was subjected to normal diet with sedentary activity and the last group had high-fat diet with exercise training (voluntary running), which started 6 weeks before and throughout pregnancy. The authors found out that maternal exercise might suppress the maternal high-fat died induced hypermethylation of *PGC-1α* rather than initiate an independent process of epigenetic modification, such as demethylation

and, therefore, maternal exercise might protect offspring from maternal high-fat diet induced metabolic dysfunction at 9 months of age in mice.[37]

Another example is a study in mice that shows evidence of mother exercise inducing muscle development of the foetus and protecting them from diet-induced obesity and metabolic disorders[38].

Skeletal muscle development undergoes a crucial phase in-utero, for this study, the authors hypothesized that maternal obesity impairs while maternal exercise enhances non-shivering thermogenesis of skeletal muscle in offspring via stimulation of apelin/APJ signaling during foetal muscle development. This study had female mice fed with a control diet or high fat diet to induce obesity. Obese mice were separated into obesity only group (group A) and obesity plus daily exercise group (group B). Foetal muscle was collected at embryonic day 18.5 and offspring mice at 3-month-old.[38]

While **maternal obesity** was found to **impair *SLN/UCP3* expression** in foetal muscle, **maternal exercise enhanced** mitochondrial activity and ***SLN/UCP3* expression**, which enhances **muscle-based non-shivering thermogenesis** and **prevents diet-induced obesity** in offspring. Moreover, the authors found out that apelin administration during pregnancy mirrors the benefit of maternal exercise via the activation of the APJ-AMP-activated protein kinase axis.

In other words, exercise during pregnancy induces muscle-based thermogenic gene expression in both mothers and foetuses and it improves metabolic-related molecular expression in the offspring challenged with high-fat diet. While obesity suppresses oxidative metabolism in skeletal muscle, activation of sarcolipin (encoded by the *SLN* gene) signaling pathways promotes mitochondrial biogenesis and oxidative phosphorylation, preventing obesity-related metabolic dysfunction.

To **sum up**, exercise in females during pregnancy has shown to revert high-fat diet induced consequences and to promote muscle development in the offspring.

### 4.5 Summary of the most remarkable findings

Here below is a summary (Table 2) to synthesize the strongest evidence found which can be extrapolated to horses in order to create a handling protocol.

*Table 2. Summary of the effect that the main exposomes in pregnant females has on the offspring, their epigenetic tags and which species will the information be extrapolated from.*

| ***Exposome*** | **Main consequence** | **Epigenetic changes** | **Evidence in** |
|---|---|---|---|
| ***Malnutrition*** *during pregnancy* | Offspring's higher risk for **type 2 diabetes** and **heart disease** in adulthood | Less methylation of four CpG dinucleotides within the ***IGF2*** DMR | Human |
| | If the embryo is **female** → Increased prevalence of **cardiovascular** and **cancer mortality** | | |
| | If the embryo is **male** → Higher risk for **neurodegenerative diseases** | | |
| | Offspring whose **mothers** suffered from famine **in utero** → **Heavier at birth** | | |
| | Offspring whose **father** suffered from famine **in utero** → **Heavier** in **adult** life | | |
| ***Low protein*** *diet* | Increased risk of **type 2 diabetes** as adults | Increases activity of histone deacetylases ***HDAC1*** **and** ***HDAC4***, increases binding of ***DNMT3a*** and ***DNMT3b***, deacetylation of **histone 3 lysine 14**, increased recruitment of **heterochromatin protein 1α**, and demethylation of **H3K9** in adult life. | Rodents |
| ***Weight gain*** *during pregnancy* | **Obesity** in children and adults | Upregulation of ***SNX5*** methylation, downregulation of ***FRAT1*** methylation, and ***KCNK3*** underexpression | Human |
| ***Overnutrition*** *or* ***obesity*** *during pregnancy* | Offspring's **obesity** | Changes in **DNA methylation** and **miRNAs** regulate expression of ***BDNF*** gene and ***HIF3A*** gene.signaling | Human, mice, rats, sheep, non-human primates. |
| ***Feeding*** *the pregnant mare* ***concentrates*** | Increased risk of **osteochondrosis** lesions | **Not yet described**. But the association has been found significant. | Mares |

| | | | |
|---|---|---|---|
| *Maternal **licking and grooming*** | Reduced **fear** and lower **stress responses** as adults | Reduced methylation of the gene encoding glucocorticoid receptor in the hippocampus | Rats |
| *Maternal psychological **stress** and **depression** during pregnancy* | Negative influence on offspring's **response** to **stress factors** | Methylation of a CpG-rich region of ***NR3C1*** gene | Human |
| | Affecting **growth/immune** function | DNA methylation of ***IGF2, FKBP51*** | |
| | Affecting **socioemotional** regulation | DNA methylation of ***SLC6A4, OXTR*** | |
| ***Uterine space** restriction* | Offspring more prone to **osteochondrosis** at six months of age | Not yet described but significantly associated. | |
| ***Exercise** during pregnancy* | **Avoids** maternal **high-fat diet consequences** in offspring | Suppresses offspring hypermethylation of ***PGC-1α*** | Mice |
| | Improves **muscle thermogenesis** | Promotes ***SLN/UCP3*** expression | Mice |
| | **Protects** from **obesity** in adulthood | | |

# 5. Discussion

Horses are being selected for their genetics. When someone wants to buy a future high performance racing horse, they ensure they have a good pedigree and they have been well taken care of, but it might be good to rethink what does that include and how does it impact the animal's future performance.

Studying epigenetics has shown that having a good pedigree isn't assurance of ultimately expressing a desired phenotype, environment can have an obvious effect on an individual's health, but it also has an effect on how the genome is expressed. Indeed, that is something to take into account when raising horses, especially if they are meant to be sold for millions of dollars.

With that on the clear, this project aims to provide a protocol, supported by scientific evidence, for handling perigestational stage of mares in order to enhance expression of desired traits in the offspring. With the results obtained, the following protocol is proposed.

## 5.1 Proposed protocol

- When choosing the mare and the stallion to breed, **size** has to be taken into account. It is advised that both individuals' sizes are **similar**, avoiding big differences. This lowers the risk of **osteochondrosis** lesions in the offspring.
- Before breeding a mare, her Body Condition Score (**BCS**) should be **5** (4-6) on a 1–9-point scale, which according to Iowa State University it shows as: "Back is level. Ribs cannot be visually distinguished, but can be easily felt. Fat around tail head begins to feel spongy. Withers appear rounded over spinous processes. Shoulders and neck blend smoothly into body". If a mare is in a lower BCS and comes out pregnant, diet should be adapted to **maintain her body condition** throughout pregnancy, being **mindful of malnourishment** and preventing it. – This action will lower the offspring predisposition to **type 2 diabetes, heart conditions, cardiovascular and cancer mortality, neurodegenerative diseases and metabolic syndrome** as an adult. These are consequences of malnutrition during pregnancy and weight gain during pregnancy.
- If a mare is either in a risk-low-BCS during pregnancy and **needs to gain weight** or in a **high BCS**, **exercise** is strongly advised. This is not aiming to regulate body fat, but to **prevent** the mare and the offspring from epigenetic changes a type of diet for that purpose can cause. Avoided consequences are **obesity** and **metabolic disorders** which

derive from high-fat diet, gaining weight during pregnancy and other exposomes proven to lead to obesity.

- If a mare has been exercising before pregnancy, even if she is in an ideal BCS, she should **keep on exercising** for three main reasons: for exercise's benefits themselves, to **avoid maternal stress** raising from a sudden drop on energy release, which has been associated to have a negative influence **on stress factors' response** in offspring**, impaired growth and immune function** and a **poor socioemotional regulation**. As for the third reason: maternal exercise is also advised to promote **muscle development** of the offspring.
- If a mare is a **high-performance athlete, exercising can continue** with lowered intensity at gestation, that is avoiding exercise that requires maximum $VO_2$, which many scientific reports have described as being safe for both maternal and foetal health[39,40]. Stress should be avoided.
- As for the **diet**: **highly available energy food** should be **avoided**, specially **concentrates**. Free pasture should be prioritized and, if not possible, *ad libitum* hay should be provided. Hay shouldn't come from monocultures, thereby risking deficiencies. It should come from a mix of legumes and grasses, which will **prevent** from an excess of energy intake and also from a **deficiency** in **protein intake**. Loss of essential fatty acids and vitamins through hay-making process should be compensated with supplementation. However, on this matter, it is advisable to ask for professional nutritional advise. Avoiding concentrates helps preventing **osteochondrosis** lesions.
- **The mare's living environment** should **change as little as possible** during pregnancy, that is: if a mare is usually kept in a group, she shouldn't be isolated during pregnancy, as that might cause stress and could set her up for a depressive mood, which should be avoided. This prevents the offspring from maternal stress and depression's consequences.
- If, on the contrary, a mare is usually kept stalled and she shows anxious behaviour when letting her loose, she should be kept stalled in the environment she is familiar to.
- Once the **foal is born**, they should be **kept with the mare**. If the mare is **rejecting** the foal and there is no way to revert it, it is advised to look for a **mare that will mother** the **foal** to groom it. This has been proven to **reduce fear and to lower stress responses** (Table 2).

It is important to remark that a racing horse, breeder and buyer all benefit from the horse having a good heart and metabolic condition, to be spared from articular conditions, to be well

behaved and easy-going (that is especially beneficial to the handler and to prevent possible injuries).

All the things listed above can still happen even when epigenetic tags leading to them are prevented, but there is **strong evidence** that **non-communicable diseases** are **highly associated** to ***in utero*** **environment** through **epigenetics** and, therefore, there are enough reasons to do everything to improve handling during this period of time.[41–43]

## 5.2 Scientific evidence associated to male handling

Additionally to this, there is also evidence on **stallion's handling**. This project has focused on pregnant mares' handling, but here below is an example on why stallions' handling protocol should be taken into account, and should be asked for, when choosing the male that will be covering the mare. This scientific report is related to **exercise** before mating.

A carried by Mega et al., 2018, was able to give strong data suggesting that father's exercise can have an impact on **offspring's cognitive development**. In this study, **hippocampal DNA methylation** was found to be **lower** in offspring whose **fathers exercised before mating**.

In this study, sixty days old male and female rats (n=20-25 each one) were initially used, they were housed in standard plexiglass housing boxes, keeping 3-4 per cage, under controlled environmental conditions and food and water *ad libitum*. Males were divided among exercise and no-exercise. In exercise groups, animals were placed individually on the treadmill with the following exercise protocol: running for 20min/day, 5 consecutive days/week during 8 weeks. After this period of time, male and female were housed together overnight to mate. Each experimental group of study was composed of 108-115 male pups from 21-25 litters.

At day 54 after birth, rats were euthanised to obtain hippocampal samples. As a result, parental-exercise's offspring group presented a significative lower percentage of hippocampus DNA methylation when compared to sedentary group ($p=0.019$ in an Unpaired Student's t test). However, this same study theorized that DNA hypermethylation is usually associated with silencing of genes with dramatic importance for cell function under homeostatic and disease conditions, but, when testing for increased hippocampal BDNF expression (a protein that may play a crucial role in regulating survival, growth and maintenance of neurons among others), it didn't match the DNA methylation results. More research needs to be done in this area in order

to see what phenotypical consequences can have on offspring exercising parental line before mating.

Anyhow, there is valuable information to be extracted out of this study: one important point is that there is a significative difference among no exercise and exercise of males in hippocampal DNA methylation of offspring, which is of crucial importance and which may probably lead to **better cognitive development** when exercise is performed before mating.[44]

This type of evidence on how male's exercise affects the offspring provides sufficient information to be mindful when choosing a stallion to sire a mare. In other words, it's not only the pedigree of the stallion and their relatives and own achievements that matter, but also how he has been handled, as the genetics that he will be passed along will be conditioned on their expression by said environment exposure.

## 6. Conclusion

There is enough scientific evidence to say that genetic expression can be regulated by environmental factors even those to which an individual is exposed in their *in-utero* phase. In fact, *in utero* environment has been highly associated with developing noncommunicable diseases. Therefore, by checking parental records or, for example, siblings' conditions, research on a horse's probable phenotype as an adult is only partially done.

This project aimed to provide breeders and buyers with a useful and scientifically supported handling protocol, so breeders know how to enhance desired traits and prevent from developing non-desires ones and buyers know what else to ask for when siring their mares or buying a foal.

Metabolic disorders, cardiac and vascular conditions, obesity (which usually leads to metabolic conditions), osteochondrosis, neurodegenerative diseases, increased fear and stress response, poor growth and immune function are some of the potential outcomes of typical handling of pregnant mares. That's explained by many reasons, for example: it's not so rare to see a mare with a BCS below 4, stressing her by changing where she lives during pregnancy, stop exercising her, feeding her concentrates because she is pregnant, etc. Pretty much all the exposomes mentioned in this project are environmental factors to which lots of breeders expose their mares with the best of their intentions. With this bibliographical research, conclusive answers have been given to common doubts on how to handle a pregnant mare when it comes to nutrition, stress and exercise. Additionally, information on what the difference among sizes of the stallion and the mare should be, as it is something that isn't always taken into account.

## 7. Bibliography


1. Genetics of Thoroughbred Racehorse Performance | Annual Review of Animal Biosciences. Accessed May 20, 2023. https://www.annualreviews.org/doi/10.1146/annurev-animal-020420-035235?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub++0pubmed

2. Rivero JLL, Hill EW. Skeletal muscle adaptations and muscle genomics of performance horses. *Vet J Lond Engl 1997*. 2016;209:5-13. doi:10.1016/j.tvjl.2015.11.019

3. Rooney MF, Hill EW, Kelly VP, Porter RK. The "speed gene" effect of myostatin arises in Thoroughbred horses due to a promoter proximal SINE insertion. *PLOS ONE*. 2018;13(10):e0205664. doi:10.1371/journal.pone.0205664

4. Cavalli G, Heard E. Advances in epigenetics link genetics to the environment and disease. *Nature*. 2019;571(7766):489-499. doi:10.1038/s41586-019-1411-0

5. Fessele KL, Wright F. Primer in Genetics and Genomics, Article 6: Basics of Epigenetic Control. *Biol Res Nurs*. 2018;20(1):103-110. doi:10.1177/1099800417742967

6. Handy DE, Castro R, Loscalzo J. Epigenetic Modifications: Basic Mechanisms and Role in Cardiovascular Disease. *Circulation*. 2011;123(19):2145-2156. doi:10.1161/CIRCULATIONAHA.110.956839

7. da Cruz RS, Chen E, Smith M, Bates J, de Assis S. Diet and Transgenerational Epigenetic Inheritance of Breast Cancer: The Role of the Paternal Germline. *Front Nutr*. 2020;7. Accessed May 20, 2023. https://www.frontiersin.org/articles/10.3389/fnut.2020.00093

8. Xue M, Zhuo Y, Shan B. MicroRNAs, Long Noncoding RNAs, and Their Functions in Human Disease. *Methods Mol Biol Clifton NJ*. 2017;1617:1-25. doi:10.1007/978-1-4939-7046-9_1

9. Meruvu S, Schutz LF, Choudhury M. Chapter 16 - Nutritional influence on miRNA epigenetic regulation: Effect of maternal diet and miRNAs on the fetal metabolic programming. In: Vinciguerra M, Sanchez PC, eds. *Molecular Nutrition: Mother and Infant*. Academic Press; 2021:401-420. doi:10.1016/B978-0-12-813862-5.00016-5

10. Gao N, Li Y, Li J, et al. Long Non-Coding RNAs: The Regulatory Mechanisms, Research Strategies, and Future Directions in Cancers. *Front Oncol*. 2020;10. Accessed July 12, 2023. https://www.frontiersin.org/articles/10.3389/fonc.2020.598817

11. Chavatte-Palmer P, Velazquez MA, Jammes H, Duranthon V. Review: Epigenetics, developmental programming and nutrition in herbivores. *Anim Int J Anim Biosci*. 2018;12(s2):s363-s371. doi:10.1017/S1751731118001337

12. Jin B, Li Y, Robertson KD. DNA Methylation. *Genes Cancer*. 2011;2(6):607-617. doi:10.1177/1947601910393957

13. Dindot SV, Cohen ND. Epigenetic Regulation of Gene Expression: Emerging Applications for Horses. *J Equine Vet Sci*. 2013;33(5):288-294. doi:10.1016/j.jevs.2013.03.006

14. Fowden AL, Jellyman JK, Valenzuela OA, Forhead AJ. Nutritional Programming of Intrauterine Development: A Concept Applicable to the Horse? *J Equine Vet Sci*. 2013;33(5):295-304. doi:10.1016/j.jevs.2013.03.005

15. Fowden AL, Forhead AJ. Hormones as epigenetic signals in developmental programming. *Exp Physiol*. 2009;94(6):607-625. doi:10.1113/expphysiol.2008.046359

16. Lillycrop KA, Burdge GC. Epigenetic mechanisms linking early nutrition to long term health. *Best Pract Res Clin Endocrinol Metab*. 2012;26(5):667-676. doi:10.1016/j.beem.2012.03.009

17. Vaiserman A, Lushchak O. Prenatal Malnutrition-Induced Epigenetic Dysregulation as a Risk Factor for Type 2 Diabetes. *Int J Genomics*. 2019;2019:3821409. doi:10.1155/2019/3821409

18. Vipin VA, Blesson CS, Yallampalli C. Maternal low protein diet and fetal programming of lean type 2 diabetes. *World J Diabetes*. 2022;13(3):185-202. doi:10.4239/wjd.v13.i3.185

19. De Rooij SR, Bleker LS, Painter RC, Ravelli AC, Roseboom TJ. Lessons learned from 25 Years of Research into Long term Consequences of Prenatal Exposure to the Dutch famine 1944–45: The Dutch famine Birth Cohort. *Int J Environ Health Res*. 2022;32(7):1432-1446. doi:10.1080/09603123.2021.1888894

20. Satterfield MC, Coverdale JA, Wu G. Review of Fetal Programming: Implications to Horse Health.

21. Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci*. 2008;105(44):17046-17049. doi:10.1073/pnas.0806560105

22. Chavatte-Palmer P, Robles M, Tarrade A, Duranthon V. Gametes, Embryos, and Their Epigenome: Considerations for Equine Embryo Technologies. *J Equine Vet Sci*. 2016;41:13-21. doi:10.1016/j.jevs.2016.03.019

23. Zhu Z, Cao F, Li X. Epigenetic Programming and Fetal Metabolic Programming. *Front Endocrinol*. 2019;10. Accessed May 30, 2023. https://www.frontiersin.org/articles/10.3389/fendo.2019.00764

24. Lau EY, Liu J, Archer E, McDonald SM, Liu J. Maternal Weight Gain in Pregnancy and Risk of Obesity among Offspring: A Systematic Review. *J Obes*. 2014;2014:e524939. doi:10.1155/2014/524939

25. Gómez-Vilarrubla A, Mas-Parés B, Carreras-Badosa G, et al. Placental epigenetic marks related to gestational weight gain reveal potential genes associated with offspring obesity parameters. *Obesity*. 2023;31(7):1903-1912. doi:10.1002/oby.23780

26. Drake AJ, Reynolds RM. Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. *REPRODUCTION*. 2010;140(3):387-398. doi:10.1530/REP-10-0077

27. Zambrano E, Nathanielsz PW. Mechanisms by which maternal obesity programs offspring for obesity: evidence from animal studies. *Nutr Rev*. 2013;71(suppl_1):S42-S54. doi:10.1111/nure.12068

28. Long NM, Rule DC, Tuersunjiang N, Nathanielsz PW, Ford SP. Maternal Obesity in Sheep Increases Fatty Acid Synthesis, Upregulates Nutrient Transporters, and Increases Adiposity in Adult Male Offspring after a Feeding Challenge. *PLoS ONE*. 2015;10(4):e0122152. doi:10.1371/journal.pone.0122152

29. Puppala S, Li C, Glenn JP, et al. Primate fetal hepatic responses to maternal obesity: epigenetic signalling pathways and lipid accumulation. *J Physiol*. 2018;596(23):5823-5837. doi:10.1113/JP275422

30. Pfeiffer S, Krüger J, Maierhofer A, et al. Hypoxia-inducible factor 3A gene expression and methylation in adipose tissue is related to adipose tissue dysfunction. *Sci Rep*. 2016;6(1):27969. doi:10.1038/srep27969

31. Epigenetic signatures associated with maternal body mass index or gestational weight gain: a systematic review | Journal of Developmental Origins of Health and Disease | Cambridge Core. Accessed July 11, 2023. https://www.cambridge.org/core/journals/journal-of-developmental-origins-of-health-and-disease/article/epigenetic-signatures-associated-with-maternal-body-mass-index-or-gestational-weight-gain-a-systematic-review/B0825701A9CB9D050E38FD14DAC0A8ED

32. Peugnet P, Mendoza L, Wimel L, et al. Longitudinal Study of Growth and Osteoarticular Status in Foals Born to Between-Breed Embryo Transfers. *J Equine Vet Sci*. 2016;37:24-38. doi:10.1016/j.jevs.2015.11.008

33. Peugnet P, Robles M, Mendoza L, et al. Effects of moderate amounts of barley in late pregnancy on growth, glucose metabolism and osteoarticular status of pre-weaning horses. *PloS One*. 2015;10(4):e0122596. doi:10.1371/journal.pone.0122596

34. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics*. 2008;3(2):97-106. doi:10.4161/epi.3.2.6034

35. Nowak AL, Anderson CM, Mackos AR, Neiman E, Gillespie SL. Stress during pregnancy and epigenetic modifications to offspring DNA: A systematic review of associations and implications for preterm birth. *J Perinat Neonatal Nurs*. 2020;34(2):134-145. doi:10.1097/JPN.0000000000000471

36. Cooper C, Fall C, Egger P, Hobbs R, Eastell R, Barker D. Growth in infancy and bone mass in later life. *Ann Rheum Dis*. 1997;56(1):17-21. doi:10.1136/ard.56.1.17

37. Laker RC, Lillard TS, Okutsu M, et al. Exercise Prevents Maternal High-Fat Diet–Induced Hypermethylation of the Pgc-1α Gene and Age-Dependent Metabolic Dysfunction in the Offspring. *Diabetes*. 2014;63(5):1605-1611. doi:10.2337/db13-1614

38. Son JS, Chae SA, Zhao L, et al. Maternal exercise intergenerationally drives muscle-based thermogenesis via activation of apelin-AMPK signaling. *eBioMedicine*. 2022;76:103842. doi:10.1016/j.ebiom.2022.103842

39. Wowdzia JB, Hazell TJ, Berg ERV, Labrecque L, Brassard P, Davenport MH. Maternal and Fetal Cardiovascular Responses to Acute High-Intensity Interval and Moderate-Intensity Continuous Training Exercise During Pregnancy: A Randomized Crossover Trial. *Sports Med*. Published online May 22, 2023. doi:10.1007/s40279-023-01858-5

40. Anderson J, Pudwell J, McAuslan C, Barr L, Kehoe J, Davies GA. Acute fetal response to high-intensity interval training in the second and third trimesters of pregnancy. *Appl Physiol Nutr Metab*. 2021;46(12):1552-1558. doi:10.1139/apnm-2020-1086

41. Peral-Sanchez I, Hojeij B, Ojeda DA, Steegers-Theunissen RPM, Willaime-Morawek S. Epigenetics in the Uterine Environment: How Maternal Diet and ART May Influence the Epigenome in the Offspring with Long-Term Health Consequences. *Genes*. 2021;13(1):31. doi:10.3390/genes13010031

42. Bronson SC, Seshiah V, Bronson SC, Seshiah V. Transgenerational Transmission of Non-communicable Diseases: How to Break the Vicious Cycle? *Cureus*. 2021;13(10). doi:10.7759/cureus.18754

43. Hobbs A, Ramsay M. Epigenetics and the burden of noncommunicable disease: a paucity of research in Africa. *Epigenomics*. 2015;7(4):627-639. doi:10.2217/epi.15.17

44. Mega F, de Meireles ALF, Piazza FV, et al. Paternal physical exercise demethylates the hippocampal DNA of male pups without modifying the cognitive and physical development. *Behav Brain Res*. 2018;348:1-8. doi:10.1016/j.bbr.2018.03.040