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# Equine Science

*Edited by Catrin Rutland and Albert Rizvanov*

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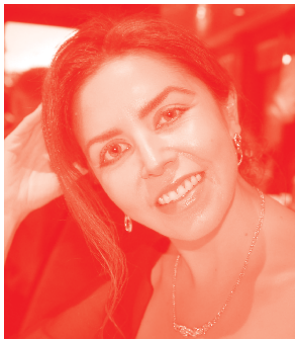

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IntechOpen Book Series

# Veterinary Medicine and Science

## Volume 5

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# Scope of the Series

Paralleling similar advances in the medical field, astounding advances occurred in the Veterinary Medicine and Science in recent decades, fostering a better support to animal health and more humane animal production, a better understanding of the physiology of endangered species, to improve the assisted reproductive technologies or the pathogenesis of certain diseases, where animals can be used as models for human diseases (like cancer, degenerative diseases or fertility), and even as a guarantee of public health. Bridging the Human, Animal and Environmental health, the holistic and integrative "One Health" concept intimately associates the developments within those fields, projecting its advancements into practice.
This book series aims to tackle a variety of fields in the animal-related medicine and sciences, providing thematic volumes, high quality and significance in the field, directed to researchers and postgraduates. It aims to give us a glimpse into the new accomplishments in the Veterinary Medicine and Science field. By addressing hot topics in veterinary sciences, we aim to gather authoritative texts within each issue of this series, providing in-depth overviews and analysis for graduates, academics and practitioners and foreseeing a deeper understanding of the subject. Forthcoming texts, written and edited by experienced researchers from both industry and academia, will also discuss scientific challenges faced today in Veterinary Medicine and Science. In brief, we hope that books in this series will provide accessible references for those interested or working in this field and encourage learning in a range of different topics.

# Contents

# Preface

Understanding equine medicine, anatomy, physiology, nutrition, and behaviors is essential for horse and donkey welfare. This book contains both literature reviews and recent research into topics ranging from reproduction and locomotion through to ancient and modern treatments. The book investigates both healthy and pathological conditions at differing stages of life. The importance of each cell and tissue through to the whole organism is explored alongside the methodologies used to understand these vital structures and functions.

As editors, we are both practicing gene therapy, anatomy, and physiology researchers and teachers, therefore we have strived to ensure that every chapter is accessible to everyone. Whether you are a veterinary professional, student, researcher, animal owner, or simply have an interest in horses and donkeys, we hope you will find a number of interesting chapters in this book.

The 'Medieval Medicine to Gene Therapy' section starts with an interesting chapter showing strategies used in Armenia, where East meets West in both traditions and medicines. This fascinating chapter bridges science and history, kings and physicians, to guide the reader through a topic rarely written about. The next two chapters show the brilliant advances being made in gene therapy used to treat lameness caused by tendinitis, desmitis, and osteoarthritis. These techniques rely on knowledge of not only how and why genes and proteins are expressed, but also on the best methods to introduce gene therapies and then the effects on healing, the cells, tissues, and the whole animal.

The next section concentrates on 'Reproduction, Locomotion, and Skin'. The first chapter reviews seasonality and the effect of photoperiod on mares, shows improvements in reproductive efficiency in mares, and also explores endometritis and twin pregnancies. The next chapter looks at proximal suspensory desmitis of the hindlimb and whether this can predispose horses to sacroiliac disease. By asking owners and bringing together published evidence and information, the authors cover both disorders. The next chapter investigates cartilage health, looking in detail at potential biomarkers for diagnosis and novel therapeutic targets, concentrating on equine joints and lameness causing issues. The final chapter delves into equine sarcoid, which is the most common skin neoplasia in the horse and also affects other equids. This chapter describes what equine sarcoid is, how and why it presents and develops, clinical and pathological diagnosis, and potential treatments.

The final section covers the digestive system, diet, and finishes with a chapter on behavior. The first chapter covers the gastrointestinal system, looking at decades of experiences diagnosing and treating horses with postoperative ileus using different methods. It explores the difficulties, outcomes, and makes recommendations based not only on a large study but also on the published literature. Continuing the theme, a morphophysiological study of the gastrointestinal tract of the donkey is presented. Although the donkey is very similar to the horse, the chapter highlights the key attributes of the digestive system in the donkey, bringing together the vast literature on the subject into one chapter. Following information on the digestive system, it

seems natural to discuss some aspects of nutrition. The next chapter looks at studies on grass in horse diets and implementing a sustainable deworming program. The book concludes with a chapter that is arguably affected by many of the aspects of equine science already discussed, behavior. Behavioral Neuroscientists look at both horsemanship and horse behavior in relation to injury, locomotion, training, in differing environments and under saddle, and highlight aspects of memory.

The chapter contributors are experts in their fields from across the world. They have conducted scientific studies, collated the published literature from across the years, and presented a number of graphics throughout in order to illustrate their work.

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Section 1

# Medieval Medicine to Gene Therapy

Chapter 1

# Medieval Equine Medicine from Armenia

*Jasmine Dum-Tragut*

## Abstract

The Armenian medieval and early modern equine medicine has rarely been noticed or researched by veterinarians, historians of science, philologists, or medieval researchers. As Armenia represents both a geographical border and cultural corridor between Muslim East and Christian West, a consideration of its hippiatric texts and their integration into the general history of veterinary medicine can only lead to a deeper understanding of equine medicine from the medieval to the early modern period. They could also contribute toward tracing the paths of knowledge diffusion and transmission across political, linguistic, and religious-cultural boundaries in the time of the Crusades. The role of Armenian manuscripts bridging the traditions of equine medicine from the Muslim East and the Christian West is examined by revealing the complicated history of Armenian horse treatises that traveled the long way from Baghdad via Sis to Tbilisi.

**Keywords:** medieval horse medicine, Cilician Kingdom of Armenia, knowledge transfer, cultural encounter between East and West, Crusades

## 1. Introduction

In recent years, much research has been dedicated to the cross-cultural and cross-religious aspects of the encounters of peoples from the Christian West and the predominantly Muslim East during the Crusades. The role of the small Oriental-Christian kingdoms and principalities both geographically and culturally located between Europe and Asia was, however, very rarely taken into account.

Equine medicine occupies a prominent role: hippiatric treatises were widely circulated, translated, and adapted. According to tradition, Greco-Roman and Byzantine works lost in Europe in the Middle Ages were preserved in Arabic libraries in translations from the original Greek to Arabic. These texts returned to Europe in the Renaissance, with a greater or lesser influence of Muslim/Eastern knowledge on European. Detailed analyses show that even if European writings provided the basis for Arabic hippiatric books, the Arabs also referred to the knowledge of Indian medicine and their own local practices. Yet none of these studies question how and where European and Eastern traditions met, and whether there were any cultural intermediaries.

Only gradually, researchers began to understand the historical significance of a corridor country and significant negotiator with the peoples of the Far East: the Armenian principality and later Kingdom of Cilicia between the eleventh and fourteenth centuries. Despite religious and linguistic differences, mutual influences were revealed, which help to identify socio-cultural parallels, particularly concerning royal courts.

In addition to the royal treasury, manuscripts, noble horses, and court physicians were among the most respected possessions in many royal and noble courts.

## 2. Medieval Armenian equine medicine

Medical works first appear in Armenian literature with the early translations from Greek into Armenian of the works of Galen (199 AD) by the Armenian philosopher David Anhałt (fifth century). During the age of Hunayn (prior to the ninth century), a translation followed from a Syriac version and the most comprehensive version of Galen's text was made from Arabic to Armenian in the period covered by the ninth to tenth centuries.

Genuinely Armenian medical literature only emerged in the medieval period, in the Armenian principality and the later Kingdom of Cilicia (1081–1375). Veterinary medicine developed later, initially under the influence of the secondary Armenian translation of the Byzantine compilation on agriculture, known as *Geoponica*. In this translation, we find the first chapters on horse medicine ever written in the Armenian language, in book 16, chapters 284–310.

From the eleventh century onward, Armenians experienced a striking acceleration of medical activities thanks to the patronage of the Cilician rulers and later royal dynasties. The number of surviving or known Armenian horse treatises is modest [1].

| MS | Title | Folios | Date | Place | Scribe | State of Art | Comment |
|---|---|---|---|---|---|---|---|
| | About the treatment of horses | ? | 1263? | Baghdad | Step'anos | Not preserved. Only mentioned in colophon of F278, BNF | Commissioned by King Het'um I |
| M10975 | Medical book for horse and mount | 185 | 1296–96 | Sis | T'oros & Farač the Syrian | Edited 1984. Translated and analyzed | Commissioned by King Smbat |
| V2385 | Copied from the Medical book for mount | 6 | — | — | — | Edited 1867. Translated and analyzed | Copy of M10975 |
| M11161 | Medical book for horses | 287 | 1504 | Sivas | — | Unedited. Superficially analyzed | Copy of M10975 |
| M459 | Medical book for horse, mule and donkey | 21 | 1696 | Hogac' Vank' | Łazar Amt'ec'i | Unedited; interlinear translation, tentative analysis | Obviously not related to M10975 |
| M550 | On the diseases and ulcers of the horse | 2 | 1710? | — | — | Unedited. Superficially analyzed | Unclear relation to M109 |

**Table 1.**
*Overview of known Armenian manuscripts on horse medicine.*

Medieval manuscripts—remarkable books painstakingly written and decorated by hand—are regarded some of the most precious objects produced in medieval times. Another precious object was the horse in the medieval aristocratic society. The horse cannot be dissociated from knighthood. The horses of the high-ranking knights and kings have been considered representatives of royal power, physical strength, beauty, and elegance. They were precious and expensive creatures; so, their training, care, and health were of paramount importance for their owners. Kings very often employed their own horse specialists, farriers, horse doctors or mounted militarists, often named by the rank of marshal or constable (**Table 1**).

And thus, it is not really surprising that kings and nobles combined the esteemed values of a medieval aristocratic society, namely manuscripts, royal steeds, and horse experts serving the king into one single prestigious object: a (professional) medical book on horses.

## 3. The Cilician medical book for horses

The Armenian institute for ancient manuscripts, Matenadaran, in Yerevan holds a unique, illustrated manuscript, codex M 10975, called "Medical book for horse and mount" [2–7].

At the beginning of the twentieth century, this manuscript was still found on the "List of Armenian Manuscripts of Tabriz" as MS 74, owned by tailor Širmazani [8]. It was donated to the Yerevan collection only in April 1987, by a private owner named Saračyan from Los Angeles.

The manuscript is a hippological and hippiatric book on 184 folios, containing 182 chapters and some illuminations. It was written in 1296–98 on behalf of the Cilician King Smbat, as can be read in the manuscript's main colophon (f.184a):

> *Well, this medical book for horses and mounts was written to recognize the good and the bad [in a horse], on behalf of the Christ-loving 184a // and wise, thoughtful, witty, God-fearing King of the Armenians, Smbat.*
>
> *And thus, I, the humble physician Farayč, on behalf of my lord, the holy king, took this [task] on me with great difficulty and translated this into a correct and clear language, for I was very well versed and have been trained in the art of healing in the big (city) Baghdad for many years. And I am a Syrian by origin and by faith, and by piety completely orthodox. And I worked on the translation of this medical book in the capital Sis [9].*

Before we unfold the general history of this manuscript, its production, reception, and provenance, the socio-historical contexts of its time of production as well as the persons involved must be investigated. The efficacy and importance of this horse book will be tracked in the subsequent horse treatises both in the Armenian and the neighboring traditions.

### 3.1 The production

#### *3.1.1 The time of the manuscript*

Toward the end of the thirteenth century, after the Armenian Kingdom of Cilicia had already fought on the side of the European Crusaders, the small Christian Kingdom was still under the protection of the Mongolian Ilkhanids. The Armenians fought on the side of the Mongols and the European Crusaders against

the Islamic Middle East. The growing conflict with the Mamluks can be seen in this context of the Armenians' involvement with the Mongols [10]. This alley brought the Armenians into first direct contact with the forces of the Mamluk Sultanate: the Mongol conquest of the Middle East in 1259 was heavily supported by the Armenian King Het'um I. The Mamluks, steadily replacing the Ayyubid masters in the Middle East, by 1250 already controlled Egypt after having it defended from the crusaders. They had succeeded in re-uniting the Muslim Middle East.

The conflict between Mamluks and Armenians broke out with the request of the Mameluk Sultan Baybars to the Armenian ruler Het'um I to break the alliance with the Mongols and give those territories and fortresses to the Mamluks that had been granted to the Cilician king through the alliance with the Mamluks [11]. From the first invasion of the Mamluks in Cilicia in 1266, decades of constant threat to the Christian Armenians began. In 1285, the Armenians had to sign a 10-year armistice under harsh conditions, but the Mamluks did not actually keep it. Already in 1292 another Mamluk invasion forced King Het'um II to abandon many towns. He abdicated in favor of his brother T'oros III and entered the monastery Mamistra. After a short interregnum of his younger brother Smbat, King Het'um II returned to the throne in the summer of 1299. Faced with a new attack by the Mameluks, he asked the Mongol Khan of Persia, Ghâzân, for support. In response, Ghâzân marched toward Syria with the support of Franks of Cyprus. The allied Armenians and Mongols defeated the Mamluks in the battle of Wadi al-Khazandar, 1299. In 1303, the Mongols tried to conquer Syria once again in larger numbers (approximately 80,000) along with the Armenians, but they were defeated at Homs on March 30, 1303. At this time, the Mongol leaders had already turned to Islam, and this put also the Armenian-Mongol alleys to the end [12].

The Armenian royal family of the Het'umids continued ruling the unstable Cilicia until the midst of the fourteenth century, but it could not resist attacks from the Mamluks any longer. The Armenian capital Sis fell to the Mamluks in 1375, and the final king, Levon V, died in exile in Paris in 1393 [13, 14].

### *3.1.2 King Smbat, the commissioner of the horse treatise*

Smbat was the king of the Armenian Kingdom of Cilicia from 1296 to 1298. He was born in 1277 as one of the 16 children of king Levon II of Armenia and his wife Keṛan of Lambron and was a representant of the Hetumid noble family.

Upon the death of King Levon II on February 6, 1289 AD, his surviving 11 children fought for control of the kingdom, and three of his sons managed to obtain the throne, mostly for relatively brief periods at a time. First to emerge as king was his son Het'um II [15]. Smbat seized the throne with the aid of his younger brother Kostandin while his brothers King Het'um II and prince T'oros visited Constantinople. In 1297, on a journey to the court of the Mongol ruler of Persia, Ghazan, Smbat received recognition of his position as king from Ghazan, which was necessary to legitimate his usurpation. He also received a bride from the Mongol Khan in order to form a matrimonial alliance. During his return to Cilicia, he came across his two brothers in the region of Caesarea and imprisoned them in the fortress of Barjraberd. In early 1298, Smbat even ordered T'oros to be strangled and Het'um to be blinded with a hot iron. This cruel action resulted in the rebellion of his former ally, Kostantin. Smbat was imprisoned, and Het'um was freed [16]. Smbat plotted again to resume the throne of his brother Het'um, meanwhile a Francisan monk, but he was imprisoned for the rest of his life.

Thus, Smbat reigned only for a period of 2 years; he, however, left essential objects to posterity that make him unforgettable: King Smbat had his own smaller bronze

coins, called *p'oł,* minted, showing him on horseback and he commissioned a medical book for horses. And this makes him unforgettable in Armenian cultural history.

### 3.2 Reception of the Cilician medical book(s) for horses

The reception of any manuscript can be measured by its output in the form of later copies and translations. In the later Armenian tradition, we do have at least two preserved Armenian copies of Smbat's horse book.

In 1867, a Mekhitarist father published the text of a horse book fragment with the title "I grastu bžškaranēn p'oxac" (copied/taken form the medical book for mount) in the armenological journal Bazmavep [17], which we could identify with manuscript Ms 2385 of the Mekhitharist Library in Venice [18]. This text consists only of three folios and was attached to a (human) medical book that was commissioned by King Het'um II and copied by a Vardapet Mkrtič' in 1294–1295, before Smbat seized his brother's throne. It is still unclear whether these three folios were written by the same scribe and in the same time or whether they were copied later and just bound into this book. Thus, at the current state of research it is uncertain whether these three folios are a later copy of Smbat's or of Het'ums horse book; the title, however, and obvious textual parallel speak for Smbat's horse book [19].

In 2008, the Institute for Ancient Armenian Manuscripts was given a voluminous, damaged codex from the private property of the Nazumlean family of Isfahan. It was catalogued as MS 11161 and contains various medical treatises and a treatise on the care of horses. It has not been explored yet, but in our first analysis after the manuscript's restoration in 2014 we discovered that the compilation's third stratum, a medical book for horses, consisting of folios 210a–261b, was copied in 1504 in the town of Sebaste, present-day Sivas (Turkey). It represents definitely a reproduction of Smbat's Cilician horse book, not an accurate copy, rather an adjusted and updated version.

If we dig deeper into Armenian medieval equine medicine, we see that the other surviving texts and fragments in Armenian language suggest that there was even an older Armenian horse treatise, written some decades before Smbat's text.

#### *3.2.1 The horse treatises of King Het'um I*

In the main colophon of an Armenian chemistry and pharmacology treatise (*Girk' arvesti k'imiakan*), codex 248, kept in the Bibliotheque Nationale de Paris, one gets the following information about another horse treatise on Ff. 34v [20]:

"King Het'um, who marched again the enemy sultan with an army of [corrupt writing] horsemen, massacred and destroyed everything, and he went with big honour to Baghdad [...] and there a certain wise man, a deacon named Step'anos, with the king. This man had learnt plenty of languages and writings just like the former philosophers, and there was no writing, this man could not find. He was much loved by the Armenian king because of his knowledge and thus was asked by the King. And he translated three writings about the farriery (i.e. medical treatment) of horses and about how to make a sword...and he took them to the Armenian lands" [21].

These few lines lead us to believe that King Het'um I, one of the most colorful figures in the Armenian Kingdom of Cilicia and grandfather of Smbat, had commissioned one or even more horse treatises based on an Arabic source, and that these had been brought to Cilicia in the 1260s.

Unfortunately, there is no trace of these Hetumian horse books; we have to rely only on the information from this colophon. But this leaves another question: are Hetum's horse book and Smbat's horse book related? Since there is no preserved copy of Het'ums horse books, a meticulous text comparison cannot answer this

question. Therefore, we have to take a deeper look into the literature of the contemporary royal courts to see whether we find any traces of Het'um's or any other Armenian horse book [22].

*3.2.2 The Arabic-Armenian horse treatises*

A certain Abū l-Faraǧ is named the author of an *Aqra'bādīn (al-hayl)* "Treatise about horses," which is kept in the Dār-al-Kutub Library in Cairo [23].

Its introduction says that this treatise was translated from Armenian to Arabic by a certain Maḫbub (al Armani) and his friend Abū l-Faraǧ, who knew Arabic thoroughly and was versed in many languages. It was commissioned by Maḫmud b. Khalīfah Ya'qūb and the philosopher Sa'd Al-Dīn b. Ẓāhir al-ʿAjamī, during the reign of Sultan Baybars (i.e., 1260–1277). The colophon tells us that the Armenian king had removed the Arabic original from the school of Baghdad during the reign of Sultan Baybars [24]. This treatise most likely refers to the lost horse book of Het'um I. The mentioned manuscript in Cairo has not been analyzed yet—also because of the complicated access— but the information given in the collection's catalogue states that many expressions to be found in the text are given in Armenian [25].

The Arabic equine literature also provides further information about several copies of this Arabic translation of Het'um's horse treatise in London [26], Bethesda USA [27], and Gotha, Germany [28].

The Manuscript or.3133 of British Library was analyzed, literally translated and compared with the existing copy of the Armenian horse book of King Smbat.

In the introduction to the Arabic text, one reads that this text is a treatise on equine medicine:

"The Armenian king took the treatise out of the Dār al-ᶜilm of the Caliph treasures in Baghdād ..... And it was an Arabic manuscript, which he brought to Armenia" [29].

In the first chapter the text continues,

"the one who translated the treatise from Armenian was called Maǧbūb, the name of his friend was Abū l-Faraǧ, who spoke excellent Arabic ... .. And when the Armenian king took this treatise away from Bagdād, this was in the realm of Baybars, the ruler of Egypt ... " [29].

The colophon gives an exact date of completion of the manuscript:

"The writing of the book was finished, with God's help, on Thursday, the 11th ǧumādā I of 1270" [29, 30].

This means, that the mentioned Arabic horse treatises kept in collections in Cairo, London, Bethesda, and Gotha are all based on the translation from the Armenian horse book of King Het'um I [31]. Historical sources additionally confirm this story. In 1258, joint Mongol-Armenian forces led by Het'um captured Bagdad.

The comparison of the Arabic translations of King Het'um's horse book with the text of Smbat's horse book of 1296–98 provokes an even greater confusion: the texts have striking similarities and textual parallels. Thus, a reverse conclusion is obvious: Smbat's horse book was probably created on the basis of the Arabic translation of Het'um's horse book.

*3.2.2.1 A multilingual Syrian physician: key to the puzzle?*

The conclusion mentioned above could be supported by the key person in the production of the equine manuscripts: the compiler and translator of the Arabic and Armenian copies, the wise Abū l-Faraǧ of the Arabic and the Syrian

physician Farač in the Armenian copies. Of course, it could be a simple similarity of names, but the description of a learned Syrian named Farač, who had an excellent command of Arabic and other languages, is found both in the Armenian and Arabic colophons and allows the conclusion that this is a single person. Thus, the Armenian designation as "wise Syrian Farač," and, especially, the Arabic form Abū l-Faraǧ point to one of the most famous Syrian scholars at the time: Gregory Bar-Hebraeus [32, 33]. The famous Syrian polymath and Bishop Gregory Bar-Hebraeus (1226–1286) wrote and compiled in his numerous and elaborate treatises research in theology, philosophy, medicine, science, and history. Being in general proficient in several languages, he was also known as gifted translator from and into Arabic.

The speculations about Bar-Hebraeus' involvement in the Armenian horse books could be confirmed not only by the name form Abū l-Faraǧ Ibn al-ʿIbrī commonly used in the Arabic sources, but also by the fact that the learned Syrian had studied medicine and also worked as a personal physician of the Ilkhan Hulegu Khan. He also had contact with the Cilician royal house and was ordained Primate of the East in 1264 in the Cilician capital Sis, in the presence of the Cilician King Het'um I. Moreover, the information provided by the Arabic colophons allows a dating of a translation of an Armenian horse book into Arabic during the reign of both King Het'um I and Mamluk Sultan Baybars and to the lifetime of Bar-Hebraeus. The argument in favor of Bar-Hebraeus may be reinforced by the fact that he was known in the Armenian tradition also very often by the Arabized form of his name, as Abu(l) Faraǰ.

Strong counterarguments are, however, the fact that there is no indication in Syrian, Arabic, Persian, Armenian, or other sources that Bar-Hebraeus ever translated from Armenian into Arabic on the one hand, and veterinary treatises, on the other.

The assumption that the same Abū l-Faraǧ was also responsible for the translation of an Arabic horse book into Armenian on behalf of King Smbat, 1296–98, is difficult to sustain due to the biographical data of Bar-Hebraeus, who had already died in Maraga, Persia, in 1286.

One can even argue that the Armenian priest T'oros may have been working alone on an already existing, earlier translation by the Syrian Farač/ Abū l-Faraǧ particularly taking the fact into account that Arabic terms describing horse coat colors, diseases, and remedies are very often rendered completely corrupt in the Armenian horse book of king Smbat. A person who is said to have an excellent command of Arabic would not use such corrupt Arabic terms in Armenian.

### *3.2.3 The Georgian-Armenian horse treatise*

Another further proof of the importance of this horse book is a late translation into Georgian. The national library of Georgia holds the manuscript T 3467, a Georgian horse treatise copied in 1791 [34].

The colophons tell us that

"On May 18th, 1788, we, the High priest of Sioni in Tiflis, Ioane Osedze and the Armenian priest Ter-Petros, were asked by Giorgi [35], the first born son and heir of the King of Georgia Irakli II, to translate this treatise from Armenian into Georgian. The copy was finished on August 23rd, 1791" [36].

From the colophon we also learn that a certain "Parač'i" had translated the original Armenian treatise from Arabic in the Armenian year 953 (1503) in Sebaste (Sivas) and that the exact name of the treatise is "Medical book for horse and mount." The Georgian translation was obviously based on the copy of Smbat's horse book in Sebaste 2004, M11161 (**Figure 1**).

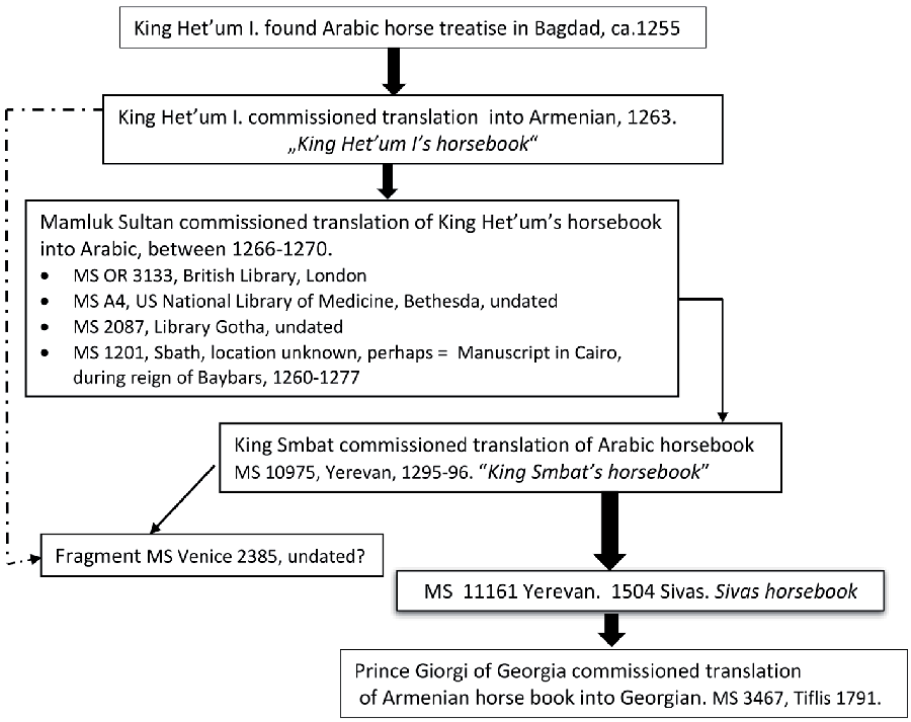


**Figure 1.**
*Presentation of the assumed transfer of knowledge and reception of Armenian equine medicine manuscripts between the thirteenth and nineteenth centuries.*

### 3.3 Provenance of the Armenian horse books

Three of the given Armenian horse treatises, the Cilician horse book of king Smbat 1296–98, its not clearly dated Venice copy, and the voluminous Sivas reproduction of 1504, had been kept by private owners in the Armenian-inhabited settlements of Persia before they were donated to the Armenian manuscript collection in Yerevan. It is still one of the many unsolved mysteries of these Armenian horse books, how and when they ended up in Persia.

## 4. To the west of Cilician Armenia, far beyond the Bosporus

The Cilician noble families have been under the constant cultural influence of the Frankish kings for many decades, particularly due to the close relations with the European noble crusaders and the Staufer kings. It was Prince Levon II (1150–1219) who profited from this situation by improving relations with the Europeans. Cilician Armenia's prominence in the region is attested by letters sent in 1189 by Pope Clement III to Levon and to Catholicos Gregory IV, in which he asks Armenian military and financial assistance to the crusaders. On January 6, 1199, Prince Levon II was crowned with great solemnity in the cathedral of Tarsus, in the presence of the Syrian patriarch, the Greek metropolitan of Tarsus, and numerous church dignitaries and military leaders. While he was crowned by the Catholicos Gregory VI of Cilicia, Levon received a banner with the insignia of a lion from Archbishop Conrad of Mainz in the name of Henry VI, Holy Roman Emperor. By securing his crown, he became the first King of Armenian Cilicia as King Levon I [13].

Cilician Armenians were attracted by European culture and art. It did not take long for them to absorb the findings and texts of European science and medicine. In addition to their local knowledge and that of the Muslim East they also started to include European knowledge, in particular also in horse breeding and training.

King Het'um I was a major player in the political struggles and shifting alliances during the Crusades, trying to keep ties with all sides, both in the West and the East. Perhaps he was not only fascinated by the Arabic equine treatises from Baghdad but also somewhat inspired by his European counterpart's hippiatric book? In Europe, Jordanus Ruffus, chief marshal and close associate of Staufer emperor Frederick II, completed his very influential "Medicina Equorum" around 1250, which was commissioned by and dedicated to his emperor. We know that this work spread quite quickly from Italy through Europe as a result of the Italian equestrian schools. Can the mere fact, that one of the most important contemporary European monarchs has commissioned a horse book, have affected the Armenian King Het'um I who was much oriented toward Europe?

The growing influence of European equestrian art and equine knowledge cannot be investigated in the Het'um's horse treatises, only guessed. Some 30 years later, however, this influence is clearly presented in the influential horse book of king Smbat, especially regarding breeding, training, and chivalrous tournaments (such as buhurt and jousting) [6]. The European influence was also increasingly reflected in some newly adopted Frankish terms in horsemanship, anatomy, and names of diseases, but hardly noticeable in farriery [37, 38].

## 5. Outlook: galloping from east to west?

In order to understand the history and interrelation of Armenian and Arabic horse treatises, not only the person of the "producer and translator," the wise Syrian Farač/Abū l-Farağ, must be investigated, but also the socio-historical and scientific historical context. Moreover, the efficacy and importance of the Cilician Horse Book 1296–98 will be tracked in all subsequent texts—both Armenian copies and foreign translations. A range of local and foreign treatises will be checked: these are the supposed translations of an Armenian text into Arabic (13th c to 14th c) and into Georgian (18th c).

The meticulous comparison of all texts in question will perhaps also prove that the main source for all texts was an unknown Arabic text, which King Het'um I had discovered in Baghdad and which he had translated into Armenian. Further investigation of Armenian equine manuscripts and fragments will clarify what can be regarded as the actual starting point of the reception history of Armenian horse medicine.

This will be the goal of an interdisciplinary, international research project in the coming years.

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(November 1273 – October 27, 1331), fully Abu Al-fida' Isma'il Ibn 'ali ibn Mahmud Al-malik Al-mu'ayyad 'imad Ad-din and better known in English as Abulfeda, was a Kurdish historian, geographer and local governor of Hama. He was a prince of the Ayyubid dynasty and the author of *The memoirs of a Syrian prince: Abu'l-Fidā', Sultan of Ḥamāh*

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Chapter 2

# Gene Therapy as a Modern Method of Treating Naturally Occurring Tendinitis and Desmitis in Horses

*Elena Zakirova, Kovac Milomir, Margarita Zhuravleva, Catrin Sian Rutland and Albert Rizvanov*

## Abstract

Tendon and ligament injuries have always been complex to treat, with recovery often taking many months, if successful at all. This chapter looks at recent work undertaken using regenerative medicine, specifically gene therapy and the advances that have been made in equine therapy. It looks at the process from plasmid construction, in vitro testing through to trialing the equine-specific plasmid construct in horses with superficial digital flexor tendon (tendinitis) and suspensory ligament branch injuries. It also looks at the rationale for utilizing vascular endothelial growth factor (VEGF164) and a basic fibroblast growth factor (FGF2) for these trials and the cellular effects and potential mechanisms of actions.

**Keywords:** horse, gene therapy, tissue regeneration, superficial digital flexor tendon, tendon injuries, suspensory ligament

## 1. Introduction

Tendon and ligament injuries are the most common traumas in horses (*Equus caballus*) irrespective of the age and breed [1]. Based on the statistics, injuries in sport horses can achieve 86% of a total morbidity rate, with 37% of them accounting for muscle, tendon and ligament pathologies. As a rule, musculoskeletal injuries require long-term recovery for 9–12 months [2]. Tendon and ligament injuries result in a loss of performance of the horses and frequently cause discomfort or pain. Therefore, the animals are unable to participate in competitions for a long period of time. Complications of these injuries include chronic musculoskeletal diseases. They result in degenerative-dystrophic damage of collagen fibers of tendons as well as adjacent and underlying tissues. Incomplete tissue recovery leads to recurrent injuries in 80% of horses with treated tendon micro- and macroruptures within 3–12 months after the first injury [3].

Methods of regenerative medicine are used for appropriate regeneration of damaged tissue in animals. These include the administration of stem cells [4, 5] and recombinant proteins, as well as gene therapy. These methods are presently the most advanced and promising approaches to manage musculoskeletal disorders [6]. However, regenerative medicine is mainly targeted toward the treatment of human disorders. Animals are mostly considered as models to test drugs and devices intended for human use. Drugs developed for human use can be ineffective for the

treatment of animal diseases due to partial homology of physiological processes. When given to animals, such products can cause long-term immunological disorders, decrease the efficacy of a subsequent treatment or even cause adverse side effects including anaphylactic shock.

In a veterinary practice, an autologous graft rejection can be avoided in 85% of cases [7]. The likelihood of immune responses in animals to the administration of allogenic or autologous species-specific stem cells is also low [8–10]. However, full homology can be of vital importance when applying more advanced therapeutic approaches such as gene therapy.

Gene therapy is a novel, rapidly developing trend in regenerative medicine and veterinary, which can provide continuous stimulation of regeneration. When this approach is used, a recipient's body constantly synthesizes its own substances instead of a multiple drug (pharmaceuticals, recombinant proteins and so on) delivery. Gene therapy has been successful in the treatment of various human disorders [11, 12], and it can be used to treat animals [13]. However, species-specific recombinant genes that would provide biological activity and at the same time have no immunological side effects should be developed for this purpose. A therapeutic potential of gene therapy for the treatment of tendinitis and desmitis in sport horses will be discussed in detail in this review, especially those related to a series of papers recently covering gene therapy in horses [14–17].

## 2. Use of a species-specific plasmid construct in the treatment of traumas in horses

### 2.1 Description of the plasmid construct

A group of scientists from Russia and Great Britain developed and tested a drug for gene therapy of soft tissue injuries in horses. This gene construct is plasmid DNA (pDNA), encoding animal-specific genes (**Figure 1**). A plasmid construct pBUDK-ecVEGF164-ecFGF2 based on a pBudCE4.1 vector contained codon-optimized sequences of horse genes, a vascular endothelial growth factor (VEGF164) and a basic fibroblast growth factor (FGF2) under eukaryotic promoters (EF-1α and CMV promoters, respectively) [14].

These genes were selected with good reason as VEGF stimulates synthesis of DNA and proliferation of cells involved in antiapoptotic signaling pathways. It promotes the proliferation and migration of endothelial cells, stimulates angiogenesis and attracts endothelial progenitor cells from bone marrow, stimulates the activity of pericytes and stabilizes newly formed blood vessels. VEGF is also a

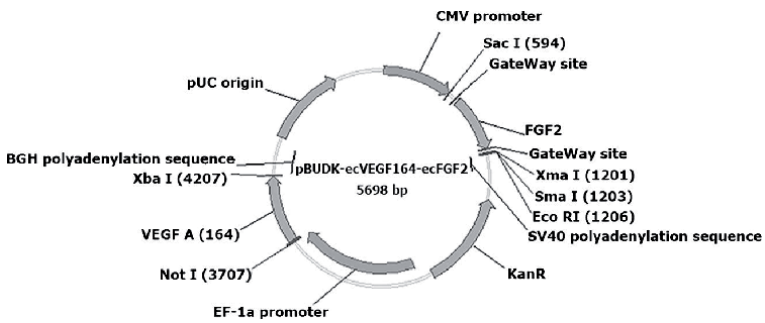


**Figure 1.**
*Map of recombinant plasmid pBUDK-ecVEGF164-ecFGF2 [14].*

chemoattractant for smooth muscle cells, monocytes, macrophages and granulocytes. All of these are involved in the process of wound healing. VEGF also increases vessel wall permeability at the site of injury that enhances the formation of granulation tissue [14].

In turn, FGF2 exerts a wide range of mitogenic and angiogenic activities and is a neurotrophic factor. In intact tissues, it is present in a basement membrane of the epithelium and in the subendothelial extracellular matrix of blood vessels. It stimulates cell proliferation, regeneration of nervous, muscle and connective tissues. Also, FGF2 activates de novo formation of blood vessels by triggering the process of angiogenesis [18].

Thus, a mechanism of action of gene therapy comprising VEGF and FGF2 is to stimulate synthesis of proteins in a recipient that enhances the vascularization of damaged tissues. This, in turn, leads to a higher regeneration rate. Both VEGF and FGF2 are well-known growth factors with a wide range of mitogenic and angiogenic activity. They also contribute to regeneration of muscle and connective tissues. What is more important is that in combination these factors demonstrate synergistic effects that surpass those of therapy with just one growth factor [19].

This gene product has been tested for identification and functional activity in mammal cells in the laboratory. Full genetic sequencing and restriction analysis with subsequent agarose gel electrophoresis demonstrated a complete compliance with the claimed structure of pBUDK-ecVEGF164-ecFGF2 (**Figure 2**).

Biosynthesis of recombinant VEGF164 and FGF2 in transfected immortalized HEK293FT cells was confirmed by an immunofluorescence assay with anti-VEGF and anti-FGF2 antibodies (**Figure 3**), which confirmed co-expression of recombinant proteins in transgenic cells [14, 14].

The biological activity of the pBUDK-ecVEGF164-ecFGF2 DNA plasmid was evaluated during *in vitro* experiments in horse stem cells. For this purpose, horse MSCs were isolated under a standard procedure by incubating a subcutaneous adipose tissue homogenate with crab collagenase. The cells obtained were identified as MSCs with flow cytofluorometry-more than 80% of them expressed MSC-specific markers (Thy-1 in 99.8% and CD44 in 83% of the cells) and no CD34 or CD45 was

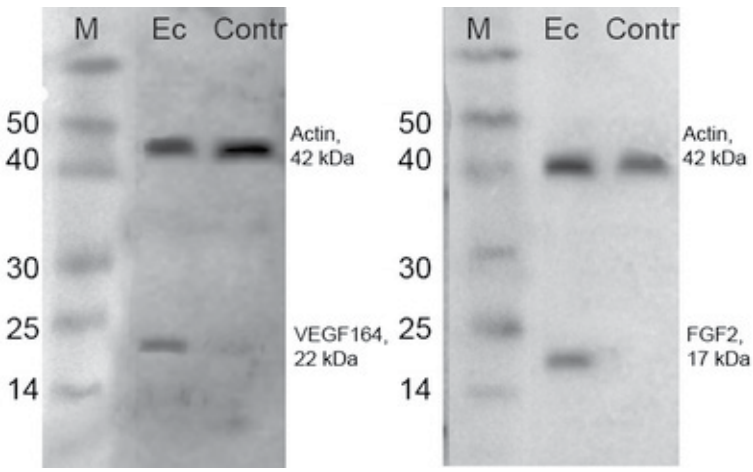


**Figure 2.**
*Analysis of VEGF164 and FGF2 biosynthesis by immunoblotting in HEK293FT cells after transfection. Electrophoresis in 12% SDS-PAGE gel was performed in Laemmli system. Antibodies against human actin, VEGF and FGF2 were used. Bands correspond to human actin (42 kDa), horse VEGF164 (22.3 kDa) and horse FGF2 (17.2 kDa). M-molecular weight protein marker (GE LifeSciences RPN756E); Ec-HEK293FT cells transfected with pBUDK-ecVEGF164-ecFGF2; control nontransfected cells [14].*

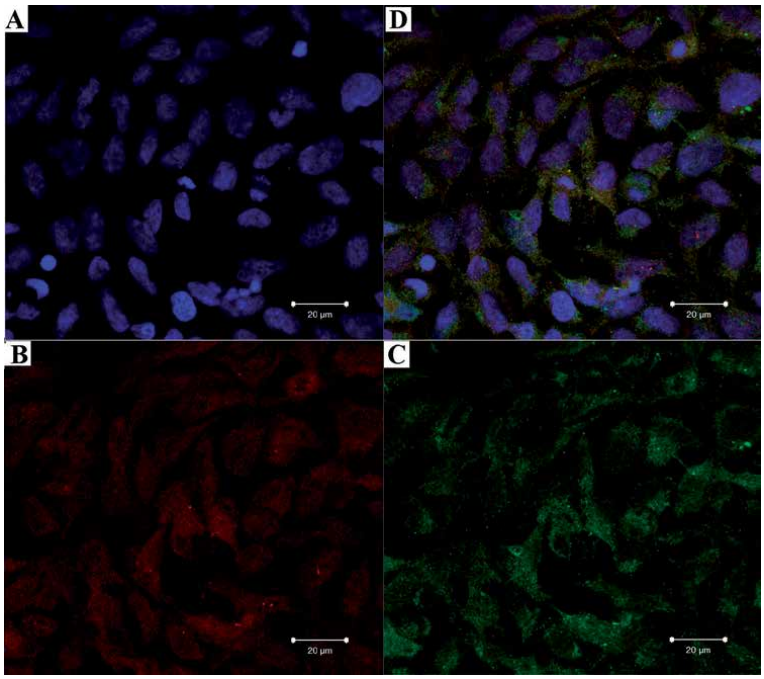


**Figure 3.**
*Immunofluorescence analysis of VEGF164 and FGF2 biosynthesis in HEK293FT cells, 48 h after transfection. (A) Negative control: HEK293FT cells without pDNA transfection, nuclei-stained DAPI (blue). (B)–(D) HEK293FT cells transfected with pBUDK-ecVEGF164-ecFGF2. b) Staining with primary antibody against VEGF and secondary antibody, conjugated with a fluorescent label Alexa Fluor 555 (red). (C) Staining with primary antibody against FGF2 and secondary antibody, conjugated with a fluorescent label Alexa Fluor 488 (green). (D) Overlay image of a, c and d: VEGF (red), FGF2 (green), cell nuclei stained with DAPI (blue) [14].*

expressed (data not provided). Thus, according to literature comparisons and based upon the laboratory data, the cells obtained were equine MSCs [20].

Genetic modification of horse MSCs with pDNA pBUDK-ecVEGF164-ecFGF2 showed that transfected cells possess a higher ability to form a capillary-like networks on the Matrigel™ matrix as compared to intact cells ($p < 0.005$) (**Figure 4**).

### 2.2 Use of the plasmid construct in vivo

Due to a high incidence of tendon and ligament injuries in horses, a high rate of recurrent traumas and a prolonged period of recovery that normally lasts for several months and up to 15 months with severe injuries, these injuries are a medical and surgical challenge. Even when modern technologies are applied, in many cases, damaged tendons and ligaments demonstrate biochemical and ultrastructural abnormalities after 12 months and preinjury biomechanical properties are not completely restored [21].

A total of 12 horses were given gene therapy [15, 16] through in vivo trials of the treatment. Out of them, eight horses had naturally occurring injuries of the superficial digital flexor tendon (SDFT; tendinitis) and four horses had suspensory ligament branch (SLB) desmitis. All the horses had spontaneous SDFT and SLB injuries and were included into the study from 2015 to 2017 undergoing treatment in the veterinary clinic "New Century" at the Moscow State Academy of Veterinary and Biotechnologies, Moscow.

Gene therapy of the four horses with injured SLBs showed that before treatment all horses had pain in the injured leg. By day 40 after treatment, no animals had any sings of inflammation at the site of injury, nor was there a change in the skin surface temperature within the area of injury, swelling or tenderness when palpated. By day 20 after treatment, lameness significantly reduced as compared to the baseline. By 12 weeks and during subsequent follow-up examinations, no horses were lame.

Ultrasound parameters in damaged SLB began to improve 20 days after the onset of treatment, this positive tendency remaining thereafter. Parameters such as changes of the zone of damage, echogenicity and fiber alignment made this especially evident. When the treated horses started doing a program of physical exercise, the ligament architecture constantly improved, as indicated by their longitudinal alignment and length (**Figure 5**).

Based on the examination results, only one horse had no significant ultrasound improvements in the first 90 days after pDNA injection. On days 20 and 40, this horse had new hypoechoic lesions that indicate a nonstable healing process. By 120–180 days after treatment, this horse had a noticeable ultrasound improvement in the site of injury.

Color Doppler ultrasonography (CDU) demonstrated evidently increased blood supply by day 20 after pDNA injection. This tendency remained up to day 40 and

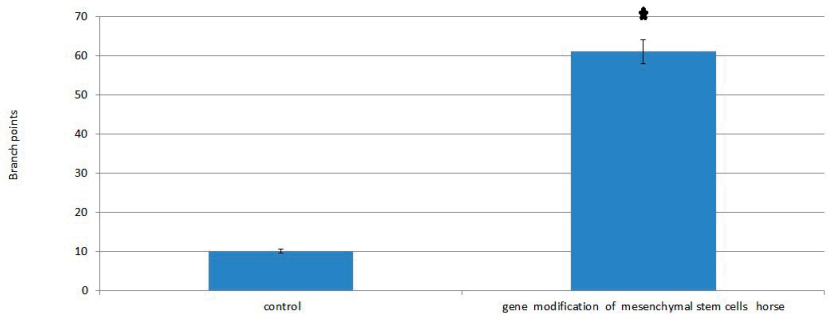


**Figure 4.**
In vitro *angiogenesis assay using Matrigel to characterize the proangiogenic effect mediated by genetic modification of mesenchymal stem cells.*

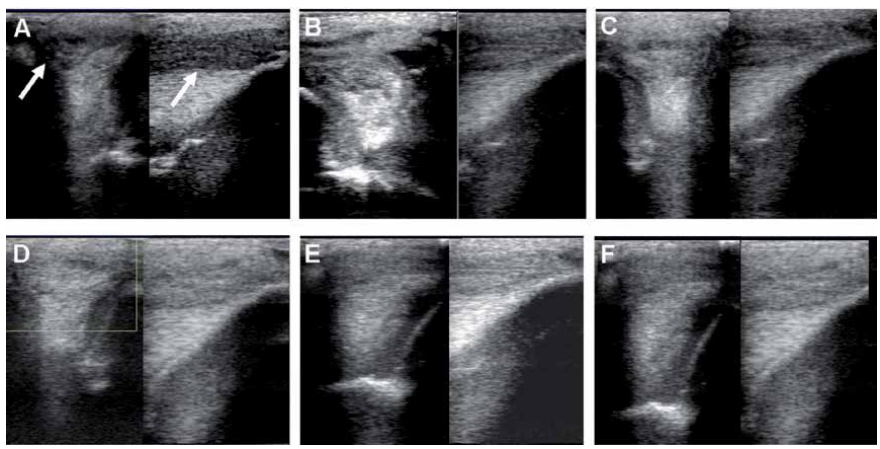


**Figure 5.**
*Ultrasound images prior to plasmid DNA encoding VEGF164 and FGF2 genes on day 0 (A), 20 (B), 40 (C), 90 (D), 180 (E) and 300 (F) after administration in horse with SLB desmopathy. Arrows indicate lesion.*

was high until day 90. By day 180 after plasmid injection, CDU parameters reduced to baseline values in most horses (with the baseline set at values within an intact limb of the same animal). There was no significant correlation between the soft tissue damage severity prior to treatment and post-treatment CDU parameters.

Ultrasound parameters of SDFT lesions in most horses began to improve 20 days after treatment [15]. This positive tendency remained during the follow-up period. With the onset of training, healing of the damaged tissue increased in the tendon treated. This manifested as a longitudinal alignment of fibers and an increase in their length [16].

After treatment, the echogenicity of the damaged SDFT constantly and significantly decreased from day 0 to day 60 in all horses except one. In 3 months after the beginning of treatment, the echostructure was more uniform in most horses, with collagen fibers arranged in parallel to the longitudinal axis.

A linear fiber pattern in horses with SDFT injuries also improved during the study, but this was happening more slowly when compared to the echogenicity. Within nine months, there were scarcely any signs of tendon damage in most of the horses with the SDFT injury. They had correct alignment and a well-arranged longitudinal pattern of fibers.

Doppler ultrasonography demonstrated a significant improvement in blood supply of the affected areas by day 20 [16]. This tendency continued for 90–120 days, with a peak that was reached on day 40. After postinjection day 180, the vascularization decreased to baseline levels (as in healthy limbs of the same animal). There was no significant correlation between the injury severity before treatment and CDU parameters afterwards. There were no significant differences in CDU images between horses with SDFT and SLB injuries after treatment; CDU changes were strictly individual. CDU changes can be due to hypervascularity being natural in the process of healing. Normally, tendons and ligaments are hypovascular [22]. A short-term increase in blood flow results in response to damage-associated tissue hypoxia. We propose that the gene therapy enhanced this effect markedly.

To identify possible side effects, all horses were constantly examined by a veterinarian in the clinic from the time of plasmid administration until 12 months later. Horses did not have any side effects to the pDNA administration, and horse age, gender and the duration of lameness had no effect on the outcome of gene therapy. The main differences in clinical outcomes were determined by the extent and site of the animal's soft tissue damage sustained before treatment. The study results showed that only one horse with a serious injury of the SLB and body did not respond to treatment, and it was lame for the first 3 months after the onset of therapy. Only one horse that recovered after gene therapy (initially with SDFT tendonitis) suffered a repeated injury at the same site 6 months after treatment [15, 16]. In the 12-month follow-up after treatment, owners of the other horses rated gene therapy results as good or excellent in terms of sporting success.

## 3. Discussion on the use of gene therapy in horses

One should emphasize that the disappearance of lameness with treated tendinitis or desmitis in a horse does not mean absolute tissue regeneration. In these studies, rapid and mostly complete regeneration of both the tendon and ligament occurred within 2–3 months of treatment, which included a single injection of pBUDK-ecVEGF164-ecFGF2. This was confirmed by increased echogenicity and homogeneity at the site of injury, as well as an increased percentage of parallel collagen fibers.

Thus, the study data are encouraging and demonstrated a positive effect of using pDNA encoding horse-specific proteins at early stages of healing of traumatic tendinitis and desmitis, when injected into the site of injury. In part, this can be explained by coincidence with conditions and stages of normal tendon healing. However, the horses included into the study had moderate or severe tendon injuries. It is well known that such injuries are associated with a poor prognosis in response to standard treatments.

A drawback of these clinical studies is that they did not identify an exact mechanism of action of direct gene therapy with pBUDK-ecVEGF164-ecFGF2 on the regeneration of damaged horse tendons and ligaments. Since the horses had fully recovered, the investigators considered possible histological interventions to take tissue samples as inappropriate. If histological samples could be taken looking at the cell types, checking for inflammatory reactions and cells associated with inflammation and immune responses would be advantageous for confirming the lack of immune response at a cellular level. In addition, investigating the healing mechanism via histology by looking at collagen type and wound repair would further the knowledge in this area. Adding RNA and protein expression studies would also help understand the mechanisms involved in this therapy. The pDNA administration used also avoids possible side effects associated with vector-mediated insertional mutagenesis when integration into the patient's genome is the long-term aim. This is not necessary in these disorders as long-term correction/replacement is not required. As previous reports of treatment results of such tendinitis and desmitis in horses are lacking, results of this gene therapy cannot be compared with those of other treatment methods.

Therefore, gene therapy, as one of the most advanced technologies in medicine, is a promising treatment for hereditary diseases and in addition offers new possibilities for a clinical management of numerous orthopedic disorders, including tendon and ligament injuries [23–25]. The use of direct gene therapy with species-specific growth factors is quite promising for the treatment of orthopedic disorders not only in horses but also in other animal species and in people [17]. The successful use of direct gene therapy with a similar plasmid construct based on dog-specific VEGF164 genes and bone morphogenetic protein (BMP2) to treat an anterior cruciate ligament injury in large dogs has been previously reported [26]. Moreover, there is a case report on using gene therapy to treat patients with critical lower limb ischemia [27]. Finally, plasmid DNA pl-VEGF165 (approved as Neovasculgen), encoding human VEGF165, has demonstrated its safety and efficacy in the treatment of atherosclerotic peripheral arterial disease in patients with chronic lower limb ischemia without side effects [28]. The high efficacy and safety of direct gene therapy have been demonstrated in all of these cases. There are also numerous benefits of using pDNA rather than recombinant viruses. Plasmids are relatively easy to construct, can be produced in large quantities and provide a safe method of delivery with low levels of immunogenicity associated with delivery. They can often be kept at room temperature for long periods of time, which is especially useful in clinical settings. Although they have lower levels of gene transfer, the studies carried out in the horse show that delivery is appropriate and efficient in these circumstances as it was delivered directly to the injured area.

VEGF and FGF2 gene therapy's direct effects on the regeneration of tendon and ligament injuries in horses should be further evaluated in a larger number of experimental animals, for a longer follow-up period and in a randomized controlled clinical study. Complete and more detailed results could also be obtained by histological examination and immunohistochemistry of samples and biopsy materials given the right conditions. Factors such as gene expression levels in tissues, collagen analysis, identification and quantification, the functional and intracellular distribution of

proteins and further studies of pathological biochemistry will help identify the main mechanisms of action.

## 4. Conclusions

The introduction of gene therapy in veterinary clinics becomes ever more possible; however, there are issues that require solutions. The future of veterinary gene therapy seems promising thanks to the studies described, and many other therapies are likely to be approved for use in both human and animal medicine [17].

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## Conflicts of interest

The authors declare no conflicts of interest.

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Chapter 3

# Gene Therapy for the Treatment of Equine Osteoarthritis

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## Abstract

Osteoarthritis (OA) is the predominant cause of lameness in horses. As in humans, the clinical symptoms of equine OA are persistent pain and dysfunction of the affected joint. Its pathology is similarly marked by progressive deterioration of the articular cartilage, subchondral bone sclerosis, marginal osteophytes, soft tissue inflammation and joint effusion. Disease pathogenesis is mediated by elevated levels of inflammatory cytokines and proteolytic enzymes in the articular tissues and synovial fluid. Existing pharmacologic agents can alleviate OA joint pain; none are able to inhibit erosive disease progression. As several gene-based treatments for human disease have received approval by the Food and Drug Administration (FDA), the transition to veterinary medicine will almost certainly follow. Several viral vector systems have demonstrated highly efficient gene transfer to the equine joint, enabling expression of therapeutic transgenes at efficacious levels for well over a year. Because of its large size, the equine joint is well suited to studies of gene-based therapies for arthritic disease. The forelimb joints are vulnerable to OA onset, and treatment and diagnostic modalities are the same in humans and horses. Here, we discuss the various gene-transfer approaches under investigation and the current progress toward the development an effective gene therapy for equine OA.

**Keywords:** osteoarthritis, lameness, interleukin-1, IL-1Ra, gene therapy, adeno-associated virus

## 1. Introduction

Osteoarthritis (OA) is a chronic, painful, degenerative, often debilitating condition common in weight-bearing joints of both humans and horses. In humans the knees and hips are predominately affected, while in the horse the metacarpophalangeal and carpal joints of the forelimb are the primary sites of onset. In both species, the pathology of OA is marked by the gradual, persistent erosion of the articular cartilage, development of osteophytes at the joint margins, sclerotic growth of subchondral bone, synovitis and joint effusion [1]. Biochemical analyses reveal that the signaling molecules and pathways that drive the inflammatory and degenerative processes in both species are identical [2]. OA is incurable, difficult to manage and often progresses to disabling joint failure. It is estimated that over 50 million people in the US alone have symptomatic OA. Spontaneous joint disease is a common

clinical problem in the horse as well where it is estimated that OA accounts for up to 60% of lameness [3], and is among the leading causes of debilitation and wastage of athletic horses. As with humans, the need for an effective treatment for equine OA is immense.

In this chapter we describe progress with an experimental gene-based therapy for OA in parallel development for both humans and horses. The concept of a genetic therapy was initially put forth as a method to replace defective genes and associated protein deficiencies from monogenic diseases, such as cystic fibrosis, severe combined immunodeficiency and hemophilia. In the present application direct intra-articular gene transfer is used as an improved system for sustained local delivery of biologic agents with anti-arthritic potential [4]. By providing for high-level, persistent production of therapeutic gene products in chronically diseased joints, long-standing obstacles impeding effective drug delivery are overcome to provide stable production of gene products with activities capable of inhibiting not only pain and inflammation, but also the progression underlying the degenerative process. We discuss various approaches for intra-articular gene delivery and promising gene products. We also discuss progress toward clinical application and remaining challenges.

## 2. Osteoarthritis pathogenesis

The pathogenesis of OA is complex and can be initiated by a wide range of factors. It is most commonly linked with aging and accumulating degradation of the cartilage matrix from the loss of cellularity and reduced metabolic activity of the chondrocytes [5–7]. In younger individuals, OA most frequently occurs as a secondary consequence of joint injury (post-traumatic OA: PTOA) either from repetitive trauma to the joint surfaces due to overloading and overuse, or acute damage to the structural tissues.

Although cartilage damage and traumatic loading are considered initiating factors, a consensus in the literature indicates that inflammatory cross-talk between the synovium and cartilage is instrumental in driving the erosive progression of OA [8, 9]. Under normal conditions, the chondrocytes, which inhabit the articular cartilage at low density, maintain the integrity and quality of the matrix through slow continual remodeling through degradation and new matrix protein synthesis. Disruption of this homeostasis from chondrocyte dysfunction or depletion from apoptosis or necrosis, leads to a reduction in matrix quality, damage to the articular surface and pathologic load distribution. The increased compressive forces among weight-bearing regions, activates stress signaling pathways in regional chondrocytes and a phenotypic shift to an activated phenotype. Stress-induced activation of nuclear factor-kappa B (NF-κB), and p38 MAPK and c-Jun N-terminal kinases and their downstream signaling cascades halts the synthesis of key extracellular matrix (ECM) proteins, stimulates the release of inflammatory cytokines and chemokines and expression of matrix metalloproteinases and aggrecanases [1, 10]. The release of cellular debris and matrix molecules from eroding cartilage stimulates cytokine and toll-like receptors in the synovial lining cells and an inflammatory response in the synovium [11, 12]. The resulting synovitis, marked by hyperplasia and hypertrophy of synovial fibroblasts, infiltrating macrophages, T cells, and mast cells, is a common feature of both early and late-stage disease. Inflammatory activation of the synovium stimulates production of enzymes and inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) that feeds back in a self-perpetuating cycle to further alter chondrocyte metabolism and the balance of cartilage matrix synthesis and degradation [9]. With increasing loss of the

protective cartilage cushion the increased mechanical forces stimulate a compensatory reaction in the calcified cartilage resulting in increased thickening and stiffness of the subchondral bone.

As an avascular tissue, injured cartilage has no mechanism for self-repair or regeneration. There is no influx of exogenous cells from ruptured blood vessels to generate space-filling tissue. Although local chondrocytes attempt proliferate and form chondrocyte clusters in an apparent regenerative, reparative response, the dense ECM limits the migration of the limited number of chondrocytes. In cases of significant damage, cartilage lesions are essentially permanent and progress to fibrillation, formation of fissures, and ultimately complete loss of the cartilage surface. Cumulatively, the slow insidious processes cause fibrillation, fissures, ulceration and over time the full thickness loss of cartilage and painful bone on bone articulation [13].

## 3. Treatment limitations

Existing medications for OA, such as analgesics and non-steroidal anti-inflammatory (NSAID) agents are palliative and only provide temporary relief of joint pain without significantly altering disease progression or restoring cartilage integrity. While there are a variety of biologic agents with activities known to inhibit pathologic signaling pathways, due to the unique anatomy and physiology of synovial joints, conventional methods of drug delivery are unable to achieve or maintain effective concentrations of therapeutic molecules in chronically diseased joints [14]. The synovial fluid which serves to lubricate the articulating surfaces and nourish the chondroctyes is a dialysate of blood plasma that enters the joint through fenestrated capillaries in the subsynovium. This "sieving effect" restricts the entry of proteins and other large molecules into the joint space from the circulation [14].

While intra-articular (IA) injection circumvents physical barriers to systemic delivery, elevated pressure causes rapid turnover of synovial fluid through the lymphatics. Continuous circulation of the synovial fluid causes injected molecules to be rapidly cleared from the joint, often with a half-life of less than 4–5 hours, depending on the size. Repeated intra-articular injection is not a useful clinically as frequent repeated needle sticks are painful, can exacerbate joint pathology and carry increased risk of infection [14].

Local intra-articular injection of corticosteroids can provide temporary relief of joint pain, but the broad spectrum anti-inflammatory effects are transient. Despite the short residence time of intra-articular therapies, studies frequently report positive effects from a number of patient-derived preparations, such as platelet rich plasma, autologous conditioned serum and various formulations of "mesenchymal stem cells" (MSCs). However due to inconsistent methods of preparation and characterization, conflicts of interest and investigator bias, the efficacy of these treatments in both human and equine medicine remains highly controversial. Indeed, the assertion that MSCs injected in suspension have an intrinsic capacity to sense and address whatever is needed for the repair and regeneration of cartilaginous tissue in the joint is not based on scientific evidence [15, 16].

## 4. OA gene therapy principles

Arthritis gene therapy was conceived as a novel protein-drug delivery system capable of exploiting the anti-arthritic properties of endogenous soluble gene products for treatment of chronic joint disease [17]. By delivering cDNAs encoding

therapeutic products to cells resident in the articular tissues, and providing for high levels of independent expression, the biosynthetic machinery of the modified cells is directed to overproduce and continuously secrete the transgenic protein into the synovial fluid and surrounding tissue. In this manner, the diseased joint becomes an endogenous site of sustained, elevated drug production, eliminating the need for repeated application, while providing the greatest concentration of the protein specifically at the site of disease. While originally envisaged for delivery of secreted proteins, similar principles can be applied to gene products that function intracellularly, including transcription factors and interfering RNAs among others. OA is an excellent candidate for a local gene-based therapy, as only one or two joints are affected in most patients, and there is an absence of significant extra-articular disease. Distinct from any existing treatment for OA, this approach has the capacity for continuous local delivery of therapeutic molecules that block painful symptoms and erosive progression of disease from a single intra-articular injection [4].

The distinct advantage of using a secreted protein is that overproduction from a relatively small number of cells can treat the entire joint. While at least in theory, gene delivery provides the opportunity to explore the application of cDNAs whose products that function intra-cellularly (e.g. transcription factors and interfering RNAs), practical application is far more challenging than it may initially appear. In order to alter the biology of a diseased tissue, a substantial portion of the cells must be modified, requiring extraordinarily high levels of gene transfer in vivo.

A variety of methods can be used deliver therapeutic gene products to joints. Once a candidate cDNA is identified, the delivery vehicle that provides efficient targeting and modification of the desired cell types in vivo and robust transgene expression that persists for a prolonged period of time. For chronic joint diseases, such as RA and OA, a minimum of 6 months to a year or more of benefit following a single injection would likely be the minimum standard for efficacy. Such a profile requires metabolically active target cells with limited turnover [18]. Additionally, the vector and genetically modified cells must avoid recognition and elimination by the immune system whose central function is to eradicate infectious viruses and virally infected cells expressing non-self, surface antigens and stress-induced signaling molecules. The immune stealth of the vector, transgene product and modified cells are essential for effective gene delivery and prolonged, functional transgene expression.

## 5. Parallel development of gene therapy for human and equine OA

Early preclinical studies showed that local intra-articular delivery of certain cDNAs could inhibit experimental arthritis in the joints of laboratory animals. Although rodents and rabbits are useful for proof-of-concept studies, their small size does not accurately reflect the environment of the human or equine OA joint. Following intra-articular injection of a gene delivery vehicle, ensuing patterns of transgene expression are dictated by the biophysical interactions between the vector and the target tissues. In the case of a recombinant virus for example, dispersion in the joint space through the viscous synovial fluid, and its subsequent penetration in the ECM of the various tissues, determines the locations, phenotype, number and density of the cells that are physically encountered by the vector and genetically modified. The composition of the cell population modified by the virus at the time of injection, determines the level and duration of therapeutic transgene expression -and, in turn, the efficacy of treatment. In this respect, the small joints of a 100–200 g quadruped rodent cannot duplicate the complex milieu of the knee of a 75 kg bipedal human, much less a 500 kg horse. The vastly greater size and internal fluid

volume, the differences in cellularity within the dramatically larger and thicker connective tissues, as well as the compressive forces generated during locomotion, have a profound influence on the biodistribution of the virus following injection [19].

To model the efficacy of gene delivery in joints of clinically relevant proportions and better assess its utility for treatment of OA, the carpal and metacarpophalangeal (MCP) joints of the equine forelimb provide highly useful targets. These joints are similar in size, function, and tissue composition to the human knee, and since they carry 60–65% of the horse's weight during locomotion, they are highly vulnerable to OA secondary to trauma and excessive training [2, 3].

Because of its large size, the equine system is particularly well suited for preclinical studies of joint disease. The horse can readily perform controlled exercise, and clinical treatment and diagnostic modalities are the same in humans and horses [2, 20]. The large joints facilitate joint function analyses, examination of internal structures using magnetic resonance imaging (MRI) and radiograph, and minimally invasive arthroscopy for visual assessment and biopsy of joint tissues. The capacity to aspirate undiluted synovial fluid permits analysis of transgenic protein content by enzyme linked immunosorbent assay (ELISA) [21], and since, joint fluids can be aspirated serially without adverse effect, patterns of transgenic expression can be monitored over time within the same animal.

By examining the efficacy of OA gene therapy in joints of similar proportion to the human knee and with similar disease, results representative of the human and equine response should be obtainable. Further, the use of the horse as an experimental subject allows practical experience with gene delivery in a relevant context and on an appropriate scale. This provides the ability to identify and troubleshoot technical and logistical problems in a clinical setting and refine working parameters for safety and efficacy prior to entering phase I human or field trials in client horses. Moreover, since OA is a significant health issue in both humans and horses, findings generated in this system can be applied to both species, allowing the development of human and equine medicines in parallel.

## 6. Ex vivo gene delivery

The initial proof-of-concept was demonstrated using an ex vivo method whereby autologous synovial fibroblasts isolated from surgically harvested joint tissues, were stably modified with recombinant oncoretroviral vector (Moloney murine leukemia virus) to overexpress a secreted IL-1 inhibitor (IL-1Ra) [22, 23]. After expansion in culture the cells were injected into the diseased joint where they engraft in the synovial lining and continuously secrete the transgene product. This method demonstrated the feasibility of intra-articular gene delivery and was used successfully in a phase I human trial [24]. However, the procedure proved to be labor intensive, time consuming and tedious; its exorbitant cost made the procedure impractical for widespread clinical application, especially for a common, non-fatal disease.

It is important to note that cell in suspension (regardless the tissue of origin), following injected into the joint space, consistently engraft in the synovial lining; they do not adhere to or colonize articular cartilage. Cells surgically implanted in cartilage defects within a support matrix will remain localized, but lacking a method of physical containment loose cells will disperse throughout the capsular lining. Along these lines, much has been made in the literature of the anti-arthritic potential of so-called "mesenchymal stem cells" or MSCs [15, 16]. These cells are amenable to genetic modification and can be used as a vehicle for ex vivo gene transfer. However, it is our experience that MSCs in and of themselves are not

immune privileged and have no more regenerative or anti-inflammatory value than any other cell type injected into the joint. Most investigators have found that allogeneic MSCs are cleared very rapidly from the joints of experimental animals, with few cells remaining beyond 1–2 weeks.

## 7. Direct intra-articular gene transfer

Relative to the ex vivo approach, direct injection into the joint of recombinant vectors dramatically streamlines the gene delivery procedure [4]. A broad range of vector systems, both viral and non-viral have been evaluated for their efficiency of gene transfer to the joint tissues in situ [25–30]. While the use of non-viral gene delivery vehicles has certain theoretical advantages (larger payload, increased perception of safety, straightforward vector production, reduced costs) extensive *in vivo* testing in our laboratory, as well as in others, has shown that non-viral delivery of nucleic acids is currently not suitable for treating chronic articular diseases; the efficiency of delivery is exceedingly low and typically persists in the joint cells for no greater than 2–3 days. While non-viral formulations are often effective transfection reagents in the context of monolayer cell culture, efficacy in vitro does not equate to performance in vivo [31]. Despite claims in the literature regarding the treatment of OA, the use of these systems should be avoided as their pharmacokinetic profile is incompatible with the pathologic progression of OA.

Similarly, there are dozens of published papers that report remarkable efficacy following intra-articular injection of shRNAs, miRNAs, and circRNAs into the joints of animals either in suspension or complexed in nano- or micro-particles. As mentioned above, for an intracellular approach to be effective in OA, an extraordinarily high efficiency of delivery is required to the cells in target tissues in vivo. Moreover as gene expression is an ongoing process, interfering RNAs must be maintained at exceptionally high levels in a large proportion of cells and be continuously replenished to sustain gene silencing. While achievable when delivered in an exogenous expression cassette, it is not possible with the delivery of soluble or complexed inhibitory RNAs. These reports should be regarded with a healthy degree of skepticism.

Viral-based vector systems exploit the natural ability of a virus to deliver its genetic payload to a target cell with high efficiency. For the generation of a viral-based vector system, the coding sequences for viral proteins essential for replication are removed from the viral genome and the products are supplied in *trans* during vector propagation in permissive engineered cell lines. Several recombinant viral vector systems have shown the capacity to deliver exogenous genes to joint tissues and enable expression of therapeutic transgene products at levels sufficient to inhibit arthritic pathologies in laboratory animals. Among these are recombinant adenovirus [27], herpes simplex virus [28], adeno-associated virus (AAV) [32, 33], and lentivirus [29, 34] among others. Each of these systems has inherent advantages and limitations that dictate the applications for which they are best suited. Currently only two viral vectors, recombinant adenovirus and AAV, are in serious preclinical development for equine or human OA.

### 7.1 Recombinant adenovirus

First generation recombinant adenovirus provides highly efficient transduction of target cells in various connective tissues both in culture and in vivo [25, 26]. Several years ago this system was the workhorse vector of the field of musculoskeletal gene therapy [35]. Adenoviral vectors showed that the concept of direct

intra-articular gene transfer was capable of providing functional levels of transgene expression in the joints of animal models. In the first generation vectors the E1 and E3 genes required for immediate early stage gene expression and initiation of viral replication were deleted from the genome to prohibit viral replication in cells infected by the vector. Their removal also provided room for the insertion of an exogenous expression cassette [36].

The relative ease of production reduced the barrier to entry and provided gene transfer technology to any laboratory with basic molecular biology capabilities. Although viral replication was crippled in non-permissive cells, the vector still retained the majority of the native coding sequences. Leaky expression of viral proteins by transduced cells caused them to be eliminated in 2–3 weeks by adaptive cellular immune responses [27, 37]. Despite its transient nature, adenoviral gene delivery provided a burst of high level transgene expression sufficient to examine the biological activity of a specific gene product in vivo. Adenovirus has the reputation of causing acute toxicity from innate inflammatory responses, but much of this is due to low quality, inconsistent vector preparations containing high levels of cellular debris. Advances in adenoviral technology include the development of helper dependent systems in which the coding sequences for all viral proteins have been removed and are supplied during propagation by a second "helper" adenoviral vector, which is removed by differential centrifugation during purification [38]. These modifications allow for increased immune avoidance and long-term transgene expression without significant reduction in infection efficiency [39–41].

### 7.2 Adeno-associated virus

Of the well-characterized viral systems, AAV offers many advantages that favor its use for the treatment of arthritis: (1) The wild type virus is not associated with any pathologic human condition. (2) The recombinant form does not contain native viral coding sequences, which reduces its immunogenicity. (3) AAV can infect both dividing and quiescent cells. (4) Persistent transgenic expression in vivo has been observed in many applications, and (5) the recombinant form does not integrate into the genome of the target cell with significant frequency [42].

A further potential advantage is the relative simplicity of the AAV vector, which is comprised of an ~5000 nucleotide single-stranded DNA genome packaged in a small (20–30 nm), non-enveloped icosahedral particle by three capsid proteins, differing only at their N termini [43]. The only required *cis* elements on the vector DNA are 145 nucleotide-long inverted terminal repeats (ITRs) that flank the transgene expression cassette.

Fortuitously, with regard to veterinary medicine, in head to head comparisons equine synovial fibroblasts in culture are significantly more receptive to AAV transduction than their human counterparts. Preliminary evidence suggests increased expression of surface receptors between the two species in culture. How this discrepancy translates to the in vivo situation is unclear, as phase I testing in humans has just begun, but transgene expression is robust in equine joints.

Additionally, humans are natural hosts to wild type AAV infection and often have high circulating titers of neutralizing antibodies (NAb) to AAV capsids of several serotypes (primarily AAV2, AAV1, and to lesser extents AAV5) from prior infections with wild type virus. Horses, however, are not common hosts for wild type AAV infection, and distinct from humans, have low circulating NAb titers to most AAV vector serotypes. While NAb to AAV5 appears relatively frequently among horses, and one report describes increased NAb titers to AAV2 capsid in a small test sample, pre-existing NAb do not appear to be prevalent in the equine population nor at sufficient titer to prohibit effective gene delivery [44, 45].

Typically, following intra-articular injection of a recombinant virus, the overwhelming majority of genetically modified cells are found in the synovium, subsynovium and supporting capsular and ligamentous tissues. Chondrocytes, while receptive to genetic modification in culture, are not efficiently transduced in vivo due to the inability of most vector particles to effectively penetrate the dense cartilage ECM. The only exception is AAV whose small particle size permits its entry and diffusion through the dense cartilage ECM enabling interaction and transduction of chondrocytes deep within the cartilage. As chondrocyte dysfunction and cartilage degeneration are the characteristic pathologies of OA, the capacity to deliver therapeutic genes to chondrocytes is a clear advantage to this vector technology. Moreover, since these cells are highly stable, their modification with AAV provides the prospect of enduring transgenic expression [46].

## 8. Therapeutic strategies

Two complementary gene-based strategies have been investigated for OA. The first is geared toward chondroprotection, and involves delivery of gene products that enhance joint lubrication or block the activities of specific inflammatory cytokines that stimulate inflammation and the subsequent degeneration of cartilage ECM by articular chondrocytes [47–49]. Most studies of OA gene therapy have involved the delivery of the cDNA for interleukin-1 receptor antagonist (IL-1Ra) a competitive inhibitor of IL-1 signaling [50–55].

The second strategy is directed toward cartilage repair or regeneration using various anabolic, proliferative or chondrogenic agents to stimulate regional chondrocytes to proliferate and elaborate cartilage ECM. While these strategies appear attractive at the outset, unfortunately, as vectors (and cells) injected into the joint primarily interact with synovial fibroblasts, many growth factors that may stimulate cartilage repair or matrix synthesis by chondrocytes will likewise stimulate the abundant fibroblast populations in the synovium to generate undesirable, often dramatic adverse side effects. For example, intra-articular delivery of adenovirus containing the cDNA for TGF-β1 induces an extraordinarily potent fibrotic, chondro-osseous response in the synovium and joint capsule [56, 57]. Systemic pathologies such as pulmonary fibrosis in rats and death in rabbits occurred when TGF-β1 was expressed intra-articularly at high levels. Overexpression of TGF-β1 and BMP-2 has also been shown to induce the formation of osteophytes, ectopic cartilage and bone formation [56, 58]. Of the growth factor genes tested thus far, only IGF-1 has not been associated with an overt pathologic response [45, 59], but it has not been evaluated extensively. Concerns over potential side effects have generally limited the use of growth factor genes to localized applications in tissue engineering for cartilage repair, whereby chondrocytes or MSCs are modified in culture to express a specific growth factor before surgical implantation into focal cartilage lesions, In this manner, the expressed protein is localized to the defect, reducing exposure to adjacent tissues.

### 8.1 Interleukin-1 receptor antagonist

A consensus in the literature indicates that IL-1, synthesized locally by chondrocytes and synovial cells, is instrumental in driving OA progression [60, 61]. Found at increased levels in OA joints, IL-1 is the most potent physiological inducer of chondrocytic chondrolysis (the major route to cartilage loss in OA) [62]. Even at trace levels, IL-1 strongly inhibits ECM production in cartilage by blocking collagen type II and proteoglycan synthesis and enhancing chondrocyte apoptosis. At

slightly higher concentrations proteolytic enzyme synthesis is induced in chondrocytes, driving enhanced production of matrix metalloproteinases (MMPs) and aggrecanases that degrade the cartilaginous matrix [63]. As a primary mediator of the inflammatory cascade, IL-1 stimulates articular cells to produce a full complement of OA effector molecules, including cyclooxygenases I and II, nitric oxide, phospholipase A2, prostaglandin $E_2$, reactive oxygen species as well as inflammatory cytokines and chemokines. Release of these molecules further stimulates cartilage matrix degradation, bone erosion, synovitis and fibrosis. IL-1 is also suspected to mediate pain in OA, the most common reason for consulting a physician [64–67].

Traditional pharmacologic approaches have failed to produce clinically useful molecules for inhibiting IL-1 activity intra-articularly [68]. However, two naturally occurring proteins exist specifically for this purpose: IL-1Ra and the soluble IL-1 type II receptor (sIL-1RII) [69, 70]. IL-1Ra functions as a competitive inhibitor by binding to the type I IL-1 signaling receptor and preventing subsequent interaction with IL-1. Once bound, IL-1Ra fails to recruit the IL-1R accessory protein (IL-1-AcP) to the complex and prevents intracellular activation and signaling. The sIL-1RII molecule, in contrast, titrates IL-1 activity by binding directly to soluble IL-1 molecules and blocking interaction with the type I receptor [71, 72]. Despite differences in their modes of action, the two molecules inhibit IL-1 signaling with equal potency. In the context of gene therapy, IL-1Ra is a smaller protein and easier to express as a transgene product. The recombinant protein (anakinra/Kineret®) is well characterized and is approved for clinical use in humans for RA and other conditions in which IL-1 is known to pay a significant role [71, 72]. As anakinra is administered daily by subcutaneous injections of 150 mg, the risk of adverse response from overproduction intra-articularly is extremely small.

Commercially available ELISAs with specificity for IL-1Ra orthologs in human, mouse and horse permit sensitive quantitation in culture media and biological fluids. Analysis of synovial fluid permits the use of IL-1Ra as a quantitative reporter of total gene transfer and therapeutic gene expression, allowing direct comparison of various delivery platforms. With respect to OA, IL-1Ra does not require sophisticated regulation. The goal is simply to express IL-1Ra at levels 10–100 fold over IL-1, where it completely inhibits IL-1 signaling activity. Once the threshold for efficacy has been achieved, expression beyond this has no adverse effect [73].

It is possible that a dual therapy combining elements of chondroprotection and regeneration could both inhibit degeneration and stimulate cartilage repair [48, 74]. Such a strategy, though, would likely require gene delivery via separate vectors to account for differences in their expression patterns for safe, effective application.

## 9. Preclinical studies

Following a series of preclinical successes in small laboratory animals demonstrating the proof of concept for direct viral-mediated gene delivery to joints, studies were performed to evaluate direct viral mediated gene delivery to equine joints using a first-generation adenoviral vector containing the cDNA for equine IL-1Ra. Administration of Ad.eqIL-1Ra in the joints of healthy horses, produced dose-dependent increases in IL-1Ra levels in synovial fluid aspirates. However, the highest viral dose tested, $5 \times 10^{11}$ viral particles (vp) produced an acute synovitis [21].

To explore the capacity of Ad.eqIL-1Ra to inhibit OA pathologies, an osteochondral fragment (OCF) model of OA was used [21, 75]. In this system, a small osteochondral chip is surgically generated off the distal radial carpal bone of the midcarpal joint. Following a brief interval to recover from surgery, animals in the

treatment group are injected in the OCF joint with the vector, while control animals receive saline [21]. The horses are then exercised 5 days/week on a high-speed treadmill, which, in the context of the osteochondral fracture, generates predictable pathologic lesions that mimic the onset of equine disease [21]. ELISA analysis of joint aspirates showed a peak in eqIL-1Ra expression at 7 days post injection which gradually diminished over a period of 28 days. Clinical examinations indicated that the expression of IL-1Ra decreased joint pain and synovial effusion relative to untreated horses, and protected the cartilage from the loss of proteoglycans.

These findings provided strong support for local gene delivery of IL-1Ra in large mammalian joints. A central limitation, however, was the use of the first generation adenovirus. In later work, we found articular tissues to be highly immune sensitive to the expression of foreign proteins, such that cells expressing foreign non-homologous transgene products or viral proteins are recognized by cell-mediated immune responses which lead to abbreviated persistence of transgenic expression in vivo [37]. Thus while the results showed promise, they indicated an intense need for an improved, immune stealthy vector system.

### 9.1 AAV-mediated gene delivery to equine joints

The results of studies of other gene therapy applications, such as hemophilia, indicated that long-term transgene expression was achievable following direct delivery of AAV vectors. The results of exploratory experiments in joints were disappointing. Transgene expression from conventional single-strand AAV vectors required several days or weeks to onset with marginal levels of protein production intra-articularly, a pattern that prevented testing in experimental disease models.

AAV transduction efficiency is known to be enhanced by mechanisms associated with intracellular stress. Certain stimuli, such as UV radiation, which increase the production of DNA synthesis and repair enzymes, significantly enhance intra-articular transgene expression from conventional AAV vectors [33, 76, 77], which indicates that second strand DNA synthesis is rate-limiting in joint tissues. Accordingly, AAV vectors that are self-complementary (sc) (i.e. double stranded, containing both + and – DNA strands) generated through the use of half-genome sized vector plasmids, or those containing a mutation in one of the terminal resolution sequences of the AAV ITRs [78, 79], provided ~20-fold enhancement of gene expression, with rapid onset in synovial and capsular cells in vitro and in vivo [80]. This adaptation was found to provide transduction and transgene expression profiles comparable to that provided by adenovirus. The requirement for a half-sized genome, however, limits the size of the transgene to about 1000–1200 base pairs [79].

Following encouraging results with scAAV vectors in the joints of laboratory animals [80], studies of AAV gene transfer shifted to the equine model to assess more clearly its utility for therapeutic gene delivery in large OA joints [19, 81]. As before, the carpal and MCP joints of the equine forelimbs were targeted for injection. In pilot studies, AAV gene delivery to healthy joints was examined using vectors containing the cDNAs for human IL-1Ra (AAV.hIL-Ra) and green fluorescent protein (AAV.GFP) [19]. In the animals receiving AAV.hIL-1Ra, synovial fluids were aspirated periodically over a period of several weeks. Animals receiving AAV.GFP were euthanized 14 days after injection and the distribution of fluorescence in the joint tissues was used to determine the number and locations of the cells modified by the AAV virus following intra-articular injection.

AAV gene delivery in the equine joints was capable of elevating the steady state hIL-1Ra in synovial fluid to levels equivalent to or greater than observed previously in rodents [19]. Analysis of GFP fluorescence showed that the vast majority of the

transgene expression originated from the fibroblasts resident in the synovial lining. Fluorescent cells in the articular cartilage, though visible, were sparse, and GFP expression was faint. Peak levels of hIL-1Ra occurred at 1–2 weeks post-injection, but steadily declined over a period of 5 weeks. Studies in nude rats indicated that the abbreviated transgene expression was due to immune elimination of the cells expressing the xenogeneic human IL-1Ra protein [37].

### 9.2 Codon optimization of the equine IL-1Ra cDNA

The commercial release (R&D Systems, Minneapolis, MN) of an ELISA kit specific for the equine IL-1Ra ortholog in 2010 proved to an enabling technology, allowing for the first time definitive quantification of the equine transgene intra-articularly [82]. Prior to this point, expression data relied on inter-species cross-reactivity between human and murine ELISAs, results which were inconsistent and highly error prone.

To examine AAV-mediated transgene expression in the absence of immune interference, the human cDNA was replaced with the homologous equine IL-1Ra. To overcome initial problems with low production levels, codon-optimization resulted in >50-fold amplification in IL-1Ra secretion [18, 82]. For use in safety studies for the FDA, the cDNAs for human and rat IL-1Ra were also codon-optimized using the same algorithm [55]. After packaging in the AAV capsid, infection of synovial fibroblast cultures over a range of doses generated exceptionally high levels of IL-1Ra protein in conditioned medium, which exceeded 10 μg/ml at a vector dose of $10^5$ vg/cell [18].

Using the optimized AAV.eqIL-1Ra vector, a series of pharmacokinetic studies were performed to establish vector dose expression profiles following intra-articular injection [18]. In each of six horses, the midcarpal and MCP joints of both forelimbs were injected with AAV.eqIL-1Ra at doses ranging from $5 \times 10^{10}$ to $5 \times 10^{12}$ vg; the remaining joint received an equivalent volume of saline and served as a negative control. Analysis of synovial fluid, peripheral blood and urine collected periodically over a period of 6 months showed dose-related increases in the eqIL-1Ra content in synovial fluid at 2 weeks of injection, with peak production between 4 and 8 weeks. At the highest vector dose synovial fluid IL-1Ra levels exceeded 40 ng/ml, ~400-fold higher than endogenous synthesis. Importantly IL-1Ra production remained at these levels for the duration of the 6-month study. Despite simultaneous injection of recombinant AAV in three forelimb joints, no adverse effects were observed. IL-1Ra in blood serum, urine and synovial fluid of control joints remained at pre-injection levels (<100 pg/ml) throughout [18].

### 9.3 AAV gene delivery in naturally occurring OA

As discussed previously, the pathologic progression of OA induces sweeping changes in the architecture, cellularity and activation of the articular tissues. In conjunction with vector dosing, a series of tracking studies were performed to examine the impact of the OA environment on transgene expression and the biodistribution of the vector DNA and transduced cells [18]. Using a dose of $5 \times 10^{12}$ vg, AAV.GFP was injected into one forelimb joint of several healthy horses, and horses with advanced naturally-occurring OA. Analysis of tissue samples 2 weeks later showed GFP fluorescence in healthy joints was concentrated in the synovial lining, with only a handful of GFP+ cells visible in cartilage shavings (**Figure 1**). In joints with advanced OA, there was a striking increase in GFP expression in all joint tissues particularly in articular cartilage. In synovium, enhanced GFP expression was due to the increased cellularity from local inflammation. Although fluorescence

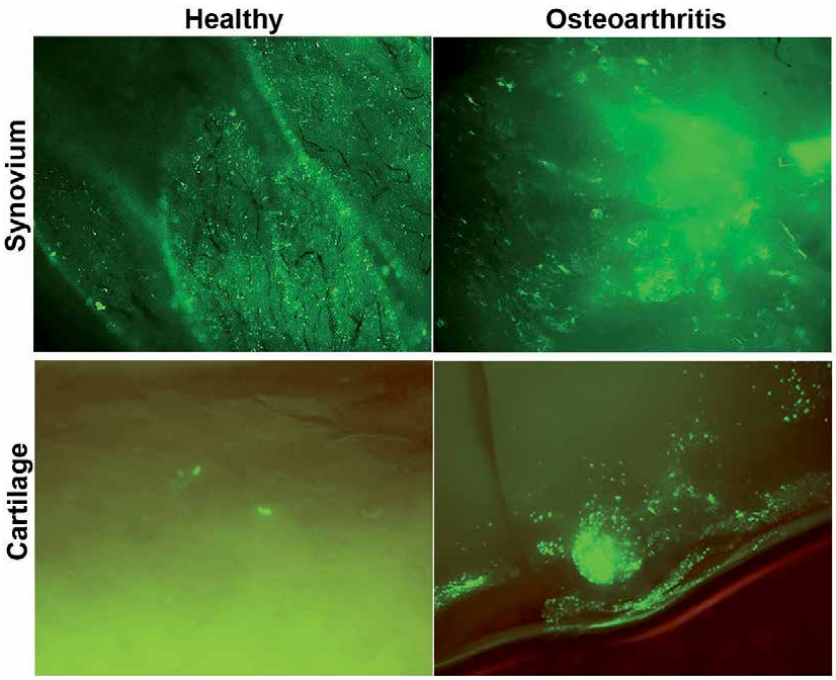


**Figure 1.**
*Representative fluorescence activity in synovium (top row) and cartilage (bottom row) following intra-articular injection of an AAV vector containing the cDNA for green fluorescent protein (GFP) into healthy carpal joints or those with naturally-occurring OA.*

was greater in all cartilage shavings, GFP expression in regions containing visibly damaged cartilage was markedly increased. GFP+ chondrocytes appeared throughout the damaged regions, particularly in clusters of proliferating chondrocytes, characteristic of OA cartilage.

In both healthy and OA joints receiving virus, no GFP+ cells were detected in extra-articular tissues, and qPCR analyses showed that 99.7% of the vector DNA was localized within the cartilage and synovium of the injected joint. Similar patterns of biodistribution were noted by Goodrich et al. [81]. While the enhanced transduction of OA chondrocytes might be presumed to arise from increased vector access from ECM degradation, qPCR analyses showed the vector DNA content in the chondrocytes of healthy and OA cartilage was essentially the same, suggesting that the increased transgene expression arose from increased metabolism due to inflammatory and stress induced activation [18].

Transcription from the CMV immediate early promoter, which drives the scAAV vector expression cassette, is induced in response to NF-κB activation and signal transduction from p38 and other stress-activated protein kinases. Similar stress-related induction of this promoter serves to re-activate human CMV from latency, and is required for expression of genes necessary for DNA replication [83–87]. In the same manner, inflammation and cellular stress can significantly increase the transcription and expression of transgenes under control of the CMV immediate early promoter [87–90]. In these respects, OA cartilage is highly enriched with stress-activated chondrocytes [91], especially at sites of cartilage degradation, where GFP expression was greatest.

Although several reports describe the generation of synthetic inflammation-inducible promoter systems for gene therapy applications, the AAV vector discussed here, at least within the context of a large mammalian joint, appears to be highly responsive to the OA environment and innately disease activated. The regional

differences in GFP expression seen in OA cartilage indicate the potential to direct therapeutic transgene expression preferentially to areas of articular cartilage under the greatest pathologic stress. This perhaps lays the groundwork for development of vectors for disease-targeted anabolic stimulation of cartilage repair and regeneration.

### 9.4 AAV.eqIL-1Ra delivery and expression in the OCF model

The OCF model was adapted to determine if the levels of equine IL-1Ra generated by AAV gene transfer were sufficient to mediate an appropriate biologic response [92]. Following creation of the osteochondral fracture, the OCF joints in the treated animals were injected with $5 \times 10^{12}$ vg AAV.eqIL-1Ra, while controls animals received an equal volume of saline. One week later the animals were placed on a 5 day/week athletic training protocol for a period of 10 weeks. In this acute injury model, mean eqIL-1Ra expression was initially ~4-fold higher than observed in healthy joints at the same viral dose and correlated directly with the severity of joint pathology at the time of vector delivery. Over the 10 week training period, eqIL-1Ra expression gradually diminished to ~60 ng/ml, similar to that seen with AAV gene transfer in normal joints. Despite variable expression among animals the steady-state eqIL-1Ra in synovial fluids exceeded that of IL-1 by >500-fold in all animals. In agreement with increased IL-1Ra, the treated horses showed a reduction synovial fluid $PGE_2$ levels and a progressive reduction in joint pain. Improved joint function was accompanied by significant reduction in joint pathology by both arthroscopy and MRI. By both diagnostics, the treated animals showed significant reductions in synovial effusion and marrow edema, local protection of cartilage and enhanced repair of the osteochondral fragment [92].

Consistent with the findings of others [45, 93], we observed an increase in AAV capsid-specific neutralizing antibody (NAb) titer over time in both the blood serum and synovial fluid of the horses receiving the AAV vector [92]. The NAb titer in synovial fluids was consistently several-fold higher than in blood but had no obvious effect on transgene expression.

Humans are natural hosts to wild type AAV infection and often have high circulating titers of neutralizing antibodies (NAb) to AAV capsids of several serotypes (primarily AAV2, AAV1, and to lesser extents AAV5) from prior infections with wild type virus. Horses, however, are not common hosts for wild type AAV infection and distinct from humans, have low circulating titers to most AAV vector serotypes. While NAb to AAV5 appears to be relatively common among horses, and one report describes increased NAb titers to AAV2 capsid in a small test sample, pre-existing NAb do not appear to be prevalent in the equine population nor at sufficient titer to prohibit effective gene delivery. As indicated above, high levels of capsid serotype specific NAb will arise from prior treatment with an AAV vector, which can inhibit the efficacy of subsequent vector administration. There is evidence that vector neutralization can be averted through the use of an alternate capsid serotype.

### 9.5 Long-term efficacy

The primary advantage of a gene-based therapy for OA lies with the capacity for sustained local delivery of anti-arthritic agents and the promise long term therapeutic benefit. To address this, we recently completed a series of studies to assess the safety and efficacy of AAV.eqIL-1Ra delivery in a chronic model of joint disease over the course of a year. For the chronic model, the 10 weeks OCF protocol was used to induce joint pathology consistent with early symptomatic disease. At the

completion of the athletic training period, $5 \times 10^{12}$ vg of the AAV.eqIL-1Ra vector was injected into the OCF joint, and a 3 day/week training regimen was instituted to maintain a slow but progressive degenerative condition for the following 12 months.

Immediately prior to injection, and then at 2 weeks and monthly thereafter, peripheral blood, urine and synovial fluids were collected, and joint pain and kinematic assessments were performed. Radiographic and MR imaging of both midcarpal joints was performed prior to injection and then at 6- and 12-month time points. Arthroscopic examination of the joints was performed at endpoint and digitally recorded, and synovial and articular cartilage biopsies were taken for histologic examination. At the conclusion of the year-long protocol all animals in the treatment group and five animals from the Control group were euthanized for biodistribution and toxicology analysis.

Analysis of synovial fluids showed that high IL-1Ra levels of 40–50 ng/ml were sustained over the 12-month course of the study. In the chronic OCF model transgenic IL-1Ra expression was far more consistent among individual animals than in joints with an acute osteochondral fracture. Relative to arthritic controls, the treated animals showed a ~40% reduction in lameness, indicative of reduced joint pain and improved mobility. By MRI assessment, joint pathology in the was reduced by ~28% relative to baseline disease, while in control joints the overall pathology was largely unchanged. Relative to pretreatment levels the treated group showed ~28% improvement in all major OA pathologies relative to baseline while in arthritic controls pathologic scores remained unchanged or increased in severity.

### 9.6 Toxicology and biodistribution

To establish a qualified biosafety profile for AAV.IL-1Ra gene transfer in a large mammalian joint, formal preclinical toxicology and biodistribution studies were performed addressing the acute and long-term phases of vector delivery. In the Acute Phase studies, early stage disease was induced in one midcarpal joint using the OCF protocol. Three horses each were injected with $5 \times 10^{12}$ vg AAV.eqIL-1Ra, 1× anticipated clinical dose, and three horses with $5 \times 10^{13}$, 10× clinical dose, intended to represent a "worst case scenario." For the long-term toxicology studies, each of the 10 horses from the treated group in the 12-month study and 5 horses randomly selected from the control group were euthanized for necropsy. Following euthanasia, samples from more than 50 tissues were collected for histopathologic evaluation or DNA extraction and PCR analysis of vector genome content.

In all animals injected with AAV.eqIL-1Ra at the 1× dose, high vector genome copies ($10^4$–$10^6$) were detected by qPCR in the synovium and cartilage, which were equivalent in animals euthanized at 2 weeks and 12 months post-injection. No vector DNA was detected in extra-articular tissues. No pathologic response associated with vector injection was observed in any tissue.

The cumulative data from these pharmacokinetic, toxicology and efficacy studies in the equine model demonstrate that a gene-based therapy using recombinant AAV can provide safe, long-term, effective delivery of anti-arthritic proteins, such as IL-1Ra, in large mammalian joints. The results of these safety and efficacy studies in horses formed the bases for a successful IND application and the initiation of a phase I trial of AAV-IL-1Ra delivery for knee OA.

### 9.7 Gene delivery with high-capacity adenovirus

Recently, studies involving gene delivery with HD.Ad have begun to move toward clinical studies, at least in human OA. Initial studies in mice using

intra-articular HD.Ad-mediated delivery of the cDNA for lubricin (Prg4) showed a marked chondroprotective effect, maintaining matrix volume and prevention of degeneration in a cruciate ligament transection (CLT) PTOA model [39].

Progressing from these results, studies were performed to examine the therapeutic capacity of IL-1Ra gene delivery via HD.Ad in mice and the forelimb joints of horses [94]. In most gene therapy applications a strong, constitutively active promoter sequence, such as the CMV promoter/enhancer or the eukaryotic translation initiation factor 1α (EIF-1α) promoter is used. In this case, however, transgene expression was driven by an inflammation-inducible promoter comprised of a minimal endothelial cell leukocyte adhesion molecule (ELAM1) promoter linked to multiple upstream NF-κB recognition elements. The rationale being that therapeutic transgene expression would be delimited specifically to inflammatory flares.

Similar to prior studies with Prg4, HD.Ad delivery of the homologous murine IL-1Ra transgene was found to inhibit osteophyte formation and cartilage erosion in the CLT defect model [94]. Importantly, vector delivery was also associated with a significant reduction in pain sensitivity. Following preliminary dosing studies in healthy equine joints, HD.Ad was used to deliver the eqIL-1Ra cDNA into the carpal joints of horses following surgical generation of the osteochondral lesion in the OCF model. IL-1Ra levels in the injected joints rose to ~20 ng/ml within the first week, but then dropped about 10 fold by week 2 and were near endogenous background by week 3. Despite the relative brevity of expression, the treated animals showed significant improvement in lameness, reduced joint effusion, synovitis and osteocyte formation and improved cartilage matrix integrity [94].

Due its relatively large size, the adenoviral particle cannot penetrate the ECM of the synovium or cartilage and remains constrained to cells residing in superficial regions [39]. Interestingly in both reports where intra-articular transgene expression was quantified, a 90–99% loss in therapeutic transgene expression was observed within 1–2 weeks of vector injection, regardless of whether an inducible promoter was used [39, 94]. While transgene expression seems to persist long-term, therapeutic protein levels appear at trace levels. Given this profile, it will be interesting to see how this platform moves ahead in the future.

## 10. Conclusions

These data altogether show that in large mammalian joints, local gene transfer can provide persistent IL-1Ra transgene expression at therapeutically relevant levels. Despite variable expression among treated joints in the context of acute inflammation, sustained IL-1Ra expression provides meaningful benefit, such that a single injection reduces joint pain and intra-articular inflammation, and improves repair of the damaged bone and protects cartilage against degradation. No adverse response to the vectors or transgene have been observed with either AAV or HD.Ad, and at least within the equine system local overexpression of IL-1Ra provides no apparent risk of systemic immunosuppression.

Having established safety and efficacy of IL-1Ra gene delivery in the equine joint, the next stage in development would be the move into field testing with client animals. Given the limited resources available for equine research, such a large and costly undertaking is likely feasible only through support from partners in the veterinary pharmaceutical industry or private investors looking to advance the treatment methods toward commercialization. In this respect, questions of market size, cost of goods and profitability move to the forefront. Currently human gene-based therapies come with a substantial price tag, and range at the high end from $450,000 per eye for Luxterna® for congenital retinal degeneration to $2,100,000

for Zolgensma®, a gene correction therapy for spinal muscular atrophy in infants. Both of these "drugs" employ AAV as a vector. As the popularity of gene-based therapies continues to advance, production costs will likely fall considerably as the field grows and therapies with greater efficacy emerge. Among these business issues, questions regarding genetic enhancement in the racing industry will need to be addressed and resolved.

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## Conflict of interest

Steve Ghivizzani is a founder and share-holder in Genascence Inc., a company pursuing development and commercialization of gene therapies for inflammatory conditions.

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Section 2

# Reproduction, Locomotion and Skin

Chapter 4

# Equine Reproduction: Seasonality, Endometritis, and Twinning in the Mare

*David A. Trundell*

## Abstract

This chapter reviews the seasonality and effect of photoperiod in mares and how, as clinicians, we can shorten the vernal transition period and improve our efficiency in getting mares in foal. Different protocols have been utilized to shorten the vernal transition, and each will be discussed. We will also examine endometritis in the mare. The role of biofilms in causing endometritis in our equine patients, and potential treatment plans, in particular breeding the dirty mare, will be reviewed. Finally, we will examine the effect of twin pregnancies in the mare, the most common cause of noninfectious abortion, and offer two management therapies for dealing with twinning in the mare.

**Keywords:** mare, endometritis, photoperiod, twins, kisspeptin

## 1. Introduction

Our knowledge of reproduction in the mare has expanded considerably in the last few decades and continues at pace. The scope of this chapter is to try to answer some of the most common questions an attending veterinarian may be asked to deal with, namely, shortening the vernal transition, dealing with postmating endometritis, and dealing with twin pregnancy, all of which can frustrate even the seasoned clinician. The aim of this chapter is to give the reader the background knowledge of why certain therapies may or may not work and to give the clinician workable solutions to some of the more common aspects of clinical reproductive work in the mare.

## 2. Mare reproduction

### 2.1 Seasonality and photoperiod

The mare is a seasonal, long-day, polyestrous animal, meaning that her reproductive status is intrinsically linked to photoperiod. In the Northern Hemisphere, the normal physiologic breeding season starts in spring (April) and continues through to autumn (September). This corresponds to increasing photoperiod (increasing daylight length). The light signals are received by the retina, processed by melanopsin [1], which, as a pigment, is located in retinal ganglion cells, themselves being photosensitive. This information reaches the suprachiasmatic nucleus via the retino-hypothalamic tract [2]. Melatonin produced in the pineal

gland is suppressed during hours of darkness. This fall in melatonin as photoperiod increases during the spring stimulates the mare to, reproductively, enter the spring or vernal transition. The classical hypothalamic–pituitary-ovarian axis, known to many clinicians, is oversimplified.

In the last decade or so, numerous authors have examined the role of kisspeptin neurons in the relation of cyclicity in many animal models, including the mare. It appears that increasing photoperiod stimulates the main kisspeptin neuron population located in the arcuate nucleus of the hypothalamus [3]. Distinctly, the horse does not appear to have a secondary population of kisspeptin neurons in the preoptic area, unlike cattle and sheep [4]. A small population of these receptors are located within the ventromedial nucleus of the hypothalamus [5]. The kisspeptin neuron fibers are found throughout the septo-preoptic region, an area that the majority of gonadotrophin-releasing hormone (GnRH) neurons are located [6]. In 2007, Smith et al. were the first to note that kisspeptin neurons may be influenced by photoperiod [7]. They saw an increase in KISS1 mRNA in the arcuate nucleus in sheep in their physiologic breeding season. Our understanding of the role of kisspeptin in the mare and effects on her reproductive status is derived mainly from studies on sheep models; there have been limited studies in the mare.

In the sheep model, it appears that in artificially decreasing photoperiod, thereby eliciting a stimulatory effect in sheep (sheep are short-day breeders), there is a corresponding increase in the number of kisspeptin neurons [8]. Kisspeptin neurons appear to form numerous synapses with other neurons that produce dopamine [9], melanocyte-stimulating hormone [10], and GnRH [11], among others. The regulation of kisspeptin is still not fully understood, but it appears that it may consist of a combination of negative feedback via estrogen in the sheep model [12] and via dopamine. The dopamine neurons in the retrochiasmatic area of the hypothalamus exerts an inhibitory effect on GnRH secretion during anestrous but not during the physiologic breeding season [13]. There is an upregulation of dopamine receptors in the kisspeptin neurons during breeding season [13]. There appears to be a seasonal difference in the number of kisspeptin neurons in the population found in the arcuate nucleus of the hypothalamus, but no seasonal difference in the preoptic population. This has been confirmed in the mare [4]. This seasonality difference appears to be driven, or at least modulated, by photoperiod. However, from sheep models, we know kisspeptin does not express melatonin receptors [14], and it is proposed that any effect of melatonin on the functionality of kisspeptin may be indirect [3]. It has also been postulated there is an indirect effect of photoperiod that is modulated via the thyroid hormones [3]. Nearly all preoptic kisspeptin neurons express thyroid receptors [15]. It has been shown that the thyrotropes (the cells secreting these hormones) located in the pars tuberalis of the rostral adenohypophysis are melatonin responsive [16, 17]. It appears these cells display dramatic melatonin-dependent photoperiodic changes; under short photoperiod, there is low level expression, while under long photoperiod, there is high level expression [18, 19].

GnRH is a 10 amino acid peptide secreted by the hypothalamus. Its secretion, regulated by decreasing melatonin during increased photoperiod, is modulated via kisspeptin neurons mentioned above. Secretion of this peptide enters the hypothalamic-hypophyseal blood portal system, which bathes over the gonadotrophs located in the anterior pituitary, cells that synthesize and secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). During the vernal transition, there are ever-increasing circulating concentrations of FSH and LH. This increase is gradual, with LH in particular remaining low prior to the first ovulation. It is thought that the low circulating LH is due to the low storage of LH within the gonadotrophs [20]. Under the regulation described above, the increased GnRH stimulates FSH secretion and thus drives the growth of ovarian follicles. However during the vernal transition,

mares undergo several follicular waves. Under these waves, follicles seemingly grow (although they rarely reach pre-ovulatory size), only to regress, and be replaced by another follicular wave. In a report by Watson et al. [21], only 31% of all FSH surges during this transition period lead to the production of a follicular wave. Only one a follicle under the influence of FSH, produces sufficient estrogen, will a follicle ovulate under the LH surge. Estrogen appears to be involved in numerous components of the mare reproductive cycle. It appears that estrogen has a negative and a positive feedback mechanism on kisspeptin within the arcuate nucleus [7, 22], while the fall in circulating estrogen levels leads to increased LH secretion at the level of the anterior pituitary. During the peri-ovulatory surge, declining FSH under the influence of increasing estrogen and inhibin production from the growing follicle, LH reaches maximum concentrations 1 day post ovulation. LH promotes the maturation and subsequent ovulation of the follicle.

Numerous issues can be seen related to this complex reproductive cycle. During the vernal transition, mares may be presented with multiple small follicles evident on the ovaries via transrectal ultrasonography. Follicles may grow and then regress. The unpredictable nature of the folliculogenesis and the exact duration of the vernal transition are not only frustrating for the owner but a headache for the attending clinician. It is imperative that the clinician has a thorough understanding of the mechanisms at play during this period. Having this knowledge will allow the veterinarian to potentially manipulate these mechanisms and the hormones involved and shorten this phase. Understanding the systems at play allows for proper management by the veterinarian and will increase the productivity of the mares under their control.

### *2.1.1 Taking control of the vernal transition*

#### *2.1.1.1 Light manipulation*

In the Northern Hemisphere, it has become standard industry practice to place mares under light regimes starting December 1 [23] in the breeding of thoroughbred racehorses. Nonetheless many breeders of other types of horses also utilize light manipulation to hasten time to first ovulation. A 200 watt incandescent light bulb in a 12 × 12 foot stall is sufficient to begin stimulating follicular activity. Typically light is added to the evening; mares are brought into their stalls from pasture before dusk and then exposed to artificial light until 23.00 hours. It is now known that light in the short wave spectrum (465–485 nm) is most effective at inhibiting melatonin production [24]. It is important to allow the mare to receive some hours of darkness and that exposing them constantly to light stimulation actually extends the anestrous period. On larger horse farms, the use of indoor schools, housing numerous barren, and/or maiden mares can be effective. However, in those large building, it is important to check the light intensity at all areas. A loose rule of thumb is that the light should cast no shadows and that you should be able to read a newspaper anywhere in the building. For those who want to be more scientific in their approach, a photometer can be utilized. Exposure to this light regimen should continue for at least 70 days. Within 60 days, most mares will show some follicular activity, with the majority expressing their first ovulation with 70 days of onset of light exposure. While exposure to artificial light does not eliminate the vernal transition, it simply moves it forward. In the natural physiologic breeding season, the mare will not display follicular activity until April, and many will not experience their first ovulation of the season until May. Moving the vernal transition forward several months allows the clinician to start breeding these mares in February and March.

There are disadvantages to this regime. The mares must be housed either in stalls or a large barn, which intensifies their maintenance. Stalls need to be cleaned out regularly, adding to staffing responsibilities. In addition mares housed in groups in barns allow for opportune risk for injury especially if they are fed together—the lowly mare has nowhere to run from her aggressive barn mate.

*2.1.1.2 Equilume™ face mask*

To counter the problems of intensified housing of mares under light, researchers in Ireland have come up with a novel way to provide the mare with enough stimulatory light to advance the physiologic breeding season, while in their pasture. These masks provide blue light to one eye. It was concluded by Murphy et al. [23] that one light stimulation to one eye is sufficient to stimulate onset of follicular activity, is as effective as stall or barn light regimes, but also has added benefits of being more economic, especially to the small-scale breeder, while increasing horse welfare. Horses can remain out in their pastures, which reduces stress on these animals. However, these are currently one-time use items (as in for one season) and cost a few hundred dollars per mask, and occasionally inquisitive mares may pull off the face mask of another. On larger studs where the infrastructure for housing numerous animals under artificial lights, and with adequate staffing, it appears that the traditional light regimes remain the favor. Despite this, there is a place of the use for such masks. Mares seem to lose interest in the mask of other horses within a few days (the likelihood of a mask being pulled off is highest at the start). Providing there is nothing in the pasture on which the mare could hook the mask on (access to tree branches or fence posts above rails), and given that it is securely fastened, the mask should remain in place. Those that have only a handful of broodmares may prefer this method, as it reduces labor costs involved with stalling the mare.

*2.1.1.3 Kisspeptin supplementation*

As described above, kisspeptin appears to regulate GnRH secretion. As of yet, no commercial kisspeptin product is available. A recent report by Australian researchers found that although kisspeptin administered to mares as a constant rate infusion elevated circulating LH levels, it did not lead to an LH surge and therefore did not evoke ovulations within their group of mares during the vernal transition [4]. It remains to be seen whether the use of kisspeptin may shorten time to first ovulation, by potentially driving follicle maturation, under influence of LH, without necessarily causing ovulation.

*2.1.1.4 Use of dopamine antagonists (domperidone, sulpiride)*

As shown, dopamine plays an essential role in the stimulation of the reproductive axis in the mare. Dopamine has an inhibitory effect on GnRH release. For completeness it appears that dopamine antagonist acts via the stimulation of prolactin. For both domperidone and sulpiride, the dose is 1 mg/kg given PO and IM, respectively. Both are administered once daily for 25 days. The reports on the efficacy of the use of these preparations to shorten time to first ovulation in the mare are conflicting. A recent study by Mari et al. [25], comparing the two products, found that sulpiride significantly shortened time to pregnancy establishment (61 days) compared with domperidone-treated mares (83 days). That group concluded sulpiride is effective in advancing the vernal transition, whereas domperidone is only effective in some mares.

*2.1.1.5 Use of progesterone*

As mentioned the long transitional phase exhibited by mares is characterized by numerous follicular waves, unpredictable follicle growth, and follicle regression. Many protocols have examined the use of progesterone (P4) to dampen down these unpredictable features of the transitional phase and to drive a follicle to become dominant and one that will ovulate. The physiological effect of exogenous progesterone supplementation is relatively simple. P4 exerts an inhibitory mechanism with regard to LH but has minimal effect on FSH secretion. As described earlier, LH is required for maturation and final ovulation of the dominant follicle. While the mare is exposed to exogenous P4, LH is blocked at the level of the anterior pituitary, while FSH continues to be secreted. Therefore the follicles continue to grow under influence of FSH. Once the exogenous source of P4 is removed, this sudden fall in circulating P4 stimulates the LH surge, leading to final maturation and ovulation of a dominant follicle. The typical regimen is a dietary supplementation with altrenogest (Regumate®) at 0.044 mg/kg PO for 10 days. Injectable P4 products are becoming more routinely available. In the USA compounded products such as progesterone in oil can be utilized. Controlled release of P4 from these compounds last between 7 and 10 days. Daily application of oral altrenogest can be time-consuming. There also is a risk of noncompliance, should a mare be difficult to catch, not to mention potential side effect for the operator. The use of these long-acting P4 BioRelease products have been shown to be effective [26]. It appears that the use of exogenous P4 has maximal benefits when the mare exhibits a follicle of at least 20 mm in diameter and when administered in deep anestrous has little effect [27, 28]. An injectable altrenogest marketed via BOVA has recently become available in the UK for the first time, although no studies on its efficacy are currently available.

## 2.2 Endometritis

*2.2.1 Endometritis in the mare*

Endometritis is a leading cause of subfertility in the mare [29] and is the third most reported condition seen in our equine patients [30]. Endometritis, simply, the inflammation of the lining of the uterus, has historically been attributed to bacterial colonization and infection of the uterus. However there are a subgroup of mares that will exhibit persistent mating-induced endometritis (PMIE), in the absence of bacterial isolation. Furthermore we will also examine in the chapter the role of biofilm formation and bacterial endometritis.

Post breeding, a normal, physiologic endometritis will be observed in all mares [31]. This normal, transient event, which peaks around 8 hours post insemination, occurs to eliminate excessive spermatozoa, seminal plasma, and contaminants from the uterus [32]. This physiologic response should be over by 48 hours post insemination [33]. The subgroup of mares that experience PMIE appear to have an altered inflammatory response to the presence of spermatozoa and seminal plasma within the uterus. These mares tend to be aged, have increased parity, may exhibit chronic inflammatory changes within the endometrium [34], and exhibit failure to clear intrauterine bacterial challenges [35]. Susceptibility rates among thoroughbred broodmares is 15% [36], and crucially the early embryonic death rate is three times higher in this group of mares [37]. A persistent inflammatory uterine environment 5 days post fertilization is incompatible with embryo survival [38].

It has long been proposed that mares are classified as either susceptible or resistant to PMIE. It has been shown that susceptible mares do have altered protein

composition of their endometrial fluid [39] and these mares also exhibit higher levels of pro-inflammatory cytokines [40, 41]. It has also been shown that these mares with a delayed uterine clearance have contractile defects of the endometrium, possibly contributing to this delay in uterine fluid clearance [42]. It has been proposed that nitric oxide mediates smooth muscle relaxation [43]. It also important that mares that fall into this subgroup tend to have poor perineal conformation and a forward tilt to the uterus, such that it sits over the pelvic brim. It is therefore paramount to be able to identify these mares and initiate appropriate therapy.

Bacterial endometritis in the mare is primarily caused by four pathogenic species: *Streptococcus equi* subspecies *zooepidemicus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* [44–47]. By far the most commonly isolated are *Escherichia coli* and *Streptococcus equi* subspecies *zooepidemicus* (**Figure 1**). Diagnosis of bacterial endometritis is based on transrectal ultrasonography, uterine culture, and uterine cytology. In transrectal ultrasonography, these mares may show increased uterine edema and increased uterine luminal fluid, which may be echogenic in nature. Any mare that has uterine fluid with an accompanying corpus luteum (CL) should be highly suspected to have a uterine infection. There are now several reports of bacterial species becoming resistant to commonly used antimicrobials especially gram-negative species. It is therefore paramount that the attending veterinarian takes cultures to identify which bacterial species are present and then to select an appropriate antimicrobial based on antibiotic sensitivities.

A list of commonly used intrauterine antibiotics and dosages can be found in **Table 1** in the therapy section.

### *2.2.2 Fungal endometritis*

Around 5 percent of infectious endometritis are attributed to fungal organisms [48], of which *Candida* spp., *Aspergillus* spp., and *Mucor* spp. are most frequently isolated. Again, mares that have anatomical defects are predisposed, and the use of previous intrauterine antimicrobial therapy is thought to increase the likelihood of fungal infections. Two schools of thought exist as to why this may be the case. Firstly, with repeated infusions, fungal organisms may be transplanted into the uterus (i.e., via contamination), and secondly, whether the antimicrobials may disrupt the normal bacterial flora of the caudal reproductive tract and subsequently

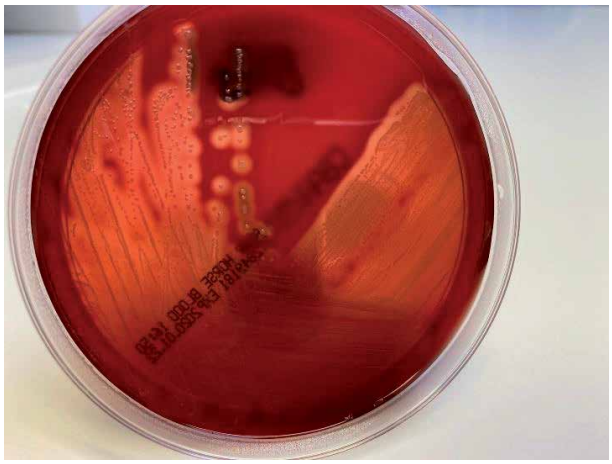

**Figure 1.**
Streptococcus equi *subspecies growing on blood agar showing distinct beta hemolysis. Image courtesy of BioTe veterinary laboratories, England.*

| | Product | Dosage | Notes |
|---|---|---|---|
| Antifungals | Clotrimazole (100 mg/ tablet) | 500 mg in 50 mls sterile saline | |
| | Fluconazole (200 mg/ tablet) | 100–250 mg in 50 ml sterile water | Add 5 ml DMSO per 1 gram of fluconazole to aid in dissolving tablets |
| Antibacterials | Ampicillin (1 g vial) | 1–2 g in 50 ml sterile saline | Mainly effective against gram-positive bacteria |
| | Ceftiofur (1 g vial) | 1 g in 20 ml sterile saline | Broad-spectrum antibiotic |
| | Ciprofloxacin (10 mg/ml) | 400–500 mg in 50 ml sterile saline | Mainly effective against gram-negative bacteria. Not first line; only utilize if strains are resistant to other antimicrobials |
| | Gentamicin (100 mg/ml) | 1–2 g, buffer with 10 ml 8.4% sodium bicarbonate | Mainly effective against gram-negative bacteria |
| | Penicillin (procaine) 300,000 units per ml | 15 ml, dilute to 50 ml in sterile saline | Mainly effective against gram-positive bacteria |

**Table 1.**
*Doses for commonly utilized antimicrobials for intrauterine administration (table reproduced courtesy of equine reproduction laboratory, Colorado State University).*

allow colonization by the fungus. These sites (such as the clitoral fossa) can then serve as a nidus for uterine infection.

In any suspected fungal endometritis, it is imperative to send the sample swab to be tested for polymerase chain reaction (PCR). Fungal growth in routine laboratory cultures encompasses a long wait for results, whereas the turnaround for PCR is relatively quick.

### *2.2.3 Therapy*

Therapy for endometritis in the mare will vary depending on whether the attending veterinarian is dealing with a bacterial endometritis, fungal endometritis, or indeed PMIE. Nonetheless there are some therapies that will be necessary in all cases, and they are dealt with first.

It is imperative to correct any caudal reproductive tract anatomical anomalies, such as poor perineal conformation. Surgical correction, such as a vulvoplasty (also known as a Caslick procedure), should be performed on these mares prior to breeding. A temporary Caslick can aid in treatment during the few days intrauterine access is required. A permanent Caslick can then be placed after treatment has ceased or after breeding (and resolution of any post-breeding fluid). An alternative is to place a permanent Caslick and to administer the treatment via a speculum, giving access to the cervix. Fixing anatomical defects in this area will prevent recontamination of the caudal reproductive tract and helps to "pull" the uterus into a more caudal position, aiding the natural mechanical cleansing mechanism of the mare.

All mares that have excess fluid should undergo uterine lavages. In cases of infectious endometritis, these uterine lavages reduce the organism load, aid in removal of biofilms (see below for further treatments), and reduce particulate matter that may interfere with the antimicrobials used.

### 2.2.4 Ecbolics

No attending veterinarian should underestimate the use of ecbolic when dealing with endometritis in the mare. The two commonly used preparations are oxytocin and prostaglandin F2α (PGF2α) in dealing with uterine fluid.

Oxytocin is by far the most commonly used of these two. Its ease of administration either given IM (intramuscular) or IV (intravenous) and its relatively short duration of approximately 30–45 minutes make it an essential product to have on standby when breeding mares. Side effects are minimal. However given its short duration of action, it does require multiple doses. Typically 1 ml either IV or IM of oxytocin given every 4 hours for 1 day, starting a minimum 4 hours post breeding, will be sufficient in treating most minor cases of uterine fluid retention.

The use of prostaglandins is not as straightforward as oxytocin. There are more side effects with the use of this preparation, and some are potentially quite serious. Prostaglandin is a known abortifacient. It is a good practice to always identify the mare in front of you for any reproductive treatment and, if in doubt, ultrasound the mare to confirm that she is indeed empty. Duration of action is approximately 4 hours. During this time, the mare may sweat, may act colicky, and may exhibit loose stools. It is recommended to monitor the mare during these 4 hours. Many clinicians are familiar with the use of PGF2α, as a luteolytic agent, and that is by far the most common use in equine reproduction. However, the veterinarian should not be afraid of its use when dealing with uterine fluid retention. Caution must be taken, however, when dosing PGF2α on the day of ovulation, as some studies have suggested that it can impact the formation of the corpus hemorrhagicum (which later becomes the CL, the source of progesterone required for maintenance of pregnancy). On the day of ovulation, it would steer the clinician away from use of prostaglandins, unless he or she is prepared to place that mare on an exogenous source of progesterone. The typical protocol initiated at my practice is that we would start with oxytocin for the first day and a half. If the mare has yet to respond satisfactorily to oxytocin therapy in that time, she is unlikely to respond. Throwing more oxytocin her way is futile. It is at this point we would consider the use of prostaglandin. In exceptional and severe cases, where there is significant fluid retention, it is not unknown to utilize both oxytocin and prostaglandin simultaneously on day 1.

### 2.2.5 Bacterial endometritis

Typically 3 days of intrauterine therapy is sufficient to see a positive outcome to therapy. It is bad practice to initiate intrauterine therapy for more than 3 days and predispose the bacterial inhabitants of the uterus to develop resistance to the antimicrobial utilized.

If the mare is presented with significant uterine fluid (in excess of 1 cm on ultrasonography), care must be taken to remove excessive uterine luminal fluid before commencement of the therapy. This is because we now know that certain antimicrobials may be affected by the fluid, but also there is a dilution factor to consider. Removal of fluid may include uterine lavages where 1–2 L of sterile fluid is distilled into the uterus and then allowed to flow back through the same giving set back into their original bags. Manual palpation of the uterus via the rectum at the same time the veterinarian is trying to remove the fluid may aid in evacuation of the uterine fluid. Ecbolics can be utilized concurrently, namely, oxytocin (see **Table 2**). For mares that present with minimal fluid, the use of ecbolics may be sufficient to remove the fluid. It is recommended to ultrasound the uterus prior to each intrauterine infusion.

| Product | Dose and route of administration | Notes |
|---|---|---|
| Lutalyse® (dinoprost tromethamine) | 5–10 mg IM once | Naturally occurring prostaglandin F2α |
| Estrumate® (cloprostenol) | 250 μg IM once | Synthetic prostaglandin F2α |
| Oxytocin (20 units/ml) | 20 units IV or IM | q 6 hours |

**Table 2.**
*Doses and routes of administration for the commonly utilized echolic agents (table reproduced courtesy of equine reproduction laboratory, Colorado State University).*

### *2.2.6 Fungal endometritis*

Further to the treatments below (**Table 1**), it is indicated to lavage the uterus with dilute acetic acid or dilute povidone-iodine.

### *2.2.7 Exercise*

The use of exercise postmating, whether pasture turnout on the use of a horse walker, is widespread, yet the efficacy and examination in control studies are lacking. It is hypothesized that increases in intra-abdominal pressure from exercise transfer pressure to the uterus to aid in evacuating the contents and improve the lymphatic drainage [49]. Others have suggested that exercise can tone the hindquarters and leads to an improvement of perineal conformation [50]. Swift et al. [51] demonstrated that exercise was an effective management technique to aid in evacuation of uterine contents post breeding in mares. In their study, they note the lack of control studies on the efficacy of exercise alone as a treatment for uterine fluid retention post breeding in the mare.

### *2.2.8 Glucocorticoid treatment*

The use of IV dexamethasone at a dose of 50 mg at time of treatment has become widespread following the classic studies by Bucca's group in Ireland [52]. It has been shown that there is a negative correlation between elevated endometrial score at time of breeding and pregnancy rates [53]. Dexamethasone has been shown to modulate the inflammatory process, possessing anti-inflammatory effects (decreasing IgG) while showing a stimulatory effect on α1-antitrypsin and transthyretin, which both enhance the defense mechanisms of the uterus.

### *2.2.9 Acupuncture*

A recent and growing addition to the treatment of endometritis in the mare is acupuncture. It has been suggested that electroacupuncture stimulates afferent nerve fibers, leading to modulation of hormone release through ascending pathways to the hypothalamus as well as reflex activation of the autonomic efferent pathways to the uterus [54]. The first control study examining the use of electroacupuncture in the mare as a treatment modality for endometritis found mare resistance to treatment was a major limitation in the use of this treatment, and that given the multiple acupuncture points, as of yet, does not appear to be an effective mechanism when treating endometritis in the mare.

### *2.2.10 Breeding on a dirty cycle*

In an ideal world, we would swab the uterus, and if found to have an infection, we would "clean" her up and wait for the mare's next cycle. However in the time-pressured breeding season, and in particular when dealing with valuable thoroughbred racehorses, time is seldom something the attending veterinarian has. This author has had great success breeding mares on dirty cycles, as long as there is at least 3 days prior to cover, to allow 3 days of intrauterine therapy. It is well established that the optimum time to swab the mare's uterus is when there is presence of uterine edema; swabbing when there is no uterine edema raises the risk of a false-negative result. An assumption is that the mare is infection-free only to be found negative on her pregnancy scan. Moreover it was inappropriate for the attending clinician to swab the uterus of a mare in diestrus (i.e., that she has a CL present). For one, the cervix will be tightly closed, and you may damage the cervix while trying to force the culture instrument through. Additionally, as the cervix is tightly closed, if you have accidently tracked bacterial isolates from the external vulva, or indeed the vaginal vault into the uterus, thereby inoculating the uterus with an infectious agent, the infection will take hold as the mare will be unable to "cleanse" herself with a closed cervix.

### *2.2.11 A frustrating scenario and the role of biofilm*

We have all been there, as attending clinicians. We swab the mare, she cultures negative, there are no ultrasonic changes to make us think there may be an infection, and she returns negative on multiple cycles. There is a caveat here, that reproduction is a complex beast, and many, many things must fall into place for successful fertilization and subsequent embryonic development to take place. As the saying goes, it takes two to tango. However as this part of the chapter is dedicated to endometritis and often we do not have access to the stallion, it is fair for the clinician to start with the mare, and indeed her uterus, when beginning to evaluate why a mare may not become pregnant.

In the short breeding season, the author recommends that any mare that is negative on two cycles (i.e., she has been inseminated twice) should undergo a full reproductive examination that includes swabbing the uterus for culture. If there is any suspicion that the mare maybe dirty, but has a negative culture, then the clinician should explore other diagnostic routes. This would, namely, be low-volume lavage.

Nonetheless there are some mares that either routinely cultured negative but fail to conceive or conversely routinely cultured positive despite appropriate therapy based on sensitivities. In these cases, the attending clinician must consider the possibility of a biofilm. A biofilm as defined by Loncar et al. [55] is a community of bacteria that are attached to an interface or to one another, encased within an extrapolymeric matrix consisting of nucleic acids, lipids, proteins, and exopolysaccharides. These biofilm plaques are inherently resistance to both antimicrobial and innate immune defenses, which leads to a persistent, chronic infection, even in the face of prolonged antimicrobial therapy. The matrix reduces the penetration of antimicrobials. Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* are all capable of producing biofilms. The workout of Colorado State University has given us incredible information on treating biofilms. The work of Loncar et al. [55] showed that no single treatment was effective against all three bacterial species named above and suggests appropriate identification of bacterial species is paramount for successful treatment. The administration of dimethyl sulfoxide to the uterus shows promise in treating biofilms caused by *E. coli* and *K. pneumoniae*.

### 2.3 Twinning events in the mare

Twin or multiple pregnancies in the mare is the most common noninfectious cause of pregnancy loss. Twin pregnancies have been reported to account for up to 30% of abortions in the mare [56, 57]. When twin pregnancies are established, the pregnancy will continue as normal to approximately 6 months, when one or both fetuses will die due to insufficient placental supply. Typically mares carrying twins will abort around 5–9 months of gestation [58]. Only 14% of twin pregnancies resulted in foals surviving into their second week of postnatal life [56]. Mares producing live twins will inevitably require intense (and therefore expensive) neonatal care. The majority of twin pregnancies are dizygotic and arise from multiple ovulations which can be either synchronous or asynchronous in nature. Ginther [59] has proposed that there are familial lines higher than normal incidences of multiple ovulations and thereby there may be a genetic predisposition. Thoroughbreds show the highest incidence of multiple ovulations, while it is low in native breeds and yet to be reported in native Shetland ponies. Older mares also seem to have high incidences of multiple ovulations [60]; however lactating mares appear to have lower multiple ovulation rates, presumably due to the suckling effect on the hypothalamic–pituitary-ovary axis. Not only do mare normally abort twin pregnancies, they also show high incidence of dystocia, damage to the reproductive tract (including the cervix), retained placenta, and delayed uterine involution. These have ramifications on the future reproductive health of these mares. In one study only 38% of mares that had a twin pregnancy in the previous breeding season produced a viable foal the following year [61]. Given the significant risks associated with twin pregnancies, detection of these is paramount. The attending veterinarian should make detailed notes of the presence of large follicles on the ovary and, at ovulation detection, note all ovulations. However do not be fooled, if only one large follicle has ovulated and another large follicle remains. If this follicle should subsequently ovulate, there is a chance of the establishment of asynchronous twins. It is advised to examine the mare in stocks and have the mare adequately restrained. Checking for twins in the field, where a mare may be fractious and/or not restrained correctly, will lead the clinician to potentially rush through examination. There is a danger element to ultrasounding mares not in stocks. Occasionally owners will state that they do not wish to transport the mare to facilities that have the required setup. If this is the case, get the mare restrained as best as possible, and advise the owner that this is not optimal. No ultrasound examination is foolproof. Begin in a systematic manner. The author starts with the left horn, runs the ultrasound probe laterally until the left ovary is seen, and then returns to the bifurcation. This is repeated twice. The same is then done for the right horn. Finally the body of the uterus is examined twice. During the examination, it is paramount to retain the uterus within the center of the screen at all times. If you feel as though a section of the uterus has been missed, repeat. As can been seen, to do this in the field without stocks in a fractious mare can be difficult. Natural reduction of unilateral twins before day 40 is reported at 85% [62, 63].

Given the limitations of this chapter, only two techniques for dealing with twin pregnancies in the mare will be described. There are numerous other techniques described and the readers are encouraged to examine these. At approximately 16 days post ovulation, the embryo (in this case the embryos) become fixed. Up until this point, the embryos are highly mobile and move throughout the uterine lumen. Typically a twin check using transrectal ultrasound takes place before this day 16. Identification of the small embryo takes place. If the pregnancies are adjacent to one another, the probe is gently oscillated to move them apart. The smaller embryo is then moved to the tip of the uterine horn, while a downward pressure

from the ultrasound probe on the selected embryo is performed. While keeping the embryo in focus on the ultrasound screen, rupture of the embryonic wall will be observed, and leakage of the embryonic fluid into the uterine lumen will also be observed. A quick check on the remaining embryo should also be performed, following this procedure. Adjunct therapy typically includes a single dose of flunixin meglumine (1 mg/kg IV) given prior to the elimination procedure. Typically these mares are placed on oral Regumate® (dose of 0.088 mg/kg SID PO), until a P4 sample is taken around the heartbeat ultrasound check (approximately day 25 post ovulation). Success rates of continued survival of the singleton pregnancy after a twin reduction around this time is in excess of 90% [64].

If you are presented with twin pregnancies beyond this stage, the clinician has a few options to choose from. After day 40, 63% of these pregnancies result in loss of both fetuses [65]. One of the authors preferred mechanism of twin reduction after day 40, which is cranio-cervical dislocation. Here the clinician is dislocating the first cervical vertebrae from the cranium along with disruption of the ligamentous attachments and severing the spinal cord via transrectal manipulation. This technique can be utilized between 60 and 110 days' gestation. The mare is sedated and placed in stocks. Buscopan (2 cc IV) can facilitate manipulation of the fetuses. Flunixin meglumine (1 mg/kg IV) is administered prior. The small fetus is selected and identification of the head performed, via identification of the mandible. Stabilize the head between the thumb and the finger and move the head side to side. Place the thumb at the base of the cranium and apply pressure proximally and dorsally; this will result in dislocation, whereby a "pop" is felt. Adjunct therapy included altrenogest (Regumate® at dose 0.088 mg/kg SID PO). Fetal death should be confirmed in 1 week post procedure via transrectal ultrasonography. Viability of the remaining conceptus should be evaluated (viz., by continued growth and the presence of a fetal heartbeat). If both pregnancies continue to be viable, then further intervention will be necessary.

## 3. Conclusions

With a thorough understanding on the physiologic events in the spring/vernal transition, the clinician can aid in hastening time to first ovulation. Most mares, if not all, will show some transient uterine fluid accumulation post breeding. Having the skills to note which mares are likely candidates to have excessive fluid accumulation, or which mares have a uterine infection, will greatly improve pregnancy rates. Identification of mares that may develop twin pregnancies is a key skill of the equine theriogenologist, but transrectal ultrasonography has its limitations if the mare is examined in the field. Twin pregnancies are easily dealt with if identified prior to fixation.

## Conflict of interest

The author declares no conflict of interest.

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Chapter 5

# Investigation into Whether Proximal Suspensory Desmitis of the Hindlimb Could Predispose Horses to Sacroiliac Disease

*Anne Skivington, Milomir Kovac, Elena Zakirova, Albert A. Rizvanov and Catrin Sian Rutland*

## Abstract

Proximal suspensory desmopathy/desmitis (PSD) of the hindlimb is a well understood condition with widely accepted treatment protocols; however, there is little research demonstrating understanding or potential correlation between hindlimb PSD and sacroiliac disease (SID). Several studies have examined the co-existence of hindlimb PSD and SID each investigating unique predisposing factors. This has led to little direct correlation of cause and effect with no definitive conclusions drawn. The need to be objective is highlighted by the limited number of studies and that two studies used anecdotal evidence to support their hypothesis and thus creating the question does hindlimb proximal suspensory desmopathy predispose horses to sacroiliac disease? This review looks at the two conditions and compares the literature for each, including the incidence, biomechanics, anatomy, and treatment. The review further discusses whether one disorder predisposes horses/equids to the other.

**Keywords:** hindlimb proximal suspensory desmitis, sacroiliac disease, lameness, equine, sacroiliac joint, interosseous, kinematics

## 1. Introduction

The objective of this review was to assess whether there is a correlation between hindlimb proximal suspensory ligament desmopathy (hindlimb PSD) and sacroiliac dysfunction (SID), and provide an understanding of the current thought process of examining these disorders. There are several studies examining the coexistence of back pain and poor performance, however for the most part, the discussion focusses on the efficacy of diagnostic techniques of the thoracolumbar region with some recognition of influencing factors [1–3]. Some authors have assumed a correlation between the two disorders in their treatment programmes [4, 5] but none quantified the association or correlation of the two conditions. There are limited studies that have looked at the structure of the sacroiliac region and applied those principles to locomotion [2] however there are many text books that describe the structure alone [6, 7]. This chapter explores the two conditions and explores the background and present theories behind hindlimb PSD and SID.

### 1.1 Sacroiliac joint structure and function

The sacroiliac joint lies deep within the pelvis of the horse, made up of the sacrum (five vertebrae fused together) and the surrounding ligaments. It is known as an atypical synovial joint [2] and a cartilaginous joint [7]. The iliac surface has fibrocartilage coverage, with the sacral surface lined with hyaline cartilage, thus creating a modified symphysis [8]. There is great variation in the joint form from L shaped to C shaped either being relatively flat or concaved, although most are at an angle of 30° [2].

The sacroiliac joint lies between the ilium wings, forming a synchondrosis that is held in place by a multitude of ligaments. These ligaments are called the dorsal and ventral sacrosciatic ligaments and the broad sacrotuberous ligament [7]. The dorsal sacrosciatic ligament has two elements, a band that runs from the dorsal tuber sacrale to the apex of the sacral spinous processes; with the lateral dorsal sacrosciatic ligament running from the tuber sacrale and ilial wing to the sacral crest on the lateral aspect. The broad sacrotuberous ligament runs from the sacrum and transverse processes of the 1st and 2nd caudal vertebrae to the ischiatic spine and tuber ischium [2, 7]. The function of this joint is to provide a relatively inelastic structure that is capable of asymmetric pelvic deformation during movement [2, 9]. The muscle structure of the back plays significant influential roles in both anatomy and biomechanics.

The movement of the horses back differs depending on the location and medio-lateral swing of body mass; dorsoventral movement is seen with the greatest being middle of the back (40–47 mm per peak per stride) with a reduction cranially and caudally [10–12]. The natural movement of the lumbosacral area and the hindlimb produce a sinusoidal movement of no more than 4° within each stride cycle. Extension within this sinusoidal curve starts just moments before ground contact with the hoof, with the hindlimb at maximal protraction. In the sound horse this means that movement of the sacroiliac joint is minimal as longissimus dorsi is inactive in the impact and support phase of the flight arc of the hoof, in theory resulting in a stable joint [12–14]. Having said that linear regression revealed a significant deviation in movement over Lumbar 1 and Sacral 3 correlated to increasing speed [12]. This indicated that the movement of the back and sacroiliac joint is complex [2] and changes with every change in pace (**Figure 1**) [11].

The movement within the joint is assumed to be little [15] due to the middle gluteal and surrounding ligaments holding it in place. Despite this, a series of studies of the human sacroiliac joint revealed adaptations to forces transmitted through the joint;

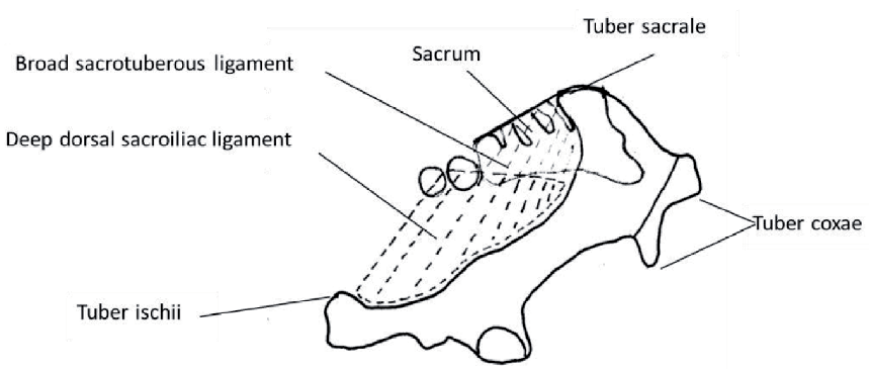


**Figure 1.**
*Schematic of the right lateral view of the pelvis showing the position of the sacroiliac joint between the wings of the ilium and wing of the sacrum and the sacrotuberous ligament (adapted from [2]).*

which was seen as roughened areas on the contrasting surfaces [16]. Comparable studies of the equine sacrum have looked at nutational forces to determine the degree of movement and suggested there is limited movement [2]. However, another investigation raised the interesting point that when the sacrotuberous ligament was cut there was a marked increase in movement [2]. This would seem obvious, as its function is to reduce movement but does suggest that ligament damage or laxity could cause increased asymmetrical movement which in itself could have an adverse effect on the soft tissue structures of the distal limb.

### 1.2 The interosseous muscle structure and function

The structure of the third interosseous muscle, also known as the suspensory ligament, the middle interosseous muscle or the interosseous ligament, is relatively straight forward. It originates from the proximal palmer surface of the metacarpal bones, running distally where just proximal to the sesamoid bones it bifurcates inserting on to each of the two sesamoid bones. From here it travels as the extensor branch joining the common digital extensor tendon. Even though it is termed a muscle, it is believed that once the horse matures it becomes completely collagenous in nature [7]. However, this is an over simplification as others describe the ligament as having a reduction of muscle fibres [17], while still retaining some which reduce with increased age [18, 19]. Muscle fibres quantitation showed a difference of 40% between the Thoroughbreds and Standardbreds with the Thoroughbred having less muscle fibres than its counterpart, with more muscle content being found in the hindlimb suspensory ligament than the forelimb [20]. It was also noted that the proximal region of the suspensory ligament contained less muscular tissue [19, 21]. This work also showed that the number of muscle fibres reduced with increased work intensity, thus suggesting that the suspensory ligament becomes less elastic and more susceptible to strain with increased work load (**Figure 2**).

The composition of the interosseous muscle is something of a hybrid, with the majority being collagen fibres but approximately 10% being type I muscle fibres and less than 5% type II muscle fibres. The suspensory ligament is defined by the infrequent fibroblasts embedded in the collagen matrix. These fibres are dispersed differently throughout the length of the ligament. Proximally, they are grouped as loose fascicles medially and laterally with the greater concentration just below the

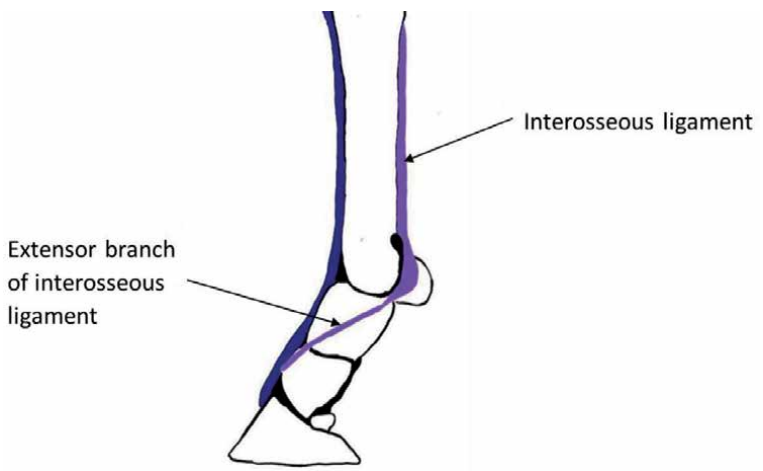


**Figure 2.**
*Schematic left lateral view showing the interosseous ligament of the hindlimb (adapted from Budras et al. [6]).*

surface. As it reaches the three quarter mark they become less distinct, fewer in number with reduced striations. Interestingly these fibres are arranged pinnately between 45 and 80° [17, 22, 23] leading to theories that high forces are created because of the greater pinnate angle in order to stabilise the joint and indications that its purpose is anti-fatigue and postural support [24]. This was supported further by the suggestion that the elasticity of the lower limb, creating a vibration of 30–40 Hz, needs damping to reduce the likelihood of damage to tendons or bones and that this is achieved through these short muscle fibres [25, 26]. Due to the elastic nature of the suspensory ligament, it is unable to cope with sudden surges in force and is not built to deal with increased amounts of fatigue [27, 28]. It has also been noted that as the age of the horse increases so does the stiffness of a tendon unit which in turn could induce a change in kinematics [29].

### 1.3 Elastic strain energy

It is commonly understood that tendons and ligaments play an important role in elastic strain energy during locomotion. Humans and ungulates have evolved to have more efficient locomotory systems; with equine evolution determining the distal limb muscle mass would not only be challenging to manoeuvre but very costly in terms of energy expenditure. Thus we see tendons and ligaments in the distal limb as a means of storing elastic energy [25, 28, 30–33]. In order for the horse to utilise this mechanism within the suspensory ligament the energy from the ground reaction force is stored as strain energy to retract the limb [27, 32] helping to produce the break over point [34].

The function of the suspensory ligament is to stabilise the metacarpophalangeal joint and hindlimb in preventing hyper flexion in locomotion but also to act as part of the stay apparatus in preventing collapse of the fetlock joint when immobile [35, 36] effectively acting as passive control [17, 28]. However, the suspensory ligament differs slightly in its role compared to the other tendons of the distal limb. For example, the maximal stress the superficial digital flexor tendon (SDFT) and deep digital flexor tendon (DDFT) functions at is 40–50 MPa (mega-pascal units) compared to the suspensory ligament functioning at 18–25 MPa when in gallop; of course this is maximal output and decreases with decreased speeds. To gain a relative perspective, muscles work at 200–240 MPa. By comparison this seems quite small but provides an elastic energy saving of 25% for the suspensory ligament and 40% for the SDFT and DDFT which translates into an energy saving of 1.23 J/Kg at trot and 6 J/Kg at the gallop [33]; thus reducing metabolic expenditure [25, 31].

Biewener [31] calculated the peak activity stress mean standard deviation on the fore and hindlimb suspensory ligament with 53 ± 14% from walk to trot and 23 ± 19% into gallop. When ground reaction forces are considered and coupled with an increase in pace, the change in stress has an astonishingly small mean of 4%. This could be due to the kinematic calculation methods or potentially due to the biomechanical nature of the suspensory ligament. As the hoof makes contact with the ground, the suspensory ligament briefly stretches as a reaction to the ground reaction force and the sinking action of the metacarpal-phalangeal joint. The ligament then shortens to create an anti-hyperextension force. This elastic strain energy depends greatly upon the tendon shape and volume. These are varied as the suspensory ligament bifurcates distally resulting in a greatly reduced cross sectional area, leaving it under greater stress and strain [25, 31]. The elastic property of the lower limb is also heavily influenced by the individual gait pattern of each horse.

### 1.4 Kinematics and kinetics of locomotion

In order to understand the process of veterinary examination and its resultant observations; it is imperative to fully understand the kinematics and kinetics of the locomotion of the horse. The structure and function of the cursorial musculoskeletal systems have evolved to provide structures and patterns of movement that favour acceleration, manoeuvrability speed and endurance [30, 37, 38] which has been harnessed over centuries for various disciplines such as racing and dressage.

It is also important to note the influence central pattern generators (CPG) and proprioception have on the biomechanics of the horse. The regulated rhythm of a pace is created by the CPG neurons which are capable of generating the stimuli and therefore a rhythmic motor behaviour. Even though some believe that the CPG neurons are capable of producing this regulatory rhythm without stimulus, sensory feedback is still required [39, 40]. Minute differences the timings or intensity of these impulses of the right and left central pattern generators cause asymmetrical movement [41]. Horses that have modified their locomotory movement in an attempt to compensate for discomfort or pain of either hindlimb PSD or SID will in effect cause the CPG neurons to adapt their "pacemaker" like outputs; thus creating a new norm for the horses locomotion [38].

Locomotion occurs as a result of torque at the hip joint [42, 43] and ground reaction forces exerted on the hoof which in gallop can be as much as 2.5 times the horses body weight [44, 45], with equal magnitude working in the opposite direction providing propulsion [46]. Therefore, it is worth considering the kinematic pattern of hoof placement, to determine how the pathology of SID and hindlimb PSD may occur. The structure and function of the cursorial musculoskeletal systems have evolved to provide structures and patterns of movement that favour acceleration, manoeuvrability speed and endurance [30, 37].

The hoof does not hit the ground with a total sole impact, but instead, as a measure of control, impacts the ground with the lateral edge. This reduces the concussive effect of the initial ground contact [47, 48]. It is important to remember that the hoof at ground contact is moving forward and downward during the initial loading phase [38]. The degree of impact when the hoof hits the ground is determined by several factors; the 57:43% split of vertical impulse for fore and hindlimb respectively [23, 38], the hoof mass, size and shape of the hoof, contact surface, type of shoe i.e. racing plate or hunter with or without grips or studs. These all influence the vertical and horizontal hoof velocity, and degree of slip [37, 38, 49]. The degree of lameness also has a large influence on interplay between hoof and ground reaction force [14].

Several studies have analysed hoof velocity [38, 44, 50], two of which have considered horizontal hoof velocity of fore and hindlimbs; one demonstrating the greatest being in the non-leading limb [49] and other the leading limb [51]. The hoof velocity and leading limb has important implications to the structures in the hindlimbs; if it is the forelimb the majority of the velocity will be absorbed by the thoracic sling, if it is the hindlimb the velocity can only end at the sacroiliac joint, although this is greatly simplified. Having said that, longitudinal velocity reduces (regardless of limb) as the horse starts to break in early stance phase. In this early phase the hindlimb suspensory ligament (third interosseous muscle) is at its peak inertial capacity to prevent hyper extension, while at the same time the pitch avoidance movement of raising the head and neck backwards increases forces on the pelvic limb, as the weight is shifted backwards in the late stance phase. This increases propulsion of the moment arms of the hindlimbs, creating oscillating forces though the hindlimb [28, 52]. These oscillating forces are created with

hoof-ground impact causing the limb to vibrate in a craniocaudal movement at 30–40 Hz, the greatest impact being distal in the limb. The muscles of the hindlimb act as adequate shock absorbers however risk of soft tissue damage increases with the increase in loading cycles [26]. This suggests that the greater the work load and discipline level of the horse, the more likely they are to sustain an injury. One method of removing force is slipping or sliding. The hoof is designed to allow an element of slip as a natural method of dissipating energy [53] however if sliding continues in the right conditions this can increase the risk of damage to soft tissue structures. Coupled with the ground reaction forces, this means that there are two opposing forces meeting at the horizontal axis, namely the sacroiliac joint [51].

### 1.5 Conformation of the horse

There are many variable factors when considering the relationship between hindlimb PSD and SID; one of which is the natural biological variation in every horse, in that no two are exactly the same in conformation which ultimately enhances or impedes function. Discipline desirable traits have been documented for enhancing performance, such as the warmblood breeds for dressage, with greater hock angle reducing the incidences of injury compare to those with smaller hock angles [54, 55]. However, this was refuted in a later study of 66 warmblood horses that had the supposedly undesirable tarsal joint angle of <155.50° [56]. This was agreed with in another study examining the hock angles of 194 Warmblood horses with hindlimb PSD [57]. Hobbs et al. [54] described a selection of horses that had variations between contralateral limbs conformation and those with bone morphology variance in contralateral limbs [58]. The results of these differences may induce compensatory movements in an attempt to redistribute the weight through the stride cycle. In an attempt to counter this, and stabilise the gait, the hindlimbs may start to load in a pattern similar to a lame horse. Having said that this load distribution pattern may come from the horses' handedness. This raises the question, if the horse is not physiologically capable of creating vertical impulsion (due to straight hocks), how and where will this affect the soft tissue structures in the hindlimb?

Asymmetries come in many forms, however each will have a marked effect on the biomechanics of the horse and more importantly the ground reaction forces; in the horses attempt to maintain equilibrium [54]. Of course, this need to maintain stability has different ground reaction forces depending on breed. Elite dressage Lusitano horses had lower vertical impulses compared to their Dutch Warmblood counterparts in collected trot with a range of 1.64 ± 0.02 N/Kg and 1.90 ± 0.08 N/Kg respectively. However this evened out with a change from collected trot to passage, with minimal difference being seen. Nevertheless, the key point in this is that the centre of mass is moved closer to the hindlimbs in the higher movements. Heim and co-authors [11] demonstrated a significant difference between Franches-Montagnes stallions ($n = 27$) and a general populous of horses ($n = 6$) in the dorsoventral movement ($p < 0.02$) and mediolateral movement ($p < 0.01$) for the spine, although to say this is a generalisation of differing anatomical parts and their role in locomotion. There is also the influence of the rider to consider here; not only as their body mass is part of the calculation but as the elite rider is capable of re-balancing even the most uneducated of horses to maintain the uphill longitudinal balance that is required of a dressage horse [59]. Dyson and colleagues [60] refuted this in their pilot study of rider weight, in that the weight of the rider had a greater significance than body mass index. Although this situation is not definitive, as there are many influencing factors in this scenario. For example, the balance of the rider and the dynamics between saddle and rider, both of which have a role in distribution of forces. In essence if the rider is displaced by an ill-fitting saddle or the rider

is inexperienced the horse has to re-balance itself in order to compensate [10, 44], which in itself produces compensatory locomotion. Another interesting factor relating to distribution of forces, body movement and rider interaction was demonstrated during the heavy and very heavy rider trials, as the horse demonstrated 3/8 lameness (based on the 0–8 grade lameness scale where 0 is sound and 8 is non-weightbearing) with these heavier riders [60]. The thoracolumbar width changed with weight of rider, from 3.9% with a light rider to 2.8% with a heavy rider. Heim et al. [11] noted that there was less mediolateral movement in the vertebrae when under saddle, with a difference of approximately 10 mm in the 3rd lumbar vertebrae as compared to an 8 mm difference in the movement of the tuber sacrale. This suggested that the horses may be bracing themselves against the movement of the heavier rider. However this was an observation and not a direct conclusion. It was also suggested that the interactive surface between horse and rider, the saddle, if not fitted correctly increased the mediolateral movement of the rider, which led to their conclusion that the closer contact the rider has with the horse the more likely they are to be working in equilibrium with them [10].

### 1.6 Conformation of the hoof and influence of shoeing

The conformation of the hoof capsule and the angle of the internal structures have a role to play in suspensory ligament desmopathy and limb kinematics. A significant level of research focusses on the correlation between the navicular bone angle and force applied to the deep digital flexor tendon [44, 61]. Although the research was not directed at the hindlimb suspensory ligament; their findings still shed light on this area due to the anatomical angle of bordering structure and limb kinematics. The shape of the hoof has been reported to change the kinetics and kinematics of the distal limb. Dyson et al. [61] reported that the distal phalanx to hoof wall angle and distal phalanx to horizontal angle were smallest for deep digital flexor tendon injuries at 52.27° ± 3.29 and 50.32° ± 3.70 (mean ± SD) respectively. However, it would seem there was no direct correlation between that and the angles of the hoof wall. Research suggests that optimal hoof angles for both front and back feet should be 50–55° [62]. In addition, minimal correlation between the dorsal aspect of the distal phalanx angle and deep digital flexor tendon injury has been found and the hoof wall angle was not the same as the distal phalanx angle [61], which could account for natural variation in hoof pastern axis.

The deviation of distal phalanx angle affects the orientation of the structures above it and subsequently the metacarpophalangeal joint; which in turn has the potential to cause soft tissue injuries [63, 64]. This is because the ground reaction forces are reduced delaying break-over to latter breaking phase [64] whereas the horse should have increased loading at this point [62, 65]. This has the potential to reduce the strain on the interosseous muscle but could also inhibit the elastic strain energy needed to create its passive force.

Kane et al. [63] identified 43 race horses with ruptured suspensory ligaments with lower heel and toe angles; for example the difference between the toe heel angle control group and those with suspensory apparatus failure was 1.3° less, a relatively small number in terms of angles but quite significant over the lifetime of a horse. In real terms this means that an increase in angle of 10° increases the chance of suspensory ligament failure by 6.75 times [63].

Shoeing has been used since domestication of the horse as a means to improve performance and help maintain hoof balance. The combination of farriery techniques like rolled toes, plus different types of shoe have a significant effect on the horse's feet and their movement [34, 45, 66]. It could be assumed that the application of the shoe would only affect the gait pattern of the horse but an 11% vertical

displacement of the trunk has been observed [66], which implies a physiological effect of the structures of the back over a lifetime of a horse. Different types of shoe also have varying effects on the horse [67]. The glue on heart bar increased strain of the suspensory ligament while the racing plate alone increased strain in the superficial digital flexor tendon, interestingly when packing was added to the racing plate the increased strain was seen in the suspensory ligament. Others demonstrated an increase force of 101 N between the unshod and the steel shod foot [45, 66]. However, when looking at this in greater detail it can be seen that there is a difference in kinetics between the two states. By comparison the shod foot remains medial throughout the entire stance phase putting greater strain on the medial aspect of the limb structures. This is due to the gripping nature of the steel shoe which effectively shortens the natural slip effect of the bare foot and increases musculoskeletal forces after impact, altering the dampening effect of the suspensory ligament and preventing hoof and frog expansion on impact [34]. The stride duration also increased with the application of a shoe from (mean) 694 to 706 ms as did the stride length from 2.78 to 2.82 m; with the stride protraction and retraction decreasing after the application of shoes. This was seen as the carpal joint extending later in the swing phase and the foot being behind the movement at impact [66]. The unshod foot lands medially to then shift laterally at mid stance to then move back again medially. The application of a metal shoe removed the hoofs natural cycle of wear from the equation, which proved to be beneficial for the horse when assessing the morphology of 100 feral Brumbies [68]. Increased substrate hardness and distance travelled reduced the likelihood of hoof wall flare, however a possible negative of this is the loading of the peripheral sole in locomotion as well as the expected loading of the hoof wall [68].

### 1.7 Influence of discipline

There are many influencing factors when taking into consideration the relationship between horse and rider; the riders ability to control their balance, the weight of the rider and the fit of the saddle, all of these factors can have an effect on the equilibrium and the physiology of the horse. The influence of rider weight on horse movement has also been investigated. Riders were classified as light, medium, heavy and very heavy; all of which were classified as experienced riders [69]. Horses were subjectively and objectively observed with inertial sensors to determine movement at the poll and pelvis, each horse was then assessed with each rider. All heavy and very heavy rider assessments were abandoned due to temporary lameness inducement, suggesting a biomechanical change with the introduction of a dynamic load. In a study that used a lead weight added to the saddle they found the addition of weight extended the spine [70]. Thoracolumbar width changes have also been observed in another study, differing by 7.3% from the lightest to heaviest riders [71]. Variables such as saddle fit were accounted for by Master Saddlers checking prior to the tests being ridden and on the days of the test being ridden. However oscillation of the saddle in trot was reported with all rider weight groups; very heavy 14.0%, heavy 50.0%, medium 76.9% and light 84.6%, although there was no depth of discussion as to the occurrence of this except to say not all saddles fitted perfectly. Saddle bounce also occurred with the very heavy rider on 4 out of 6 horses, although this was associated with the horse being crooked in canter. Having said that, in the objective gait analysis a pelvic minimal difference of 2.2 ± 4.8 (mean ± SD) was observed [72].

Influential factors also include rider height and leg length, as this affects the fit of the saddle for both horse and rider, plus the rider's core strength for which it is assumed that an increase in core strength would reduce rider movement in the

saddle. One of the stark conclusions drawn from this study was that lameness was observed in most of the horses when being ridden regardless of rider weight (that was not apparent in hand) and that the heavier riders consistently induced severe lameness [71, 72]. This research did not answer the question of rider weight ratio but it highlighted the importance of a well-fitting saddle and the role that it plays in maintaining normal gait patterns for that horse.

An important consideration is also the discipline of the horse and the movements they are required to perfect. An example of this was elite dressage horses which are required to produce collection; "maintaining impulsion from behind to allow a lighter shoulder", to carry out higher level movements thus distinguishing the important factor of higher proportion of bodyweight carried by the pelvic limb [73]. Although this was recognised there was no appreciation that the movement must originate in the sacroiliac joint. Furthermore the link between tarsal joint compressions was made but not associated to orthopaedic injury. However this point was contradicted by the description that the greatest movement of the SIJ to be on the transverse plane [2]. This allowed for a wider overall viewpoint comparing the likelihood of SID by disciplines; with dressage horses and show jumpers being more susceptible [2]. This suggested that SID is induced by the greater degree of collection required of each discipline and increased angles of the moment arms of the hindlimbs, in effect reducing stability of the joint.

Data analysis primarily segregates elite and non-elite horses in order to classify gross morphology [73], demonstrating the understanding that each discipline has a differing physiological impact. This is then subdivided to location or type of injury. Conversely, they did not make the distinction in forelimb and hindlimb suspensory ligament injuries, and although there were a significant number of classifications observed, it was not stated whether these were distinct individual injuries or if the horses had sustained more than one [73]. However Barstow and Dyson [1] went a step further and subdivided their cohort into sacroiliac pain only and sacroiliac pain with hindlimb lameness; thus starting to demonstrate a correlation between the two. In comparison, others recognised the presence of other abnormalities but mainly focussed on osseous changes [74]. Dyson [61] considered an alternative perspective of tarsal conformation predisposing horses to PSD and acknowledged biomechanics as a possible influencing factor but again with no correlation to SID.

### 1.8 Surface variables

The surface that horses work on have to be taken into consideration as they directly influence the impact on hoof loading (hoof sliding and the declarative longitudinal forces) and therefore the reaction of the limb structures [38]. Surfaces vary based on their composition, a ménage situation will have a hard under layer with surface applied to a specific depth, while some race tracks will run on turf. The most important element here is the cushion depth as this has the potential to absorb some of the concussion [75, 76]. Having said that, a softer surface encourages the toe to pivot causing a rotational force on the distal limb structures [38]. In a human based assessment it was found that peak forces reduced with an increase in compliant surfaces [76]. The compliance of track surfaces has also been examined, each type of surface had a distinct effect on the hoof velocity and swing phase, with the greatest deformation coming from the most compliant surface [75]. Even though it was noted that this surface caused significant increases in stance time and angle of hoof on landing, they did not draw any conclusions from this or discuss the soft tissue implications for the horse. However, it does imply that the suspensory ligament would have to sustain its force for a prolonged period and thus potentially fatigue if longer stance time occurred. This concept was looked at in greater detail with the use

of a dynamometric shoe applied to three race horses which showed that turf surfaces had a greater ground reaction force (42.9 ± 3.8 g; mean ± SEM) compared to synthetic surfaces which reduced the ground reaction forces significantly (28.5 ± 2.9 g; mean ± SEM) [77]. This implies that there will be less impact on the soft tissue structures of the hindlimb and subsequently the sacroiliac joint.

### 1.9 Lameness and evaluation

In order to gain a full understanding of the relationship between hindlimb PSD and SID, the way in which the horse works, its discipline and level, plus the rider influence and ability must be considered [73, 78, 82]. Barstow and Dyson [1] used rider colloquialisms to aid quantification of lameness; this is very subjective even when well versed in this terminology [12]. This highlights the need to be objective and specific in pinpointing lameness. Similarly another study used anecdotal evidence to support their hypothesis of sports performance level and orthopaedic injury diagnosis, suggesting that this is frequently seen in practice but not yet documented [73]. Having said that, some studies [4, 5] have noted that some horses may suffer concurrent injuries of the sacroiliac joint or proximal suspensory (respectively) but did not draw conclusions from this regarding cause and effect or relationship.

As already stated, it is difficult, if not impossible to ascertain where the pain is coming from within the sacroiliac joint; one of the possibilities is the articular surface. As the horse ages there is an increased likelihood of cartilaginous deterioration irrespective of breed type or discipline. This deterioration and possible changes may be the result of long term laxity of the surrounding ligaments [83] which in itself could cause instability of the sacroiliac joint or degenerative suspensory desmitis which would alter the gait pattern of the horse permanently [84]. Another factor, of course, could be the ground reaction forces and the impact of hard work on hard ground for sustained periods.

It is recognised that lameness of the hindlimb creates compensatory movements within the lumbosacral region [74, 85]. Signs of subtle discomfort or pain are not so easily detected. A reduction in equine motivation to work or refusing jumps or bolting with their rider can be seen [4]. However, use of inertial measurement units can make the process of assessing asymmetry objective. The assessment of 60 horses used for polo showed 36 horses (60%) demonstrated an asymmetrical movement in the head, pelvic or both [86]. Statistical analysis linear regression revealed none of these measures had a slope greater in difference than zero. This tells us two things; that inertial measures are able to quantify small asymmetries in the horse but the value of this in a lameness evaluation must be left with the veterinary professionals to interpret. In reality this technology is not commonly used in practice and the standardised approach is to use diagnostic nerve blocks to determine the area of pain. However, this is not straight forward as they need to be used in conjunction with clinical examination and imaging modalities. In fact Pilsworth and Dyson [87] described clinically sound horses receiving a palmer nerve block to have a change in gait. This was echoed by Denoix and co-authors [88] when describing the pitfalls of sacroiliac nerve blocks, in that potential error could cause a false positive. In contrast others focussed on the biomechanics of the entire vertebral column [11, 82] but limited the discussion of the limbs to kinematics. This was echoed following assessment of the dynamic asymmetry of polo ponies, which again reverberated the question of correlation and cause [89].

The need to be more specific was demonstrated by Murray et al. [73] in their results making reference to thoracolumbar and pelvis but not specifically the SIJ. Goff and co-authors [90] advanced this to identify degenerative changes of the SIJ causing poor performance. However there is no correlation to unilateral or bilateral distal

limb lameness. To emphasise the need to be unambiguous Murray et al. [73] used a large sample size (1069 horses), which potentially could be representative of the equine population. However, as the study was conducted at a referral hospital it would not represent primary veterinarians seeing acute injuries or stages of disease; emphasising the need for a retrospective study of primary veterinary practices.

In a study by Barstow and Dyson [1] 296 horses were assessed for SIJ pain, of which 203 (80%) showed hindlimb lameness with 181 specifically identified with proximal suspensory desmitis (89% [94% bilateral, 6% unilateral]). Although this represents relatively small numbers by comparison to sports performance studies [73] its findings are significant and showed a direct correlation. Furthermore, the work up of the horses was carried out by the same veterinarian reducing the likelihood of subjectivity in gait analysis.

In a similar study the prevalence of orthopaedic injuries was examined, classifying the horse by injury alone [91]. Having said that, discipline was acknowledged but no relationship established; although the kinematics of the show jumper's pelvic limb were noted. A limitation of this study was that the information was extracted from yard records rather than from veterinarian's records. Furthermore the initial assessments were made by several veterinarians potentially providing greater diversity in objectivity of lameness detection. In contrast, a unique perspective examining the likelihood of heritable degenerative suspensory ligament desmitis in the Peruvian Paso was published [92]. Dyson [61] demonstrated an understanding of this but also questioned conformation as a predisposing factor.

All of this begs the question as to how a horse with sacroiliac dysfunction and hindlimb PSD can be identified? Generalised pain detection using facial expressions has been used for many years with infants. Langford et al. [93] took this principle and adapted it to form the mouse grimace scale for those used in biomedical research, this was hailed as a great success as a pain indicator. Miller et al. [94] developed this further to include pain behaviours. The assessment of pain has always been subjective and relative to the experience of the practitioner, formalising a grimace scale for horses [95] has made this an objective process for the equine veterinarian. There are general indicators of pain as seen in the horse grimace scale whereby an assessment of the horses facial postures are calculated on an ethogram to determine general level of pain. For example, a horse with tension above the eye alone may not be indicative of pain, but coupled with ears stiffly backwards and prominent chewing muscles, it may indicate a level of pain [95]. The facial grimace scale alone has been identified as limiting an ethogram for equine pain behaviours both ridden and in hand has been developed [60]. Importantly this study ensured its efficacy by refining its use with a "within observer repeatability study" to confirm this as a suitable tool for quantifying pain behaviours. This concept was taken a step forward in order to develop a scale for the ridden horse, for example the horse moving on three tracks in trot or canter could be an indicator of sacroiliac pain [69–96]. Some other indicators are a direct reflexion of the location of pain such as bucking going into canter demonstrating pain in the sacroiliac region; however, a horse at the very start of its education may resist the rider and buck out of frustration. Having said that, persistent displays of these behaviours are a direct indicator of pain [69]. There are many more subtle signs including asymmetry of the tuber coxae and the tuber ischii that can be visually assessed by the practitioner, asymmetrical muscle mass of the superficial gluteal and holding the tail to one side can also be seen as pain indicators [97]. Saddle slip has also been identified as an indicator of hindlimb lameness with a direct correlation between bilateral and unilateral lameness ($p = 0.344$ and $p = 0.286$ respectively) [98]. This advancement could improve criteria in determining the subtle variations in lameness between sacroiliac dysfunction and hindlimb PSD.

## 2. Conclusions

Research in the last 10 years has focussed on poor performance and diagnostic techniques, back pain and biomechanics or suspensory ligament disease. The correlation of information to demonstrate that lameness may be from one or more sites in the horse is limited. This indicates the necessity for further studies to determine whether there are correlations between hindlimb proximal suspensory desmopathy and sacroiliac disease. Understanding whether correlations are present between the two disorders could have an impact on evaluation and diagnosis, treatment and recovery, prognostics and welfare.

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## Conflicts of interest

The authors declare no conflicts of interest.

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Chapter 6

# ADAMTS Proteases: Potential Biomarkers and Novel Therapeutic Targets for Cartilage Health

*Sinan Kandir*

## Abstract

The equine locomotor system's health plays a key role on athletic performance. Bone and joint diseases are the major causes of lameness. Poor performance and diseases lead to great economic loss to equestrian sports and horse breeders. Therefore, prevention, early diagnosis, and therapy of joint diseases are important. A disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) proteinase family plays an important role in many physiological processes such as tissue reorganization, coagulation, and angiogenesis. Aggrecan proteinases ADAMTS-4 and ADAMTS-5 are physiologically responsible for the restructuring with enzymatic cleavage of the cartilage, specific biomarkers in the synovium or body fluids for early diagnosis, and potential specific therapeutic targets in order to their role on degenerative joint diseases physiopathology in humans and various animals.

**Keywords:** ADAMTS, aggrecan, equine, lameness, metalloproteinase, proteoglycan

## 1. Introduction

A disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) protease family plays an important role in many physiological and physiopathological processes. The ADAMTS family is an important potential biomarker for the evaluation of early diagnosis due to its roles in the physiopathological mechanisms of many diseases such as cancer, arthritis, and atherosclerosis. ADAMTS-4 and ADAMTS-5 have been reported to play an important role in the pathogenesis of osteoarthritis in humans and various animals, following their first molecular purification and cloning.

Articular cartilage is structurally composed of partially chondrocyte cells and a large number of extracellular matrix components. Many macromolecules have been identified in cartilage tissue, including collagen fibrils, aggregate proteoglycans, and glycoproteins. Although joint damage is caused by oxidative metabolism-induced free radicals and hypoxic conditions, the main reason is the increase in proteolytic enzymes. Matrix metalloproteinases (MMPs), pro- and anti-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), and retinoic acid are the main biomarkers recommended for the diagnosis of joint damage [1–8].

In this chapter, we focus on ADAMTS proteases and their role on cartilage health for joints' stability, their possible usage as damage biomarkers for early diagnosis, and novel therapeutic properties.

## 2. ADAMTS proteases

Metzincins are a superfamily of zinc-dependent metalloproteases, metalloproteinases, or metalloendopeptidases, which are responsible for many physiological functions that also include cartilage turnover due to their regulatory activities on the extracellular matrix (ECM). The metzincins constitute by the zinc-binding catalytic motif consensus sequence HEXXHXXGXX (H/D) exactly; the binding of a zinc molecule is modulated by the three histidines (or an aspartic acid in the third position), the acid-base catalysis is facilitated by the glutamic acid residue in general, and the steric flexibility is acquired by the small glycine residue in the catalytic motif [9].

The first referenced equine gene mapping project was initiated in October 1995 by the "First International Equine Gene Mapping Workshop," in order to search answers of the main questions "speed gene" and "evidence of heritable trait", and the first construction of a low density, male linkage map in 1999 was reported [10, 11]. Thereafter, the first domestic horse gene map (EquCab2.0), a thoroughbred mare Twilight's gene sequence, was released in 2007 and published in November 2009 [12–16]. The latest version of high-quality equine gene map EquCab3.0 is available and enables to detailed data about genes and encoding proteins [16]. The Vertebrate Gene Nomenclature Committee (VGNC) [17] has standardized names to genes in vertebrate species including horse [18, 19]. According to these accessible latest data versions, the equine ADAMTS and ADAMTS-like family members with chromosomal locations are listed in **Table 1**.

### 2.1 ADAMTS family

Towards the end of 1990s, Kuno et al. [20] described a new family of metalloproteinase, which consists of sequence similarity with snake venom disintegrin that was upregulated in colon adenocarcinoma cell line as a specific gene for cachexigenic tumor and was named a disintegrin-like and metalloproteinase with thrombospondin type-1 motif (ADAMTS). The equine ADAMTS and ADAMTS-like proteins are a superfamily comprised of 19 and 7 members, respectively (**Table 1**). The ADAMTSs are secreted proteinases and multidomain enzymes constituted of zinc-binding active site motif similar to adamalysin (ADAMs) and ensued by a metalloproteinase domain with that of reprolysins (snake venom metalloproteinases) and disintegrin-like domain (**Figures 1** and **3**). ADAMTS-like (ADAMTSL) family and papilin are newly identified and secreted ECM-related proteins which are relatives to ADAMTS proteases. Additionally, they lack catalytic activity due to the absence of prometalloprotease and the disintegrin-like domain which are found in the ADAMTSs [21–24]. ADAMTSs differ from ADAMs with the lack of a transmembrane domain and the inclusion of well-conserved thrombospondin 1-like repeats, a cysteine-rich domain, and the CUB (complement subcomponents C1s/C1r, Uegf, BMP1) domain, thus being soluble extracellular proteases [25–31].

The main physiological functions of equine ADAMTSLs and papilin are extensively unknown; thus they have some troubles and need to further detailed investigations. However, recent studies on genome-wide association analysis and transgenic animals have indicated that ADAMTS, ADAMTSL, and papilin gene mutations cause lethal embryonic defects and autosomal recessive Mendelian disorders such as human Ehlers-Danlos syndrome [32], bovine dermatosparaxis

| Gene/protein name | Chromosomal Location | VGNC_ ID | ENSEMBL | UniProt |
|---|---|---|---|---|
| ADAMTS1 | 26 | 15061 | ENSECAG00000016339 | F6YLN3 |
| ADAMTS2 | 14 | 55397 | ENSECAG00000016328 | F6X633 |
| ADAMTS3 | 3 | — | ENSECAG00000019061 | F6ZC90/F7A3A4 |
| ADAMTS4 | 5 | 15070 | ENSECAG00000024172 | F6YRD3/ A0A3Q2H7G6 |
| ADAMTS5 | 26 | 59233 | ENSECAG00000006500 | F7ACI3/ A0A5F5PZN1 |
| ADAMTS6 | 21 | 15071 | ENSECAG00000029347 | F6X9L7 |
| ADAMTS7 | 1 | 15072 | ENSECAG00000007527 | F6W0M1 |
| ADAMTS8 | 7 | 15073 | ENSECAG00000014164 | F6ZXN7 |
| ADAMTS9 | 16 | 15074 | ENSECAG00000019880 | F6VTC7 |
| ADAMTS10 | 7 | 55772 | ENSECAG00000016210 | F6QIB9 |
| ADAMTS12 | 21 | 15062 | ENSECAG00000016121 | F6TW13 |
| ADAMTS13 | 25 | — | (NCBI Gene ID: 100069281) | — |
| ADAMTS14 | 1 | 15063 | ENSECAG00000014713 | F7D1G5 |
| ADAMTS15 | 7 | 15064 | ENSECAG00000015715 | F6V0J9 |
| ADAMTS16 | 21 | 15065 | ENSECAG00000000787 | F6W504 |
| ADAMTS17 | 1 | 15066 | ENSECAG00000000579 | F7DNJ9 |
| ADAMTS18 | 3 | 15067 | ENSECAG00000019006 | F7A7V7 |
| ADAMTS19 | 14 | 15068 | ENSECAG00000023694 | F6YNK0 |
| ADAMTS20 | 6 | 15069 | ENSECAG00000020835 | F6PZV0 |
| ADAMTSL1 (Punctin-1) | 23 | 15075 | ENSECAG00000015972 | F6W1K7 |
| ADAMTSL2 | 25 | 15076 | ENSECAG00000011887 | F6TEW7 |
| ADAMTSL3 (Punctin-2/ SH3GL3) | 1 | 22941 | ENSECAG00000012008 | F6T6C4 |
| ADAMTSL4 | 5 | 15077 | ENSECAG00000019154 | F7A7L3 |
| ADAMTSL5 | 7 | 50328 | ENSECAG00000009642 | F6X928 |
| ADAMTSL6 (THSD4) | 1 | 51434 | ENSECAG00000022944 | F6UWV1 |
| PAPLN (Papilin) | 24 | 21150 | ENSECAT00000008176 | F6VT48 |

**Table 1.**
*The equine ADAMTS and ADAMTS-like family members with chromosomal locations and accession numbers.*

[33, 34], human Weill-Marchesani syndrome [35], canine ectopia lentis [36], human Geleophysic dysplasia [37], canine Musladin-Lueke syndrome [38], and thrombotic thrombocytopenic purpura [39]. In consideration of this knowledge, ADAMTSs, ADAMTSLs, and papilin could be responsible as most commonly screened genetic disorders among horses as early embryonic death and abnormalities, junctional epidermolysis bullosa [40], hereditary equine regional dermal asthenia [41], thrombocytopenia and, von Willebrand disease [42].

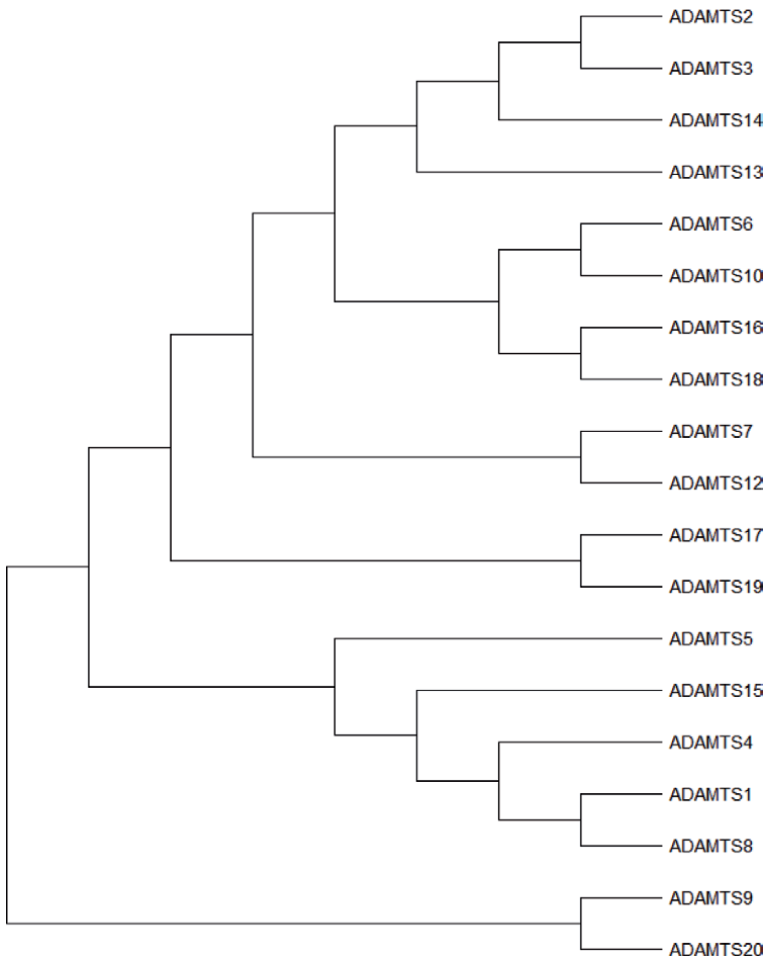


**Figure 1.**
*Phylogenetic analysis of equine ADAMTS protein family. The evolutionary history was inferred using the maximum parsimony method. The most parsimonious tree with length = 9505 is shown. The consistency index is (0.751174), the retention index is (0.551189), and the composite index is 0.440603 (0.414039) for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [43]. The MP tree was obtained using the subtree-pruning-regrafting (SPR) algorithm (p. 126 in Ref. [44]) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Branch lengths were calculated using the average pathway method (see p. 132 in Ref. [2]) and are in the units of the number of changes over the whole sequence. They are shown next to the branches. This analysis involved 19 amino acid sequences. There were a total of 2768 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [45].*

## 3. Role of ADAMTS family on equine cartilage health

Musculoskeletal system health is crucial for equine locomotion. This system is responsible to deploy the mechanical energy to the joints for efficient movement and specific biomechanical functions. There are many types of joints presented, where a majority of the free movements are managed by diarthrodial or synovial joints in the body. Cartilage tissue, which is the most important part of the diarthrodial joints, is absorbed and the loading energy throughout locomotion is distributed. Alterations due to inaccurately loading or metabolic disruptions could lead to acute or chronic damage, namely as arthritis, osteoarthrosis, or osteoarthritis to the joint and its critical component cartilage tissue, and restrict the locomotor functions. It is important to understand the molecular mechanisms of healthy and damaged cartilage tissues by the novel candidate molecular biomarkers in order to

early detection, easily clinical application, and therapy. Hence, we will focus on the cartilage health and importance of the ADAMTS family in this section.

### 3.1 Healthy cartilage tissue

Cartilages are divided into three major types as histological and biochemical properties in the body as hyaline, elastic and fibrocartilage. The distinctive features among these cartilage types are water content/dry matter balance and fiber types. In the diarthrodial joints, hyaline cartilage is existed on the articular surfaces and is well resist to pressure stress during various locomotion by its unique structure [46, 47].

Avascularized, unnerved, alymphatic hyaline cartilage (Latin words "hyălĭnus" meaning "glassy; made of glass; transparent") tissue's matrix is fundamentally constituted by water (%63–70), collagens (the majority type II in normal hyaline cartilage), non-collagenous proteins, and proteoglycans (the majority of aggrecans), while the most compounds are glycosaminoglycans (GAG) [48, 49].

Proteoglycans are classified into four subgroups related to their function: intracellular, cell-surface, pericellular, and extracellular. In the cartilage tissue, hyaluronan- and lectin-binding proteoglycans (hyalectans; aggrecan, versican, neurocan, and brevican) and small leucine-rich proteoglycans exist. Hyalectans are compromised with a similar structure in their tridomain structure; the N-terminal domain binds to hyaluronan, a central domain with the core protein for attachment of GAG chains, and the C-terminal region that binds lectins [50, 51].

The major proteoglycans in the diarthrodial joints aggrecans are the crucial elements for the biomechanical function with well-balanced load distribution and transmissions in order to provide the viscoelastic properties, the tight junctions, and the bridges of the extracellular matrix (ECM) [50, 52–55]. Aggrecans have a large molecular mass that contains GAG side chains comprising of the mostly chondroitin sulfate and keratan sulfate. They have three globular domains (G1, G2, and G3) to maintain the stabilization of protein complexes and to ensure mechanical features of cartilage. These highly conserved globular domains among the vertebrates have specific cleavage sites for proteases such as ADAMTSs (**Figure 2**) [50, 55–57]. The GAG's chondroitin sulfate and keratan sulfate contents of aggrecan could directly affect the aggrecanase activity by ADAMTSs [58].

Although, the hyaline cartilage consists of the chondrocytes which are the only cell type; this cell population has shown different morphological properties under the microscope and has been identified as dark, light, and adipocyte-like

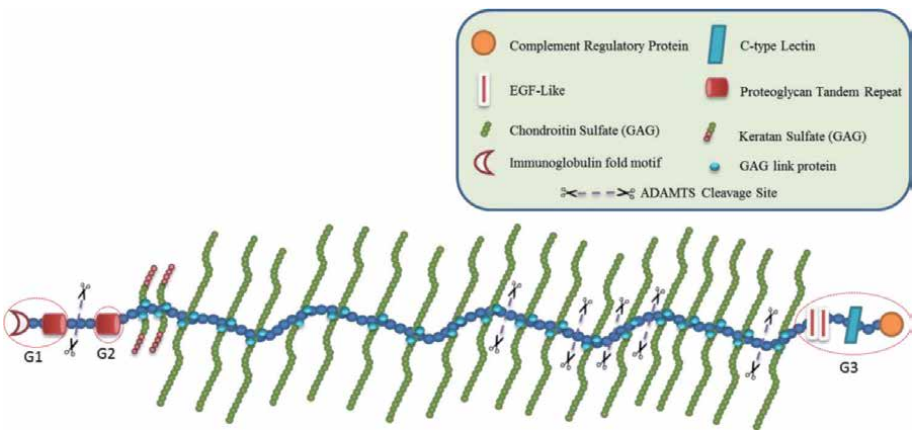


**Figure 2.**
*Schematic view of aggrecan proteoglycan [50, 55, 56].*

(adipochondrocytes) [59]. Chondrocytes manage the functional regulation of joint. These cell groups provide the synthesis and degradation of the ECM compnents, to support growth and regeneration, through the maintenance the gene expression and metabolism responded by mechanical stimuli. Extracellular matrix and proteoglycans are expressed by chondrocytes in articular joints [60–64].

The interleukins (ILs) and tumor necrosis factor α (TNF-α) cytokines are synthesized by chondrocytes, synovium, or inflammatory cells [7, 65]. Proteoglycan depletion has been stimulating physiopathologic processes, which is the main cause of degenerative joint diseases, e.g., osteoarthritis [66, 67]. Interleukins and TNF-α exert their effects on many diseases nonselectively from dental to cancer [68, 69], despite that a biomarker must be tissue-specific [70, 71]. Thus, in the last decade, the equine orthopedic researches have been deeply focused on proteoglycans, especially aggrecanases.

### 3.2 Aggrecanases on cartilage physiopathology

As it is well known, the proteases are responsible for proteolysis processes, which are catalytic enzymes to breakdown of the proteins into small polypeptides and amino acids by cleaved peptide bonds. The metalloproteinase superfamily members show their proteinase activity on osteoarthritis formation throughout the physiopathological processes. The matrix metalloproteinases (MMPs) and their endogenous inhibitors tissue inhibitors of metalloproteinases (TIMPs) are extensively studied; besides this, recent advances have indicated that the role of ADAMTS proteinase family is more considerable due to its abundant and specific aggrecanase activity by ADAMTS-4 and -5.

The aggrecan residues which cleaved at the glutamate 373-alanine 374 bond between the G1 and G2 interglobular domains were found at the synovial fluid analysis from various joint diseases (inflammatory or noninflammatory) in humans [72]. The first aggrecanase was purified and cloned by Tortorella et.al and named as aggrecanase-1 (currently termed as ADAMTS-4). They showed that ADAMTS-4 cleaves the aggrecan at the glutamic acid-373-alanine-374 bond [73]. After a while, Abbaszade et al. described aggrecanase-2 and named ADAMTS-11 (presently known as ADAMTS-5) [74]. ADAMTS-4 and ADAMTS-5 cleave the aggrecan at five common aggrecanase specific sites (Glu373-Ala374, Glu1480-Gly1481, Glu1667-Gly1668, Glu1771-Ala1772, and Glu1871-Leu1872,); nonetheless, ADAMTS-5 cleaves an additional site (Glu1480-Gly1481). Moreover, ADAMTS-5 is approximately twice slower than ADAMTS-4 [75, 76].

ADAMTS-4 and -5 are distributed in equine hoof lamina [78] and joints [79] and are expressed more in cartilage tissue than other tissues [80]. In our study, we observed concour horses after 50 minutes of a regular exercise program. As a result the serum ADAMTS-5 levels significantly increased but ADAMTS-4 did not. We concluded that ADAMTS-4 and ADAMTS-5 are using different pathways to physiologic and physiopathologic response [81]. Additionally and interestingly, the owners, whose horses had higher individual ADAMTS-5 serum levels, called the local veterinarians to complain about an orthopedic problem two or three weeks after our observations (unpublished data).

ADAMTS-4 needs to interact with sulfated GAGs that are attached to aggrecan core protein in order to effectively aggrecan degradation [58, 82]. ADAMTS-4 lacks the thrombospondin repeat domain on C-terminal region (**Figure 3**). This unique configuration allows bind to the adhesive glycoprotein fibronectin [82, 83]. Fibronectin is a glycoprotein that is found in low levels under physiologic conditions at the articular surface of cartilage and increases on pathologic conditions by activating innate immune response with toll-like receptors that are responded to

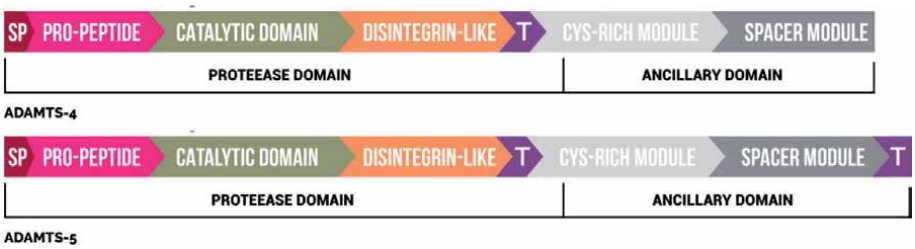


**Figure 3.**
*Domain organization of ADAMTS aggrecanases. SP: signal peptide; T: thrombospondin type 1 motif; and CYS: cysteine [77].*

regulate the innate immune system in case of pathogen-related inflammation [84, 85]. Hashimoto et al. reported that fibronectin is a novel inhibitor of ADAMTS4 activity in addition to its original endogenous inhibitor TIMP-3. Hence, fibronectin could be a potent preventive therapeutic against aggrecan degradation related to degenerative joint diseases [82]. While ADAMTS-4 is mediated by TNF-α, IL-1β, and nuclear factor-kappa B (NFκB) released from synovial macrophages, the regulation of ADAMTS-5 is not totally but predominantly independent of the these cytokine response [86].

## 4. Conclusion

The differences between synthesis pathways of ADAMTS-4 and ADAMTS-5 have to be taken into consideration on the TNF-α and IL-1 neutralization-targeted cytokine inhibitor therapies throughout degenerative joint diseases. In addition to classical therapy strategies, novel gene therapies are arising nowadays. An exciting work on this subject is a knockout murine model by the correction of ADAMTS-13 gene, which causes von Willebrand disease and leads to thrombotic thrombocytopenic purpura [87]. Transgenic animal studies with ADAMTS-4 and -5 double knockout mice [88, 89] revealed that aggrecan deletion protects from progressive osteoarthritis. These results have indicated that ADAMTS-4 and -5 may be potent therapeutic agents against laminitis and osteoarthritis, tendon, and ligament injuries for equine gene therapy.

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Chapter 7

# Equine Sarcoid

*Beatrice Funiciello and Paola Roccabianca*

## Abstract

The equine sarcoid is the most common skin neoplasia in the horse. It has a worldwide distribution and can also affect other equids such as donkeys, zebras, and mules. All breeds can develop the disease at any age, with no sex predilection, although geldings seem to be overrepresented. This fibroblastic neoplasm has several clinical presentations and microscopic features and has a nonmetastatic behavior but can be severely locally invasive. In many cases, multiple sarcoids may develop simultaneously or sequentially during their life and spontaneous remission is rarely reported. The etiology is multifactorial and involves bovine papillomaviruses, genetic, and environmental factors. Treatment options include different modalities depending on multiple factors: lesion type, location and extent, individual patient, facilities, owner, and financial issues.

**Keywords:** sarcoid, neoplasia, tumor, skin, horse, equids, donkey, mule, zebra

## 1. Introduction

The equine sarcoid is the most common skin neoplasia in the horse. This fibroblastic neoplasm has a multifactorial etiology and is nonmetastatic but can be severely locally invasive. First described in 1936, it has a worldwide distribution and can also affect other equids such as donkeys, zebras, and mules as well as other mammals [1–3]. Prevalence of sarcoid varies among published studies; however, many reports include cases from referral clinics that may not exactly reflect the entire equine population. Reported percentages of sarcoid among skin diseases and skin neoplasms vary from 13% to 90% and 8% to 38% when considering ocular neoplasms. There are also some geographical variations that may correlate with variations of risk factors, including the presence of cattle and vectors near horses [1, 4–6]. Horses of all breeds and colors can develop the disease at any age, most presenting a first lesion between 2 and 9 years of age. There is no demonstrated sex predilection, although geldings seem to be overrepresented [3, 5, 7]. Data about the incidence of sarcoids in the population are available only for donkeys and not for horses [8].

Affected animals can never be considered free of the disease even after successful treatment and presence or history of sarcoid can lower the likely sale value of the animal [2].

## 2. Etiopathogenesis of the equine sarcoid

### 2.1 Bovine papillomavirus infection

To date, it is widely recognized that sarcoids are associated with the presence of bovine papillomaviruses (BPV), typically BPV-1 and/or BPV-2. In two Brazilian

studies, newly proposed BPV 'BsR-UEL-4' and BPV-13 were found in some equine sarcoids, suggesting the need for further research regarding BPV serotype involvement in the development of these tumors [9–12].

The prevalence of BPV-1 and BPV-2 types seem to vary among geographical areas. In Europe and Australia, BPV-1 is most detected. In eastern USA, an almost equal proportion of both virus types was found, whereas in Canada and Western USA, BPV-2 was demonstrated in most of the samples [12–17].

The bovine papillomavirus genome comprises early and late coding regions. The early (E) genes encode nonstructural proteins involved in viral replication, maintenance of the episomal state, and activation of cell proliferation. The late (L) genes encode structural proteins (viral capsid) produced only in the life cycle of keratinocytes in natural hosts. A non-coding long control region is also present playing a role in viral replication and transcription [18, 19]. The main factors identified in sarcoid oncogenesis are the E2, E5, E6, E7, and p53 proteins. The E2 protein has regulatory effects on viral transcription and on the expression of matrix metalloproteinases (MMPs) that may be implicated in neoplastic cell invasiveness. The E5 protein exerts its function by binding to platelet-derived growth factor-β receptor (PDGFβ-R) thus activating p38 mitogen-activated protein kinase (MAPK) to induce fibroblastic transformation in sarcoids and down-regulate the major histocompatibility complex (MHC) I to facilitate the evasion of the immune system. The E6 protein can interfere in the activity of the p53 protein and has anti-apoptotic activity. The E7 protein cooperates in evading innate immunity [19–27]. The oncogenesis of equine sarcoids also involves loss of expression of the Fragile Histidine Triad (FHIT) and of the $O^6$-methylguanine-DNA methyltransferase (MGMT) tumor suppressor proteins [28, 29]. Recent studies have evaluated the role of small non-coding RNAs that regulate gene expression (microRNAs) in the development of sarcoids, and the role of aberrant methylation (S100A14 gene) is under research [30–34].

It seems that BPV infection in horses starts in the epidermis, where it can remain latent, with a subsequent presence of viral material within sub-epidermal fibroblasts where full transformation takes place [35–37]. Latency seems to take place also in peripheral blood mononuclear cells (PBMC) [38]. The infection in horses is abortive, the virus is present episomally but intact virions have never been detected. Furthermore, intralesional viral load seems to be correlated to disease severity [39, 40].

### 2.2 BPV transmission

Viral transmission between animals has not been completely elucidated yet. Direct contact with cattle, contaminated surfaces, and flies are presumably the most common routes of transmission [35, 36, 41, 42]. Infected equids may possibly spread BPV infection to horses and donkeys through contact. Appropriate fly protection and hygiene should be basic control measures in the presence of cattle and sarcoid-affected animals [35, 36].

### 2.3 Genetic risk factors

Bovine papillomavirus infection alone is not sufficient to promote normal cells transformation into sarcoid tumors, the presence of genetic factors and trauma are associated with the disease [43]. All breeds can be affected but Quarter Horses, Appaloosas, and Arabian horses are reported to be at greater risk than Thoroughbreds. Standardbreds have an even lower risk of developing sarcoids [5, 44, 45]. Certain equine families have an increased prevalence of sarcoid lesions and an association between the disease and equine leukocyte antigen (ELA) alleles has been observed in several breeds. The ELA W13 allele associated with the MHC

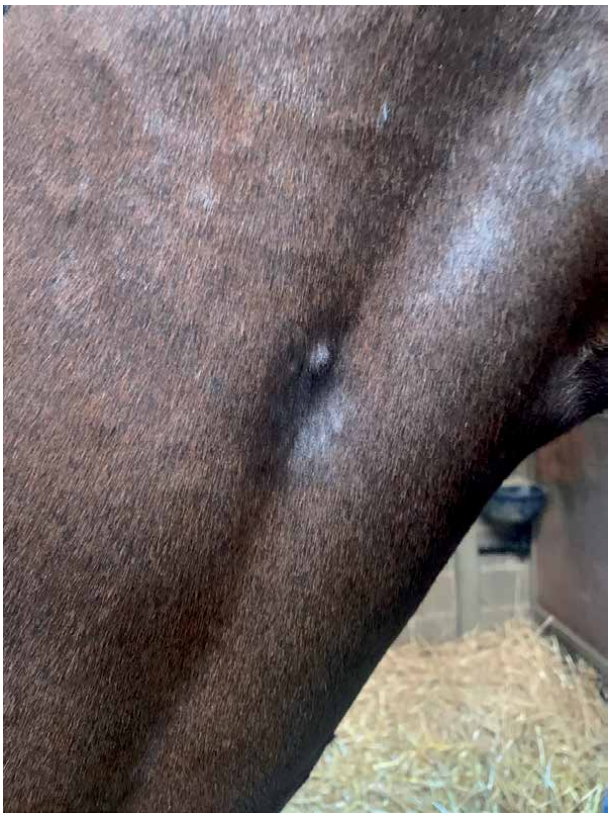

**Figure 1.**
*Sarcoid development on the jugular groove, possibly triggered by injection micro-trauma.*

II has been linked with sarcoid susceptibility in studies involving different breeds such as Swiss, Irish, French, and Swedish Warmbloods, and Thoroughbreds. The ELA W13 allele is not expressed in Standardbreds, a breed at lower risk of developing lesions [43, 46, 47]. Other MCH-encoded antigens are reported to play a role in sarcoid development: W3, B1, A3, A5, A16, A20, W5, W11, and W21 [1, 48–50]. A breed specific antigen, the Abe108, has been associated with sarcoids in Freiberger horses that lack A3, A5, and W13 antigens [50].

### 2.4 Trauma

Skin trauma is involved in sarcoid initiation, progression, and possibly recurrence. Micro-trauma due to injections (**Figure 1**) or even insect bites can be followed by sarcoid development, even long after apparent healing. Furthermore, sarcoids are a well-recognized possible complication and cause for delayed healing in both traumatic and surgical wounds in horses [2, 5, 51].

## 3. The biological behavior of the equine sarcoid

An individual animal may present only with one sarcoid, but most commonly, affected horses develop multiple sarcoids during their lives. These neoplasms may remain static for months or years and then, slowly or suddenly become aggressive and progress in type and/or extension without apparent reason (**Figure 2A** and **B**). Sarcoids tend to be locally invasive, sometimes extending into subcutaneous and


**Figure 2.**
*(A) Ear sarcoid slowly grown over years. (B) Same horse (hair clipped) few weeks after, the sarcoid underwent rapid growth at the beginning of the fly season. (C) Fibroblastic sarcoid development on a recently treated occult sarcoid. Note the "healthy" scar on the right where a similar occult sarcoid was successfully treated simultaneously.*

muscular planes, especially periocular lesions. They do not metastasize, however, with the exception of the malignant form that can spread to lymphatics and cause the formation of multiple masses along the lymphatic vessels and at remote sites such as lymph nodes [52].

Spontaneous regression is rarely reported and usually these horses do not develop new sarcoid tumors. Only in one recent study on a population of Franches-Montagnes horses in Switzerland has a high proportion of spontaneous remission been observed [53]. The mechanisms for spontaneous regression are not clear and antibodies have been detected only in donkeys [5].

The equine sarcoid has high frequency of recurrence after treatment (**Figure 2C**), especially following surgical excision. Recurrent tumors are usually more aggressive than the initial lesion and tend to grow rapidly and be more invasive. Recurring sarcoids can appear within a few days or weeks to months or years. The recurrence is often due to incomplete removal or spread of sarcoid cells during the procedure [1, 2, 52].

## 4. Clinical presentations of the equine sarcoid

Sarcoids have been classified into six different types depending on their macroscopic appearance (**Table 1**). This clinical classification is important because different types require different therapeutic approaches and have differing prognoses. One subject may carry more than one type of sarcoid and commonly, though unpredictably, milder forms can progress to more severe types [52, 54, 55].

### 4.1 Occult sarcoid

The occult sarcoid presents as an area of hairless skin, generally roughly circular. The skin may be thinned and/or have variably hyperkeratotic or roughened areas and contain one or more nodules, usually about 2–5 mm in diameter (**Figure 3A** and **B**). Occult sarcoids may involve extensive surfaces and individual horses may carry several lesions (**Figure 3C**). In some cases, only partial alopecia with thin hair and mild changes in skin and/or hair pigmentation (darker or paler) can be detected. Pruritus and pain are not present. These sarcoids have a slow progression toward verrucose growth.

Occult lesions can develop at any site but with predilection for the skin around mouth, eyes, the neck, and areas with less hair such as the medial thighs (**Figure 3D**) and forearms. They rarely affect the limbs.

Differential diagnoses for occult sarcoids are: idiopathic hypotrichosis/alopecia, dermatophytosis, alopecia areata, rub marks, chronic rubbing and scarring, bullous conditions (pemphigus foliaceous, and vasculitis), and burns.

| Type | Subtype | Features |
|---|---|---|
| Occult | — | Roughly circular, hairless thinned and/or hyperkeratotic skin, may contain nodules |
| Verrucose | — | Warty, hyperkeratotic area, may have nodules and/or occult halo |
| Nodular | | Subcutaneous spherical masses |
| A—no cutaneous involvement | A1 | Deeper tissues are not involved, loose capsule and defined margins |
| | A2 | Deep tissue involvement with poorly defined margins and invasive 'bound-down' nature |
| B—cutaneous involvement | B1 | Deeper tissues are not involved, loose capsule and defined margins |
| | B2 | Deep tissue involvement with poorly defined margins and invasive 'bound-down' nature |
| Fibroblastic | | Fleshy, ulcerated appearance, fibrocelullar scab |
| 1—pedunculated | 1a | Distinct pedicle without palpable or histological presence of tumor extensions |
| | 1b | Distinct pedicle with palpable root, poorly defined margins, invasive 'bound-down' nature |
| 2—sessile/broad-based | | Poorly defined margins, invasive 'bound-down' |
| Mixed | — | Verrucose, nodular and fibroblastic features present in variable proportions within the same lesion |
| Malignant | — | Multiple, locally invasive nodular and fibroblastic sarcoids with subcutaneous connections, may spread to lymphatics |

**Table 1.**
*Summary of the clinical classification of sarcoid types and features.*

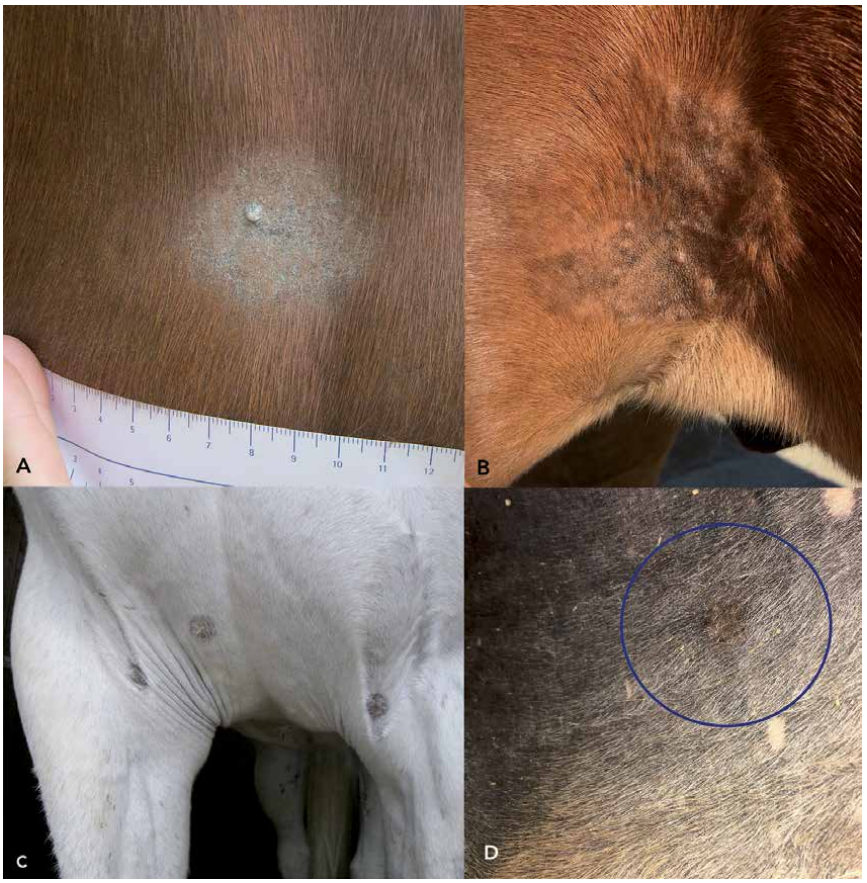


**Figure 3.**
*(A) Occult sarcoid in the pectoral region: circular roughened hairless area with one small nodule within. (B) Large occult sarcoid: note mild hair loss, alterations in skin pigmentation, and presence of nodules. (C) Multiple occult sarcoids, the central one has a verrucose central area with occult halo. (D) Early occult sarcoid (blue circle) on the medial thigh. Sparsely haired shining skin with mild pigmentary changes.*

### 4.2 Verrucose sarcoid

The verrucose form has a characteristic "wart-like" appearance, which is the main reason for calling sarcoids "warts". These lesions are alopecic and neither pruritic nor painful unless secondarily infected (**Figure 4A**). Some may ulcerate and bleed (**Figure 4B**). Thickness and size vary, small nodules may develop in the hyperkeratotic area and some lesions may present a pathognomonic occult margin/halo (**Figure 4C**). They usually grow slowly but progression to a more aggressive form is possible, especially with trauma. As occult sarcoids, the verrucose ones can coalesce and cover large body areas (**Figure 5**).

Verrucose sarcoids can develop in any region with predilection sites being face (periorbital), axillae, groin, body, and sheath. Limbs are rarely affected.

Differential diagnoses for verrucose sarcoids are: papillomatosis (warts), linear keratosis/epidermal nevus, dermatophytosis, chronic blistering.

### 4.3 Nodular sarcoid

Nodular sarcoids are firm and well-defined subcutaneous masses, usually spherical with variable diameters from few mm to 7 cm. In many cases, the nodules may be multiple and coalescing. Pain and pruritus are not typical features.

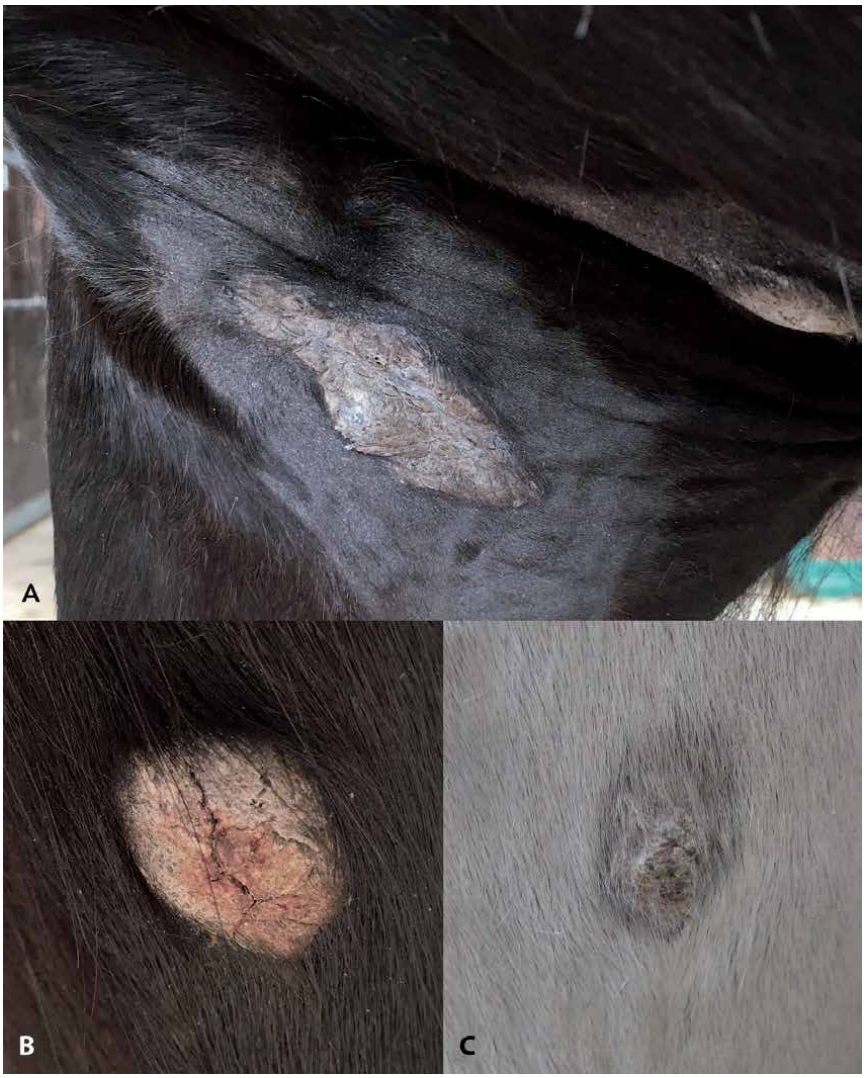


**Figure 4.**
*(A) Verrucose sarcoid in the axillary region (hair has been clipped), note another one in the sternal region. (B) Verrucose sarcoid with fissures and mild bleeding. (C) Verrucose sarcoid with occult halo.*


**Figure 5.**
*(A) Large verrucose sarcoid with nodular formations on the side of the neck. (B) Same horse after hair clipping: note extended hyperkeratotic and occult areas that were not previously visible because of coverage by hair.*

Similar to other forms, they very rarely develop on the limbs and the predilection sites are the groin, sheath, and eyelids.

A further classification has been suggested for these sarcoids based on skin and deep tissues involvement.

- Type A nodules do not involve skin that is not altered and can be freely moved over the nodule. Two subtypes exist:
    - Type A1: the nodule can be moved from both the skin and the underlying tissues, usually has a fibrocelullar capsule. In some lesions, a skin pedicle is palpable.
    - Type A2: no skin involvement but the nodule cannot be moved independently from the underlying tissues, it has a 'bound-down' nature. Very common around the eye.
- Type B nodules are characterized by visible and/or palpable alterations of the skin. They cannot be freely moved from the overlying skin that may look normal or be alopecic, thinned, hyperkeratotic, or ulcerated. Some may have adjacent occult changes. Also, two subtypes are recognized:
    - Type B1: no involvement of the deeper structures (**Figure 6A**).
    - Type B2: locally invasive with 'bound-down' nature and no separation from deeper layers (**Figure 6B**).

Differential diagnoses for nodular sarcoids are: fibroma/fibrosarcoma, neurofibroma, eosinophilic/collagenolytic granuloma, melanoma, equine cutaneous mastocytosis/malignant cutaneous mastocytosis/congenital mastocytoma, lymphosarcoma/lymphoma/cutaneous histiocytic lymphoma, dermoid cyst, and *Hypoderma* spp./foreign body cyst.

### 4.4 Fibroblastic sarcoid

Fibroblastic sarcoids are a more aggressive form with fleshy and ulcerated appearance, often covered by a fibrocellular scab and possibly secondary infection.

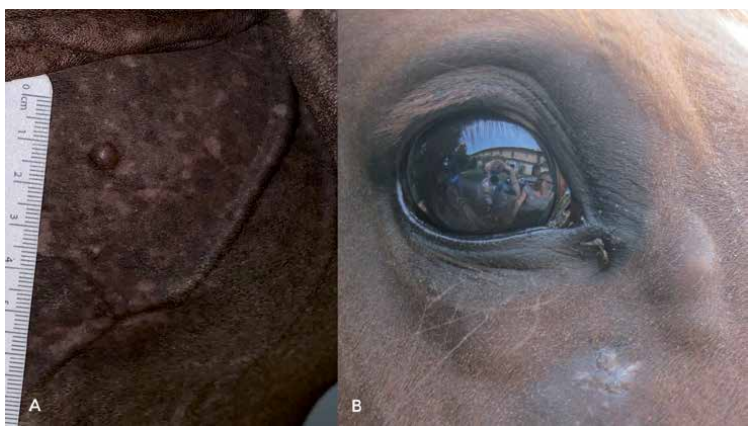


**Figure 6.**
*(A) Type B1 nodular sarcoid on the medial thigh. (B) Three type B2 nodular sarcoids around the eye, an occult area is also present.*

Bleeding and serum exudation are common and can be heavy with trauma. These surface characteristics attract flies that may contribute to irritation and self-trauma. Fibroblastic sarcoids commonly develop at wound sites (both traumatic and surgical), on the site of other sarcoids, especially if treatment attempts have been unsuccessful (**Figure 2C**) and they are usually more difficult to manage. Excessive granulation tissue may develop especially at wound sites thus complicating the diagnosis of sarcoid. Pruritus and pain rarely characterize these lesions. Predilection sites for fibroblastic sarcoids are groin, eyelid, wounds, coronets, and distal limbs. At some of these sites they carry a very poor prognosis. The classification of this form includes:

- Type 1 pedunculated fibroblastic sarcoids: characterized by a narrow pedicle with apparently normal skin and a fleshy crown. Subtypes are:
    - Type 1a: pedunculated with no palpable tumor and thickening at the base, no extensions detected on histology (**Figure 7A**).
    - Type 1b: this is pedunculated and rooted, where palpable alterations are detected beneath the pedicle, sometimes alteration are also visible (**Figure 7B**).
- Type 2 sessile fibroblastic sarcoid: the lesion is broad-based with invariably ill-defined margins and extensive invasion of the lateral and deeper tissues (**Figure 7C**).

Differential diagnoses for fibroblastic sarcoids are: exuberant granulation tissue, habronemiasis, pythiosis, botryomycosis/pyogranuloma/pseudomycetoma,

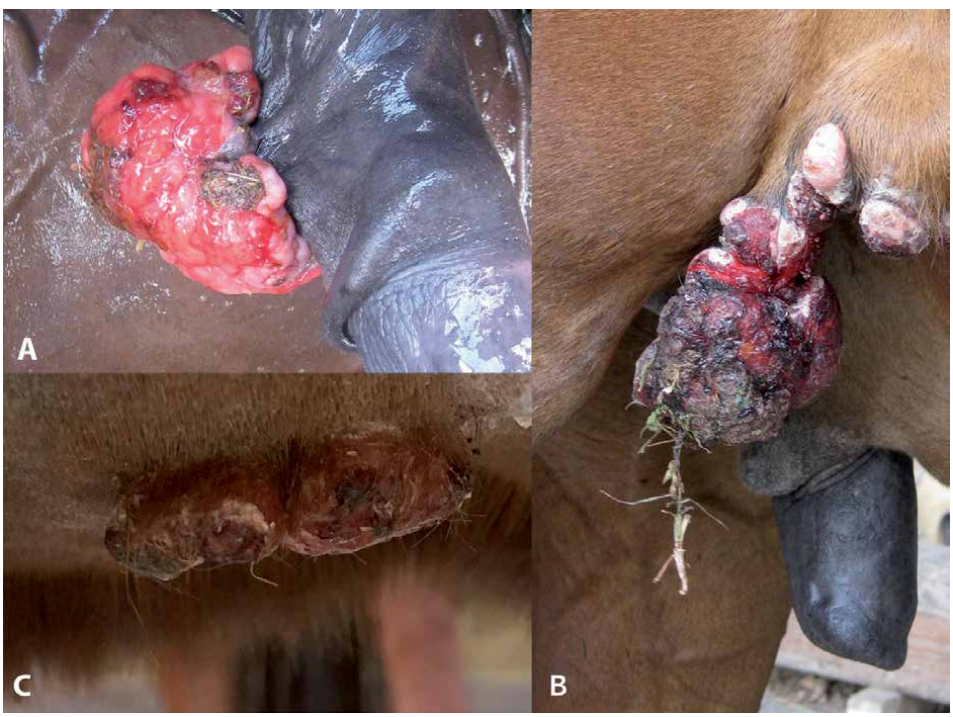


**Figure 7.**
*(A) Type 1a fibroblastic sarcoid on the penis. (B) Type 1b fibroblastic sarcoid with clear tumor involvement of the pedicle. (C) Type 2 sessile fibroblastic sarcoid.*

**Figure 8.**
*Mixed sarcoid: a type B2 nodule with a small fibroblastic sarcoid within an occult area.*

hemangioma/hemangiosarcoma, cavernous hemangioma/vascular hamartoma, neurofibroma/neurofibrosarcoma (ulcerated), fibrosarcoma, squamous cell carcinoma, sweat gland tumor, giant cell sarcoma, and mycosis fungoides.

### 4.5 Mixed sarcoid

Most sarcoids could be classified as mixed since different types (verrucose, nodular, and fibroblastic) are often present in variable proportions within the same lesion. Nevertheless, the definition of mixed sarcoid is usually reserved for those where a specific sarcoid type is not considered predominant. These cases may probably represent the transition/progression phase between one clinical type into the other. The combinations and extents of the various types are multiple, and they usually tend to become more aggressive, especially as the fibroblastic type grows (**Figure 8**).

Predilection sites for mixed sarcoids are the face, eyelids, groin, and medial thigh but mixed sarcoids can appear everywhere.

Differential diagnoses for mixed sarcoids are mixtures of granulation tissue within verrucose or fibroblastic lesions, habronemiasis, pemphigus complex.

### 4.6 Malignant sarcoid

The most recently described form of sarcoid tumor is the malignant type. It is usually, but not always, characterized by a history of repeated trauma or interference (also with inappropriate treatments) with another type of sarcoid. The particular behavior of the malignant type is the development of multiple, locally invasive nodular and fibroblastic sarcoids. Often cords of nodules and ulcerated lesions are visible and/or palpable, when these connections are subcutaneous the classification should be that of malignant sarcoid. They can be localized or spread through the lymphatic vessels invading local tissues with possible associated lymph node enlargement. No disseminated metastasis has been reported even for this form. A rare particular and dangerous form presents with a ring of nodules surrounding a verrucose or occult central area, especially on the neck/jugular and buttock regions. Predilection sites include jaw, face (**Figure 9**), elbow, and medial thigh.

Differential diagnoses for malignant sarcoids are squamous cell carcinoma, lymphoma/lymphosarcoma, subcutaneous mycosis, lymphangitis, glanders, epizootic lymphangitis/histoplasmosis, and hypertrophic scarring/cheloid.

**Figure 9.**
*Malignant sarcoid on the face: 'bound-down' invasive nodules with a central area with occult to verrucose changes, ulceration, and a fibroblastic component.*

## 5. Clinical examination and diagnostic procedures

The clinical examination should include signalment and a full thorough history with details on lesion development, especially about the behavior and progression. The clinical presentation of sarcoid lesions and their features are usually clearly recognizable, especially if multiple tumors of different types are present on the same horse. The confirmation of the diagnosis is not always straightforward and possible differential diagnoses and concurrent conditions should be considered [55, 56].

Depending on the sarcoid type, the full list of differential diagnoses should be considered when choosing the diagnostic procedures. The diagnosis of sarcoid is confirmed by histopathology, thus a biopsy sample is needed. Partial or excisional biopsy should provide sufficient information but a risk of exacerbation due to the surgical trauma should always be taken into account. If possible, a total excisional biopsy is preferable, the owner should be carefully advised about the implicit risks and a proper therapeutic plan should be prepared when taking the biopsy to avoid any exacerbation triggered by the procedure. If benign neglect is the plan, the opportunity of taking a biopsy should be carefully evaluated [2, 55].

### 5.1 Equine sarcoid pathology

Histopathology is deemed necessary to confirm the diagnosis of many equine sarcoids [57]. It is important to stress that due to the variable microscopic features of equine sarcoids, small biopsies may not provide enough tissue to differentiate sarcoids from other lesions such as granulation tissue, fibromas, or fibrosarcomas.

This is especially true if samples are obtained from ulcerated areas of the tumors [58]. Notably, trauma and reparative processes (wound healing) may activate cell growth and facilitate the development or heighten the progression of equine sarcoids [57–59], particularly for verrucose, occult and small nodular sarcoids [60]. Thus, excisional biopsies with wide margins should be favored for clinical reasons and because they provide with the most diagnostic material [46, 58]. If a non-excisional biopsy must be performed, sites within the mass must be carefully chosen to minimize the confounding factors of surrounding inflammation and granulation and to include intact epidermis [46].

Sarcoids derive from the proliferation of two components: the dermal fibroblasts and epidermal keratinocytes. They are regarded as biphasic tumors. Histopathology is heterogeneous and microscopic aspects and number of components varies according to the type of sarcoid [61].

Microscopic features of the epidermis may include orthokeratotic to compact hyperkeratosis, parakeratosis, irregular hyperplasia with epithelial proliferations producing long and pointed branches, termed rete pegs or rete ridges, extending deep into the dermal proliferation (**Figure 10A**) [46]. Epidermal ulceration is variable but frequent in nodular and fibroblastic sarcoids.

The amount of epithelial cell proliferation varies according with the type of sarcoid and ranges from severe hyperplasia to epidermal atrophy [1, 61]. Overall up to 46% of sarcoids lack epidermal hyperplasia and 54% lack rete peg formation [61]. Epidermal changes are maximal in verrucous sarcoids [58] and can be minimal to absent in nodular and occult sarcoids. Epidermal ulceration is common especially in nodular sarcoids [58].

All sarcoids are characterized by variable substitution of normal dermal components by neoplastic fibroblasts embedded in variable amounts of collagen. Histopathological findings consist of poorly demarcated, unencapsulated, variably infiltrative proliferation of large spindle to stellate, bland to highly atypical fibroblasts with plump, oval, nuclei with granular chromatin and variable hyperchromasia and with prominent nucleoli. Cellular atypia is low to absent and increases with time, number of excisions, ulceration, inflammation and type of sarcoid, being higher in malignant and mixed sarcoids. Number of mitoses is generally low (0–1 per HPF) if excluding malignant sarcoids. Density of neoplastic fibroblast is oftentimes higher in the superficial dermis [61]. At the dermal-epidermal junction, fibroblasts may be oriented perpendicularly to the basement membrane in the so-called "picket fence" arrangement (**Figure 10B** and **C**) [61–63]. This feature is considered highly diagnostic but is missing in up to 52% of sarcoids [61]. Additional patterns that can been seen at all levels of the dermis are whorling (**Figure 11A**), present in over 86% of tumors [64], parallel to interlacing short bundles (**Figure 11B**), storiform, herringbone, tangles or fibroblasts may be haphazardly arranged, this latter arrangement occurring more often in flat sarcoids (**Figure 11C**) [46, 62]. Amount of collagen matrix varies from minimal to abundant and can be dense, edematous, or myxoid (**Figure 11D**). Adnexal structures are variably reduced in density or obscured by the neoplasm [46].

Of all types of equine sarcoids, flat/occult sarcoids at initial stages can be easily overlooked at histopathology [63]. For this type of sarcoid, the only histopathologic finding may be an increased density of subepidermal neoplastic fibroblasts infiltrating between a reduced number of hair follicles and sweat glands [61]. The density of dermal fibroblasts is lower compared with the other types of sarcoids [58].

Immunohistochemistry can assist in the diagnosis of sarcoids although protein expression patterns are not considered highly specific. Fibroblasts in sarcoid express vimentin, the intermediate filament identifying mesodermal origin, and may be variably positive for laminin, smooth muscle actin, and type IV

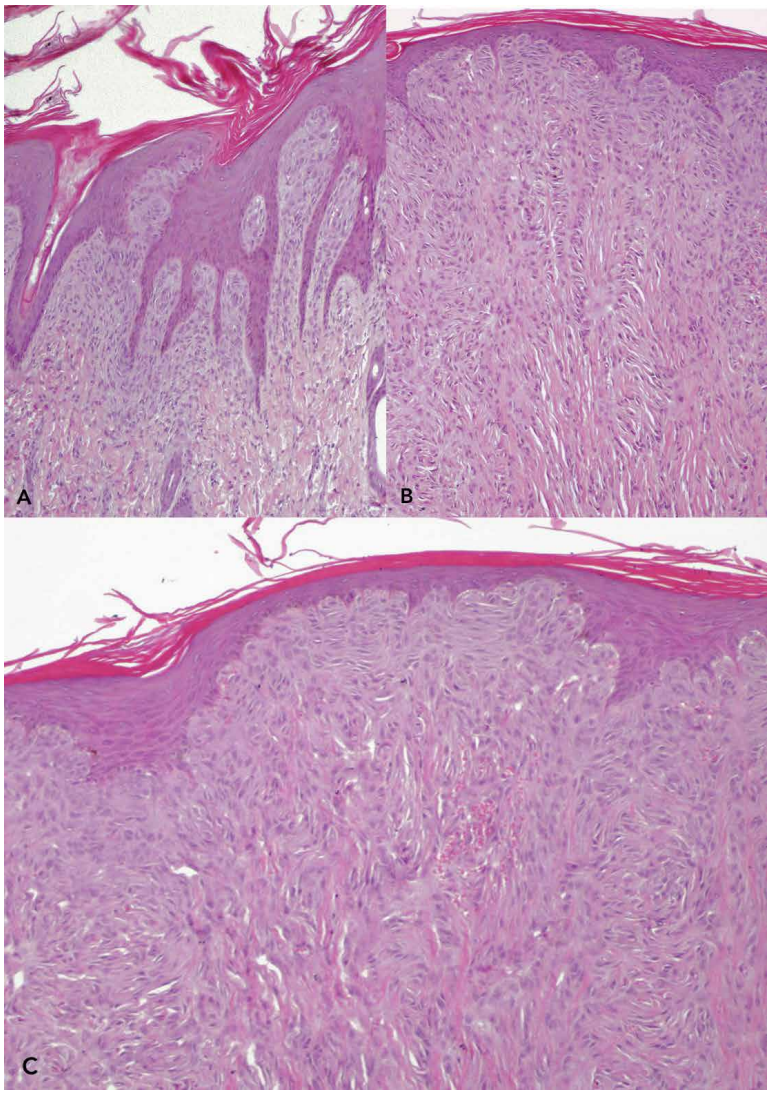


**Figure 10.**
*(A) Moderate hyperkeratosis and severe epidermal irregular hyperplasia with rete peg formation. In the superficial dermis higher density of neoplastic fibroblasts compared to mid dermis is evident. Hematoxylin and eosin, 200×. (B) Moderate compact hyperkeratosis with mild epidermal hyperplasia and mild rete peg formation. In the superficial and mid dermis, typical picket fence arrangement of fibroblasts is present. The picket fence pattern is considered a highly diagnostic pattern but is observed in less than 50% of equine sarcoids. Hematoxylin and eosin, 20×. (C) Moderate compact hyperkeratosis with mild epidermal hyperplasia and rete peg formation. In the superficial dermis, high cellularity and typical picket fence arrangement of fibroblasts are present. The picket fence pattern is considered a highly diagnostic pattern but is observed in less than 50% of equine sarcoids. Hematoxylin and eosin, 100×.*

collagen [59, 65, 66]. Sarcoids are generally S100 negative [65], however, S100 focal expression has been observed [66]. Bovine papillomavirus is involved in the pathogenesis of equine sarcoids, however, BPV infection of fibroblasts is mainly nonproductive [10]. Therefore immunohistochemistry against BPV is mostly negative [62].

BPV DNA can be detected by in situ hybridization and PCR on formalin fixed and paraffin embedded tissue sections of biopsy samples [36, 65, 67] or by PCR fresh cytological specimens obtained by swabbing or scraping of equine sarcoid tissue in non-healing wounds and recurrent cases and following recurrence after surgery [9].

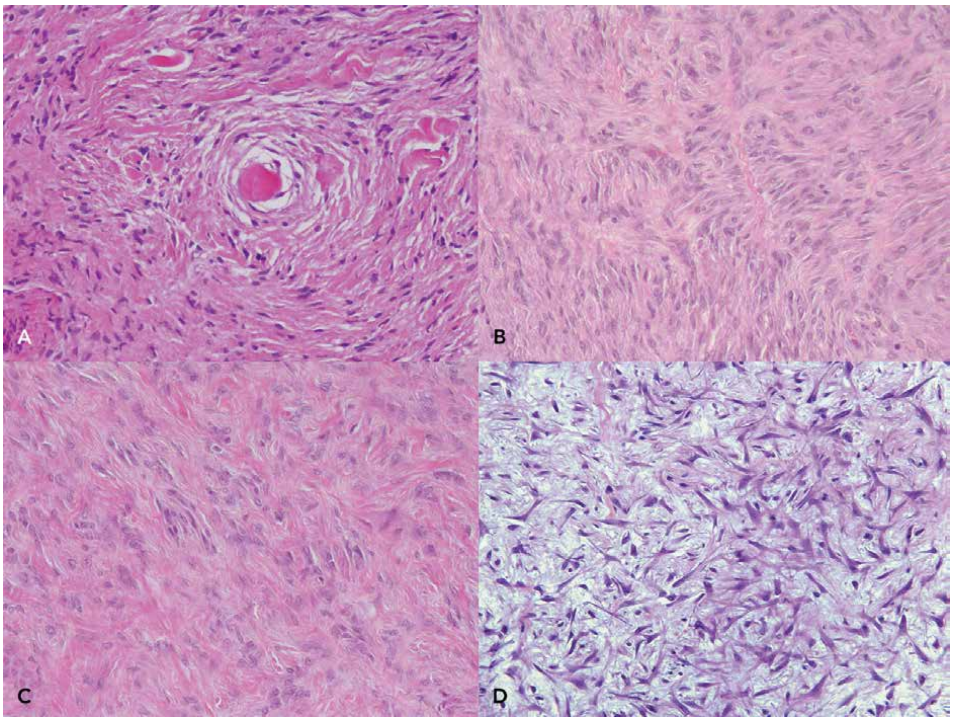


**Figure 11.**
*(A) Bland neoplastic fibroblasts whorling around a thick collagen bundle. Whorling is considered a highly diagnostic pattern described in over 85% of equine sarcoids. Hematoxylin and eosin, 200×. (B) Area of high cellularity with plump neoplastic fibroblasts embedded in finely fibrillar to dense collagen and organized in parallel and perpendicular rows. Moderate anisocytosis and anisokaryosis are evident. Hematoxylin and eosin, 400×. (C) Area of moderate cellularity with parallel to haphazardly arranged plump fibroblasts embedded in abundant finely fibrillar to dense collagen. Moderate anisokaryosis is evident. Hematoxylin and eosin, 400×. (D) Area of high cellularity with haphazardly arranged highly atypical fibroblasts with spindle to stellate morphology embedded in finely fibrillar to myxoid stroma observed in a recurrent sarcoid. Hematoxylin and eosin, 200×.*

DNA from BPV 1, 2 [14, 67] and 13 [12] is detected in up to 90% of equine sarcoids by in situ hybridization in the nuclei of fibroblasts and keratinocytes [37, 67]. Disadvantages of DNA detection are the unsuitability for diagnosing occult sarcoids, the lower sensitivity compared to clinical diagnosis, and the low specificity due to high prevalence of BPV DNA positivity in normal equine skin samples [35], cutaneous inflammation [68], and in other skin-associated spindle cell soft tissue tumors such as peripheral nerve sheath tumors (PNSTs), fibrosarcomas, myxosarcomas, and fibromas [66].

In summary, the most diagnostic histopathologic features, when present, are the epidermal changes of hyperkeratosis, hyperplasia with elongated rete pegs and "picket fence" aspect in conjunction by proliferation of fibroblasts [9, 35, 62]. However, common to most sarcoids are the fibroblastic dermal proliferation and presence of BPV DNA [10, 35, 67].

Microscopic features of sarcoids can overlap with other lesions. Differential diagnosis may be challenging because of the variable histological configuration of the dermal proliferation especially in cases with extensive ulceration or lack of distinctive epidermal lesions. Major histopathological differentials include granulation tissue (proud flesh), fibroma, fibrosarcoma, and peripheral nerve sheath tumors (e.g. schwannoma and neurofibroma) [57, 63]. Granulation tissue is characterized by fibrous tissue oriented at right angles to newly formed capillaries and is often associated with edema

and a prominent inflammatory component. When fibroblastic sarcoids are ulcerated, it may not be possible to differentiate them from granulation tissue and clinical follow-up becomes necessary. Fibromas can be differentiated morphologically as well-circumscribed, expansile, sparsely cellular tumors composed of a monomorphic population of mature fibroblasts with no epidermal proliferation. Fibrosarcoma is more pleomorphic with higher cytological atypia but multiple patterns are rarely observed, and the epidermal component is absent. Peripheral nerve sheath tumors (PNST)/Schwannoma are characterized by variable presence of highly cellular often palisading areas (Antoni A pattern) and low cellular myxoid areas (Antoni B pattern). These areas are associated with the presence of typical Verocay bodies composed of acellular areas between areas of nuclear palisading. Immunohistochemical staining for S-100 protein may be useful in differentiating PNSTs from sarcoids; however, focal S100 positivity has been reported in sarcoids [62, 66].

## 6. Management of the equine sarcoid

A treatment should be prompted as soon as possible following diagnosis, and in some cases, suspicious lesions could be treated immediately after biopsy [5].

Several treatment modalities for the management of equine sarcoids are historically 'known' and anecdotal reports and retrospective studies on more or less effective therapies exist, but valuable prospective double-blinded trials are lacking in the literature [3].

### 6.1 General considerations

Before choosing a proper therapeutic plan, some general considerations must be made [3, 55]:

- The prognosis is usually very guarded and owners must be thoroughly informed about possible complications associated with the condition.

- Sarcoid-affected animals can never be considered free of the disease, even after successful treatment.

- Each lesion can require a specific treatment and can react in a different way compared to other sarcoids even on the same horse.

- The extent and location of the tumor greatly affect the decisional process. Periorbital sarcoids (**Figure 12**) tend to penetrate the underlying musculature. Function of the upper eyelid must be preserved and any possible deformation in the healing process must be avoided. Sarcoids over tendons, joints or the facial nerve can have severe complications. The worst sites are the elbow and the face, where sarcoids much more tend to local invasion and progression to the malignant form.

- The duration of the lesion is important as early intervention usually requires less aggressive treatments. It is also easier to treat small lesions that extensive ones that may also be under transformation from one type to the other.

- Previous therapies and/or interferences influence the response to a new treatment course and possibly a different approach may be indicated.

**Figure 12.**
*Periorbital sarcoid, eyelid function must be preserved when treating these lesions.*

Wrong interference is a major cause of exacerbation and the prognosis significantly reduces with each treatment failure.

- Planning combined, prolonged or repeated treatments can be necessary for many sarcoids.
- Costs and logistics can have a great influence on the choice of the therapeutic modality.
- Professional skills and experience of the veterinarian can also affect the rate of success and the same treatment used by different clinicians can result in different outcomes.
- Animal and owner compliance for the best treatment: some are very painful, some sites (e.g. ear) are more sensitive, general anesthesia may be necessary in certain cases.
- Careful fly protection, wound management and regular checks must be part of the long-term management of any sarcoid-affected horse.
- Spontaneous remission is reported but rare, the decision to delay treatment based on a possible spontaneous remission is discouraged.

### 6.2 Benign neglect

As previously discussed, a proper treatment should follow the diagnosis of equine sarcoid, but in some cases benign neglect may be an option. Horses may present with such extensive lesions that any treatment method would be impractical. In other patients, the sarcoids may be small enough to render the procedure too expensive. Clinicians should opt for benign neglect with caution, both patient welfare and the lesions should be strictly monitored as sarcoids can progress. Furthermore, their presence may contribute to spread to other sites and horses [3, 55].

### 6.3 Surgical methods

- *Sharp surgical excision*: this technique is often appealing to practitioners and, in some cases, easy and successful but carries rates of recurrence as high as 70%, with recurrences occurring mostly within few months or even during the healing process and being much more aggressive (commonly fibroblastic) than the original sarcoid [2, 3, 46]. Wide excision is necessary to reduce the risk of recurrence, but it is not always practical or feasible and a safe margin is impossible to define. The principle of smart surgery should be applied to minimize cell contamination during surgery. Protecting the tumor with adhesive dressings before surgery reduces contamination and in case of recurrence another therapeutic method or combined treatments are indicated [3, 55]. Occult and verrucose sarcoids can be effectively removed with wide margins, nodules in the eyelids are invasive thus very dangerous, whereas other nodular lesions may respond better. However, the prognosis is usually very guarded when using surgery alone [55].

- *Cryosurgery*: this method causes tumor necrosis and is commonly used but has the same limitations as surgical excision. It can be used successfully on superficial lesions but restriction of blood flow, a defined safety margin and adjuvant chemotherapy (intralesional or topical) during the procedure can improve outcome. It can be repeated if necessary until the tumor is completely removed but the ability of the patient to resist the cold can be a limitation [3, 55, 69].

- *Hyperthermia/radiofrequency hyperthermia*: the tumor, being more sensitive to temperature than normal tissue, is heated for 30 s to 50°C weekly for up to 5 weeks. Very few cases are reported using this technique that is not generally recognized in equine practice [55].

- *Surgical electrocautery*: this method was recently reported with a high rate of success, its advantages are the minimal bleeding into the wound site with a reduced risk of tumor cell contamination and usually limited scarring. Electrocautery is one of the few options for sarcoids on the ear pinna [3, 55, 70].

- *Laser surgery*: surgical ablation with $CO_2$-YAG laser or diode laser devices is reported with success rates as high as more than 80%. When accurately used, this method is associated with the ability to sterilize the wound, no bleeding and avoids seeding tumor cells during the procedure. $CO_2$ lasers cause less thermal injury than diode ones. Primary closure may be possible, but a high rate of wound dehiscence and slow healing are disadvantages. Careful selection of the lesion is important: recurrence is most likely in verrucose sarcoids with poorly defined margins, whereas localized pinnal sarcoids and fibroblastic type 1a tumors around the eye may respond well [3, 55, 71, 72].

- *Ligation*: this method can be used only on pedunculated sarcoids where no tumor extensions are present in the pedicle below the ligature. This means that it is suitable for nodular type A1 and B1 or fibroblastic type 1a sarcoids, or any sarcoid where an artificial tumor-free pedicle can be created. The pedicle is ligated with castration/elastration bands, it works better if several bands can be placed and if adjunctive intralesional or chemotherapy are combined. The use of plastic ties or suture material that cut the lesion and partial ligation should be avoided as it carries a poorer prognosis and is associated with exacerbation or recurrence [3, 55].

### 6.4 Chemotherapy

Different chemotherapeutic agents and compounds can be used to treat sarcoids, usually they are topically or intralesionally administered with little or no systemic effects [55]. Systemic doxorubicin was used only in one study, but limitations and constraints to its use reserve this treatment only to very extensive or wide-spread lesions referred to specialist centers [3, 73].

- *Topical and intralesional 5-fluorouracil*: this cytotoxic and antimitotic agent can be topically applied as 5% ointment with a twice daily protocol over a few weeks. It is usually successful on small occult and verrucose lesions, or to control large areas that cannot be treated with other modalities. During treatment an inflammatory reaction can be marked but usually minimal scarring follows. It can also be combined with surgery [3, 55, 74]. The intralesional injection of 5-fluorouracil at the dose of 50 mg/cm$^3$ every 2 weeks for up to 7 weeks is reported with a successful rate of 61.5%, sarcoids larger than 13.5 cm$^3$ had a poorer prognosis compared with smaller lesions [75].

- *Topical imiquimod*: this agent is an immune response modifier with potent antiviral and antitumor activity and is used to treat human genital warts. The reported protocol for equine sarcoid is to apply the cream three times a week for 16–32 weeks. The treatment is usually associated with inflammation, alopecia and depigmentation. Administration of oral phenylbutazone can be helpful in some cases to control the discomfort [55, 70, 76].

- *Topical AW5*: it is a cream containing heavy metal salts, fluorouracil, thiouracil and steroid. Its use is restricted to veterinarians only, protocols include repeated applications but it can be contraindicated in some cases such as around the eye or other structures (facial nerve) that can be damaged. The reported success rate is around 74% depending on lesion and previous treatment history [3, 52, 55].

- *Topical acyclovir*: topical 5% acyclovir cream has been used to treat sarcoids with some benefits reported in one study [77]. A subsequent retrospective case-series and a double-blinded placebo-controlled trial resulted in no advantages from this agent compared to other treatments or placebo [70, 78]. The cream is used without prescription for human herpes virus infection and this may be attractive for owners that desire to treat horses without looking for veterinary advice with deleterious effects [3].

- *Silver nitrate*: silver nitrate caustic pencil is an old-fashioned treatment that can be applied to very localized small lesions [55].

- *Intralesional cisplatin*: this chemotherapeutic agent has been used in several studies in the form of injectable solution, emulsion and of biodegradable beads. Resolution rates are up to 93% in sarcoids less than 5 cm in diameter, larger lesions can be cured combining surgical debulking and intralesional cisplatin [3, 55]. A general protocol includes repeated injections of cisplatin oily emulsion at the dose of 1 mg/cm$^3$ every 2 weeks for four times but intervals may change upon patient needs. The material does not diffuse more than 5 mm in tissues so several injections every 6 mm–1 cm of tumor and margin of normal tissue are necessary. The aqueous solution has a clearance of minutes whereas the medical-grade sesame seed oil emulsion has the advantage of a lower

concentration and a slower release. Due to the high toxicity, self-protection measures must be strictly respected when handling cisplatin [3, 55, 79]. The use of biodegradable beads containing cisplatin is also reported with or without surgical debulking, the latter is usually necessary in tumors larger than 1.5 cm in diameter. Beads are placed at 1–1.5 cm intervals along the wound or tumor margins [3, 55, 70, 80].

- *Bleomycin*: bleomycin is a glycopeptide antibiotic with antineoplastic activity, it has been used to treat sarcoids intralesionally and with the use of electrochemotherapy. Recently the topical use on occult and verrucose sarcoids of an ultradeformable liposomal preparation of bleomycin, alone or following 5-fluorouracil or tazarotene application, has shown good efficacy with the absence of pain and inflammation as an advantage [3, 81].

- *Electrochemotherapy*: electrochemotherapy is based on the use of electrically induced increases in cell membrane permeability to increase the effects of cytotoxic agents such as cisplatin, carboplatin, and bleomycin. It requires repeated general anesthesia, up to 8 treatments, and specialist equipment [3, 79, 82, 83].

### 6.5 Photodynamic therapy

This method is based on photosensitization of tumor cells with a topical or intralesional photosensitizer (e.g. hypericin or 5-aminolevulinic acid and derivatives) followed by the application of a specific light wavelength emitted by a proper light source, for a defined time (minutes). The mechanism is complex and takes advantage of the production of reactive oxygen species that kill sensitized cells, so it is very localized. The literature on its use on sarcoids is limited but significant benefits are reported, with or without surgical debulking [3, 84–88].

### 6.6 Immunotherapy

Since the involvement of BPV infection, much research is being focused on immunologic methods but without practical results so far. Moreover, horses do not seroconvert for BPV and vaccination does not prevent sarcoid development [3].

- *Spontaneous remission*: it is generally reported as rare, however, a recent study just reported a high proportion of spontaneous remission in a population of Franches-Montagnes horses in Switzerland [53]. The mechanism is not clear yet and antibodies have been detected only in donkeys [5]. Long-term immunity appear to occur in horses with sarcoids that undergo spontaneous remission [3].

- *Immunomodulation*: the use of intralesional injection of the bacillus Calmette-Guérin (BCG) is reported in different studies with high success rates, especially around the eye. Sarcoids on the distal limbs respond less or may even exacerbate [3, 69, 89, 90]. This method gives best results on nodular and fibroblastic sarcoids but may be associated with anaphylaxis, especially when repeated injections are performed [55, 90].

- *Vaccines*: attempts to stimulate sarcoid regression or potential preventive effects through autogenous and BPV-1 L1virus-like particles vaccines have been made but further studies are needed [3, 91].

- *Autoinoculation/autografting*: the inoculation of sarcoid tissue is reported in two studies, but doubts are raised about this method due to the risk of complications and the fact that it is not described for other cancers in any species [3, 92].

- *Hemotherapy*: no literature is available describing the effectiveness of this method. However, it is widely used in Central America and consists of withdrawal of venous blood and its intramuscular injection with anecdotal success [3].

### 6.7 Gene therapy

Mediator-governed therapy and genetic manipulation are under research but no practical treatment for the equine sarcoid has been reported yet [3, 27].

### 6.8 Radiotherapy

Facilities and special equipment are required for radiation therapy, which contribute to its high costs and limited availability. Different techniques exist:

- *Teletherapy*: it is expected to be effective, but few reports exist [3, 93].

- *Brachytherapy*: using radioactive radon, iridium and gold isotopes, it has become the gold standard for sarcoids, especially periorbital lesions [3, 93–96].

- *Plesiotherapy*: this surface brachytherapy method uses beta radiation from strontium$^{90}$ and is reported on small superficial sarcoids [3, 93].

### 6.9 Adjunctive therapy

To remove secondary epidermal changes in sarcoid tumors, tazarotene can be used as adjunctive treatment. It is a retinoid 0.1% gel commonly used in human medicine for the management of keratinization disorders [3].

### 6.10 Phytotherapy

- *Viscus album austriacus*: the use of the injectable extract of the white mistletoe plant is reported to have immunomodulating effects in humans and was used in one double-blinded placebo-controlled trial in horses with sarcoids. Repeated subcutaneous injections for 15 weeks provided a positive outcome compared to placebo [3, 97].

- *Sanguinaria canadensis/zinc chloride*: commercially available compounds containing bloodroot (*S. canadensis*) and zinc chloride are anecdotally used for the treatment of equine sarcoids. Although high rates of success are reported on the internet, the use of this material by owners without veterinary advice carries risks. The use of this product on horses is not supported by scientific literature and dangerous toxicity in humans is reported [3].

### 6.11 Other remedies

Several 'natural' or herbal or homeopathic remedies are often used to treat sarcoids, usually with a delay in proper treatment and a risk of interference causing exacerbation of the tumor. Caution should be used considering the use of any material suggested to treat every condition in every species [3].

## 7. Sarcoids in other equids

Sarcoid tumors are reported also in animals other than horses. Donkeys, mules, and zebras can be affected, but reports of sarcoid tumors associated to BPV infection exist also in cats, giraffes, sable antelopes, and captive tapirs [98–104].

As far as equids are concerned, the reported prevalence of sarcoids in zebras is 25–53%, whereas incidence in UK donkeys is 0.6 per 100 animal years with apparent increased risk for young males [5, 105, 106]. The equine sarcoid is reported as the most common tumor in donkeys and presence of sarcoids among these equids and zebras is sometimes reported as outbreaks [105–109].

Diagnostic and treatment methods are the same as for horses, one study reports the use of surgical excision, intralesional 5-fluorouracil, allogenous vaccine or 5-fluorouracil in combination with autogenous vaccine in zebras [98, 110].

## 8. Conclusions

The equine sarcoid is a locally invasive skin neoplasm commonly encountered in practice. It has different clinical presentations, and early diagnosis with prompt treatment can improve the prognosis, but their importance is often underestimated. Several treatment options are available with variability in lesion and patient response. Spontaneous regression is rare, recurrence is common, and exacerbation is a possible complication, especially when a wrong therapy is attempted. Sarcoid-affected animals can never be considered free of the disease and horse owners must be correctly informed about the features and behavior of this tumor.

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Section 3

# Digestive System, Diet and Behavior

Chapter 8

# Current Strategies for Prevention and Treatment of Equine Postoperative Ileus: A Multimodal Approach

*Milomir Kovac, Ruslan Aliev, Sergey Pozyabin, Nevena Drakul and Albert Rizvanov*

## Abstract

Equine paralytic (postoperative) ileus generally refers to an acute condition of impaired gastrointestinal motility. Paralytic ileus is most frequently seen following abdominal surgery on the small intestine in horses. Three main mechanisms are involved separately or simultaneously in its causation, namely neurogenic-endocrinic, inflammatory-endotoxic and pharmacological mechanisms. Regardless of the cause, equine paralytic ileus can be fatal, if not properly diagnosed and treated. Over the past 22 years (1997–2019), we have diagnosed and treated more than 180 horses with postoperative ileus using differing methods. Based on our results and experience, and that of others, we have developed a multimodal strategy to reduce the incidence of postoperative ileus. This has resulted in effective treatment of ileus-diagnosed patients in 94% of cases, a significant improvement in survival rates over the last 20 years. In this review, we described pre-, intra-, and postoperative multiple supplementary preventative and treatment procedures that cure this condition. These methods are dependent on individual cases but include the control of endotoxemia and inflammation, as well as using the least traumatic surgical techniques, carrying out the pelvic flexure colotomy, improved anesthesia techniques, treating with continuous postoperative peritoneal lavage, the use of fluid, antibiotic and NSAIDs therapy, according to a scheme the use of different prokinetic agents (including metoclopramide, neostigmine methylsulfate and domperidone), nasogastric decompression, management to minimize the surgical and postoperative stress reaction and judicious timing of postoperative feeding of horses.

**Keywords:** postoperative ileus, paralytic ileus, horse, prevention, treatment

## 1. Introduction

Visceral abdominal pain of the horse, defined as equine colic, is one of the most acute life-threatening problems facing equine practitioners [1]. The incidence of equine colic has been reported as between 4 and 10 cases/100 horses/year [2]. Colic in horses can be caused by more than 70 pathological processes in the gastrointestinal tract and manifests itself in many forms [3]. The diseases that

accompany colic in horse are often characterized by ileus. The Greek physician Soranus defined an ileus as "a severe and dangerous twisting of the intestines." Currently, an ileus can be referred to as a symptom characterized by a complete or partial disturbance passage of contents through the intestinal canal, due to obturation, strangulation, spasm, ischemia, adhesions and impaired motor function (paralytic ileus) [4].

The definition of paralytic ileus is somewhat controversial. Paralytic ileus is mostly defined as a temporary or permanent cessation of propulsive contractions of the gastrointestinal tract, irrespective of pathogenetic mechanisms, with subsequent gut dilation and accumulation of secretions and gas within its lumen [5]. Paralytic ileus in the horse is not a primary disorder but rather an underlying cause and can be classified on the basis of its etiology. More than 95% of all paralytic ileus cases in horses, seen after abdominal surgery, are primarily in the small intestine [6, 7]. Precisely for this reason, the paralytic ileus is often signified as postoperative ileus (POI). Once in a while, equine POI can be classified more precisely according to anatomical localization, for instance POI of the small intestine or POI of the cecum and colon. POI of the small intestine is easy to diagnose, through the presence of gastric reflux (i.a.), and impaired motor function seldom occurs in other parts of the gastrointestinal tract in horses too [8]. In the latter rarer cases, diagnosis is more of a challenge, because the presence of gastric reflux in the postoperative period is relatively uncommon after surgery on the large intestine in horses [9]. However, it must be considered also that the dysmotility in equine POI of small intestine may mask large intestine involvement.

In people following surgery, the return of the small intestine's action generally begins around 4–8 h postoperatively and generally completes in around 24 h [10]. The colon resumes its function between 48 and 72 h postoperatively [11, 12]. In humans, based on this observation, various additional qualifying terms have been applied to POI, such as physiological POI, prolonged POI and recurrent POI [13]. This classification system can also be applied to horses, but it must be emphasized that the recurrent form of POI is very rare [14].

Regardless of determination or classification, equine paralytic ileus is a common and serious complication of surgery associated with highly increased odds of death. Reported fatality rate in horses with POI also varies widely, from 13 to 86% [15–18]. In one study, horses that developed postoperative ileus were nearly 30 times less likely to survive than horses that did not develop ileus [19]. Additionally, equine POI leads to increased hospitalization time and treatment costs. It is for these reasons that since the first modern attempts to undertake abdominal surgery 50 years ago through to today, prevention and treatment of POI are widely discussed topics in equine medicine [20, 21]. In human medicine, enhanced recovery after surgery (ERAS) programs exist, which include multiple pre-, intra- and postoperative interventions, aiming to reduce the occurrence of POI [22]. Currently, in equine medicine, no universally-accepted approach exists for the management of equine POI.

Over the past 22 years, we have diagnosed and treated more than 180 horses with POI, using, in two veterinary clinics "Hochmoor" (Germany, 1997–2007) and "New Century" in Moskow (Russia, 2007–2019). In the latter times, with multiple pre-, intra- and postoperative procedures, not only was POI prevalence reduced significantly, but also following occurrences of equine POI successful treatment and survival were possible in more than 94% of cases. The purpose of this chapter review is to clarify some of the proposed key mechanisms in the pathophysiology of POI, share our experiences and make proposals for the prevention and treatment of equine POI.

## 2. Normal physiology of equine gastrointestinal motility

An appreciation of the basic mechanisms that regulate gastrointestinal motility is a key component to understanding paralytic ileus. The musculature of the gastrointestinal tract in horses is made up of smooth muscle cells that are intimately associated, thus allowing them to conduct electrophysiological functions. There are three distinctive electrical potentials in the equine intestine: resting potential, slow-wave and spike potential that trigger contractions. Slow waves are rhythmic pacemaker currents initiated by the interstitial cells of Cajal (ICC). Normal gastrointestinal motility in horses results from very complex interactions among the enteric nervous system (ENS), autonomic and central nervous systems, ICC, gastrointestinal hormones, immune cells, glial cells and local factors that affect smooth-muscle activity [21, 23–25]. Extrinsically, the sympathetic nervous input through noradrenaline has an inhibitory effect on gastrointestinal motility, whereas parasympathetic input increases motility. The ENS is involved in all aspects of gastrointestinal function, not only motility, as well as by enteric processes such as immune responses, detecting nutrients, microvascular circulation, intestinal barrier function, and epithelial secretion of fluids and ions [10]. The neurons of the ENS are collected into two types of ganglia: myenteric (Auerbach's) and submucosal (Meissner's) plexuses. The enteric nervous system influences the gastrointestinal tract either directly through neurotransmitters or indirectly through intermediate cells, such as the ICC, cells of the immune system or endocrine cells [10]. These intestinal neurons communicate through more than 25 different neurotransmitters, including stimulatory neurotransmitters (acetylcholine, neurokinin A, adenosine, substance P, motilin, serotonin and cholecystokinin) and inhibitory neurotransmitters, for instance, vasoactive intestinal peptide (VIP), nitrous oxide (NO), neuropeptide Y, calcitonin gene-related peptide, GABA and neurotensin [26–29]. The endocrine system also indirectly affects regulation of the gastrointestinal tract motility. The hormones related to stress activity (glucocorticoids, cortico-realizing peptide, thyroid hormones and somatotropic hormone) have the most pronounced inhibitory effect on gastrointestinal tract activity. Additionally, the intestinal cells produce a range of hormones and hormone-like substances, some of which are also neurotransmitters. These substances regulate the motility of the gastrointestinal tract (motilin, enteroglucagon, cholecystokinin, pancreatic polypeptide and peptide YY) and secretory activity (gastrin, secretin, cholecystokinin, pancreatic polypeptide, gastric inhibitory peptide and neurotensin) and also regulate the production of other hormonal substances (somatostatin and gastrin-releasing peptide) [30].

## 3. Prevalence and risk factors for equine postoperative ileus

The etiology of paralytic ileus in the horse is multifactorial, and various factors contribute either simultaneously or at various times during the development of this entity. In the current literature, the incidence of POI in horses undergoing surgical treatment of all types of colic has been reported to range from 10 to 21% [31–33]. The incidence of POI in horses undergoing surgical treatment for small intestine lesions varies widely from 10 to 73% [1, 15, 16, 18–20, 31, 34–38]. The large variation in the reported rates can be at least partly explained by the criteria used to define postoperative ileus. Other forms of paralytic ileus, those that do not present due to equine surgery, are much less common than in humans. These include forms that result from metabolic derangements, acid-base abnormalities, electrolyte

imbalances (hypokalemia and hypocalcemia), severity stress syndrome, peritonitis, bacterial infection, uremia, hypoalbuminemia, abdominal trauma, burns, botulism, grass sickness, atrophic visceral myopathy and application of drugs and anesthetic agents [6, 7, 26, 32].

According to our investigation, there was no significant age or breed dependence associated with the incidence of postoperative ileus in horses, but stallions more often had POI than geldings [39]. Our observations showed that the horses with a so-called hot temperament (i.e., horses with more excitable demeanors) had increased risks of developing POI than warm- or cold-blooded horses. The preliminary results (unpublished data) also showed that horses with behavioral symptoms of stress and high concentrations of cortisol in their blood in the pre- and postoperative time more often had POI than horses with normal concentrations of cortisol.

According to our observations, if pre- and during surgical intervention the equine jejunum had a high degree of intraluminal distension (more than 8 cm), postoperative ileus was more likely to occur and did so in more than 70% of the cases observed [32]. This is partly confirmed by other authors [15, 18]. A possible reason for this finding is the long onset of colic disease and high degree of endotoxic shock, which leads to enteric nervous system damage and a high degree of intraluminal jejunum distension. It has been demonstrated that decreases in intestinal motility through the distension of equine jejunum are partly due to decreases in motilin receptor synthesis [21].

Horses with small intestinal strangulating obstruction (for instance, by hernia inguinalis, entrapment in foramen omentalis) are at increased risk of developing POI, compared with obstructive ileus (for instance, by ileum or jejunum obstipation) [40, 41]. A basis for higher concentration endotoxins is that horses with entrapment in the foramen omentalis have lower blood pressures during abdominal surgery than horses with ileum obstipation [42]. It has also been shown that horses suffering from pedunculated lipoma obstruction are three times more likely to suffer from POI when compared with horses suffering from other intestinal pathologies [3]. This fact was confirmed in our own observations [4]. Many factors are associated with increased risk of POI in horses with pedunculated lipoma obstruction, for instance age-related decreases in intestinal density of the enteric neurons and glial cells, rapidly developing endotoxic shock and perhaps also high colic pain, which lead to enormous activation of the sympathetic nervous system.

Numerous studies have shown that long duration of colic disease, with evidence of endotoxin shock (high pulse rate and hemoconcentration), the presence of >8 l of reflux at admission, anesthesia for longer than 2.5 h, and the performance of a small intestinal resections pose enormous risks for POI development [15, 31, 43, 44]. Based on our results, the risk of developing POI and other fatal complications were associated with increased duration of surgery [14]. Surgical techniques also affect the incidence of POI in horses. The ability to perform a safe bowel resection and anastomosis techniques also affects the incidence of POI in horses; for instance, the use of jejunocecostomy has been associated more often with the development of POI when compared to horses in which end-to-end jejunostomy is performed [45]. One possible reason for this fact is possibly that the end-end jejunostomy is done more rapidly, and therefore duration of surgery and anesthesia is shorter than by the jejunocecostomy.

Other postoperative complications such as primarily postanesthetic myopathy and peritonitis increased the rapid risk of POI development in horses [46]. Other postoperative complications after colic surgery for example incisional infection, herniation and dehiscence, jugular vein thrombophlebitis, laminitis, adhesions and

diarrhea had no important influence on the development of POI; however, these complications tend to develop later during the postoperative period in horses [39].

## 4. Pathophysiology of equine postoperative ileus

The pathogenesis of postoperative ileus is complex, with multiple factors contributing either simultaneously or at various times during the development of this entity. The classical view in the pathogenesis of POI involves two phases: an initial neurogenic phase resulting in immediate postoperative impairment of intestinal motility and a subsequent inflammatory phase lasting for several days [24, 30, 37, 47]. On the basis of our observations, we expanded this view into three (or five) main mechanisms, which either independently or in combination are involved in the causation of equine postoperative ileus, namely inflammatory-endotoxic mechanism, neurogen-endocrinic mechanism and pharmacological-anesthetic mechanisms. However, the importance of each contributing mechanism may vary over time, with considerable overlap and possible interactions; therefore, this division is conditional.

### 4.1 The role of inflammation and endotoxemia

Postoperative inflammation of the small intestine (and nearly imperceptibly of equine large intestine) is an important factor in the pathophysiology of equine POI [9, 48]. It is well known that inflammation is a biological response of the immune system, blood vessels and molecular mediators to a broad range of different stimuli such as pathogens, endotoxins, and physical and chemical irritants. There are a lot of inflammatory agents to take into consideration in POI, for instance, specific intestinal pathology and tissue injury (including obturation, strangulation and adhesion), bacteria, endotoxins, surgical trauma and manipulation [49–53]. The classical intestinal inflammation following paralytic ileus occurs by the duodenitis-proximal jejunitis (DPJ). This syndrome is caused primarily by toxic and infectious agents (e.g., Salmonella and *Clostridium perfringens*) [4, 44].

Horses with strangulating lesions of the small intestine have been shown to have various degrees of serosal and neuromuscular inflammation and high numbers of apoptotic cells (including smooth muscle, enteric neurons and glia), possibly due to intestinal ischemia and reperfusion injury [23, 54–57]. According to different studies, equine POI might actually be triggered by a primary disturbance of the smooth muscles' ability to contract, and how the number of smooth muscles or

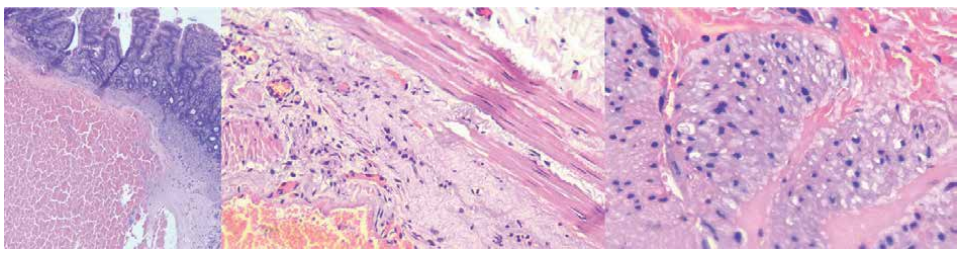

**Figure 1.**
*Histological appearance of the jejunum in a horse affected by postoperative ileus. Note the mucous membrane maintaining its correct structure. In the submucosal layer and on the border with the inner muscle, extensive delaminating hemorrhage and leukocyte reaction were determined. Muscle fibers of the inner muscle layer with pronounced dystrophic changes in the cytoplasm were present. Ganglion cells in the intramuscular layer were not detected; in their place, a loose, weakly basophilic fibrous connective tissue with single mononuclear cells was present. The serosa was moderately edematous. H&E stained, ×10 magnification.*

neural receptors has changed through a leukocytic and macrophage inflammatory response, primarily within the intestinal muscularis externa (**Figure 1**) [58–60]. Potentially due to different inhibitory mediators (NO, SP, VIP and NPY), cytokines (TNF, IL-1b, IL-6 and IL-10), monocyte chemotactic protein-1, prostaglandins, histamine, mast cell proteinase-1, tryptase, reactive oxygen intermediates, defensins and adenosine secreted from the muscularis externa during intestinal inflammation and abdominal surgery [49, 61–64]. This local molecular inflammatory response increases prostaglandin E2 levels in the peritoneal cavity that correlates temporally with the development of postoperative ileus [13, 65, 66]. According to our former investigations, in most cases of equine abdominal surgery, during three postoperative days, the concentration of leukocytes is markedly increased (sometimes up to $100 \times 10^9$/l) as are total plasma proteins in the peritoneal cavity [39]. In the horse, postoperative neutrophilic and eosinophilic inflammation of the jejunum has been identified up to 18 h postoperatively [55, 67].

In intestinal inflammation, pathogen-associated molecular patterns or PAMPs have important roles. These molecules can be referred to as small molecular motifs conserved within a class of microbes. Bacterial lipopolysaccharides or endotoxins found on the cell membranes of Gram-negative bacteria are considered to be the prototypical class of PAMPs. The endotoxins are very potent and are widely spread inflammation-inducing substances. One of the basic characteristics of almost all gastrointestinal disorders in horses (primarily by different forms of strangulation ileus) is the development of the endotoxic shock [68]. The mucosal barrier of the equine intestine normally efficiently restricts the transmural movement of endotoxins and bacteria. However, whenever the integrity of the mucosal barrier is lost, as occurs with inflammation or ischemia of the intestinal wall, endotoxins cross into the portal blood and peritoneal cavity [69]. The generally accepted scheme for endotoxin binding is to CD14-bearing receptor cells (monocytes, macrophages, dendritic cells, and possibly vascular endothelial cells), which then associates with the TLR4-MD-2 complex to initiate a downstream signal, causing a proinflammatory response, such as leukocyte recruitment [53]. Macrophage-derived cytokines (such as IL-1b and TNF), as well as arachidonic acid metabolites (i.e., prostacyclin and thromboxane), are responsible for many of the pathophysiologic consequences of endotoxemia and tissue injury in equine colic cases. Endotoxins among other things activate inducible nitric oxide synthase (iNOS) in intestinal macrophages [66]. The resultant increase in NO release stimulates decreased smooth muscle contractility. In healthy ponies, IV infusion of endotoxin also resulted in inhibition of motility in the stomach, cecum, left dorsal colon, and small colon [70]. However, no nasogastric reflux was observed. Although motility in the small intestine was increased, its myoelectric pattern was abnormal. The effects of endotoxins on motility were partially mediated by $PGE_2$ possibly stimulating alpha-2 adrenergic receptors [28, 51, 71]. A platelet-activating factor (PAF) antagonist suppressed some of the endotoxin-induced inhibition of motility in horses. These findings led to the conclusion that the PAF may also play a role in the development of POI [68].

The degree of endotoxic shock in horses is directly dependent on the forms and time span of gastrointestinal disease [72]. As the concentration of Gram-negative bacteria is highest in the large intestine of horses, the release of endotoxin and development of endotoxic shock are logically expected in pathologies of this part of the gastrointestinal tract, for instance by volvulus or colitis [43]. In the small intestine of horses, different population of Gram-negative bacteria exist, but in lower concentrations than observed in the large intestine. It would therefore be expected that in this case the endotoxins would not play a decisive role in development of POI. In contrast to this theory in one study of colic cases, the highest endotoxin concentrations were found in horses with entrapment in the foramen omentalis,

pedunculated lipoma obstruction and volvulus (torsion) of the large colon [72]. A significant impairment of small intestine transit has been shown in a rat model of colonic manipulation, which occurred even when the small intestine was surgically isolated [73]. These findings led to the conclusion that colonic manipulation induces an inflammatory response in the muscularis of the small intestine that is initiated and maintained by the release of endotoxins from the colon.

One potential trigger for intestinal inflammation not only endotoxins, as well the damage-associated molecular patterns (DAMPs) which realase by extensive surgical intestinal manipulation, luminale distension and resection [6, 7, 48, 52]. DAMPs are host biomolecules that can initiate a noninfectious inflammatory response. DAMPs are mostly cytosolic proteins and materials derived from the extracellular matrix (including hyaluronan fragments, ATP and heparin sulfate) and are generated following tissue injury [6, 7]. An activation of resident muscularis macrophages in the small intestine through DAMPs results in recruitment of intracellular signaling pathways (p38, JNK/SAP) and the release of pro-inflammatory cytokines [6, 7, 37]. Inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) upregulation then facilitates the production of NO and prostaglandins, both of which impair the contractile activity of the small intestine [52, 74, 75]. It additionally reduces lymphatic drainage with the occurrence of intestinal edema, which further impairs intestinal motility [76].

### 4.2 The role of the neurogen-endocrinic factors

The sympathetic division of the autonomic nervous system maintains internal organ homeostasis and initiates the stress response. In addition, sympathetic (adrenergic) hyperactivity results in the reduction of propulsive intestinal motility [8, 77, 78]. In the early component of ileus, the sympathetic neural pathways are activated already in the preoperative period, primarily through intestinal distension or strangulation (i.e., through initial colic pain), but inflammation and surgical manipulation and incision of the intestines and abdomen wall additionally stimulate afferent nerve fibers that subsequently activate peripheral, spinal and/or supraspinal reflex pathways [8, 15, 24, 27]. The sympathetic hyperactivity is amplified through various preoperative stressors (i.e., transport to clinic, unfamiliar surroundings with unknown caretakers, restraint of the horse for examination and rectal investigation) and also in the postoperative period of horse, initially by the recovery from anesthesia, but also in many postoperative diagnostic and management procedures (such as tying in stall, fasting, gastric decompression and blood collection) [24, 79]. There is overwhelming experimental and clinical evidence that different stress paradigms influence gastrointestinal motility [47, 80–82].

Sympathetic hyperactivity in horse primarily depends on the intensity of the nociceptive (pain) receptor stimulus [83]. Numerous nociceptors of sensory intestinal neurons by tissue damage or surgical manipulation send signals to the spinal cord and further to specific hypothalamic and pontine-medullary neurons. Within this pathway, corticotropin-releasing factor (CRF) plays a central role in inhibiting gastric and small intestinal motor function (but not of the colon) via interaction with the CRF-R2 receptors [82, 84–88]. The CRF stimulates neurons in the supraoptic nucleus of the hypothalamus, which send projections to the spinal cord, including the intermediolateral column of the thoracic cord, where sympathetic preganglionic neurons are located [88, 89]. At this point, inhibitory sympathetic efferent neurons are activated. Norepinephrine is released by sympathetic neurons at the enteric ganglia, which inhibit the release of the excitatory neurotransmitter acetylcholine by stimulating α2-receptors located presynaptically on cholinergic neurons [21]. This causes a depression of smooth muscle contractions in the gastrointestinal tract.

Activation of CRF receptors in the hypothalamus of horses mediates almost the entire repertoire of behavioral, neuroendocrine, autonomic, immunologic and visceral responses characteristic of stress syndrome [77, 90]. CRF release is the first step in activation of the hypothalamic-pituitary-adrenal axis (HPA axis) involved in stress response. The magnitude and duration of the activation in the HPA axis are proportional to the initial tissue damage and surgical injury, but also in other perioperative stress conditions. The pituitary gland responds to CRF by synthesizing a larger precursor molecule, proopiomelanocortin, which is metabolized within the pituitary into ACTH, β-endorphin and N-terminal precursor. Growth hormones and prolactin are also secreted in increased amounts from the pituitary in response to a surgical stimulus. Surgery is one of the most potent activators of ACTH and cortisol secretion; therefore, increased plasma concentrations of both hormones in human can be measured within minutes of the start of surgery [91]. Usually, a feedback mechanism operates so that increased concentrations of circulating cortisol inhibit further secretion of ACTH. This control mechanism appears to be ineffective after surgery resulting in elevated concentrations of both hormones [80]. Cortisol has known complex metabolic effects on the metabolism of carbohydrate, fat and protein. Cortisol impairs inflammation, which is helpful in the short term during conditions such as 'fight-or-flight,' also referred to as hyperarousal, or the acute stress response. In response to surgical trauma, massive levels of catecholamine (adrenaline, noradrenaline and dopamine) and glucagons are also released, while serum insulin concentrations decrease relatively [91]. The overall metabolic effect of the hormonal changes is increased catabolism, which mobilizes substrates to provide energy sources, and a mechanism to retain salt and water and maintain fluid volume and cardiovascular homeostasis [86]. According to our own preliminary research results (unpublished data), the cortisol levels increased from a baseline in the postoperative days after colic surgery, but more remarkably in POI group horses.

It seems that upon inflammation, there are numerous neurotransmitters that are mediated through surgical and postoperative stress, which caused disturbances in the motility of the gastrointestinal tract [92]. In an experimental model in ponies, using jejunal trauma through sympathetic reflexes and inflammation, electrical activity was decreased and the normal synchrony of gastric and duodenal MMCs was disrupted [8]. Intestinal manipulation of the small intestine in rodents impairs intestinal transit, through an inhibitory adrenergic pathway, because its sympathetic blockade is not always successful in reversing the inhibition of gastrointestinal motility induced by abdominal surgical procedures [93, 94]. In addition to sympathetic reflexes, surgical manipulation of the intestines activates inhibitory non-adrenergic, non-cholinergic (NANC) neurons in the gastrointestinal tract, resulting in the release primarily of NO and VIP, the consequences of which result in decreased gastrointestinal motility [93, 95, 96]. Substance P, which is a neurotransmitter involved in pain, has also been hypothesized to have a role in postoperative ileus [85]. In a model of POI in rats, where mechanical trauma to the small intestine and cecum was used, reserpine (which depletes catecholamine stores) and L-nitroarginine (a nitric-oxide synthase inhibitor) completely reversed the inhibition of ingesta transit. This finding supported the involvement of adrenergic and nitrergic neurons in the pathogenesis of POI [93]. As blockade of the calcitonin-gene-related peptide resulted in a similar effect, this peptide may be one of the neurotransmitters released by these afferent fibers and partly mediate postoperative ileus [97]. Additionally, endogenous opioids are also released after surgery and contribute toward postoperative ileus [92].

Other changes also occur following surgery stress, notably an increase in cytokine production. In human patients after surgery, cytokines IL-1, TNF-α and IL-6 may augment pituitary ACTH secretion and subsequently increase the release of cortisol.

A negative feedback system partially exists; therefore, glucocorticoids inhibit cytokine production and inflammation [98].

Most studies concentrate on central mechanisms whereby a stressful event perceived by the brain triggers neuronal and hormonal reflexes that influence the gastrointestinal motility. According to one study, the intestine produces the same stress peptides that are present in the central nervous system [99]. A local stressor, in this case endotoxins, results in the local generation and action of stress peptides that mediate inflammation without involving the central nervous system. In other words, the peripheral stressors induce local release of CRF possibly from enteric neurons and immune cells [88]. Peripherally derived CRF may act on the enteric nervous system and mast cells to induce inflammation and control motility and secretion [89].

### 4.3 The role of drugs and anesthetic agents

The pharmacological mechanisms of postoperative ileus are well described in the literature. Xylazine and detomidine are α2-adrenergic agonists and are commonly used in horses for sedation and pain control. Activation of presynaptic α2-adrenergic receptors within the enteric nervous system inhibits ACh release from cholinergic neurons, thereby suppressing intestinal contractions in normal ponies, primarily of the distal jejunum, pelvic flexure, cecum, and right ventral colon [100–102]. Although the use of α2-agonists has been reported to suppress intestinal motility, no direct significant associations have been made between POI and sedation or type of sedative used.

Anesthesia gases do have an effect on intestinal motility, and the longer anesthesia lasts, the greater the actions [85]. Based on our observations during 1997–2000, the incidence of POI was greater than after the year 2000. One of the reasons for this was that the active use of halothane was stopped, and we began to use isoflurane as an anesthetic gas. Other studies have also shown that anesthetic drugs such as halothane and atropine tend to decrease gastric emptying and inhibit intestinal motility, with the greatest effect on the colon and cecum and they can initiate cecal impaction in horses [103–105]. Interestingly, the cecal impaction occurs more commonly after orthopedic procedures [106]. Therefore, general anesthesia herein appears to be a less likely primary cause of cecal and small intestine motility dysfunction [104, 107]. Possibly, persistent pain after orthopedic procedures, resulting in sympathetic overstimulation, is a significant contributing factor.

## 5. Diagnosis of equine postoperative ileus

Large intestinal dysmotility is commonly recognized following a delay in defecation and also by rectal and/or ultrasonographic examination. There are different criteria for the diagnosis of equine small intestine POI in the literature [15–17, 108, 109]. Based on our previous experience and regardless of the rare cases of exclusion, the main criteria for diagnosis of POI of small intestine are as follows:

1. Postoperative period during 1–7 days after abdominal surgery. Most cases of POI occur within 12–48 h after recovery from anesthesia.

2. Postoperative reflux of ≥2 l upon any given intubation, or > 2 l/h on repeated intubation, of gastric contents with pH ≥ 6.0. Another study defined cases of postoperative ileus as horses with >20 l during a 24-h period, or >8 l during any single refluxing event [110]. Merrit and Blikslager [111] suggested the adoption

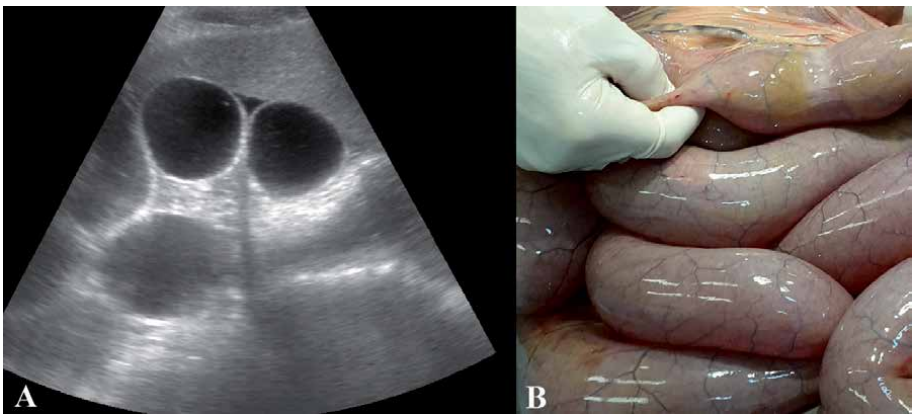


**Figure 2.**
*(A) Transcutaneous ultrasonogram in a horse with POI before surgery in region 3 l with evidence of multiple fluid-distended small intestinal bowel loops [113]. (B) Jejunojejunal distension identified during repeat celiotomy in the same horse.*

of a consensus on the classification of clinical criteria for POI, which included ≥4 l on any given intubation or > 2 l/h on repeated intubation. In most of our cases of POI, an average of 8–12 l, if intubation is performed every 4–5 h (i.e., approximately 2 l/h of fluid accumulate in the stomach). If more reflux is noticed, other pathologies are possible (e.g., mechanical obstructions and anastomotic leaks).

3. Moderate abdominal discomfort, which intensifies every 4–5 h after the last intubation. The response to nasogastric decompression provides an important clue that the problem is functional (i.e., POI). If a high degree of pain is noticed and continues, other gastrointestinal pathologies are possible.

4. Heart frequency 40–65 beats/min, if intubation is performed every 4–5 h.

5. Hematocrit 0.40–0.50 l/l, if standard infusion therapy is performed. If a high hematocrit is noticed, other gastrointestinal pathologies are possible.

6. Evidence of multiple fluid-distended small intestinal loops on rectal examination.

7. Ultrasonographic evidence of multiple fluid-distended small intestinal bowel loops (≥3 cm), edema and lack of motility in different parts of the equine abdomen (**Figure 2**) [112–114].

8. Borborygmi are usually decreased, especially the absence the ileocecal noise dorsally behind the right costal arch [48, 115].

## 6. Prevention and treatment of equine postoperative ileus

Since the treatment of this condition is very complex, and the complications are often fatal, the prevention strategy of POI is a very important way to improve the survival rate of horses that have undergone abdominal surgery [34, 109]. There are many methods and procedures and prophylactic and therapeutic choices for equine POI, depending on each individual case. The preventive strategies come from better

understanding the pathogenesis of this condition and treatment of POI must first address the underlying cause(s). The prophylactic and treatment strategies of the equine POI we are currently proposing are a multimodal regimen, which can be divided into three phases pre-, intra- and postoperative. The proposed multimodal treatment approach should include limiting factors, which are known to contribute to postoperative ileus. Each phase has the same significance in survival rate of equine POI.

### 6.1 Preoperative strategies in the prevention of equine POI

The long onset of colic disease producing high degree of endotoxic shock, in accordance with our earlier findings [32], are strongly associated with an increased risk for POI development. In this regard, it is appropriate to again highlight the importance of timely referral and prompt surgical intervention in surgical (strangulation) colic cases for the prevention of POI. Additionally, time from onset of colic to surgery has a decisive role not only in prophylactic but also in terms of a successful treatment by occurrence of equine POI. Failure to refer promptly leads to not only POI but also the occurrence of other perioperative complications by abdominal surgery in the horse. Approximately, every hour of tardiness with surgical interventions in a horse with strangulation ileus (for instance, by small intestine or large colon volvulus) reduces the survival rate from 5 to 10%, due to the rapid development of endotoxic shock [4, 116]. We found a significant correlation between the occurrence and survival of POI with colic duration in horses with inguinal hernia ($r$ = 0.72) and epiploic foramen entrapment ($r$ = 0.78), and partially (not significant) due to ileum obstipation ($r$ = 0.41) [40, 41, 45]. According to our recent study in 33 horses with entrapment in the epiploic foramen, surgery performed within 6 h from onset of colic had a survival rate of 87%, compared with 25% survival with surgery 10 h or more after onset [40].

It is important even before the onset of surgery to prepare, applying medicaments that reduce endotoxin release and alleviate inflammation effect, for instance, the application of NSAIDs (flunixin meglumine), corticosteroids (prednisolone) and antibiotics. Administration of corticosteroid drugs to critically ill surgical colic horses results in a significant reduction of shock symptoms. The use of these drugs should certainly continue in the postoperative period [26]. Antimicrobials should be administered intravenously, ideally within 30–60 min before the first surgical incision. For horses undergoing abdominal surgery in the perioperative period, we introduced the following antibiotics: cobactan® 2.5% (cefquinome) (IM 3 mg/kg BW) for 5 d; gentamicin (6.6 mg/kg BW, IV, q24h) and metronidazole (20 mg/kg BW IV, four times daily) for 3 days. Additionally, if time permits, during urgent transport to the equine clinic, horses should have a balanced polyionic intravenous fluid applied in order to reduce hemoconcentration. It is advisable that before abdominal surgery horses should have a hematocrit level of below 0.45 l/l. In cases of metabolic acidosis, 5% sodium bicarbonate solution should also be administered. We used pre- and intraoperative the hypertonic saline (NaCl 8.0%) only in horses with severe endotoxic shock and if in doubt on the presence of intestinal edema [40].

### 6.2 Intraoperative strategies in the prevention of equine POI

Surgical procedures and anesthesia affect the development of POI in horses (as discussed above). Operative management should be aimed at reducing duration of surgery and anesthesia in addition to other preventative strategies. In this aspect, an important role is played by a high-performing multidisciplinary surgical team with experience and knowledge as this optimizes surgical procedures. During the

abdominal surgery, the least traumatic surgical methods should be selected and performed and these should be carried out as efficiently and therefore quickly as possible. The degree of inhibition of circular muscle contractility is related directly to the magnitude of leukocyte and macrophage infiltration, which in turn depends on the intensity of intestinal manipulation; therefore, every effort should be made to reduce intestinal trauma. One surgical method able to reduce surgery time is the use of the stapled technique for jejunal resections [117]. As already described above, other postoperative complications in horses undergoing abdominal surgeries have an impact in developing POI, notably by postanesthetic myopathy [118, 119]. Therefore, special attention must be paid to optimizing blood pressure during abdominal surgery. For these purposes, anesthesia monitoring should be carried out at all the time, and if a decrease in blood pressure (defined as mean arterial pressure <70 mmHg) is observed, a dobutamine injection should be administered [42, 46]. In cases of severe anesthetic hypoxia ($PO_2$ < 70 mmHg), one ought to have the issue resolved in a timely manner with intermittent positive pressure ventilation (IPPV) with constant positive end-expiratory pressure.

Several methods have recently been developed to decrease the rate of other surgical complications [35, 117]. The methods of minimizing postoperative adhesions are the application of meticulous atraumatic surgical technique, use of a bioresorbable hyaluronate-carboxymethylcellulose membrane [118, 120], administration of heparin [40], omentectomy [121] and performing intraoperative peritoneal lavage [39]. In the case of strangulating obstruction of the small intestine, the bowel to be resected and discarded should be placed over the edge of the surgical field while removing the contents of the small intestine. Performing a pelvic flexure enterotomy may also reduce POI risk [110], which has also been confirmed during our observations [45]. The protective influence of these procedures may be attributable to a reduction in the intraluminal source of endotoxin, but the potential value of evacuating the colon should be weighed against the increased anesthesia time required to perform the surgery, as both factors have been associated with an increased risk of POI [106].

### 6.3 Postoperative strategies in the prevention and therapy of equine POI

Postoperative strategies in the prevention and treatment of equine POI are numerous and dependent on each individual case [26]. This can be divided into standard supportive postoperative procedures and procedure by risk for the patient. Under standard management, the following should be considered: regular basic clinical measurements (every 4–5 h) including heart and respiration frequency, temperature, auscultation of bowel sound, and of laboratory parameters including hematocrit, total plasma protein and acid-base state of the blood. In the standard postoperative procedures, several checks should be undertaken. We used at least 3 days administration of balanced polyionic intravenous fluid. The amount and length of time of the infusion solution are dependent on blood parameters; on average, we applied 2.5–3 l/h/500 kg BW. Dehydration and electrolyte imbalances are commonly encountered as a result of abdominal disorders and surgery. Even though a horse is stabilized in the perioperative period and the primary problem is corrected, continued replacement of previous and ongoing fluid losses is critical for a successful outcome. If a horse has gastric reflux, the use of the infusion solution should be continued throughout this condition. Given that the introduction of a large number of solutions provokes the development of thrombophlebitis of the jugular veins, it is recommended that a central catheter is installed through the abdominal vein.

In all horses without and with POI after abdominal surgery, NSAIDs should be administered such as flunixin meglumine (1.1 mg/kg BW, IV, q12h initially for

2 days, then 0.55 mg/kg BW, IV, q12h for at least 2 days). Flunixin meglumine controls postoperative pain and improves the cardiovascular manifestations of endotoxemia. Additionally, flunixin meglumine has been shown to significantly attenuate the disruption of gastric, small intestine, and large colon motility elicited by endotoxin infusion [49]. Additional treatments include anti-oxidant medications, which prevent the generation of chemoattractants: DMSO (20 mg/kg BW in 1 l saline IV bolus, q12h) and sodium heparin (20,000 IU, SQ, q12h). In all postoperative horses without gastric reflux, we applied obligatory laxatives (2 l liquid paraffin, p.o.) after abdominal surgery. For the prevention of incisional infection, horses received abdominal bandages during hospitalization. The bandages consisted of sterile absorbent cotton padding next to the incision secured by elastic adhesive tape.

### 6.4 Postoperative peritoneal lavage

Postoperative peritoneal lavage has been used in an attempt to reduce the rate of postoperative adhesions [119], but this procedure decreases occurrence and increases survival rates of equine POI [39]. Thus, in horses with a high risk of POI and who additionally showed symptoms of peritonitis, we performed retrograde peritoneal lavage through a Foley catheter, which was installed into the abdominal cavity prior to closure of the abdominal incision. For abdomen lavage, we used sterile physiologic saline or Ringer's lactate solution (10–15 l) containing amoxicillin (5 g) and 20,000 units of sodium heparin.

### 6.5 Prokinetic drugs for the treatment of equine postoperative ileus

The use of prokinetics in horses with POI is only part of the treatment and is not defined as a unique technique toward the survival rate of this disease, only working in combination with other methods [5]. The effectiveness of some prokinetic drugs in horses is associated with the difficulties of conducting a well-designed, randomized clinical trial with homogenous groups of animals [38]. None of the intestinal prokinetic agents have been subject to rigorous clinical efficacy trials [122]. This statement is supported by the fact that the contractile response of intestinal smooth muscle to prokinetic drugs is significantly impaired in many horses with POI. Prokinetic motility drugs are also commonly used following abdominal surgery in humans to prevent ileus, although a Cochrane review examined 39 randomized controlled trials and found most medications to be of little or no benefit [6, 7]. There are numerous prokinetics drugs that can be used by POI in the horse, which have differing mechanisms of action and different efficiency rates [26].

#### *6.5.1 Parasympathomimetic agents (cholinomimetics)*

Parasympathomimetic agents (cholinomimetics) are drugs that mimic the effects of the parasympathetic nervous system activity. Directly acting parasympathomimetic agents, bethanechol chloride, improve myoelectric activity in the stomach, jejunum, ileum, and large and small colons of horses, but produce significant cholinergic side effects (increased salivation), and therefore are not used as a standard in equine praxis [71, 101]. In horses with POI, applications are mostly indirectly acting parasympathomimetic agents such as neostigmine methylsulfate. Neostigmine is a cholinesterase inhibitor that prolongs the activity of acetylcholine by retarding its breakdown at the synaptic junction [102, 123]. Neostigmine has been shown to delay gastric emptying and decrease jejunal myoelectric activity, but enhances myoelectric activity in the ileum, cecum, right ventral colon and

pelvic flexure activity in healthy ponies [71, 122]. These results suggest that the drug would not be appropriate for gastric and small intestinal problems but may be beneficial for large intestinal motility dysfunction. However, neostigmine increased the amplitude of rhythmic contractions in both the resting and distended jejunum in anesthetized ponies, and it induced contractile activity in the ileum, supporting its use for motility dysfunction in both the small and large intestine [26, 29, 124]. Based on our clinical impressions, neostigmine if used as monotherapy repeated at 60 min intervals (during 24–48 h) has significant beneficial effects in the treatment of colitis cases, but not in POI of the small intestine [43].

*6.5.2 Sodium channel blockers*

Sodium channel blockers—lidocaine is currently a prokinetic agent, which is most frequently used for the treatment of POI in equine practice, although scientific evidence on its prokinetic and analgesic effectiveness is limited [33, 124–128]. Lidocaine has antinociceptive, antihyperalgesic, and anti-inflammatory effects [6, 7]. In an investigation within a UK hospital population, lidocaine therapy had no effect on the prevalence of postoperative reflux, total reflux volume or duration of reflux and as well as no effect on postoperative survival in horses undergoing abdominal surgery [129]. According to our observations, lidocaine if used as monotherapy has little positive effect on the treatment of equine POI and is significantly inferior to a combination of prokinetic drugs [32].

*6.5.3 Drugs acting as 5-hydroxytryptamine receptors*

Drugs acting as 5-hydroxytryptamine receptors include metoclopramide, cisapride, mosapride citrate and tegaserod [36, 130–132]. Metoclopramide hydrochloride (MCP) is a first-generation substituted benzamide whose prokinetic activity is both through dopamine 1 (DA1) and 2 (DA2) receptor antagonism and through 5-HT 4-receptor (5-HT4) agonism and 5-HT3 receptor antagonism [11]. Stimulation of DA2 receptors inhibits the release of acetylcholine, and stimulation of 5-HT4 receptors enhances the release of acetylcholine from the myenteric ganglia. MCP is a drug, which for a long time has often been used in the prevention and treatment of equine POI, but results in published studies have been variable [5, 8, 21, 130]. The prokinetic capacity of metoclopramide appears substantial in the equine stomach, duodenum and jejunum, but not in the large intestine [128, 133].

*6.5.4 Motilin agonists*

Motilin agonists include erythromycin lactobionate, and a macrolide antibiotic has been shown to significantly increase solid phase gastric and dose-dependent cacal emptying and is thought to exert prokinetic effects via activation of motilin receptors [20, 21, 36]. The prokinetic effects of erythromycin reported in healthy horses were not the same in horses with gastrointestinal disease [110, 122, 128].

*6.5.5 Adrenergic antagonists*

Adrenergic antagonists include acepromazine maleate, a nonselective α-adrenergic antagonist, and yohimbine, tolazamide, and atipamezole, which are selective α2-adrenergic antagonists. Their use as prokinetics is based on the assumption that sympathetic hyperactivity contributes to POI, but their beneficial effects are not well understood [6, 7].

### *6.5.6 Dopamine antagonist*

Dopamine antagonist—domperidone is a selective peripheral $DA_2$ receptor antagonist [26]. In a preliminary experimental model of POI in ponies, domperidone was effective in restoring transit time, electromechanical activity, and coordination of gastric and intestinal cycles [134].

### *6.5.7 Combination of prokinetic drugs*

Combination of prokinetic drugs—based on our research, the best medicinal method for prevention and treatment of equine POI is a combination of three drugs, according to the needs of the individual scheme of each case [5, 14, 32]:

1. Neostigmine methylsulfate (in a dose of 0.004 mg/kg per 2 h, i.e., 2 mg per 500 kg BW, subcutaneously)

2. Metoclopramide (in a dose of 0.01–0.02 mg/kg per 2 h, i.e., 5–10 mg per 500 kg BW, subcutaneously or intravenously)

3. Domperidone (in a dose of 0.16 mg/kg orally, every 8 h)

We found that the prophylactic perioperative use of these drugs in risk horses to reduce the incidence of POI, and by occurrence of ileus significantly improved survival rate [5, 14, 32]. These prokinetic drugs should not be applied at the same time (little benefit), but strictly in turn. Why these drugs benefit only in turn in combination and not at the same time is unknown. Neostigmine methylsulfate and metoclopramide were applied alternatively between each other in 60 min intervals, so that every horse received each of the drugs every second hour (i.e., 1 h neostigmine methylsulfate was administered and in the second hour metoclopramide was given). We used this therapeutic regimen for POI horses continuously for several days until a result was obtained (complete absence of gastric reflux), and usually, this happened within 24–90 h. The withdrawal of these drugs should take place gradually throughout a few days. On average, this occurred 5–6 days after the onset of equine POI. If sharp withdrawal of these prokinetics is undertaken, a relapse of gastric reflux is possible.

## 6.6 Nasogastric decompression

Nasogastric decompression is a classic supportive treatment that prevents gastric dilation in horses with POI. We performed this procedure in horses that showed gastric reflux, it was applied regularly every 4–6 h, and most horses begin to show clinical signs (colic) associated with excessive fluid accumulation in the stomach. Retaining an indwelling tube for 12–15 h in horses with POI was performed only in cases where animals showed extensive stress syndrome by intubation.

## 6.7 Judicious timing of feeding

Horses without gastric reflux were allowed access to water within 12–18 h after abdominal surgery and were provided with small amounts of feed at 18–30 h after surgery. Initially, small amounts of grass hay or small amounts of bran mash with 100 ml laxatives were fed every 3–4 h. The quantities were gradually increased daily until the horses were allowed to freely eat hay by choice (usually by 21 days after surgery).

Freeman and coworkers were able to show that of the horses taken to surgery for small intestinal disease, only 10% developed postoperative ileus [17]. According to the authors, one key management factor in prophylactic procedures of POI was early re-feeding, where horses were offered water and small amounts of hay within 18–24 h of the completion of surgery for small intestinal disease. Early feeding following abdominal surgery is a commonly applied prophylactic approach in human medicine, as well. It is hypothesized to promote restoration of gastrointestinal motility via the release of neuropeptides in response to solid feed ingestion. In humans, it is known that chewing gum is a type of sham feeding that promotes intestinal motility through cephalic-vagal stimulation [6, 7, 135].

According to our opinion, the judicious timing of feeding in horses with POI is when no signs of reflux are apparent or when motility is regained. Horses with evidence of gastric reflux are unlikely to tolerate enteral feeding and should receive intravenous nutritional support (i.e., glucose solutions and amino acids). In addition to the intravenous administration of glucose solutions, it is necessary to use insulin subcutaneously at a dose of 0.08 U/kg every 12 h in order to block the lipase enzyme responsible for releasing triglycerides from fat depots. As is well known, if the fasting regime lasts more than 3 days, this may provoke development of a severe form of equine hyperlipidemia, notable in obese horses. Hyperlipidemia is associated with periods of negative energy balance and physiologic stress [136]. For this reason, in horses with POI at 48 h after abdominal surgery, regardless of the presence of gastric reflux, we allowed the horses, after nasogastric decompression, to be fed with a small amount of bran mash with ranitidine oral tables ($H_2$-antihistamine). Additionally, for horses with gastric reflux for which the provision of enteral nutrition is not possible, the provision of a lick (e.g., mineral block) has been suggested as a form of sham feeding, equivalent to gum chewing in humans.

### 6.8 Stress reduction strategies

Suppression of parasympathetic activity and hyperactivity of the sympathetic nervous system with activation of the hypothalamic-pituitary-adrenal axis (stress syndrome) has a very important role in the development of equine POI (as discussed above). Causes of equine stress syndrome in perioperative period can be varied, primarily pain and inflammation, but also recovery from anesthesia, postoperative diagnostic and management procedures and fasting, as well as different psychological (fear) factors. It is generally considered or hypothesized that a more invasive surgery, with extensive tissue trauma and noxious stimuli, triggers a more significant stress response. After surgery, the horse is placed in an unfamiliar environment with unknown caretakers and probably starved while having additional pain to deal with. Postoperative pain can originate from peritoneal inflammation and abdominal incision. Consequently, it is important on all occasions to consider minimizing sympathetic activity, primarily pain and inflammation control of the horses after abdominal surgery. As is well known, anti-inflammatory drugs lead to lower pain scores and lower plasma cortisol levels [82, 91]. This amount of stress modulates the pain perception and adds further to the perceived pain. This in turn increases appetite, so the horse does not enter a catabolic state in order to produce substrates for healing.

Given all of the stress, it is therefore also extremely important to take care of the horse psychologically. All therapeutic procedures in postoperative period should be performed with minimal stress. Stress-enhancing procedures may include introduction of a nasogastric tube into the stomach, which causes discomfort and the release of catecholamine, and this process is necessarily carried out with the imposition of

a twitch and without sedation. It is believed that a twitch calms the horse by releasing endorphins as pressure is applied, thus reducing stress and pain. Administration of corticosteroid drugs results in the reduction of stress syndrome, but non-pharmacologic mechanisms for reducing the stress response are quite successful, for instance, regular visits from the owner or a familiar caretaker, frequent contact and grooming preferably by the same handler or veterinarian, short periods of hand-walking, treats given from time to time, short periods of grazing (1–2 min around 24–48 h after abdominal surgery) and minimal enteral nutrition. As described above, early re-feeding has been attributed to possible downregulation of the metabolic stress response [35]. Additionally, all other external stress factors for horses (including transport, loud noises, bright light and rudeness of medical staff), as far as possible, should be abolished during the postoperative period in an equine clinic, both in horses with a risk of development and also in horses that already have a POI.

### 6.9 Repeat celiotomy and postoperative ileus

Relaparotomy (repeat celiotomy) is widely accepted as a treatment option in the management of postoperative colic and ileus [6, 7]. A repeated surgical intervention in the abdominal cavity may correct technical errors that occurred during the first surgery and solve conservatively unsolvable motility disorders as well as pathological conditions that occur in the post-surgical period without a clear relation to the first intervention [1]. Previously, authors considered that intestinal manipulation (massage) and repeated enterotomy likely have beneficial effects to equine POI [103]. However, the potential benefit of limiting the degree of intestinal manipulation in equine surgery must be weighed against the increased risk of other postoperative complications (postanesthetic myopathy, wound infection and hernia). According to our observation, repeat celiotomy did not increase survival rate in horses with POI; for example, surgical cases had a lower survival rate than medically managed cases of POI [32].

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## Conflicts of interest

The authors declare no conflicts of interest.

## Dedication

This chapter is dedicated to Prof. Dr. Dr. H.C. Bernhard Huskamp (1932–2018) the founder of the Veterinary clinic Hochmoor in recognition of his extensive contributions to equine colic surgery.

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Chapter 9

# Morphophysiological Study of Gastrointestinal Tract of the Donkey (*Equus asinus*)

*Arbab Sikandar*

## Abstract

In most of the developing countries, donkeys are used to carry goods and water and to guard herds as a livestock guardian. Donkeys possessed a good digestive system and are being offered only low-cost fibers diet like hay and straw. Despite the biological potential of the donkey, only a few studies have focused on the morphophysiological aspects of their digestive system. A series of tubular organs and associated glands are present in the digestive system. Although generally the morphology of the donkey digestive system is comparable to the horse, few dissimilarities exist among such species. In this chapter, we tried to highlight the anatomy, histology and physiology of the digestive system of domestic donkeys including tongue (mucosa, papillae, muscle, taste buds), teeth, pharynx, esophagus, stomach, saccus cecus, descending part, an ascending part and transversal part of the duodenum, jejunum, ileum, cecum, colon (right dorsal and ventral; left dorsal and ventral), rectum and anal canal. The microarchitecture of the tunica mucosa, next to the lumen, is focused upon. Morphology of the large accessary digestive glands viz. salivary gland, liver and pancreas were also highlighted. These structures are situated away from the gut-tubular system but are attached to its lumen through their specified duct system. Furthermore, peculiar microstructures of the internal layers, immune system and microbiome of the gut were correspondingly highlighted in the chapter.

**Keywords:** anatomy, histology, physiology, oral cavity, stomach, intestine, glands, microscope, donkey

## 1. Introduction

### 1.1 Donkey in the past, present and future perspective

Donkey (*Equus africanus asinus*) belongs to Equidae family like horse and is domesticated in most parts of the world [1]. The African wild ass living wildly has been declared as wild ancestor of today's donkey. The life span of donkeys is generally ranging from 25 to 50 years. It is known to have been used as a beast of burden. "The first findings of donkey came from ancient art and archeological records. Donkeys bred and produced mule's offspring which were used by the Spanish during their occupations and defeats. By the same time the donkeys got progressively significance in America and were used for shipping gold around

mountainous mines. Although the world has move toward the mechanization, but donkey (an ancient animal) is being used as biological vehicle and is known as beast of burden. In arid and semi-arid places, it serves man in carrying luggage and used for transportation purpose [2]. It can live on low quality high fiber diet and scarce amount of water. It can bear harsh climatic conditions. It is the source of bread and butter for the poor laborer of the developing country and is called the horse of poor man available in low price. It is the source of earning on daily bases for many poor families. Along with mules, donkeys are also used as a means of transportation by those armed forces who are deployed in the large mountainous areas [3]. Donkeys are used to guard sheep, as they are more inclined to stand and fight than to run from a predator. They provide a means of transportation for agricultural goods, building materials, droughts, tracking carts and riding humans themselves all over the world. Donkey is also named as and will remain an inexpensive horse [2]. It can go places where cars and other vehicles cannot go, so it can be used for transportation such as hilly areas [3]. In addition, Donkey milk is used as an alternative to human breast milk, as it has many of the same important qualities viz. low in fat contents, promotes healthy intestinal flora, have anti-inflammatory properties, contains immune enhancing compounds which protect the body against pathogen [4]. Donkey meat is eaten in many places of world. Italy is the largest consumer of donkey meat in Europe [5]. Donkey meat is considered tastier than horse meat and is a delicacy in most of the Chinese restaurants. Donkey meat burgers are a favored way of eating the meat and are eaten in Canada and Mexico for example. Donkey milk components are used in making cosmetics soaps and skin creams. Donkey is also used as test animal in pharmacokinetics [4]. Donkey hide gelatin (aka ass hides glue) is used in traditional Chinese medicine to treat bleeding dizziness insomnia and dry cough and a source of raw material for Shoes company. Donkeys also played role in reproduction and produced fertile or infertile mules which helped and used by humans for many purposes [2]. Based on loyal behavior, such animal holds a position as noticeable companions and guard of pet animals. Scientists of the advance countries like America are planning to use donkey in Artificial Intelligence by modifying its brain function and so it can be used in secret missions. Due to its importance, veterinarians and other researchers are interested in discovering further benefits out of it. Only a few studies highlighted and focused the anatomical and physiological aspects of the donkey alimentary system. Unlike other individuals, such animals are being offered low-cost fibers diet [1]. High fiber forage diets are better digested by donkeys than horses. Donkeys are said to possess a better digestive system than horses, it comprises of a series of tubular organs and associated glands [6]. Its function is to cut down the ingested complex food materials and converted into the valuable energy source and removing the wasted portion for maintaining the health and growth of the organism. The digestive system of the donkey is explored in detail in this chapter.

## 2. Anatomy and physiology of the digestive system of donkey

The donkey gut can be apportioned into two segments including the foregut made up of the stomach and small intestine and hindgut or large intestine is consists of cecum and colon. The overall digestive system is a hollow tube, starting with the mouth and oral cavity leading through to the anus with structures including the esophagus, stomach, small and large intestine, rectum and the anal canal in between [7]. The digestive glands like liver, pancreas and salivary glands are also

associated with the system. The entry of the gut (buccal cavity) is surrounded by the lips anteriorly and are present posterior to the nostrils, by the cheeks and teeth laterally, by the hard and soft palate dorsally, by the movable tongue ventrally which is present in the floor, and posteriorly it opens into the pharynx. The structures in the oral cavity include the tongue, teeth and gums, salivary glands, palates and immune tissues.

### 2.1 Lips

The lips are the main prehensile organ in donkeys, they are lined by stratified squamous keratinized epithelium and comprise of skin, glands, hair follicles, and tactile hairs. Microscopically lips are composed of epidermis, subcutaneous tissues thick layer of connective tissue, orbicularis oris muscle fibers (of skeletal type). The labial glands and a layer of adipose tissue are present in lamina propria and submucosa. The lips of donkey are mobile which help in collection and direction of grass toward the incisors for cutting and the premolar and molar teeth for mastication [8].

### 2.2 Dental anatomy in donkeys

The equine tooth is made up of the same substances as human dentition i.e. cementum, enamel, dentine and pulp but the matrix is different, and our teeth are encapsulated in enamel whereas the donkey occlusal surface shows a cross section of all materials except pulp. Pulp is innermost layer contains vital structure like nerves, blood supply, lymphatics and bone forming cells the odontoblasts [9]. This soft structure is protected by outer layer. The next layer is which occupy main portion of the tooth but is less mineralized than enamel is the dentin. By this reason the dentin wears out more than enamel which is the hardest portion of the tooth. Enamel does not have ability to heal up if it is scratched and injured like the other tissue in tooth can, but it remains protected between cementum and dentin. Cementum is the outer layer and is similar to bone and assists connection between periodontal ligament and the tooth. This structure defends the tooth, maintaining it in the socket within the gum and provides care as the animal masticates. Only 11% of infundibula are completely cementum filled. The developing bone is externally lined by stratified squamous non keratinized epithelium followed by a primitive connective tissue. The incisors are located at the front of the mouth and are visible when you lift up the animal's lips [10]. The permanent incisors have crescent shaped depression called infundibulum that is filled with cementum. The incisors are used to cut the grass during grazing and also aid in assessing the animal age. Canine or bridal teeth are located between incisors and premolars. Lower canines are positioned more rostral than the upper ones. Male donkeys generally have four canines, but these generally do not fully develop in females. Wolf teeth are located just medial to first cheek teeth in both upper and lower jaws. They may be absent or four in number. Cheek teeth are most caudal group of teeth. Three molars and three premolars make up each row. Upper cheek teeth have infundibula that wear out with time. The molar are permanent teeth only. Based on closed location, collective premolars and molars performance is like a specific unit for the breakdown of food. Total number of equine teeth is as per the following formula:

$$\text{Deciduous I3 C0 P3/I3 C0 P3} = 12 \text{ and the long – lasting are as } 3\,1\,3(4)\ 3/3\,1\,3\,3 = 20\ (21) \quad (1)$$

The deciduous incisor teeth are rounded at the top and are whiter in color while the long-lasting are adopting square shape at the margin of the gum and appears yellower. Teeth eruption processes are as follows.

| Teeth | Date of deciduous teeth eruption | Date of permanent teeth eruption (years) |
|---|---|---|
| Central incisors | 0–2 weeks | 3–3.5 |
| Middle incisors | 5–8 weeks | 4 |
| Corner incisors | 1 year | 5–5.5 |

## 2.3 Donkey tongue

It is a strong muscular organ enclosed in thick mucosa. It is very sensitive organ with a groove between inner part of it which is connected to underlying tissue and a free part in front. This organ has spatula shaped having torus linguae (extended torus and muscular distinction), which is distinctive for Equidae. Stratified squamous keratinized epithelium lining the external surface followed by connective tissue and a layer of skeletal muscles [11]. The muscle layers are arranged in various forms which help in rotation of the tongue during feed mastication. Epithelium lining ventrally is the non-cornified. Filiform, fungiform, foliate and vallate papillae are present at three parts of tongue (apex, body, and base). All types of papillae are lined by partial to complete carnified epithelium. Filiform papillae are mechanical and almost cover major portion on the dorsal surface. It is short and thin at apex, pointed and rough at body, and elongated at the caudal portion. Fungiform mainly scattered at lateral surfaces, around the filiform and are round to lobulated. These are larger, wider, taller but less in number than filiform. The vallate papillae with circular grove and central spherical bulges are positioned caudally in the body and 3–4 times larger than fungiform. Group of foliate are located near the base of palato-glossal arch and are organized like leaves alienated by variable grooves. Fungiform and filiform are devoid of taste buds but vallate and foliate has taste buds. Basal cells are present at the base of each taste bud and act like the stem cells. As compared to the tongue of horses, the feature is the occasional occurrence of the dorsum cartilage (cartilago dorsi linguae) of the tongue. Lymphatic nodules are special aggregated lymphoid cells and are present dorsally. Small mucous secreting labial glands are also present in connective tissue, secretion of which moistens the oral mucosa. The connective tissue in the lamina propria is richly supplied with blood vessels, lymphatics, nerves and adipose tissue.

## 2.4 Cheek of donkey

The structure of the cheek is like that of lips, designed principally of buccinator muscles and contains some minor glands (salivary). The powerful muscles of cheek help in mastication, grinding and mixing of food. These includes masseter, pterygoid medially, pterygoid laterally [12]. These muscles get their nerve innervation from the mandibular branch of the trigeminal nerve. Furthermore, cheek muscles involved in closing the mouth through elevation of the mandible [13].

### *2.4.1 Masseter muscles*

These muscles have wide multipennate muscles with numerous tendinous connections. In the donkey, it is the largest muscle involved in mastication. It is grouped into; "Proper (first, second superficial, middle and deep) masseter

coatings and improper masseter muscles groups (zygomatico-mandibularis and maxillo-mandibularis)". Its main function comprises of the movement for chewing, achieved by the masseter group (proper) and definite shutting of the oral cavity is executed by the improper type of masseter group [14]. The improper muscle moves the mandible in lever style. Arteries of masseter muscle include masseteric artery, transverse facial artery, buccal artery, facial artery. The arrangement of the vessels which supply blood to the masseter muscle defined its importance.

*2.4.2 Pterygoid muscles*

These muscles routed through the bottom of the skull via mandible medially. Pterygoid muscles accompany the masseter during function. Upon bilateral contraction the pterygoid muscles caused elevation of the mandible and upon performing unilateral action they attract the mandible sidewise of the contracting muscle [13]. Its lateral portion is capable to direct the rostral direction of the mandible, particularly when the oral cavity is opened.

*2.4.3 Temporal muscle*

The temporal muscle is originated from the temporal crest and occupies the temporal fossa. It is inserted on the coronoid process of the mandible. Its functions include elevations of the mandible and help other muscles mutually during mastication's [13].

*2.4.4 Digastric muscle*

These caudle and rostral bellied muscles are not the actual muscle of mastication but may also add partially to the jaw movements during opening to the oral cavity. It prolongs among the process of paracondylar of the occiput and the mandible medially [13]. The facial nerve innervates the caudal part while the mandibular nerve innervated the rostral part. The lateral portion is formed from the extension of the caudal belly, attached on the mandibular angle and attracts the mandible bone backward. Below the basihyoid bone it develops the rostral belly, which attaches medially to the mandible body. This muscle opens the oral cavity by pushing the mandible [11].

**2.5 Palate**

The palate has mucosa on both oral and nasal sides and has soft and hard parts. Oral mucosa is lined by tough cornified squamous epithelium. Hard palate is formed by union of palatine, maxillary, and incisive bones with no muscles [13]. Soft palate is a muscular structure made of intrinsic paired palatine muscles paired extrinsic tensor and levator veli palatine muscles along with palatine glands present in it. Trigeminal nerve supply to the palate and glosso-pharyngeal and vagus nerve supply the muscles of soft palate. Lymphoid follicles are present in the lamina propria along with FCT [14].

**2.6 Pharynx of donkey**

Pharynx is about 15 cm in an adult animal. It is present at the back (posterior) of the mouth and is located between the skull at base and the initial two cervical vertebrae at dorsal portion and ventrally the larynx. On lateral side, two pairs of palatopharyngeal arches are present from the soft palate to esophagus. Wall of

pharynx consist of striated muscles. It includes the nasopharynx which is entrance to auditory tubes, oropharynx and laryngopharynx [15]. Nasopharyngeal mucosal epithelium is composed of pseudostratified columnar epithelium with goblet cells. Lymphoid follicles can also be seen in the lamina propria and submucosal area. Nasopharynx is innervated by cranial nerves V, IX, X, XII. It is also composed of sensory receptors of glossopharyngeal and trigeminal nerves. During swallowing, the soft palate is raised which divides pharynx into dorsal and ventral sections [13]. It plays important role in deglutition. It serves as pathway of food from mouth to esophagus. It consists of rostral constrictor muscles including the hypopharyngeal, pterygoid, and palatopharyngeus. The stylopharyngeus are the muscles responsible to dilate the area while pterygopharyngeal muscle and palatopharyngeal muscles shorten the pharynx. Palatopharyngeal muscles also close the pharyngeal arch. There are some tactile receptors which detected the air flow and cause dilatation of the air way by stimulating the gag reflex. Augmented action of such receptors stabilizing the muscles which improves the dilatation of upper respiratory tract and prevent it from being collapsed. It is advised that glossopharyngeal nerves should never locally anesthetized otherwise there be dysfunction of oropharyngeal muscle which may causing collapse of dorsal nasopharynx and ultimately inspiratory obstruction in exercising donkey. Failure of pharynx or neuromuscular activities will result into the severe respiratory disorders.

### 2.7 Tonsils

This tissue is responsible for defense, located at the rare area of the throat. A tissue of soft lymphoid follicle like the lymph nodes surrounded by a layer of stratified squamous epithelium. The mucosa invaginates deep in the lamina propria forming crypts and fundi [11]. Both defuse and nodular arrangements of the lymphoid tissues are present.

### 2.8 Esophagus

The length of the esophagus depends upon the body of animal. It consists of cervical, thoracic and abdominal parts. It moves lateral to trachea as moving down and becomes ventral again at thoracic inlet. Unique feature of donkey esophagus is its pigmentation at different parts. Esophageal obstruction is also common in donkey due to different anatomical entrance to stomach [16]. Cervical part of the esophagus is located dorsal to trachea and ventral to cervical vertebrae and the thoracic part is located dorsal to sternum, medially in the thoracic cavity. The esophagus ranges from 125 to 200 cm in length in average adult animal. It lies dorsally on trachea in the cranial third then turned toward left in the middle third of the neck [17]. In the area of thoracic inlet, it lies ventrally to the trachea. Under microscope its wall is divided into mucosa, submucosa, muscularis and tunica adventitia. Mucosa is the innermost part of the esophagus toward lumen and is lined by keratinized stratified squamous epithelium. In the lamina propria, the glands are present along with a layer of FCT and some lymphoid follicles, blood vessels and other vasculature. Lower layer of the mucosa is surrounded by smooth muscle called muscularis mucosae. The submucosa contains elastic fibers, adipose tissue and seromucous gland. The muscularis externa is composed of skeletal muscle in the proximal two-thirds and turns to smooth muscle in the distal third. The skeletal muscle layers are adapted in inner circular and outer longitudinal arrangements. The cervical pleura and peritoneum add to tunica adventitia in all portions of the esophagus. The loose attachments of the esophagus with the adjacent tissue permit the neck movement during swallowing. At abdominal portion the esophagus has a serosal covering.

The function of esophagus is to provide pathway to partially digested food into the stomach. There is no digestion in esophagus [18].

### 2.9 Stomach

Lower portion of the esophagus and the stomach lies toward right side in the abdominal cavity. Between esophagus and stomach there occurs a junction i.e., esophagus-gastric junction [17]. The stomach has three sections, saccus caecus, fundic and pyloric regions. The saccus caecus is a non-glandular portion on stomach and is located close to the esophagus entrance in the stomach [16]. It is situated ventrally to the diaphragmatic left crust and is underneath the dorsal portion of 16th and 17th ribs. It relates to pancreas, present behind the great colon extinction and situated laterally to spleen bases. This portion is covered by keratinized stratified squamous epithelium. The keratin layer thickness differs with degree of stomach distension, age and diet of the animal. The lamina propria normally has plasma cells, lymphocytes, mast cells and neutrophils. The muscularis mucosa is continuous and the submucosa hold nerves plexus and lymphatics. The muscularis externa is comprised smooth muscle arranged in three layers viz. oblique (inner), circular (middle) and longitudinal (outer) layers. Between the inner circular and outer longitudinal layers of muscle there is the myenteric nerve plexus. In this area the HCL initially combine with the ingested food mass and reduces the prior process of fermentation that initiated with the discharge of sugars (soluble) from the food in donkey's oral cavity. It is imperative that in the stomach the fermentation is very sparse because it leads to the gas formation. In donkey there is a slight experience to belch or otherwise to dispel collecting gas. Histologically, the lining epithelium at junction of stomach is abruptly transit to columnar epithelium from stratified squamous form. This junction acts like a valve that does not allow acidic contents of stomach to enter in esophagus. Due to any abnormality, this junction is not performing its proper function; it can result in reflux esophagitis. Externally diaphragmatic crura and internally C-shaped sling fibers of stomach make it possible to perform its pinchcock like action. Grossly we can say that proximal cardiac portion of stomach and distal end of esophagus makes this muscular junction. The stomach of donkey is like horses in its conformation. The average weight of an empty stomach in donkey is 1.5 kg. The comparative stomach capacity of donkey is 14 and the caecum and colon is about 80, whereas ruminants have the stomach capacity around 80 and that of caecum and colon is only 13. Hence the stomach of donkey and caecum of large ruminants are similar. Donkeys have monogastric type of small stomach that bounds the feed portion which can be got at a time. It attempts incessant foraging as numerous slight feedings are superior than few big meals since the stomach starts to unfilled when it is 2/3 full irrespective the food is processed or not in the stomach. The mucosa has folds which flattened when the stomach fills and has gastric pits and glands. The cardiac glandular region of the stomach has short, coiled tubular glands that are lined by simple cuboidal epithelium. Proper gastric (fundic) regions of the stomach is containing straight, branched tubular glands of which narrow neck, long body and dilated blind ended fundus [19]. The pyloric region has deeper pits. The mucosal glands are lined by the chief (zymogen secreting) and parietal cells (acid secretion) along with mucous neck cells. The chief cells are larger in number while the parietal cells are larger in size. Although the digestion by microbial happens in caecum and colon in donkeys while stomach temporarily stores food because of its emptying behavior. Overall compared to other animals, larger area of the donkey's stomach is covered by the non-glandular regions [20]. A small amount of food is digested in the stomach and then goes to caecum and colon for microbial digestion.

### 2.10 Intestine

It is positioned ventral to the vertebral column in the abdominal cavity and has the following three parts. The duodenum is the initial and shortest portion of the small intestine located at left side of the abdominal cavity [17]. Duodenum joins the jejunum and the stomach together and is divided into the following four parts:

1. Superior (first) part also called ampulla duodeni

2. Descending (second) part

3. Horizontal (third) part

4. Ascending (fourth) part

Superior part of duodenum: It is in interaction with the liver through the visceral surface and forming ampulla which is a dilated portion and a sigmoid flexure. The initial curve of the flexure is dorsally convex and the other also called cranial flexure is ventrally convex which provide the site of attachment for body of the pancreas. The first 2 cm of superior part of duodenum, immediately distal to the pylorus has mesentery and is mobile. This free part called the ampulla (duodenal cap). The distal 3 cm of the superior part have no mesentery and are immobile because they are retroperitoneal. The duodenal superior segment ascends from pylorus and is overlapped by the liver. Peritoneum covers its anterior aspects, but it is bare of peritoneum posteriorly, except for the ampulla.

The major duodenal papilla is a rounded projection at the beginning portion of the mutual pancreatic and bile duct into the duodenum and is the primary source of bile and other enzymes secretion that ease the process of digestion. Mucosa forming protruding papillary folds at ampulla where the lining epithelium transitions from common gut surface type to pancreatobiliary type like distal ducts. The lamina propria mucosa contains infrequent plasma cells, lymphocytes and mast cells. Little ductless mucous glands ductules lie beneath the mucosa. Sphincter of Oddi represented by smooth muscles possibly ranged into mucosal surface folds and might have some neighboring acini (pancreatic), but typically the islets are not seen nearby major papillae. The development of major duodenal papilla begins with evaginations of the gut tube lies caudal to the stomach. The dorsal mesogastrium and the ventral mesogastrium pancreatic buds are formed. Few of the epithelium fail their associations to the emerging pancreatic duct system and lead to develop into the endocrine portion in the form of islets of Langerhans in pancreas. The minor papilla (duodenal) is positioned typically about 2 cm ventroproximal to the major duodenal papilla. Jejunum is the longest portion in the small intestine. It is situated in the middle part of the intestine [21] and is present in abdominal left side. A large number of digestive glands are present in the jejunum responsible for releasing buffers and enzymes into the gut lumen. In this largest luminal absorptive area, most of minerals and nutrients are absorbed [22]. Ilium is the last part of the small intestine and is present also in the abdominal left side and is the final section of small intestine. The ileocecal fold is situated between the antimesenteric side of the ileum and the tenia dorsalis of the cecum. Its role is to absorb all the remaining bile salts vitamin B12, and other digested stuffs that were available un-absorbed in the lumen. Ileal and cecocolic ostia generally have a small opening or orifice. A muscular layer circular in shape is the sphincter which is connection of the ileum and the cecum called ileal ostium (ileocecal valve). During dissection of the gastrointestinal tract of donkey, these are the macroscopic structures. In the terminal portion

of the gastrointestinal tract (GIT) at the cecal basis, the ostia (ileo-ceco-colic) are detected undoubtedly [23]. The ileal ostia inhibit the large intestinal luminal contents (rich in bacteria) refluxes back to the small intestine. The Peyer's patches located at the ileal submucosal tunics are the distinguishing histological items [24]. The ileocecal and cecocolic folds (peritoneal) set the cecum with other intestinal portions. Through the *ceco-colic ostium* the substances present in the ceca are drained directly into the colon (ventral). Gas accompanied ingesta are also eliminated across this ostium. Mucosa of the small intestine is lined by simple columnar epithelium. It covers the longest villi and the highest number of Goblet cells related to other parts of small intestine [22]. Sub-mucosa of duodenum contains Brunner's gland that secrete a serous secretion. Two layers, circular (inner) and longitudinal (outer) arrangements of muscularis externa and the outer serosa is present in its wall. The cecum is a portion of large intestine having pouch-like region present in pelvic portion of abdominal cavity located laterally and inferior to the ileum [17]. It is a very large chamber. The cecum has comparatively thicker mucosa, lined by simple absorptive columnar epithelium having plentiful goblet cells and entero-endocrine cells. Its lamina propria and muscular mucosae is identical to that of small intestine and the glands are packed tightly and lengthier. They lack Paneth cells. The cecum further absorbed the salt and remaining digested fluids through its thick mucosa and also add mucous to the remaining intra luminal contents [20]. The colon is present in abdominal cavity [17] and pushes all other organs cranially to thoracic part of abdominal cavity. The hindgut of the equine keeps similar job to that of other animals' large intestine viz. retention, further mixing and forward movement of the intraluminal contents. Such cecal movement is based on forced contractions of the wall.

### 2.11 Immune cells of the intestine

Lamina propria lymphocytes are B-cells that secrete IgA (Antibody A). IgA comes into lumen through epithelial cells; here it performs the function of adhesion and invasion of bacteria. Intraepithelial lymphocytes are present in the basolateral spaces between luminal epithelial cells [25]. Microfold cell (M-cell) is present in mucosa-associated lymphoid tissues [26]. Its main objective is to conveyance luminal antigen to the cellular immune system. Intestinal macrophages are heterogeneous and have the ability to locate and engulf bacteria [24], virus, fungi and parasites. Intestinal macrophages are mainly located in sub-epithelial area. Activated macrophages are important source of cytokines (IL-10). These prevents large intestine from excessive inflammation during bacterial infections. Paneth cells are present just beneath the intestinal stem in intestinal gland (crypts) in colon. These cells produce great amount of alpha defensins and other antimicrobial peptides such as secretory phospholipase A2 and lysozymes.

### 2.12 Colon of donkey

The ascending colon is splatted into left dorsal, left ventral, right dorsal and right ventral portions by the flexures (sternal flexure, pelvic and diaphragmatic). Location of the sternal flexure linking to the pair portions of the ventral colon [18]. In the border between dorsal and ventral colon the pelvic flexure is located, and the diaphragmatic flexure location is between the pair portions of dorsal colon. The ascending mesocolon is attached with ventral and dorsal parts. Ventral and dorsal colon is similar in length and is a part of the gastrointestinal tract of donkey. The transverse colon is positioned between the descending and ascending colon. The descending colon has extended mesocolon (descending).

This portion of large intestine has typical similar histological structures to that of cecum including mucosa, submucosa, muscular and serosa. Extensive mucus layer and crypts in the mucosa supports the feces passage. Colon is the lengthiest segment of large intestine and collects nearly entire digested material from the cecum, absorbs the remaining nutrients and water, and permits the drainage of feces to the rectum [7]. The roll of ascending colon is to absorb the remaining water and other key nutrients from the indigestible material, solidifying it to from stool. The waste material (feces) temporarily stored in the descending colon will finally be emptied into the rectum [17]. The rectum is present in pelvic cavity and is dorsal to reproductive tract. It lies between the terminal portion of colon and anus. It is usually found empty except when there is movement of feces with the help of mass movement through large intestine. It may also happen when animal is in the state of hyper aesthesia. Rectum is situated dorsal to genital and urinary tracts. Hence, it is also used for palpation. There is recto-genital pouch at dorsal side of rectum. It is the place where rectum and vagina in female and urethra in male are attached. Meso-rectum is the ligament that is attached to rectum. The rectum has pressure sensitive cells that are activated when it is filled with feces. These special cells are involved in initiation of defecation reflex. This starts the forceful contraction of rectal muscles and internal anal sphincter relaxation. This is the way that feces are passed out. Donkey lacks the ability to control the external anal sphincter. Hence whenever stretch receptors are activated there is a sure or confirmed defecation reflex. In the rectum the columnar epithelium with goblet cells turn to stratified squamous epithelium at recto-anal junction. Circular muscles of tunica muscularis form the internal anal sphincter while that of the other anal sphincter (external) is made up of skeletal muscles that are somewhat of voluntary control. The most terminal portion of the lower GIT is the anal canal which lies between the verge of the anal portion in the perineum bellow and above the rectum (below the level of the pelvic diaphragm) and located in triangular perineum of left and right ischioanal fossa and ultimately it open into the anus. On the basis of the structure, anal canal may be apportioned into two segments (lower and upper) separated by pectinate line or dentate line. Mucosa of the zona columnaris (upper zone) is lined by simple columnar epithelium and the elevation of the mucosa layer produces a valve. It is supplied by superior rectal artery (a branch of the inferior rectal artery). The lower zone is divided into two smaller zones, separated by a line known as Hilton line. The stratified squamous non-keratinized epithelium lining the zona hemorrhagica while the zona cutanea lined stratified squamous keratinized epithelium which blend with the perianal skin. The inferior rectal artery supplies this zone. Anal gland is small gland near the anus in many mammals [27]. Sebaceous gland at the lining of the anal glands secretes some liquid. The medium number of the anal glands in each anus is ranging from (3–10) 85% anal glands were found in the sub mucosa, 7% extended to the internal smooth muscle sphincter and only 2% in the intersphinteric space. Hence these anal glands found in sacs form in the anus and these secret special types of hormones that encourage the other members of that species of opposite sex.

### 2.13 Microbial digestion of rough and fibrous food in colon of donkey

Like rumen of the ruminants the microbial digestion mostly accomplished the cecum and colon of the equines. The stomach of ruminants and the large intestine of the donkey are therefore functionally similar. The donkey although is not more efficient in digestive process (grazing) compared to ruminants but has a combination of a large cecum and colon where the process of absorption and fermentation happens. Bacterial counts remain higher in equines where most of the fibrous and

rough food digestion occurs [28]. The higher counts of hemicellulytic and cellulytic bacilli are present in the donkey cecum and in colon the luminal bacterial counts are even more. The intestinal microflora may prevent infection by fighting with pathogens. It is a complex ecosystem containing many bacterial species, protozoa, fungi and yeast. There are five types of microbes present in large intestine includes proteolytic bacteria that cause breakdown of protein, lactic acid bacteria that digest starch, protozoa make volatile fatty acids, cellulytic bacteria and yeast/fungi that digest/break fibers and few vitamin-B producing bacteria. The bacteria that is present in it includes Lactobacillus & Firmicutes in the ileum, Lachnospiraceae, Ruminococcaceae, Bacteroidetes and Spirochetes in the proximal part of large intestine and Prevotellacea in the distal part of large intestine etc. The donkey receives much of its dietary supplement through hydrolysis and by fermentation of these microflora.

## 3. Fecal ball formation

First of all, animal eat food and its whole digestion process is like other animals. Mastication of food occurs after prehension. Digestion depends on good food grinding by teeth. During mastication saliva is produced and it depends on food which type of food is eaten by donkey. In stomach digestion is minimal and its main function is liquefaction of food then food is drained into small intestine. However, there are many types of enzymes released by stomach. Food particles are broken by gastric acid that produce by stomach. While protein digestion is due to enzyme pepsin. Pancreas release an enzyme called amylase, when food drains into duodenum part of small intestine. This enzyme is less produced in donkeys, so digestion of starch is minimal. The end product of protein is amino acids, done by enzyme released such as pepsin, and it absorb into blood [29]. Volatile fatty acids are produced by process of fermentation and then blood absorbs it. Actually, this volatile fatty act as source of energy. The proteins that remain undigested in large intestine are broken down by enzyme released by microbes. Ammonia is produced by this protein and it is beneficial for growth of beneficial bacteria [20]. Water is absorbed by large intestine, when whole grinded food enters into colon, more reabsorption occurs, and semi-solid feces formed. In colon end step occur as formation of fecal ball and then move into rectum and then anus [7].

## 4. The nervous system of the gastrointestinal tract

The digestive process like gut motility, absorption, secretion and the blood flow is influenced by the nervous system [30]. Although there is a bit links between the CNC and the digestive system, but the gut is capable of having their own nervous system called as the enteric nervous system (ENS). Like the spinal cord, this system holds numerous neurons. This system alongside with parasympathetic and sympathetic nervous systems establish the autonomic nervous system. The prime constituents of the ENS based on two neurons plexuses (networks) which is implanted along the length of gut wall. The submucosal networks embedded in the submucosa while the myenteric plexus is positioned in muscular externa which regulates motility of the gut. Its key function is in-sensing the intraluminal situation, controlling the mucosal epithelium function and regulating the gut blood flow. In esophagus the submucosal plexus are spars and its function are minimal. Sensory neurons of the mucosa and muscularis receive information from sensory receptors. Almost five diverse mucosal receptors are being known to act to the stimuli

including chemical, thermal, mechanical and osmotic origin. The chemoreceptors are sensitive to intraluminal glucose, acid, and amino acids. The muscular sensory receptors are reacting to all kinds of tension and stretch. The ENS are collectively gathering the evidence on condition of the gut wall and its luminal contents and motor neurons controlling the intraluminal absorption and secretion along with gut motility. Motor neurons act directly on many effector cells, including secretory cells viz. parietal, chief, enterocytes, mucous, gut endocrine, pancreatic exocrine cells and the smooth muscle cells [31]. The interneurons of the intestine are liable for assimilating information from sensory neurons and delivering it to motor neurons. In autonomic nervous system the T5, T6, T7, T8 make greater splanchnic nerve of which splanchnic ganglion and celiac ganglion are formed that further form celiac plexus (that supplies the stomach). The T11, T12 make least splanchnic nerve of which superior enteric plexus and inferior enteric plexus are formed that further innervates intestines. The L1, L2, L3 also innervates intestines. Sympathetic nervous system includes S2, S3, S4 forms pelvic nerve that supplies intestines and C10 innervates both stomach and the intestines. Ganglions including celiacomesenteric ganglion and caudal mesenteric ganglion and the lumbosacral plexus (hypogastric nerve) also innervates stomach.

## 5. Accessary glandular structures

All three major salivary glands are composed of either serous acini, mucous acini, or a combination of both. While parotid gland is largest of the three. All glands function is to produce saliva to moisturize the mouth and assist in the breakdown of carbohydrates in the mouth. The submandibular gland is the primary source of basal saliva secretion [32], while the parotid gland is the main source of stimulated saliva secretion. Salivary glands also play a crucial immunologic role as their secretions contain many immunoglobins, namely IgA, that help fight bacteria and other foreign antigens in the oropharyngeal environment. The sublingual glands lie inferolateral to the tongue, below the floor of the mouth and above the mylohyoid muscle [18]. Sublingual tissue is also palpable and is an oval shaped when sectioned transversely, however, its shape is longitudinal and lentiform when sectioned parallel to the body of the mandible. The sublingual gland differs from the other major salivary glands, because it lacks intercalated or striated ducts, so the saliva secretes directly through the ducts of Rivinus. These ducts empty along an elevated ridge called the plica fimbriata formed by the sublingual folds, which are oblique to the frenulum linguae bilaterally. The sublingual duct of Bartholin joins Wharton's duct to form the draining orifice on each side of the lingual frenulum. The sublingual tissue is predominantly a mucous gland, however, is considered a mixed serous and mucous gland. It is made up of mainly mucous acini with serous demilunes at periphery. It is the only unencapsulated major salivary gland. Sublingual tissue primarily produces a thick mucinous fluid and lubricates the oral cavity which allows for swallowing, initiating digestion, buffering pH, and dental hygiene. It retains both serous and mucous acinar cells while parotid salivary glands possess predominantly serous acini and produces watery fluid [17]. Myoepithelial cells are present around the acinar cells. The mandibular is tubule-acinar seromucous gland. The myoepithelial cells are present around the secretory units. Cells of the mucous acini have a pale-staining foamy cytoplasm which pushed the nuclei toward the basal lamina. While, the serous cells cytoplasm has zymogen granules (markedly eosinophilic) and their nuclei having rounded shape. The secretory acini which is made up of collection of secretory cells are categorized into serous and mucous category. The serous acini have just spherical shaped serous cells and

the mucous acini have only tubular shaped mucous cells. The sero-mucous (mixed) acini hold a combination of mucous and serous cells [6]. In histological set sections of the tissue, the swell mucous cells push the serous cells into a marginal area forming cap like structure recognized as serous demilune (demilune = "half-moon"). The submandibular gland obtains the supply of blood from lingual and facial arteries and emptied by shared lingual and facial veins. The parotid is supplied through the carotid artery (external) and its terminal branches including the superficial temporal and the maxillary artery and emptied by the retromandibular veins. The sublingual glands receive its blood supply from the submental and sublingual arteries.

## 6. Liver

Liver is the principal gland having no gall bladder. Its left side lobe is divided further. It is found underneath the diaphragm and protected by the ribs. It is covered by a fibrous connective tissue capsule, known as Glisson's capsule, which penetrates deep into the organ parenchyma to form septa that divide the main organ into lobes. The liver is formed from an invagination of the digestive tube during the embryonic development; therefore, it is an epithelial derivative [6]. The cellular organization of the liver is relatively simple based on the repetition of a basic structure called hepatic lobule. Lobules are separated from each other by connective tissue. The morphology of lobules is like polygonal prisms, of about 1–2 mm in diameter, and, in cross-sections, the lobules are similar to a hexagon containing a central vein of large diameter [33]. Hepatocytes represent more than 75% of the liver and are organized in anastomosed layers, or trabeculae. These layers of hepatocytes are usually one-cell thick and fused together to form a complex structure similar to a sponge. Small diameter sinusoids run between the layers of hepatocytes [34]. Between the endothelium of sinusoids and hepatocytes there are free-cellular spaces known as perisinusoidal spaces or spaces of Disse. Hepatocytes release two types of substances: endocrine toward sinusoids and exocrine toward the bile canaliculi. Hepatocytes are relatively large (around 20–30 μm) with a rounded nucleus, some are binucleated, and most of them are tetraploid. Hepatocytes are epithelial cells polygonal in shape having rich eosinophilic granular cytoplasm and centrally placed large spherical nuclei with conspicuous nucleolus. Hepatocytes having two nuclei are also common. Cells having numerous SER and RER, several mitochondria and Golgi apparatus. Hepatocytes are arranged in form of cords and the cells are separated by sinusoidal spaces called sinusoids. These are capillaries and are lined by flattened nucleated endothelial cells. The portal tirades constituted hepatic artery, portal vein and bile duct within the connective tissue are located at the portal areas between nearby lobules [35]. Portal triads are constituted by a branch of the portal vein (venule), a branch of the hepatic artery (arteriole) and a bile duct. In addition, lymphatic vessels and nerve fibers are found in the portal areas. The bile ducts of the portal triad collect the exocrine content, or bile, produced by the hepatocytes. Bile flows in the opposite direction to the blood that runs through the sinusoidal capillaries, in other words, it is directed from the hepatocytes to the bile ducts of the periphery of the hepatic lobule (the portal areas). This is possible because the plasma membranes of adjoining hepatocytes form interconnected spaces, the bile canaliculi, which are organized in an anastomosed network that finally fuses with the bile ducts. Ito cells (stellate cells/lipocytes) exist in space of Disse (between hepatocytes and endothelial cells). Kupffer cells are round in shape positioned in the sinusoids at vascular space within sinusoids [36]. Oval cells (pluripotent stem cells) are also present in the liver. Short lived lymphocytes (pit

cells) are situated in the sinusoids. The coeliac artery branched in hepatic artery and portal vein is fashioned by tributaries draining the pancreas, digestive tract and spleen [37]. Blood flows from the portal areas into the central vein lined by simple squamous epithelium. The blood vessels, nerves and bile duct leave and enter the liver at the hepatic porta.

## 7. Pancreas

The pancreas is encapsulated and lobulated organs having both the endocrine and exocrine portions. The color of the pancreas of the donkey is reddish cream. The connective tissue stroma divides the parenchyma into various lobules having secretory units and the intralobular duct. The pancreas is triangular, tubuloacinar gland and is present aside from the duodenum [35]. It consists of a body, right lobe, and left lobe. The pancreas has pyramidal acinar cells. Apical portions of these cells have secretory granules (zymogen granules). Exocrine portion produces several enzymes while the Islets of Langerhans are the endocrine portion of this gland. Alfa, beta and delta cells of the islets produces glucagon, insulin and somatostatin respectively [36]. Glandular tissue from the caudal end of the right lobe extended over the portal vein to the left lobe thus forming a ring. The pancreas secretes digestive enzymes into duodenum such as amylase, lipase and trypsin through pancreatic duct. These enzymes digest the carbohydrates, lipids and protein part of feed. The main pancreatic ducts which empties into the duodenum is the extension of interlobular duct, intralobular duct, and intercalated duct. The body of the pancreas received its blood supply from pancreatic branches of the gastroduodenal artery, the first branch was the larger one and originated from the gastroduodenal artery just after its origin from the hepatic artery, the second smaller branch has originated just before the gastroduodenal artery distributed into cranial pancreaticoduodenal and right gastroepiploic arteries. The left lobe received its blood supply from hepatic and splenic artery. The right lobe established its supply of blood from the cranial mesenteric artery.

## 8. Mucosa associated lymphatic tissues

Mucosa-associated lymphoid tissue (MALT) defends the body from gut invasion of pathogens. The mucosae of the respiratory, urinary and digestive tracts often have few aggregated lymphocytes called MALT or lymphoid follicles [38]. It is situated in different portions of the body viz. nasopharynx, lungs, breast, thyroid, eye, salivary glands, skin and GI tract. MALT is made-up of B and T lymphocytes, macrophages and plasma cells. In the case of intestinal MALT, there are also M cells that take antigen from the lumen and deliver it to the lymphoid tissue. MALT constitutes about 50% of the lymphoid tissue in animal body and its components are sometimes divided into the following areas/types: GALT (lymphoid tissue associated with the intestine. Peyer patches are a component of GALT, which is found in the lining of the small intestine), BALT (lymphoid tissue associated with bronchi), NALT (nasal associated lymphoid tissue), CALT (conjunctiva-associated lymphoid tissue), LALT (lymphoid tissue associated with the larynx), SALT (skin-associated lymphatic tissue), VALT (lymphoid tissue associated with vulvovaginal) and TALT (lymphoid tissue associated with testicular). It can also be distinguished by the degree of tissue organization: O-MALT (lymphoid tissue associated with the organized mucosa), D-MALT (diffuse lymphoid tissue of the mucosa). The MALT that is not organized as a mass, tissue or organ anatomically identifiable separately macroscopically (like

the O-MALT mentioned above) is diffuse MALT. Due to its function during food intake, the mucous membrane is superficially slim and performed as permeable barrier in the body. Likewise, its permeability and delicateness make it susceptible to infection, and in fact most infectious agents that enter the body practice this way. GALT as protection mostly depends on plasma cells that produce antibodies. The lymphatic tissue associated with the intestine is found throughout the intestine and histopathology is the better option to study those [39]. Like thymocytes, GALT containing intestinal Peyer patches (lymphoid follicles made up of lymphocytes) are responsible to safeguard the animal health from the gut luminal side [40, 41].

## 9. Conclusion

The morphophysiological study both (gross and microscopic) of the gastrointestinal tract and associated structures of domestic donkeys are very important to document. The microstructure of internal luminal layer of the gut, luminal ecosystem, immunity and function of the gut is highlighted in this chapter.

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Chapter 10

# Promoting Grass in Horse Diets and Implementing Sustainable Deworming: 'Équipâture' Programme

*Pauline Doligez, Marie Delerue, Agnès Orsoni, Bathilde Diligeon, Céline Saillet, Hervé Feugère, Guillaume Mathieu, Jean Baptiste Quillet and Stéphanie Cassigneul*

## Abstract

The Équipâture programme examined the grazing regimes and parasite statuses of horses on 12 study farms. Its research yielded useful results. Rotational grazing of mares, foals, and school riding horses allowed animals to meet their nutritional needs without any supplements (50 ares/LU in the spring; 80 ares/LU in the summer). During the winter, haylage met the high demands of mares and foals. Late-cut hay could not, and there was a risk of P, Cu, and Zn deficiencies when horses were given a 100% hay diet. A sustainable approach to deworming was implemented on the farms. Based on faecal analysis, animals were assigned a parasite excretion status. As a result of this categorisation, only half of the animals were dewormed. This method helped limit deworming costs and the development of parasite resistance to dewormers.

**Keywords:** horse grazing, stocking rate, hay, haylage grass analysis, deworming, faecal egg count

## 1. Introduction

Grass, although being the most adapted food and considered as the least costly for herbivores, is nevertheless not always duly promoted in equine diets. The lack of data on equine pasture management and systematic deworming practices are recorded as factors hampering the efficient and sustainable management of grazing horses [1]. Systematic and frequent deworming of horses encourages the development of parasite resistance to dewormers. Three active dewormer families against cyathostomins, the most prevalent parasite in adult horses, are currently available on the market in France. Among the said three families, cyathostomins are known to resist two of them. In order to counteract the development of such resistances, it is important to change deworming practices by adopting faecal egg count as a determining element whether or not to worm. Sustainable deworming could also include the implementation of management measures in order to reduce the parasitic pressure on pastures [2]. Nevertheless, literature provides very little data

in this regard. In this context, the 'équipâture' programme was initiated through the monitoring of 12 equine study farms located in the regions of Centre Val de Loire, Limousin and Normandy (France) between 2016 and 2017, with the aim of analysing pasture management, promotion of forage intake, and implementation of sustainable deworming. In collaboration with Agricultural Chambers, local Horse Councils, and the French Horse and Equestrian Institute (IFCE), this study has permitted the compilation of data on horse grazing and pasture management. This summary illustrates the technical results of feed and pasture management, as well as the monitoring of animal infection.

## 2. Material and methods

At the national level, 12 study farms were monitored for over 2 years—2016 and 2017 (grazing seasons and in the winter). The selected equine farms were chosen according to the different breeds (draft horses, racehorses, sport and leisure horses, and cattle-combined) and the category of horses at grass (breeding stock and adult horses at rest or for schooling). The idea was to study and monitor a panel of structures with different production targets, using all grass surfaces for equine feeding and wishing to engage in more sustainable parasitic management practices. The 12 farms were located in 3 abundant grass regions [Centre-Val de Loire (4), Limousin (4), and Normandy (4)] and were comprised of 7 stud farms (2 draft horse + suckling-cow farmers, 1 pony breeder + crop farmer, 1 thoroughbred stud, 1 French Trotter stud, and 2 sport-horse breeders) and 5 riding establishments (1 riding school + sport-horse breeder, 2 riding schools + livery + breeder, 1 trail-riding centre, and 1 'active stall').

Batches of 10 to 25 adults (aged 3 years or more in 2016) per farm, i.e., 204 horses in total, were monitored by the study team (2 persons) with the aim of defining their parasitic status, measuring their body condition score (BCS), and analysing their feed pattern and grazing during the two 2016–2017 seasons. Since it has been illustrated that acquired immunity against cyathostomins is reached at age 3, adults can be grouped according to their strongyle egg excretion level. It is considered that 15 to 30% of horses over the age of 3 are responsible for excreting approximately 80% of all eggs [2].

### 2.1 Monitoring animals

For each animal over the two-year period, faeces were collected individually and their body condition score (BCS) and weight were recorded at three intervals (May, August, and November). Faecal sample, upon individual identification, was despatched within 24–48 hours after refrigeration to the same county veterinary analysis laboratory for faecal egg count [quantitative numeration of the number of strongyle and tapeworm eggs per gram of faeces (epg)]. For each visit, the INRA 1997 [3] grid was used to evaluate the body condition score of each monitored horse (score ranging from 0 to 5, 3 being the optimum score). The faecal egg count (FEC) results and BCS figures were regularly transmitted to the farmers, along with personal advice per animal as to whether or not to worm, in addition to guidance on feed and grazing.

In spring and summer, only horses whose faecal egg count resulted in an egg count exceeding 200 epg were dewormed with, respectively, ivermectin in spring and pyrantel in summer (such threshold being traditionally recommended in literature, according to [2]). All of the horses, regardless of the faecal egg count results, were dewormed at the end of autumn using a molecule association (moxidectin and praziquantel), thus enabling to eliminate strongyles, whether adult or larvae, as well as tapeworm.

At each farm visit, some horses were absent or gone definitely (sold, owners changed, died, etc.); that is why, 6 (FEC + BCS) data/horse were not possible to collect over the 2 years.

### 2.2 Monitoring the feed pattern and grazing

Three to four visits over the two-year period, from March to September, were conducted in order to draw up a grazing programme, evaluate pasture management, and carry out grass and fodder sampling. At the beginning of the season, the grazing forecast was estimated using the 'prév'Her' application (tool devised by the Creuse Chamber of Agriculture and adapted to take account of French equine LU references: one saddle mare and its foal = 1.2 LU [4–6]). The grazing base area for each batch was calculated for horses under rotational grazing. Forage, hay, and haylage samples over the 2 years, in addition to fresh grass samples in 2017, were taken from the grazing plots at various intervals, and they were tested using near-infrared spectrophotometry at the LANO 50 agronomy laboratory in order to determine their nutritive values [HFU/kg DM (net energy horse feed units), INRA 2011, (g HDCP) horse digestible crude proteins g/kgDM, Ca, P, K, Na in g/kg DM and Mg, Cu, Zn, Mn, Fe in mg/kg DM (XLStat statistics' analysis, Student Test)]. From 2 to 11 dried forage samples (2016 and 2017) were collected depending on the numbers of hay or haylage harvests per farm. From 2 to 6 fresh grass samples per farm (2017) depending on the numbers of grazing cycle exploited were taken across the various seasons from the grazed areas (rough areas excluded) on the same plots of permanent pastures, free of nitrogen fertiliser. Winter diets for the different equine categories were calculated with INRA system according to the nutritional values collected from the analysis of forage harvested in 2016.

### 2.3 Influence of stud management and the age of the horses on parasite excretion

Only the results of the faecal egg counts gathered in spring and summer of 2016 and 2017 were used. Indeed, studies have shown that parasites lay fewer eggs outside the grazing season, i.e., when the climate is less favourable to parasitical transmission [7]. Nevertheless, any such faecal egg count in November remains interesting in practice, in order to give an overall picture of the farm's parasitical situation at the end of the grazing season.

Five explanatory variables were retained in relation to stud management and to the age of the horses:

- Foals (< 1 year) on site: two criteria—present or absent
- Accommodation type: two criteria—horses living out at pasture 24/7 or horses turned out daily (stable/pasture combined)
- Annual stocking rates: three criteria—low (less than 0.6 LU/ha), medium (between 0.6 and 1.0 LU/ha), and high (more than 1 LU/ha), (LU: livestock unit)
- Age of horses: three criteria—young (under 10 years), medium (between 10 and 15 years), and old (over 15 years)
- Significant movement on site: two criteria—few or many new arrivals

Among the 204 horses monitored, data from 83 horses were used for the analysis, the result range of the others being incomplete. For the horses used, five explanatory variables were applied in addition to the 2016 and 2017 spring and summer faecal egg count results. The five explanatory variables underwent a multiple correspondence analysis (R software), followed by agglomerative clustering (AHC), in order to compile groups of variable criteria. The clusters derived from the AHC are those that maximise the difference of one group in relation to another, while ensuring the best homogeneity among individuals within the same group. The best division singles out four groups:

- Group 1: stud farms with a high turnover (many new arrivals, high stocking rate, presence of foals, living out 24/7, and horses mostly aged between 10 and 15 years)
- Group 2: horses over the age of 16
- Group 3: riding establishments with a low turnover (absence of foals, few new arrivals, living out 24/7, and horses aged under 10)
- Group 4: stud farms with a low turnover (horses turned out daily, presence of foals, low or medium stocking rate, and few new arrivals)

A principal component analysis (PCA) was conducted in order to observe a possible influence of the explanatory variables on parasite excretion in the spring and summer of 2016 and 2017.

## 3. Results of the feed patterns and pasture management

### 3.1 Stud farm stocking rates

The indicator retained for evaluating the stocking rate is calculated according to the number of LU (livestock units) per equine equivalent (one saddle mare and its foal = 1.2 LU, INRA 2012) in relation to the volume of the breed main forage areas (MFAs) in hectares. The 12 stud farms monitored are characterised by a medium to low stocking rate (from 1.05 to 0.6 LU/ha of MFA), on par with the data of equine farms monitored in the context of the REFErences' network (7). The most intensive systems can be found in multi-production farms, comprising horse breeding alongside another production (beef cattle or crop farming). The most extensive farms (<0.5 LU/ha of MFA) breed exclusively top pedigree horses (2/12). For such farms, the productivity of grassland is not a priority when considering the real economic value of the animals [8].

### 3.2 Stocking rate and conditions of pasture management

Pasture management for the 33 batches of animals was duly monitored on the 12 study farms. Grazing rotations were recorded by noting the number of animals present per cycle.

Pasture management was split into three different types (**Table 1**).

Seven batches of horses monitored out of 33 (21%) were reared in rotational pastures with a stocking rate comprised between 40 and 60 ares/LU in spring and 80 ares/LU in summer. Sixty per cent of the farms mulched the herbage rejected by the animals.

| Stocking rate observed | No. of batches of horses observed and overall % | Horse types | Feed pattern and pasture management |
|---|---|---|---|
| <20 ares/LU in spring/ summer | 4/33 (12%) | Stabled horses<br>Horses at rest | The so-called paddock void of feeding role<br>several hours/day for exercise<br>24/7 for overweight horses with restricted forage rations |
| 40–60 ares/LU in spring<br>80 ares/LU in summer | 7/33 (21%) | Broodmares and foals<br>School horses | Rotational grazing |
| > 100 ares/LU in spring and summer | 22/33 (67%) | Horses at rest<br>Broodmares | Grazing 24/7 |

**Table 1.**
*Stocking rate observed and management conditions on the 12 study farms.*

*For example:* 14 thoroughbred yearlings and 10 cows with calf at foot were taken in the spring to a 9-ha pasture divided into 5 separate plots of 1.5 to 2 ha. This combined batch then grazed 18 ha in the summer.

Among 2/3 of the batches (67%), grazing 24/7 is generally conducted at low intensity (>100 ares/LU) across large areas, with small batches of horses (2 to 3) given extra fodder in summer and/or autumn [9]. Rough was mulched several times during the grazing season.

To end, for 12% of the batches, small surface areas (<0.2 ha), mainly located near the farm buildings, were used as 'exercise paddocks' for stabled horses. Such paddocks may also serve to accommodate horses that should have limited grazing (overweight horses and ponies at rest or retired). These paddocks are not considered as a nutritional source for the animals.

### 3.3 Estimation of a horse's body condition

A body condition score (BCS) was recorded for 132 adult horses at three separate intervals (May, August, and November) over the 2 years.

The BCS of the school horses was >3.6 for 48% of them in spring and 39% in summer. In autumn, they gained weight with 16% becoming quite overweight (BCS > 4.1). In such a case, forage supplementation in winter was delayed. Eighty-three per cent of the horses at rest were overweight (BCS > 3.6) in summer, 37% of which showed a BCS > 4.1. Grass restrictions were imposed on certain horses (BCS > 4.6 in summer) by placing them in drylots in order to limit the risk of laminitis. Fifty per cent of the retired horses with a BCS < 2.4 in summer were at least supplemented in forage. Twenty-nine per cent of the thoroughbred broodmares, some of which being supplemented with concentrates, became overweight in autumn (BCS > 3.6). Eighty-eight per cent of draft horse broodmares that attained a BCS > 4.6 in autumn were not given extra fodder during that period, nor even in winter.

### 3.4 Grass analysis

#### *3.4.1 HFU content and digestible crude proteins/kg fresh grass DM*

The mean HFU energy value of grass was exactly the same in April and in May (0.718 vs. 0.72 HFU/kg DM). In June, the mean energy value significantly dropped

to 0.67 HFU/kg DM ($p < 0.01$), proving to be more heterogeneous, and then increased again in July to 0.71 HFU/kg DM ($p < 0.01$). The higher energy values in July were due to regrowth following a more prominent period of rain than in June 2017 (**Figure 1**). Hence, other mean grass HFU comparisons in April and May among the three regions illustrated superior energy values in Normandy by +0.04 ($p < 0.01$) and +0.06 ($p < 0.01$) HFU points, respectively, in relation to the HFU value of grass in the Limousin and the region of Centre Val de Loire.

Concerning the protein values in gr HDCP/kg DM (**Figure 2**), the averages observed during the grazing season hardly differed ($p > 0.5$) on a monthly scale. Hence, the protein value comparisons among the three regions failed to show any difference ($p > 0.5$).

When grass was abundant and could be compared with a minimal to maximal *ad lib* intake level of DM/kg, the nutritional needs (in intake HFU and gr HDCP/kg DM) of the broodmare during the 1st month of suckling and of the 18-month yearling are basically covered between April and June across the three regions.

### 3.5 Analysis of the harvested dry forage and the consequences on winter diets (HFU and g HDCP/kg DM)

#### *3.5.1 Energy and protein*

The harvesting conditions in 2016 (late first cut in July) resulted in slightly lower energy and crude protein values by, respectively, 0.04 points HFU and 8 g HDCP/kg of DM in relation to the values of forage harvested in 2017 (early to mid-June cut) (**Table 2**).

The HFU and HDCP nutritive values of 62 hay and 10 haylage samples were compared with the recommended dietary needs [4] for three categories of animals receiving essentially forage-based rations in winter (**Table 3**).

Haylage harvested at the end of May or as second cut seems better adapted than hay for animals with high nutritional needs (broodmares and foals). For horses at rest or having light exercise, hay seems more adapted despite a 5- to 15-gr HDCP/kg DM crude protein deficiency in relation to dietary needs (**Table 3**).

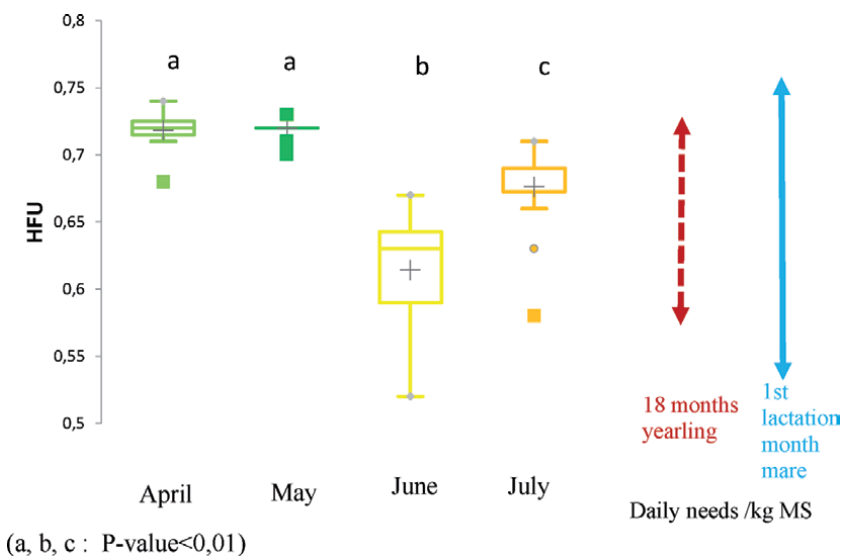


**Figure 1.**
*Net energy values (net energy horse feed units: HFU/kg DM) of grass samples depending on grazing period (2017 season, n = 52).*

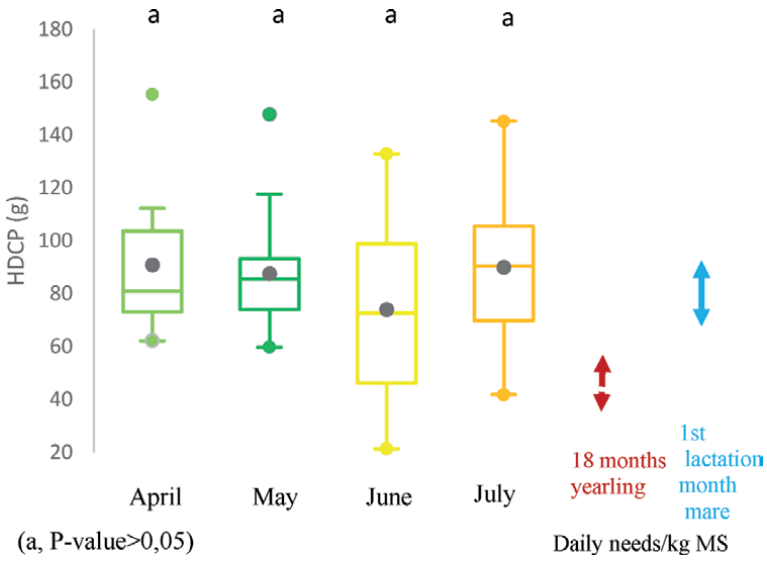


**Figure 2.**
*Horse digestible crude protein values (g HDCP/kg DM) of grass samples depending on grazing period (2017 season, n = 52).*

| | HFU/kg DM | | g HDCP/kg DM | |
|---|---|---|---|---|
| **2016 (n = 40), 2017 (n = 22)** | **2016** | **2017** | **2016** | **2017** |
| Minimum | 0.33 | 0.38 | 12 | 16 |
| Maximum | 0.65 | 0.62 | 76 | 73 |
| **Averages** | **0.48** | **0.52** | **25** | **33** |
| Standard deviation | 0.07 | 0.06 | 13 | 14 |
| | + 0.04 p < 0.1 | | +8 p < 0.1 | |

**Table 2.**
*Nutritional values (HFU and g HDCP) of hay depending on harvest year.*

For each stud farm, a report on the winter rations was drawn up based on the forage analysis.

If the net energy and digestible crude protein needs of animals with high nutritional needs (broodmares) are covered by haylage-based rations, those based on hay harvested in 2016 (with or without concentrates) fail to cover such needs (**Table 4**).

For animals with minimal needs (horse at rest or in light exercise), a winter ration of 100% hay, or 90% hay +10% cereals, based on forage analysed in 2016, failed to cover the digestible crude protein needs. A 100% haylage ration for a horse at rest was far too rich (**Table 4**).

### *3.5.2 Minerals*

The Ca and Mg content of the three forage types (grass, haylage, and hay) (**Table 5**) covered the overall daily needs for the three animal types (broodmare, 18-month foal, and horse at rest or in light exercise). The mean phosphorus content of the hay (Me = 1.8 g/kg DM) was deficient for animals with high nutritional needs (broodmare: 3.2–4.3 g/kg DM; 18-month foal: 2.7–3.4 g/kg DM). The Ca/P calcium-phosphate ratio showed an average of 2 instead of 1.5 (mean reference of all horse categories [4]). The potassium content was in excess in relation to needs (**Table 5**).

| | | HFU/kg DM | | g HDCP/kg DM | |
|---|---|---|---|---|---|
| | | **Haylage** | **Hay** | **Haylage** | **Hay** |
| Energy and protein values/kg DM of hays (n = 62) and haylage (n = 10) | Minimum | 0.62 | 0.33 | 35.2 | 11.8 |
| | Maximum | 0.73 | 0.65 | 76.8 | 75.9 |
| | **Averages** | **0.67** | **0.49** | **55** | **28** |
| | Standard deviation | 0.04 | 0.07 | 12 | 13 |
| | P-value | +0.18 ($p < 0.01$) | | +27 ($p < 0.01$) | |
| Daily needs/kg DM according to horse's low and high intake | 9th month pregnant mare | **0.55–0.67** | | **43–54** | |
| | 18–24 month yearling | **0.58–0.73** | | **33–41** | |
| | Adults at rest or having light exercise | **0.46–0.60** | | **33–43** | |

**Table 3.**
*Nutritional values (HFU and g HDCP) of hay and haylage compared with the animals' dietary needs.*

| | Ninth month pregnant mare | | Adult at rest or in light exercise | |
|---|---|---|---|---|
| **% of net energy and protein needs covered by the four ration types observed (/kg DM)** | **in HFU** | **in g HDCP** | **in HFU** | **in g HDCP** |
| 100% hay | 80 | 50 | 98 | 70 |
| Hay (90%) + cereals (10%) | 110 | 50 | 133 | 70 |
| 100% haylage | | | 174 | 194 |
| Haylage (87%) + industrial concentrates (13%) | 92 | 120 | | |

**Table 4.**
*Winter rations calculated according to the nutritional values of forage harvested in 2016.*

### *3.5.3 Trace minerals*

If the Cu and Zn content was deficient for all forages, the manganese and iron content was significantly in excess (**Table 5**). Among the stud farms monitored, some administered mineral and vitamin supplementation to the winter diet (5/12) or make it available in the grazing period (3/12).

## 4. Results of parasite excretion monitoring

Throughout the grazing season (spring and summer 2016 and 2017; **Figure 3**), the results of the faecal egg counts carried out on the 83 adult horses (**Table 6**) aged over 3 enabled, on average, to simply worm 50% of the horses, i.e., those excreting more than 200 epg. Such horses are nevertheless responsible for excreting 94 to 99% of the eggs, depending on the period concerned, thus significantly contributing to pasture contamination.

### 4.1 Definition of the excretory status

Three excretory statuses of horses were defined:

- 23% of the horses have a low excretory status: horses excreting less than 200 epg in all faecal egg count analysed in spring and summer of 2016/2017.

| Mineral g/kg DM and trace minerals mg/kg DM from forages | Grass (n = 53) | | Haylage (n = 10) | | Hay (n = 62) | | Early pregnant mare, 18–24 month yearling and light exercise adult average needs [4], per g or mg per kg DM intake |
|---|---|---|---|---|---|---|---|
| | Median | σ | Median | σ | Median | σ | |
| Ca (g) | 6.2 | 1.9 | 6.4 | 1.4 | 4.0 | 1.1 | 2 at 5 g |
| P (g) | 2.9 | 0.6 | 2.9 | 0.3 | **1.8** | 0.5 | 1.7 at 4.3 g |
| Ca/P ratio | **2.2** | 0.9 | **2.1** | 0.6 | **2.2** | 0.9 | 1.35 at 1.8 |
| Mg (g) | 1.7 | 0.4 | 1.5 | 0.5 | 1.3 | 0.3 | 0.7 at 1.1 g |
| K (g) | **23.8** | 5.5 | **25.1** | 6.5 | **14.6** | 4.6 | 2.5 at 5.5 g |
| Na (g) | 0.8 | 0.8 | 0.7 | 1.4 | 1.2 | 1.5 | 0.9 at 2 g |
| Cu (mg) | **5.5** | 1.6 | **4.9** | 1.3 | **3.2** | 1.1 | 10 mg |
| Zn (mg) | **23.9** | 7.6 | **25.0** | 6.2 | **17.9** | 5.2 | 50 mg |
| Cu/Zn ratio | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 |
| Mn (mg) | **155.2** | 120.1 | **195.3** | 86.6 | **158.1** | 150.6 | 40 mg |
| Fe (mg) | **147.6** | 215.3 | **152.5** | 94.8 | **116.6** | 507.0 | 50 at 80 mg |

**Table 5.**
*Mineral content in gr or mg/kg DM of forage (grass, haylage, and hay) for the 2 years 2016 and 2017. Data over or under the average needs.*

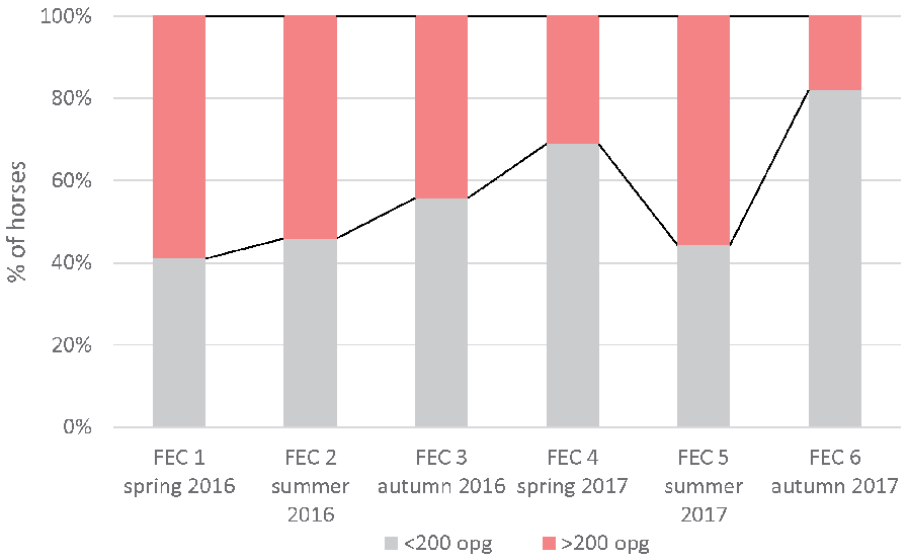


**Figure 3.**
*Percentage of the 83 horses excreting more or less 200 strongyle epg at each faecal egg count (FEC).*

- 13% of the horses have a high excretory status: horses excreting more than 200 epg in all faecal egg count analysed in spring and summer of 2016/2017.
- 64% of the horses have an unstable excretory status: horses excreting alternatively more than 200 epg or less than 200 epg.

| Region | Centre Val de Loire | | | | Normandie | | | | Limousin | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Farm No.** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| Nb of followed animals with full FEC results in spring and summer 2016/2017 | 14 | 18 | 0* | 5 | 4 | 0* | 14 | 11 | 0* | 8 | 0* | 9 |

*Horses with less than 6 FEC/2 years.*

**Table 6.**
*Followed animals per farm distribution.*

### 4.2 Study of the influence of stud management and horse's age on the level of equine parasitic excretion

In the principal component analysis, four groups were identified:

- Group 1: stud farms with a high turnover (many new arrivals, high stocking rate, presence of foals, living out 24/7, and horses mostly aged between 10 and 15 years), 20 horses
- Group 2: horses over the age of 16, 18 horses
- Group 3: riding establishments with a low turnover (absence of foals, few new arrivals, living out 24/7, and horses aged under 10), 18 horses
- Group 4: stud farms with a low turnover (horses turned out daily, presence of foals, low or medium stocking rate, and few new arrivals), 27 horses

The first and second axes of the PCA are those that best resume the data contained in the five variables; they are thus retained for the analysis. Axis 1 represents the general tendency of the faecal egg count (FEC) for an individual: on the left, an individual shows basically low results, while on the right, the results are generally high. Axis 2 represents the results from spring and summer 2016: at the top, the individuals show high results in spring 2016, while in summer 2016, the results are low. **Figure 4** thus enables to distinguish a high excretory profile in spring 2016 and a low excretory profile in summer 2016 (in the direction of 'FEC 1'), a high excretory profile in summer 2016 and a low excretory profile in spring 2016 (in the direction of 'FEC 2'), and a high excretory profile in 2016 and in 2017 (in the direction of 'FEC 4' and 'FEC 5').

**Figure 5**, on its part, enables to identify certain individuals belonging to one of the profiles highlighted in the graph of the variables. The individuals (grey dots) surrounded by a continuous line illustrate high FEC in spring 2016, though with low results in summer 2016. The individuals surrounded by a dotted line illustrate high FEC in summer 2016, though with low FEC in spring 2016. The individuals surrounded by dashes illustrate high FEC for all FEC. These different ellipses were hand drawn for educational purposes. All other individuals illustrate low or average FEC. The four groups of horses (black boxes), identified in accordance with their management type, appear in the box in the centre of the graph. None of these groups particularly stand out in relation to the four axes. No significant difference can be observed among the faecal egg count results for the different groups.

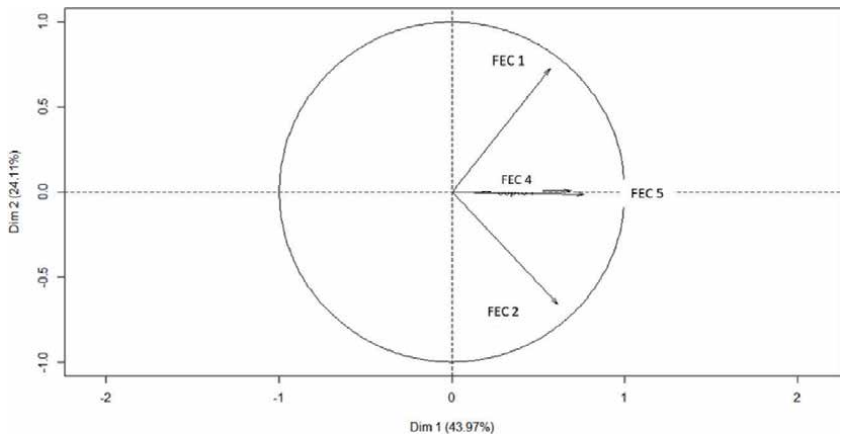


**Figure 4.**
*Principal component analysis on the effect of the five explanatory variables on the results of spring and summer 2016 and 2017 faecal egg count (FEC).*

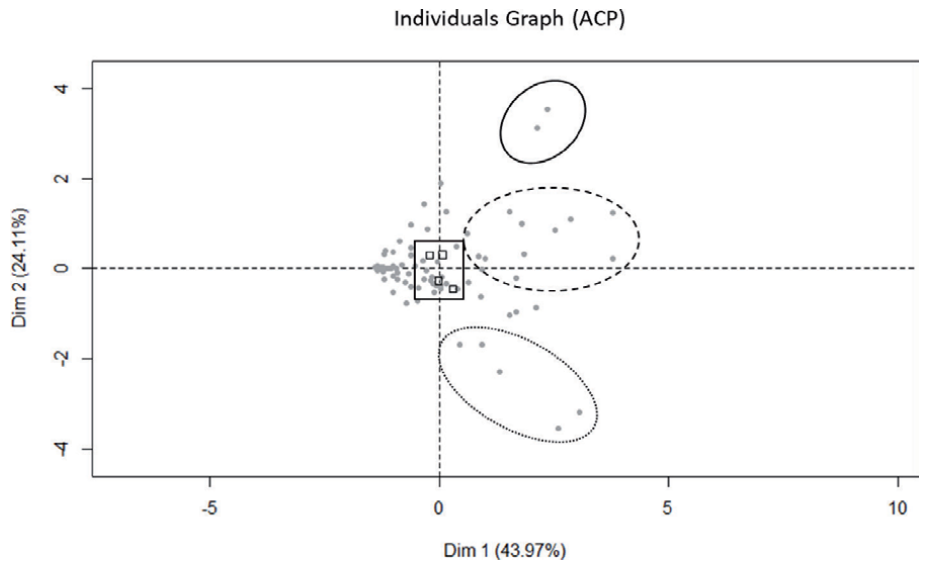


**Figure 5.**
*Principal component analysis on the effect of the individuals on the results of spring and summer 2016 and 2017 faecal egg count (FEC).*

## 5. Discussion

### 5.1 Stocking rate and pasture management

The stud managements observed illustrated a very low stocking rate in spring, thereby requiring regular mechanical maintenance in order to limit the development of roughs. The management of certain batches of animals (mares with foal at foot to be covered by a stallion and requiring individual attention and school horses exercised daily) and the need for shelters and secure fencing entail extensive pasture management and 24/7 grazing, where manipulation and care take priority in relation to a sustainable management of grassland. Such low stocking rate is, for some batches, linked to the use of pastures for wintering horses, thus reducing the available grass stocks early in the season and the productivity of grasslands [9].

When the stocking rate in the spring exceeds 100 ares/LU (22/33 of the batches studied), practices aiming to intensify the farming system, such as the sale of forage or taking on boarding cattle, were proposed to breeders. Nevertheless, the lack of human resources, the necessary investment in harvesting equipment, and additional infrastructures (fencing and wintering barn), as well as the lack of economic production attractiveness (suckling cows), are the main blockers exposed by the stud farms for developing other activities to enable optimum use of the grassland.

Since few references already exist on the interest of an equine-cattle grazing combination in grassy areas [6, 10], the French Horse and Equestrian Institute (IFCE) and the National Institute for Agricultural Research (INRA) are currently conducting studies in order to pinpoint its effects in relation to a farm's biotechnical, economic, and social (labour) performances [11].

Rotational grazing is considered as the most appropriate practice for providing an adequate energy and protein balance for animals requiring high nutritional needs (broodmares and foals), though without the necessity for concentrate supplementation and without body condition loss. The grass available must be of adequate quality (in leaf) and quantity (height between 5 and 20 cm) [12]. For those stud farms having thus invested, such practice should be long-lasting.

For horses at rest, their living conditions take priority over the optimum use of grassland. Pasture management thus becomes somewhat tricky when trying to prevent overweight and consequential metabolic illnesses (laminitis) in adult horses having little or no exercise. Indeed, as a monogastric herbivore, diets with a low starch and sugar content are more adapted to horses, who have the ability to continuously ingest vast quantities of rough forage in order to satisfy their nutritional needs and maintain a healthy digestive system [13]. When grass resources are abundant, ingestion increases beyond the normal capacity in terms of the nutritional needs for maintaining a 3/5 body condition score, and the lack of exercise results in weight gain. Restricting food in winter, in order to encourage weight loss and to limit overweight just prior to the grazing season, could be a solution. Nevertheless, such practice is scarcely applied by breeders, the latter generally offering ad libitum forage in winter.

In spring, the grass available in leaf can represent a far too rich food resource (in energy and proteins) with over 50% of the grass analysis attaining + 132% of the HDCP needs and + 110% of the HFU needs for such horses already in good condition (BCS > 3.6) at the end of winter.

Drylots are temporarily used for part of the day, or even 24/7, in order to restrict the food intake of overweight animals (horses and ponies) (body condition score corresponding to 4/5 at the end of winter). Low-protein fibrous forage is often administered in order to prevent metabolic illnesses (laminitis), notably when grass grows abundantly (spring, autumn). Such management raises the question of how to optimise the maintenance of such areas, not only in order to limit the propagation of weeds but also to maintain the weight-bearing ability of the ground in a manner not to damage the horses' hooves. An alternative, in order to prevent the degradation of drylots, sacrificed due to overgrazing, could be the creation of stabilised sandy areas where such horses could be parked during sensitive periods (spring, autumn).

### 5.2 Analysis of forage and winter rations

None of the stud farms monitored undertook forage analysis, nor calculated routine rations, despite the diet administered to their horses consisting of forage, for the most part. An essentially forage-predominant diet in terms of proportion

and quantity, thus enabling to further reduce the amount of concentrates, would not only control feed costs but also promote digestive health and the overall well-being of the horse [14, 15]. A simulation of the feed costs on a farm enabled to illustrate that a ration consisting of haylage + hay results in savings of up to 25% in relation to a standard ration of hay + concentrates.

Diets consisting of just hay, or hay + cereals, are often less balanced (~40 HDCP/HFU ratio) compared with diets consisting of haylage (90 HDCP/HFU ratio, significantly more in line with the needs of the horse). Nevertheless, few stud farms (2/12) produce haylage. Eighty-three per cent of the stud farms monitored do not produce such forage, either because they have no knowledge of the harvest techniques (4/12) or because such forage constitutes too richer feed in relation to their animals' needs (at rest, light exercise, draft horse) (6/12).

Adding a vitamin-mineral supplement (VMS) to the ration is not systematic. Having said that, P, Cu, and Zn deficiencies were recorded on the forage analysed. A vitamin-mineral supplement is necessary not only in winter rations but also when out at grass.

### 5.3 Sustainable deworming

The implementation of targeted deworming above the threshold of 200 epg in such equestrian structures enabled to simply worm half of the adult horses present on site during the grazing season. Such 200 epg threshold thus enables to preserve a parasite population not subject to anthelminthic treatment, the so-called refuge population [2]. The larger the refuge population, the less rapidly resistances progress [16].

Having said that, these dewormed horses were responsible for excreting more than 94% of eggs across the pastures. Targeted deworming thus enables to rupture the cycle of most parasites and hence to safeguard the health of all the horses on site.

One of the main hindrances to implementing targeted deworming within a structure seems, aside from the time spent in collecting individual stools, to be its cost. Indeed, Sallé et al. [17] illustrated that such targeted deworming can be financially viable in relation to systematic deworming insofar as the cost of a faecal egg count is less than 5 Euros. In the stud farms monitored, approximately ¼ of the horses had a low excretory status. Literature shows that such low excretory status is stable from one grazing season to the next [18, 19], for a healthy horse accommodated in stable conditions. In this study, 90% of the horses with this status in 2016 had the same status in 2017. For such horses, deworming once or twice a year (in autumn and possibly in spring) was recommended, without annual faecal egg count testing; only one faecal egg count approximately every 2–3 years in order to verify that the on-site epidemiological situation has not evolved and that the horse has not changed status. Annual faecal egg count monitoring is moreover recommended in the case of suspicion of immune deficiency (senior horse over the age of 20 or illness affecting the immune system (e.g., Cushing's disease)).

The cost of sustainable deworming could thus be reduced over the seasons due to the stability of the low excretory status requiring less strict faecal egg count.

Having said that, the high excretory status was much less stable in between the two grazing seasons, since only 37% of the horses with such status in 2016 also had the same status in 2017, and the remaining 63% passed from a high excretory status to an unstable status. For such unstable and high excretory statuses, it was advised to continue faecal egg count in order to adapt the frequency of deworming.

In terms of faecal egg count results, the situations varied considerably among the stud farms, such as illustrated in the following two examples:

- The first structure is a French Trotter breed farm with significant breeding and foaling activity, taking in many broodmares during the breeding season, these outside mares being lodged with the home-based horses. We noted a very high proportion of the horses with an unstable excretory status (80%) with only 20% of the horses with a stable status, of which only half, i.e., 10% overall, had a low excretory status. In such structure, targeted deworming has little interest, since most of the horses need to be dewormed following the faecal egg count. We notably observe high excretion levels in summer (1000 epg per horse in 2016, compared with 272 epg in spring 2016). Prior to introducing targeted deworming, certain stud-management measures should be implemented, in order to reduce contamination of the plots and infestation of the horses in summer, notably by separating the outside mares from the rest of the herd.

- The second structure is an 'active stable', wherein the horses (essentially over the age of 15) benefit from mixed accommodation (a central stabilised area with rotational pastures during the grazing season, the dry areas being very regularly cleared of all dung). In this structure, ¾ of the horses had a stable status (54% with a low excretory status and 21% a high excretory status).

It is thus difficult to implement an appropriate and acceptable targeted deworming protocol for all equine structures. This programme should be adapted to each stud farm, not only in accordance with the objectives of each farm (protection of the environment, health safety, economic considerations, and breeder implication) but also according to the epidemiological situation, such as the presence of foals and youngsters, or the frequency of movement, among others [2]. Nevertheless, good practices of stud management, enabling to reduce parasitic pressure across pastures, have been the subject of few studies in relation to horses, with the exception of dung removal [20] or composting manure [21]. We have attempted, during this project, to highlight the influence of certain types of stud management (presence or not of foals, accommodation, 24/7 grazing VS mixed rotational grazing, stocking rates, and importance of movements) on parasite excretion; nevertheless, no correlation was able to be established. Perhaps this was due to the limited number of horses selected for the study, or maybe due to the age of the horses (3 years and over), for which parasite immunity is deemed as being established [2]. Additional studies are thus necessary in order to research such risk factors within farms and to preach good practices of stud management [11].

## 6. Conclusion

Monitoring feed and pasture management in horses on 12 study farms for 2 years highlighted the necessity to alert horse breeders on the regular recording of the body condition score in order to optimise the balance between the needs of the animals, notably those with low nutritional needs, and the grass available in the pastures. Forage analysis and the calculation of a ration need to be more commonly accepted in order to ensure dietary balance and more targeted grazing. The parasitic monitoring in horses illustrated very heterogeneous situations among the structures. It seems very difficult to propose a sustainable deworming protocol without first carrying out a parasitic audit and ensuring strict monitoring of the farm by the treating veterinarian.

Several pasture management studies are currently ongoing in order to optimise feed and cost controls and to limit equine parasitic pressure, with notably a combined cattle-equine grazing study.

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Chapter 11

# What Are They Thinking? Scientific Horsemanship and the Mind of the Horse

*Ian Q. Whishaw and Candace J. Burke*

## Abstract

Horse behavior in an arena is examined to determine their *Umwelt*, or point of view. When in an arena singly, horses displayed home base behavior, spending their time near the entrance, and excursion behavior, trips into the arena. At home bases, horses paced against the wall, pushed against the gate, looked out, and rolled. On excursions, they displayed a "sniff, look, and loop" pattern; sniffing the ground on the outward leg, looking with ears forward down the arena at the apex, making a faster return with ears back. When free with a pair mate, the area of its excursions expanded and if a pair mate was tethered at the far end of the arena, a horse shifted its home base to that location. When ridden, horses displayed similar sniff, look, and loop behavior centered toward the entrance. Experiments on memory for the arena showed it was good but was reset each day. A model suggests that behavior is shaped by a spatial gradient, in which stress expands in proportion to distance from home, and an exploratory gradient, in which patrolling is a part of each day's outing. Science-based horsemanship can provide insight into a horse's view of its world and is relevant to safe horse handling.

**Keywords:** exploration of horse in arena, horse excursion, horse exploratory gradient, horse home base, horse spatial gradient

## 1. Introduction

The question, "what are they thinking?" in reference to horses has been addressed in many horse monographs and by many clinicians. Our intent is not to choose amongst suggested answers but to address the question by presenting a few of our own scientific studies. Science-based horsemanship can improve insight into horse behavior, contribute to the welfare of the horse, and improve safety for those interacting with a horse.

The statistics on injury related to handling or riding horses are consistent in every country in which they have been collected. The incidence of horse-related injury is due to the things that the horse does and to things that people do. The former includes things like "the horse spooked" or "the horse ran off" while the latter include things like broken or unsecured tack.

Horse-related injuries outnumber injuries obtained in other sports, including contact sports such as rugby, football, and hockey [1–3]. Injuries occur almost as frequently when a person is at home, on a farm, or at an equestrian center.

Injury is equally likely when a person is on the ground handling a horse as it is when they are riding a horse. Injury is more likely if a person is a beginner than if experienced. The average age of an injury is about 30 years of age, but injuries can happen to people of all ages, with injuries more severe in females than in males. The highest risk of injury is to young females, perhaps because so many are engaged in equestrian sports.

Although a good deal has been written on the incidence and type of injury related to handling and riding horses, less attention has been given to prevention [2]. There are ways to reduce the chance of having an accident, and in the case of an accident, to reduce severity. Inexperienced riders can take lessons from a coach who teaches safety and riders can wear helmets. Owners or buyers can ensure that a horse is well trained. But even with such precautions the statistics on horse-related injuries do not seem to change, except that head injuries are less severe if a helmet is worn.

A science-based approach to handling and training horses can improve safety with horses. Starling et al. [4] outline a 10-point approach in which understanding horse ethology is the first point. Horse ethology is the study of the natural behavior of horses. For example, ethological studies show that feral horses are herd animals. They spend up to 16 h a day feeding. They are on the move much of the day. They are flight animals and run when frightened or threatened. But a central question is, how does horse ethology translate into a relationship with humans in typical equestrian interactions?

The purpose of the present chapter is to elaborate one aspect of horse ethology that has not received much attention, the horses' *Umwelt*, its point of view. Umwelt is the biological term used to describe the world view of an animal [5]. We certainly do not know the full dimension of the horse's world view [6, 7]. The following sections present some experiments that describe behavior in an equestrian arena that provide insight into a horse's umwelt in conditions in which it is being handled or ridden. These experiments will present ideas about how humans can shape their own umwelt to that of the horse and so develop habits to improve horse handling.

## 2. Horse behavior in an arena

We took 18 horses, varying in age and sex, individually into a riding arena, released them, and filmed them for 30 min [8]. This is a test that has been given to other animals in laboratory studies and it reveals how they adapt their behavior to an environment that is different from their home. We found that the horses spent most of that 30 min at the end of the area near the door through which they had entered the arena. **Figure 1** shows a sketch of the movement of one horse during the 30-min test. The horse did periodically go out into the arena, each of its excursions initially got a little longer than the first one, but soon the number of excursions decreased as did the size of the excursions until finally the horse remained near the door. This representative horse did not make it past the midpoint of the arena on any excursion.

We did this experiment with our 18 horses and we have also informally watched many other horses in similar situations. The behavior of the horses was similar whether they were geldings or mares, whether they had frequently been ridden in the arena, or had only occasionally been ridden in the arena. Their behavior was similar when the arena was completely new to them, having been hauled to the arena from another farm. The behavior was similar for horses stabled inside, stabled right beside the arena, or at some distance away. Some of the horses were Thoroughbreds, some were American Quarter Horses, and some were mixed

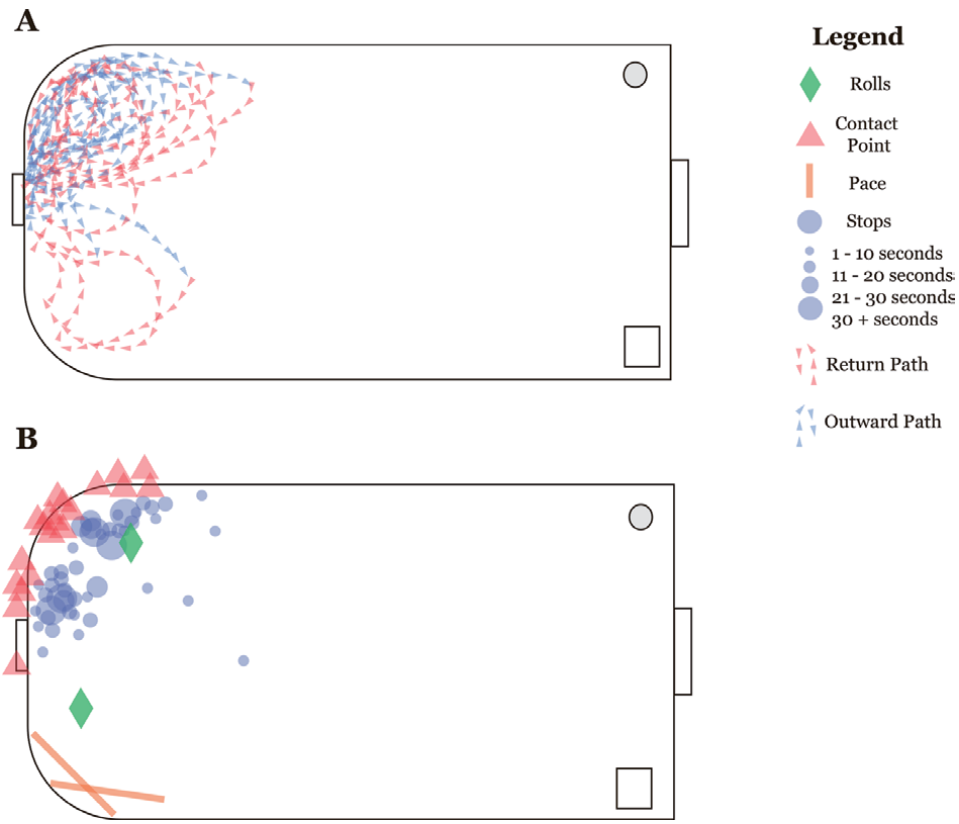


**Figure 1.**
*The organization of the exploratory behavior of a single horse released into an arena for 30 min. (A) The horse spent most of its time near the entrance gate, its home base. Periodic excursions consist of an outward leg (blue) and a homeward leg (red) but none of the excursions went past the center of the arena. (B) Activities centered on the home base consisted of stops, pacing, pushing against the gate, and rolling.*

breeds. They all behaved in much the same way. On many occasions we have observed handlers turn their horse out in the arena to exercise only to find them loitering by the gate. To encourage them to exercise, the handler might then chase them away from the gate.

The location at which an animal hangs out when placed in a novel environment is called a home base. Many different species of animal have been found to choses one or more locations—home bases—in the test area in which they spend most of their time. The behavior was first described in laboratory rats [9, 10]. Rats placed in a new environment initially remain in an area close to the entrance point and make excursions from there only to return again. If they find a more secure location, as defined by a corner or a part of the arena where a dark object is located, they move their home base to that location. Rats like areas beside walls and they like dark places. The home base for the horse in our study was the entrance point. The horses appear to otherwise avoid walls and avoid dark locations. People display home base behavior as well. Scientists have observed the behavior of small children who were taken to a novel room with their mother [11]. The children made excursions away from the mother but always returned to her. The mother's location defines the child's home base.

Behavior in a home base is characteristic. This is where a horse paces back and forth against the wall, looks out over the gate in a direction away from the arena, leans against the gate, and rolls (see **Figure 1**). Home base behavior for the horse is organized and it is different from behavior that takes a horse away from a home base.

## 3. Sniff, look, and loop

When horses leave a home base, their away behavior is also organized. Each trip forms a loop, in which a horse ventures away from the starting point and then returns to it. The outward trip is generally slow and sometimes features stops. The homeward trip is faster with stops less likely. If the loop takes a horse well into the arena, it may trot or even gallop back. On an outward trip a horse will often lower its head and sniff the ground. When reaching the apex of an excursion, it may stop and look toward the far end of the arena with head erect and ears pointing forward. It may then put one ear back, indicating the direction in which it will turn, drop its head, and with ears in a relatively neutral position or back, make the homeward trip. To highlight major features of this organization we call the behavior "sniff, look, and loop" and it is illustrated in **Figure 2**. Note that the horse in **Figure 2** has its tail up at the sniff and look points, suggesting waryness.

Sniff, look, and loop describe the organized ways that a horse investigates the area surrounding the home base. Sniffing the arena likely helps it to determine what other horses may have been there. A horse has one of the largest eyes of all animals and excellent vision and so it need not go to the far end of the arena to visually investigate it [12]. Its ears forward posture allows it to investigate sounds both inside and outside the arena. Its homeward trip is quicker because its investigatory

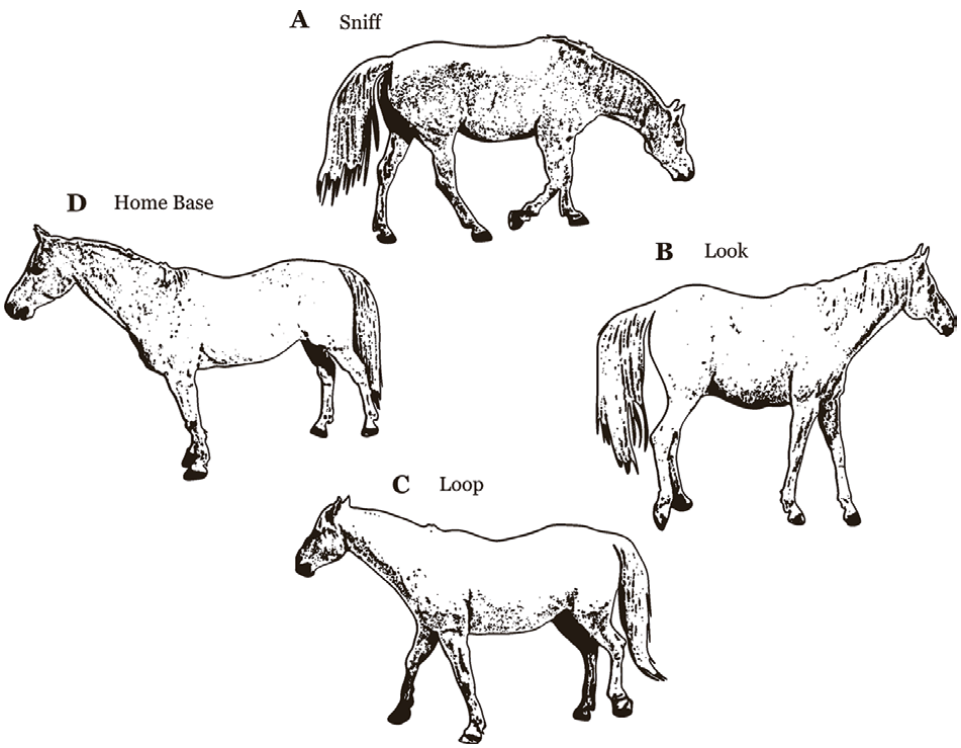


**Figure 2.**
*Stop, look, and loop. Activities that occurred on a single excursion. (A) Sniff, the horse's head is lowered as it sniffs the ground on the outward leg of an excursion. (B) Look, a horse looks and sometimes stops and looks, with head erect and ears forward toward the far end of the arena. (C) Loop, the horse turns, often signaling the direction of turn with the retraction of the ipsilateral ear, and returns to its starting point, usually with ears back and head somewhat lowered. (D) Home base, the horse stands and looks outward. Note: tail up postures at sniff and look.*

excursion is over and it can hurry back to the home base. According to the principles of optimal foraging theory, when business is done on an outward trip, it is safest not to tarry on the homeward trip [13]. Having its ears neutral or back on the homeward trip seems to suggest that a horse may be attending to what might be behind it, perhaps the unexplored arena, with which it is not comfortable. It may also be relaxing as it returns to its home base.

## 4. The home base as a surrogate for the herd

One explanation of home base behavior is that a horse stays near the gate because that is where it entered the arena. That location might be perceived as the shortest way back to its home paddock and its herd, which is its actual home. In short, it wants to "be at home with its buddies," as every horse person can attest. We examined this idea by giving four horses the same 30 min test as described above and observed that these horses set up their home base near the gate. We then brought a pair mate into the arena and tied it at the far end of the arena for another 30 min test. The horse that was free to move immediately moved to the far end of the arena, the area of the arena that it previously avoided, and spent the half hour near the pair mate (**Figure 3**).

This experiment suggests that what motivates the horse to remain near the gate end of the arena is that this is a place that is closest to its herd. For horses that were stalled individually in the arena, the herd explanation may still apply because they can see neighboring horses and so treat them as the herd.

The home paddock may also be attractive to a horse, however, because that is where it ordinarily finds safety and where it is fed. We tested this idea by turning out pair mates at liberty in the arena. When free the horses still displayed home base behavior and spent most of their time near the door where they also rolled. Rather than pacing, however, they spent time investigating objects near the door. Their loop excursions were much larger and frequently much faster. **Figure 4** illustrates the movements of one horse when it was in the arena alone and the movements of the same horse when it was in the arena with a pair mate. These experiments show that one reason a horse may form a home base near the door is that it wants to return to its pair mate but another reason is that it wants to return to its home territory. In its natural ecology, the two coincide.

**Figure 3.**
*Movement in two 30-min tests. When a horse was alone, it spent its time near the gate (red paths). When a familiar horse was tethered at the far end of the arena, the free horse moved to the far end of the arena (blue paths).*

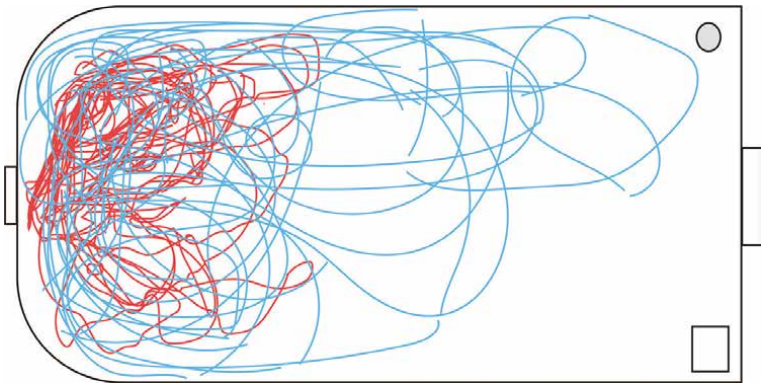

**Figure 4.**
*The movements of a horse when in the arena alone (red paths) and when with a pair mate (blue paths). The area of movement of the horse expanded with the pair mate present but movement was centered on the "home base" door area.*

## 5. Behavior under saddle

We asked what a horse's spontaneous behavior would be like if it were ridden but otherwise left alone. We used reining horses for the experiment because they are well schooled. All were familiar with the arena because it was their home arena and they had been frequently ridden there. We asked whether a horse would display elements of sniff, look, and loop behavior when ridden? We had riders do our 30-min test. We asked them to encourage the horse to leave the area entrance but once the horse began to do so, put down the reins and let the horse proceed as it wished. If the horse returned to the entrance, then after a pause, again ask the horse to leave. All of the horses made an excursion into the area when asked to do so and then they spontaneously stopped and looked down the arena, turned and returned to the starting point more quickly. For one of the horses the outward leg of the excursion was quite long and this was the only horse that went past the midpoint of the area. For all of the horses, successive excursions initially got a little longer and then progressively got shorter (**Figure 5**). The horses also came back more directly and more quickly than they went out. One horse first trotted back but on successive trips its speed increased until on one excursion, it galloped back. Two of the horses also sniffed the ground on the way out and all were more likely to have their ears up on the outward leg of a loop and their ears back on the homeward leg of a loop. These results suggest that the exploratory behavior of a horse under saddle reflects its behavior when it is at liberty.

We investigated whether stop, look, and loop behavior would influence a more typical riding session. We had riders enter the area singly and trot their horse around the edge of the arena, with the horse on a loose rein, so that the horse was free to choose its speed. At the same time, we timed the away and back legs of each of 10 trips. All of the horses spontaneously slowed their trotting speed as they left the gate end of the arena and they spontaneously increased their trotting speed as they left the far end of the arena on their trip back toward the gate. Consequently, the times taken to return were statistically shorter than the times to venture out. In addition, as a horse left the near end of the arena, it most often had its ears forward and looked toward the far end of the arena to which it was going. On the homeward leg of the trip, it noticeably lowered its head and frequently had its ears back (**Figure 6**). Thus, even though the horses were willingly circling the area under the guidance of a rider, they were noticeably engaging in behavior that they displayed when making

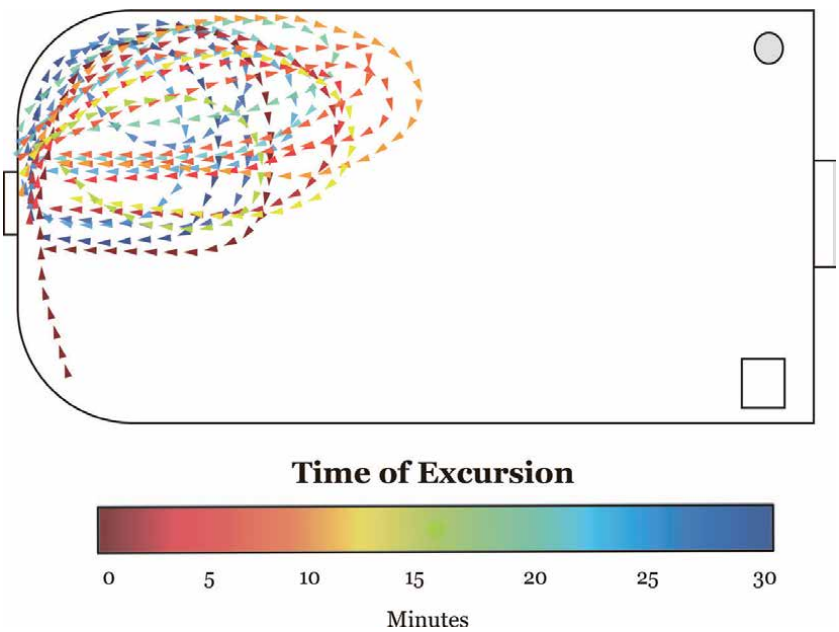


**Figure 5.**
*Loops made by a horse under saddle. The horse was asked to walk into the arena and then released from control. Note: the horse made repeated loops near the gate area of the arena. The colored bar indicates time.*

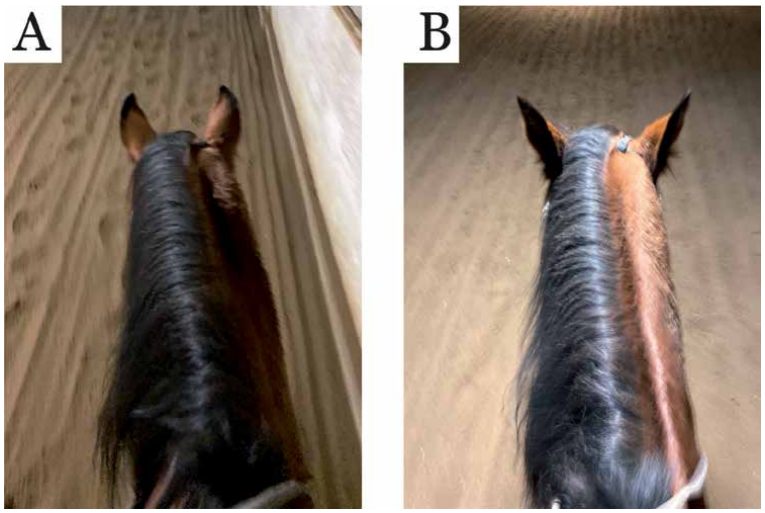


**Figure 6.**
*Ear position on outward and inward directions when circling an arena under saddle. (A) On the outward leg of the circle the horse frequently directs its ears forward. (B) On the homeward leg of a circle the horse frequently directs its ears backward. Ear position may signal caution on the outward leg and relaxation on the homeward leg.*

sniff, look, and loop trips when on their own—their going out was slow and their coming back faster and their ear position reflected their relative concern with the two ends of the arena.

The observation that ear position is a marker of the inward and outward loops of spontaneous excursions and excursions under saddle suggests that ear position could be a marker of behavior in the show pen. We used videos from the nonpro National Reining Horse Association reigning futurity held in Oklahoma

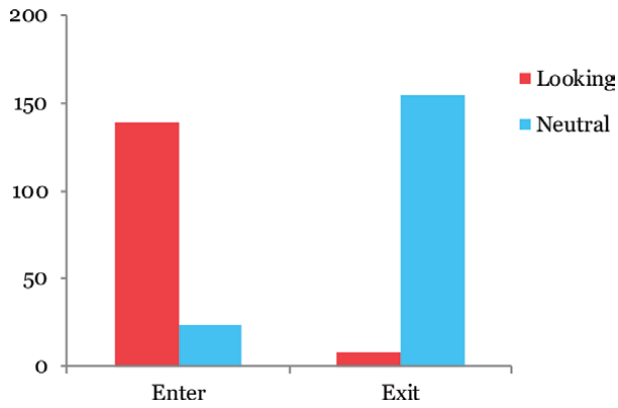


**Figure 7.**
*Number of horses displaying either mainly ears forward or neutral position when walking into an arena or walking out of the arena as a part of reining Pattern 6. Results obtained from the non-pro National Reining Horse Association futurity in Oklahoma City in 2015.*

City in 2015. The horses were performing Pattern 6, a pattern in which they walk to the center of the arena to begin the pattern and walk much the same path to the entrance gate after making their last stop. We rated ear position on the inward and outward walks. As is illustrated in **Figure** 7, inward walks were overwhelmingly associated with periods of ears forward position whereas outward walks were associated with a relatively neutral or ear back position. It is noteworthy that many riders try to minimize "look" behavior on the outward walk by collecting their horse. These results suggest that just as horses treat the outward portion of a spontaneous loop as stressful, even when well-trained they display the same behavior when performing in an arena.

## 6. Memory

The similarity of home-base behavior of horses that were familiar with the arena and those who were taken to the arena for the first time might suggest that horses have a poor memory of the arena. Horses that are familiar with the arena seemed to behave as if they are being introduced to it for the first time, as judged by a comparison of their behavior to the behavior of horses that were new to the arena. Many studies have noted that horses have good memory [14–19], but our question related to the memory for an arena they had previously visited. We tested arena memory with five horses that had been ridden in the arena a number of times each week for many weeks. The arena baseboard was painted white but was covered with dust and scuff marks from being hit by the tires of the tractor that was used to groom the arena. We placed a novel object on the arena wall, a three-inch wide two-foot long strip of cloth. If the horses were treating the arena as a completely new place, they should not notice the cue because it would look to them like other marks on the wall. If they had a memory for the arena, they might notice the cue. The riders were unaware of our experiment. We took any especially attentive or avoidance behavior of the horses toward the cue as a sign that they recognized that the cue was there.

All of the horses immediately noticed the cue when the riders first circled the arena past the cue, and two of the horses shied noticeably, surprising the riders who did not seem to have noticed the cue themselves. The results of this experiment suggest to us that the horse have an excellent memory for the arena—excellent in the sense that they recognize something new against a background that is familiar to them.

Accordingly, their home-base behavior and seeming avoidance of the far end of the arena on the exploratory tests cannot be explained in relation to poor memory for the arena. They were not avoiding the far end of the area because they had no memory of ever being there.

In the course of studying why horses might sniff the ground during a warm-up for riding, we observed that the horses would notice objects on the ground, go toward them and sniff them. The objects could be as small as a cigarette butt or a blade of hay, a sunbeam from a window, or the droppings left by a previous horse. We collected observations of sniffing and checking behavior as a way of assessing visual attention and memory. We found that horses would notice a small object as far as 10 feet away and a large object, such as the dropping from another horse, from as far as 30 feet away. When given the opportunity, the horses were very attentive to the ground and their inspection of the arena did not just consist of looking at objects in the distance but also consisted of inspecting the ground on which they were walking and approaching objects that they saw there.

In the course of studying this sniffing behavior we observed that a horse very seldom returned to an object once it had sniffed it. That they did not return to objects indicated that they remembered them. To further examine this form of object memory, we purposefully manipulated the delay between the first approach to sniff of an object and subsequent responses to the same object. We had a rider allow a horse to approach and sniff an object and then return along the same path to see whether the horse would again approach the object. We varied the return time by minutes, as measured by a complete circle around the arena at a walk, to a half an hour, as timed with a watch. We found that the interval did not matter, of 297 instances of return visit opportunities, only 9 were associated with a second visit to an object (results collected from four horses). We also did tests of having the horses approach the object from a different direction. Again, of 75 instances of returns, only four were associated with the second inspection of an object. The second visits were all associated with visits to droppings.

Accordingly, we made droppings a focus of examination. We allowed a horse to walk directly toward a dropping and sniff it and we timed the duration of the sniff. We then varied the time of our next visit on which we allowed the horse to walk directly toward the dropping. Of 150 such samples, on 137 occasions the horses did not sniff the dropping on the second trip but passed by. On the few occasions on which they sniffed on a return visit, the duration of sniffing was shorter than on the previous visit. There was no effect of the intertrial interval, as horses mainly ignored a target that they had recently sniffed as much as they ignored a target that they had sniffed a half hour previously.

We placed two plates containing droppings approximately 30 ft. away from each other and had a rider walk a horse toward the center of the space between the objects (**Figure 8**). Even at quite a long distance away, the horses veered toward one of the objects to sniff it. Then within a few minutes to as long as 30 min later, the test was repeated. Each horse then got a third trial, with the expectation that once they had examined both objects, they might ignore them on the third trial. The horses were given one test each day—with test at the short interval and the test at the 30-min interval alternated each day. For the tests, the objects were at different locations in the area each day. Thus, over 20 days the horse had 10 tests at the short interval and 10 tests at the long interval. The results are shown in **Figure 9**. One horse got 10/10 (they alternated on each of 10 trials) at both the short and the long interval and the other horse got 9/10 at the short interval and 9/10 at the long interval. On their third trial, both horses ignored both objects on 10/10 trials, so indicating that they remember that they had explored them. This experiment indicated that horses have an excellent short-term memory of objects that they get to sniff.

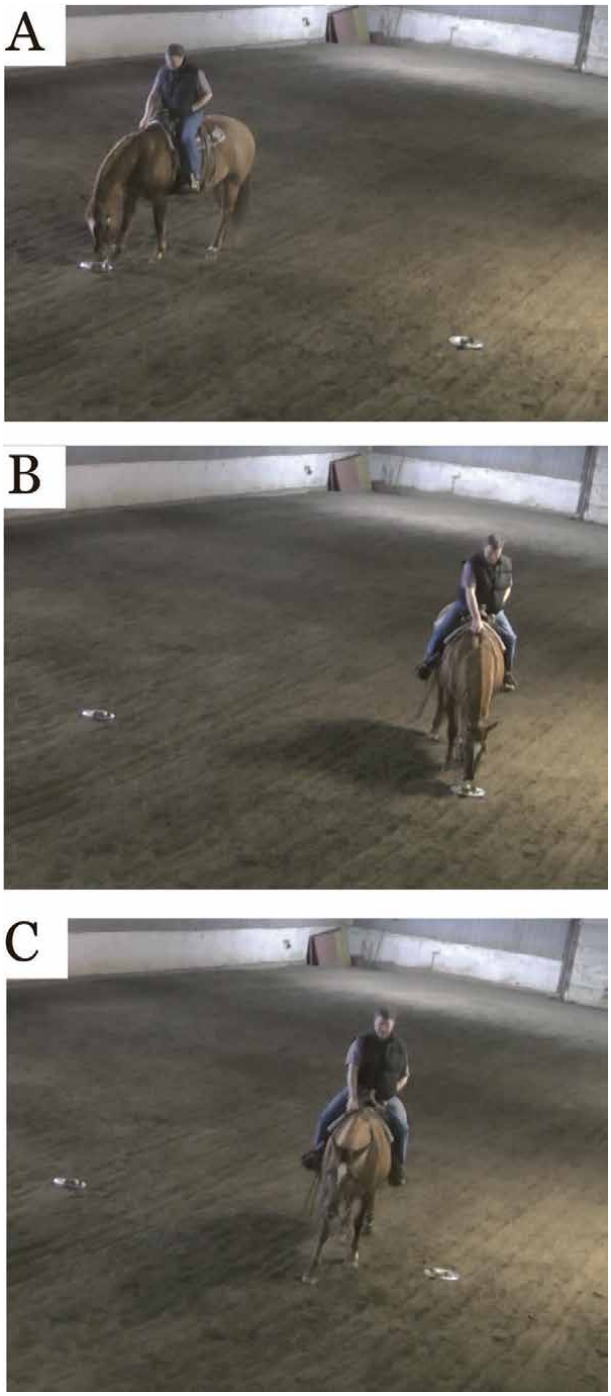


**Figure 8.**
*Two choice memory test. (A) A horse ridden to the center point between two plates containing droppings, approaches the right plate and sniffs the target. (B) About 5 min later, the horse is given a second choice and chooses the left target. (C) About 5 min later, the horse is given a third choice and passes both targets without investigating either.*

Our memory experiment shows that the horses always treated objects as novel on each day's encounter. We also tested horses in an outdoor arena, where droppings and other objects tended to be left because the arena received infrequent grooming. There, we found that the horses explored as many as six objects and remember them

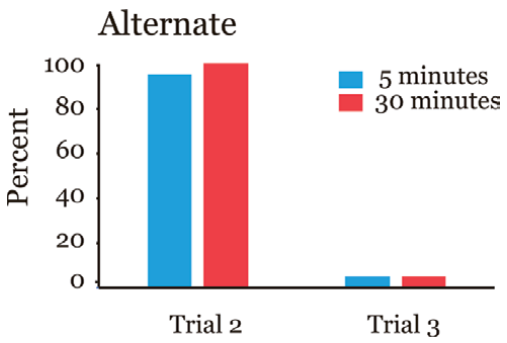


**Figure 9.**
*Two choice test results (percent choices). On the second choice given either 5 or 30 min after the first choice there is a high probability that the horses choose the target not chosen on the first trial. On the third choice, there is a high probability that the horses choose neither target. Both results show that a horse remembers targets that it has investigated.*

on a same day test. There too, when they were returned on the following day, they behaved toward the object as if they had never previously seen them. This experiment suggests that when removed from the arena for a day, a horse resets its memory and treats the objects in the arena as new.

## 7. Checking and shying

The experiments on memory show that horses are motivated to investigate/check small objects on the ground and they then remember those objects during the period of time that they are in the arena. It is well known that horses shy at novel objects and we reasoned that if we increased the size of the objects, their behavior should transition from investigatory to avoidance behavior. We made round cut outs of cardboard of various sizes and measured approach and avoidance behavior as we rode the horses around the arena. We found that as the size of the object increased, the probability of avoidance behavior increased. This behavior is very similar to that described by Ewart [20] for toads, which approach to eat small objects that he presented to them and who avoided larger objects that he presented, treating them as predators. We also varied the location of the objects in the arena and found that objects were avoided with more vigor at the far end of the arena. Often, an intermediate size object that was avoided at the far end of the arena was investigated at the near end of the arena. Interestingly, the horses were still likely to shy at large objects when returned to the object a short time later. Since their memory for objects in an arena is good, repeated shying appeared unlikely due to poor memory.

## 8. Discussion

These experiments tell us two main things about what a horse is thinking when it is taken into an arena. First, the arena is a source of stress and it is likely that it is anxiety provoking. Second, a horse views the arena as a place that is novel and that requires inspection and when not novel a place that must be patrolled and checked. In responding to these two influences, horses display a *spatial gradient* and an *exploratory gradient*. These gradients, if attended to, allow a rider to read the mind of their horse and adjust their ride and their training. We will point out some of the ways that more expert horse handlers show that they are aware of a horse's opinion of an arena to which they are taken.

### 8.1 The spatial gradient

**Figure 10** illustrates our model of a horse's spatial view of the world in relation to its actual home, the location of its herd. The model is constructed in the shape of a loop with the base of the loop representing a horse's actual home, its paddock or stall. The blue color of the spectrum of colors in the loop indicates low stress and is associated with the actual home. The color spectrum becomes redder as distance from that home increases to signify an increase in a horse's stress in proportion to the distance from its home. The model is shaped as a loop not only to signify an actual loop but also to signify avoidance of walls or other large objects that will also provoke an increase in stress.

Research on horses in herds that have been together for some time show both that a herd is stable and within the herd social relationships are structured, with horses maintaining favorite relations [21]. Substantial information suggests that that removing an animal from its social group is stressful and remains stressful even after repeated removal [22]. The loop model when superimposed onto an arena explains why the horse chooses the gate area of the area as a home base, why it avoids the walls of the arena, why its movement pattern forms a loop and why it limits its excursion to the near end of the arena. The model also explains why its behavior remains much the same even after attempted adaptation to an arena. It will attempt to confine its activities to the blue regions that are less stressful because they are perceived as closest to its actual home.

On the basis of our model, we have experimented with the idea that when beginning a ride or when warming a horse up for a ride when the horse is alone, a rider mimics the horse's natural behavior. Accordingly, a ride begins with small loops each of which bring the horse back to the starting gate and then extend to include

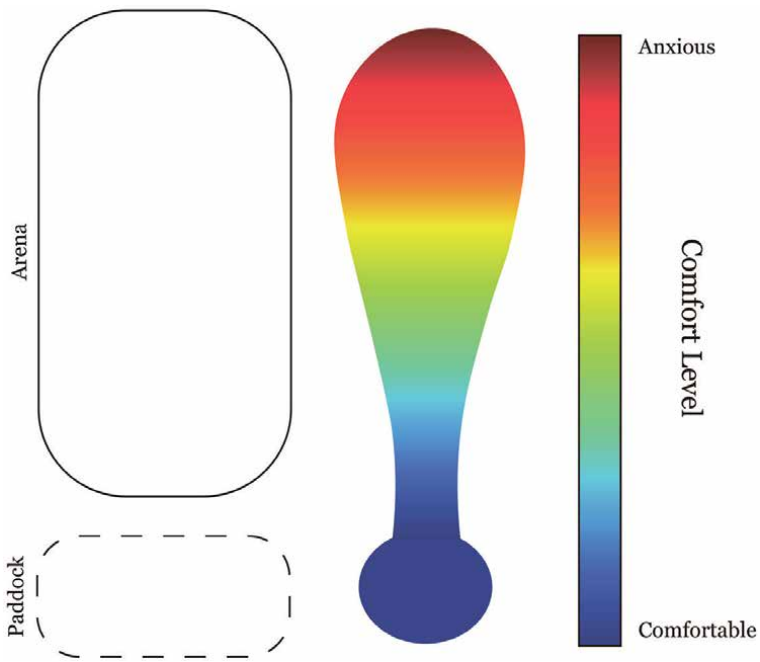


**Figure 10.**
*A model of the spatial gradient. The colored bubble represents a loop pattern of excursion color coded to represent comfort (blue) to anxiety (red). Maximal comfort is in the home paddock and maximum anxiety is at the apex of the loop. When superimposed on the arena the anxiety gradient indicates the entrance, nearest to the paddock, features lowest anxiety and the far end of the arena indicated maximum anxiety.*

more of the arena. On each outward leg of a loop, the horse's anxiety likely increases but then on the return leg to the starting point its anxiety decreases. Over a training session and over days of training, a horse learns that outward excursions will always end with homeward excursions and in this way its behavior becomes managed. A rider might not force a horse down a wall but build up to approaching walls as the ride proceeds.

We have seen aspects of our suggestion in play when young horses are first started. Some trainers first halter-break and lead-break a horse when it is in its stall and adapt a horse to a saddle while it is in its stall. We have also observed one horse trainer making the first mount and taking the first ride with horses in their stall. The stall is home and the location of a horse's lowest level of anxiety. Some trainers, when taking a horse to an arena for training might begin the training from the back of another horse. The other horse is a surrogate for its herd and serves to reduce anxiety. A trainer might begin the first ride in arena by making small circles when first asking the horse to move forward under saddle. It is likely that experience has led to training strategies that are integrated into a horse's spatial gradient. The spatial gradient also suggests that any added stress to a horse, including first separation from pair mates or pressure to perform more correctly or quickly, will shift the color gradient in our model from blue to red. It is also likely that when stressed, a horse attributes the stress to the environment and not the handler and so resistance to walls, shying at objects, and moving through the far end of an arena increase in proportion to stress [23].

We have observed handling behaviors that are inconsistent with a horse's spatial gradient. A rider might force a horse to go to the far end of an arena even though it resists. A rider might begin a ride with a horse collected and unable examine the area visually or to examine the ground by sniffing. A handler might take a horse to the center of an arena and lunge it there. Lunging will likely not substitute for arena inspection and object checking. These handling methods might maximize anxiety and result in horse/handler conflict. It is likely that rides taken outside an arena are also subject to the spatial gradient, the further a horse is taken from its paddock, the greater the stress. Many riders taking a horse out alone have experienced the anxiety gradient in a number of ways. If a horse is going to "act up" it is likely this will happen on the outward leg of a trip. A rider might also notice that a horse returns more quickly than it embarks on a ride.

### 8.2 The exploratory gradient

The second point raised by our experiments is that each day that a horse is taken to an arena it treats the arena as new. This is not because it does not remember being in the arena or because it does not remember objects in the arena. Rather, it is likely that it wants to ascertain that the arena is safe. Many animal species that maintain home territories patrol and check their territories regularly [24]. It is likely that they want to be certain that the representation that they have of their environment matches the environment. Therefore, to ensure that the arena is safe, a horse needs to sniff the ground and objects in the arena as well as look at them. One clinician explained a horse's display of anxiety as, "there might be a bear there." What could be more threating, however, is the presence of an unknown horse. The many new smells on the ground of the arena, the dropping of another horse left in the arena, are a sign that other horses have been there. Ecological studies of many animal species suggest that daily conspecific aggression is much more likely than is predatory aggression [25]. Previous studies of olfactory memory in horses show that a horse's memory of others is particularly good for horses that have been aggressive toward them [26–29].

## 9. Conclusion

With this description of our experiments we suggest that a handler can appreciate a horse's thoughts with respect to an arena into which it is taken. In the area, the further a horse goes from the entrance, the greater the stress and the more it will want to leave. Most riders will confirm that even a well-trained horse will need to be encouraged to go into an area and during a ride and will speed up when moving back in the direction of the starting point. Many horses will appear afraid of the far end of the arena and so it will be difficult to get them to go there. Once there, they will not perform as well as they do in the close end of the arena. Many horses will also avoid the wall of the arena and beginning riders may have difficulty getting their horse to stay near the wall when circling an arena. These behaviors are reflected in our model of the horse's spatial gradient. In adapting a horse to an arena, a rider might find that if given the chance, a horse will explore using vision, olfaction, and touch and it will do so each day that it comes to the arena. It has to check or patrol. Allowing a horse to explore might reduce its anxiety by making an arena more like the home paddock. In short, being aware of what a horse is thinking when it is taken out of its paddock to work will improve a horse handling experience as well as improve the chances that the handling experience is accident free. We view the present contribution to scientific based horsemanship as preliminary [30]. There are many aspects of horsemanship that can be further investigated with the arena/home base model, including sex differences, which have only been touched upon here, and genetic [31], developmental [32], and brain influences.

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