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GASTROINTESTINAL PARASITES IN PRZEWALSKI´S HORSES (*Equus ferus przewalskii)*, IN PENTEZUG WILD HORSE RESERVE, HORTOBÁGY NATIONAL PARK, HUNGARY

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Dissertação de Mestrado Integrado em Medicina Veterinária

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“We have the choice to use the gift of our life to make the world a better place

*--or not to both*er”

Dr Jane Goodall

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Resumo: Parasitas gastrointestinais em cavalos de Przewalski (*Equus ferus przewalskii)*, na reserva de cavalo selvagem do Pentezug, Parque Nacional de Hortobágy, Hungria

Equus ferus przewaslkii é classificada como uma subespécie de *E. ferus*. Presentemente, encontra-se em perigo, segundo a União Internacional para a Conservação da Natureza (IUCN), apesar de já ter assumido o estatuto de Extinta na Natureza. Por este motivo, a conservação *ex situ* tem tido um papel crucial na conservação desta subespécie. A reserva de cavalo selvagem do Pentezug, inserida no parque nacional de Hortobágy, na Hungria, tem uma das maiores populações *ex situ* de cavalo de Przewalski, com os principais propósitos de conservar a paisagem característica da região e aprofundar os conhecimentos sobre a subespécie, fazendo parte do seu programa de reintrodução. Atualmente, esta população é composta por cerca de 280 cavalos de Przewalski, que partilham a pastagem com uma população de bovinos domésticos (*Bos primigenius taurus*), selecionada de forma a apresentar um fenótipo semelhante ao auroque (*Bos primigenius*). Foram colhidas 79 amostras fecais e os métodos coprológicos (McMaster, flutuação de Willis, sedimentação natural, Baermann e coprocultura) e a respetiva identificação microscópica foram executados para todas as amostras. Os resultados demonstraram um nível de parasitismo médio de 1286,7 ovos por grama (OPG), o que representa um valor elevado. Todas as amostras foram positivas para ovos do tipo estrongilídeo (100% de prevalência) (79/79), com uma dominância dos ciatosmíneos, comparando com estrongílideos e tricostrongilídeos, e revelaram uma diversidade de 15 espécies ou morfotipos de L3 dentro da ordem Strongylida. Adicionalmente, 27,8% (22/79) foram positivas para *Parascaris* sp., 2,5% (2/79) dos animais continham *Oxyuris equi* nas fezes expelidas e numa amostra foi detetado um ovo de trematode (1/79). As L3 de ciatostomíneos de tipo A revelaram uma prevalência de 100%. Na subfamília Strongilinae, *Strongylus vulgaris* foi o mais prevalente (40.5%), seguido de *Triodontophorus serratus* (12,7%). Estes resultados são consistentes com outros estudos efetuados na espécie e representam o 1º estudo parasitológico na população de Pentezug, realizado com este nível de detalhe. Estatisticamente, este estudo revelou que animais positivos a *Parascaris* spp. tendem a ser positivos para ciatostomíneos do tipo C. O mesmo acontece entre *T. serratus* e *Poteriostomum* spp., possivelmente devido à predisposição dos juvenis para estes dois parasitas revelada neste estudo. Do mesmo modo, os machos têm mais infeções por *S. vulgaris* do que as fêmeas e os animais positivos para este parasita tendem a ter níveis mais baixos de OPG. Estes resultados demonstram a importância da monitorização parasitológica em populações *ex situ*, especialmente as que fazem parte de um programa de reintrodução, com o objetivo de aprofundar o conhecimento sobre o poder patogénico dos agentes, possíveis coinfeções, fatores de risco e consequências para a conservação da subespécie.

Palavras-chave: cavalos de Przewalski, parasitas gastrointestinais, *Cyathostomum* spp., *Strongylus vulgaris,* Pentezug, Hungria

Abstract: Gastrointestinal parasites in Przewaslki's horses (*Equus ferus przewalskii*), in Pentezug Wild horse reserve, Hortobagy National Park, Hungary

Equus ferus przewalskii, currently assumed as subspecies of *E. ferus,* is considered as endangered by the International Union for Conservation of Nature (IUCN). The *ex situ* conservation has been crucial for the continued preservation of this subspecies, once considered extinct in the wild. The Pentezug Wildhorse Reserve, located in the Hortobágy National Park, in Hungary, has one of the biggest *ex situ* populations of Przewalski's horses and it's aimed to preserve its typical landscape and to study wild horses in a semi-wild habitat, making part of this subspecies reintroduction plan. Currently, this population comprises almost 280 Przewalski's horses, sharing the area with a population of domestic cattle (*Bos primigenius taurus*), carefully bred to reconstructed aurochs (*Bos primigenius*). In this study, 79 faecal samples were collected and the coprological methods (McMaster, Willis floatation, natural sedimentation, Baermann and coproculture) and corresponding microscopic identification were performed in all the samples. Results show an average level of 1286.7 Eggs per Gram (EPG), which is considered a high level of parasitism. All the 79 samples analysed were positive for strongylid-type eggs (100% prevalence) (79/79), with a dominance of the cyasthostominae, when compared to strongylinae and tricostrongylidae. Moreover, a total of 15 different morphological L3 types and/or species identified of the order Strongylida. Additionally, 27.8% (22/79) were positive to *Parascaris* sp. and 2.5% (2/79) contained *Oxyuris equi* in their expelled faeces. By the sedimentation method, we could only evidence a Trematoda egg (1/79). In the subfamily Cyathostominae, L3 of cyathostomins type A showed 100% prevalence. In Strongilinae, *Strongylus vulgaris* is the most prevalent (40.5%), followed by *Triodontophorus serratus* (12.7%). These results are consistent with the other studies performed in the same subspecies and represent the first survey of gastrointestinal parasites performed with this level of detail in this population of Przewalski's horses. Statistically, this study revealed that animals infected by *Parascaris* spp. tend to be positive in association with cyathostomins type C. In the same way, animals infected by *T.serratus* tend to be positive for *Poteriostomum* spp., possibly due to the propensity of juveniles for these two parasites revealed in this study. Furthermore, males have more *S. vulgaris* infections than females and animals infected by *S. vulgaris* tend to have lower levels of EPG. These results reveal the importance of parasite monitoring in wild *ex situ* populations, especially those that can be part of a reintroduction program, to better-knowing their pathogenic potential, possible parasite associations, predisposition factors and consequences for the subspecies conservation.

Key-words: Przewalski's horses, gastrointestinal parasites, *Cyathostomum* spp., *Strongylus vulgaris,* Pentezug, Hungary

Introductory note

Part of this work was accepted as Full text to a poster communication on the IZW/EAZWV/ECZM conference, on 12th to 15th June 2019, in Kolmarden, Sweden, with the title “GASTROINTESTINAL PARASITES IN PRZEWALSKI’S HORSES (*Equus ferus przewalskii*) AT HORTOBÁGY NATIONAL PARK, HUNGARY – PRELIMINARY RESULTS”. A copy of the Full text for the Proceedings Booklet is presented at the end of this document (Appendix H).

As a complement to the present study, 11 blood samples were collected from euthanised or anaesthetized individuals to analyse the presence of blood parasites. The samples were sent to Vienna University, to Doctor Hans-Peter Führer, who is currently responsible for their molecular analysis. The publishing of the global results is planned.

In parallel to this study, the author is collaborating with Pentezug technical team to implement a protocol of continuous parasite monitoring program for the population, with the consultancy of Prof. Doctor Luís Madeira de Carvalho.

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Table of abbreviations and symbols

IUCN – International Union for Conservation of the Nature

HNP – Hortobágy National Park

Pentezug - Pentezug Wild Horse Reserve

GI – Gastrointestinal

CEZ – Chernobyl Exclusion Zone

GG SPA – Great Gobi B Strictly Protected Area

EPG – eggs per gram (eggs/g)

FEC – Fecal egg counts

µm – micrometer

cm – centimeter

mm – milimeter

ha – hectare

spp. – species (plural; diverse species inside the genus)

sp. – species (singular)

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1 – Activities developed during the curricular traineeship

The 6th-year curricular traineeship was accomplished at the Budapest Zoo and Botanical Garden, in Budapest, Hungary. The trainee period started on 21st September 2018 and finished on 18th January 2019, with a total duration of 600 hours of practical training.

Doctor Endre Sós, the clinical director of Budapest Zoo, was the traineeship supervisor and Prof. Doctor Luís Madeira de Carvalho, from Faculty of Veterinary Medicine, University of Lisbon (FMV-ULisboa), was the co-supervisor.

Budapest Zoo is one of the oldest zoos in Europe, with 152 years old, with more than 1.000 animal species. Besides the animal collection, Budapest Zoo has a wildlife rescue centre, where 1.500-2.000 different animals from local fauna are treated and have a possibility of reintroduction. Furthermore, the zoo has important partnerships with several institutions as the Hortobágy National Park (HNP) or the Budakeszi Wildlife Park. In this context, the veterinary team is often called to intervene with very diverse species, that belong or not to the zoo collection.

Due to the described partnerships, the trainee was able to participate actively in very different clinical cases and to be involved in three critical parts of the wildlife medicine: the zoological medicine (mainly); the recovery medicine, in the rescue centre; and the field medical management, in the HNP, where this project was developed.

Concretely, these are some of the medical procedures most developed during the trainee period:

- Handling and restraining mammals, birds and reptiles.
- Performing and interpreting diagnostic imaging procedures, with or without handling, of several body parts.
- Performing necropsies of wild or zoo animals.
- Discussing the clinical cases, diagnoses, therapies and medical decisions.
- Managing and administrating distinct drugs.
- Inducing and monitoring fixed or volatile anaesthesia, inside the surgery room or in a zoo enclosure.
- Cleaning different wounds, doing bandages, performing laser therapy in various skin lesions.
- Performing medical training sessions.
- Collecting blood from mammals and raptors.
- Performing coprology diagnoses in different animals.
- Helping in different surgeries and complex procedures as ray sedation and surgery, rhino artificial insemination or an elephant standing sedation.

As mentioned above, the partnership between the Budapest Zoo and the HNP gave the trainee the possibility of developing this project with one of the most typical species of the national park. Due to this partnership, she had access to an important semi-wild population of Przewalski’s horses, and it was possible to perform the analysis in proper laboratory installations.

During her four trips to the HNP, she was able to perform the sample collection of her project, as mentioned and, additionally, she had the opportunity to help the Pentezug team on the implementation of a monitoring program for parasite infections in Przewalski’s horses. Moreover, knowledge was shared with other master and PhD students who were also developing their projects at the HNP, for instance, a monitoring study on Przewalski's horse body condition and a behaviour study on the social bonds between harem members, using video drones and also usual standing observations. Furthermore, we were able to participate in a skin biopsy, performed with an empty anaesthetic dart, to complement the genetic database project created with the University of Davis for the genetical characterisation of the individuals.

In summary, in 600 practical hours, the trainee had the opportunity to develop a fieldwork conservation project, while she learned about clinical and rehabilitation medicine of wildlife and zoological animals. In both parts, the trainee had a very supportive, and educational, leadership, which actively contributed to her learning process.

2 – Introduction

The *ex situ* conservation is a complex concept that covers many different environments and strategies of population management. Essentially, it's defined as maintenance of individuals under different pressures than those found *in situ*, in other words, in their natural habitat, for conservation purposes. *Ex situ* always assumes a more or less controlled or modified environment, within or outside the species' geographic range, which includes from highly artificial environments, as genome resource banks, to reserves, where the individuals live under near-natural conditions (International Union for Conservation of the Nature Species Survival Commission [IUCN SSC], 2014), like the ones of the studied population.

The *ex situ* conservation creates a possibility of avoiding or, in some cases, reverting an extinction in the wild status. In this status, the *in situ* strategies were not enough for a diversity of reasons, and there are no genuinely wild individuals to preserve in the natural habitat. In this case, the *ex situ* populations represent a possibility for reintroduction and reestablishment of a stable wild population. The examples of previously extinct in the wild species, currently presented in their natural habitats, are the Przewalski's horse (*Equus ferus przewalski*) (King, Boyd, Zimmermann, & Kendall, 2017) and the Scimitar-horned oryx (*Oryx dammah*) (Gordon & Gill, 1993). With this purpose, in some *ex situ* populations, the reproduction and genetics are controlled, using artificial insemination or frequently exchanging animals with other collections, with the aim of renewing and improving the genetic composition of the population to have the best individuals for planned reintroductions.

Furthermore, the *ex situ* populations can be valuable opportunities for studying the species, since they often provide easier access to the animal and, consequently, perform good observations or sample collections. A well-developed *ex situ* research can give important information to understand the population floatations and improve *in situ* strategies, regarding, for example, potential infectious diseases. Moreover, they can represent an opportunity of education of the citizens for the species threats and good human habits (IUCN & SSC, 2014).

This study is aimed to perform a survey of the gastrointestinal parasitology of the Przewalski's horses from the Pentezug Wild Horse Reserve, at the HNP, in Hungary, through the collection of faecal samples, with the main purpose of contributing to a better understanding of the health status of an *ex situ* population that often participates in reintroduction projects. Therefore, this work is also expected to provide a base knowledge to the professionals responsible for the Pentezug population, and enables the design and implementation of continuous monitoring and control strategies in a brief future.

2.1 – Hortobágy National Park (HNP)

The Hortobágy National Park (HNP) is the biggest and first proclaimed national park from a total of four in Hungary, with 82.185 ha. It is recognised by a flat plain landscape, with the biggest European continuous grassland area and wetland mosaics, also known as “Puszta”, which means “uncultivated land” in Hungarian (Gyarmathy & Kolláth, 2017), and proclaimed as a World Heritage Cultural Landscape since 1999 (Aplin, 2007). Located in the northern area of the Great Hungarian Plain, closer to the Carpathian Mountains and crossing the Tisza river (Fig.1), the Hortobágy region is considered unique in Europe for being the westernmost protrusion of the Asian steppes. This landscape starts there and extends to Mongolia and Manchuria, which has a clear reflex on the fauna, flora and ecology that can be found in the national park (Hilbers, 2008).

Figure 1 - HNP geographical location (Hilbers, 2008)

The river Tisza and its effluents create densely vegetated forest areas with a lot of similarities with other parts of southern Europe. However, on the other hand, the different kinds of “Puszta” suggest a central-Asian landscape, with its grass swards and temporary marshlands (Fig.2). Besides these two, the HNP also have two fishpond complexes. While the river flood plains contain habitats like forests, reed beds, oxbow lakes and riverside meadows, the “Puszta” can be divided into three areas: the north area, with large areas of tall-grass steppe with a small number of woodlands inside, the south area, dominated by short-grass steppe, and the western area, a “Puszta” interrupted by multiple marshes of different sizes. However, part of the “Puszta” is now cultivated land, due to agricultural and regional development, so part of its original fauna currently uses agriculture areas and structures as part of its habitat (Hilbers, 2008).

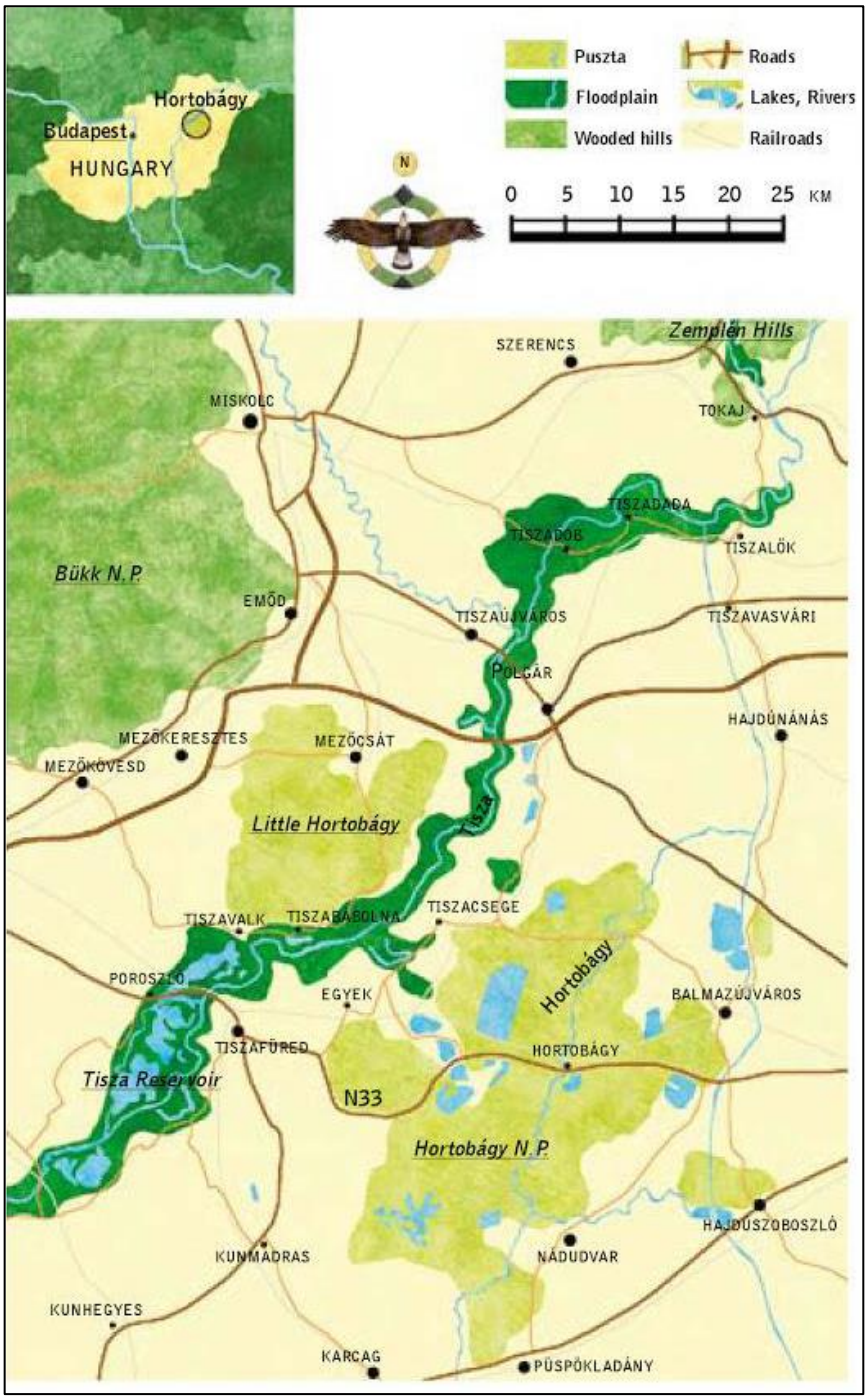


Figure 2 - Distribution of the different landscapes in the Hortobágy region (Hilbers, 2008)

Starting with amphibians and reptiles, they can be considered species of lowlands. The side meadows and oxbow lakes of the river Hortobágy represent habitat for reptiles and amphibians. Some of them include the common spadefoots (*Pelobates fuscus),* European tree frogs (*Hyla arborea),* fire-bellied toads (*Bombina* spp.) and green toads (*Bufo viridis)* in the class Amphibia, and grass snakes (*Natrix natrix*) and European pond terrapins (*Emys orbicularis*) in the class Reptilia. However, no species of these two classes have been described as endemic of the HNP (Sándor, 1981).

Recognised by its rich avifauna, for being one of the best places for bird watching in the Carpathian Basin, the HNP has about 159 nesting species and other 178 bird species are regular or irregular migrants, which a detailed description would make this chapter colossal (Végvári, 2002). Illustrating this, in the wetlands, the flood forests are breeding sites for common kingfishers (*Alcedo atthis*) and sand martins (*Riparia riparia*) and nesting sites for species like the black stork (*Ciconia nigra*) and the black kite (*Milvus migrans*). The denser forest areas include great spotted woodpeckers (*Dendrocopos major*), chaffinches (*Fringilla coelebs*), goldfinches (*Carduelis carduelis*), greenfinches (*Chloris chloris*), hobbies (*Hypotriorchis* spp.) and a diversity of broad-winged raptors; the meadows and oxbow lakes, due to their richness in fish and aquatic invertebrates, are feeding areas for black storks, herons (family Ardeidae), kingfishers and little egrets (*Egretta garzetta*). In the cultivated fields, it is possible to find short-toed larks (*Calandrella rufescens*), collared pratincoles (*Glareola pratincola*) and great bustards (*Otis tarda*), which is one of the most common bird species in "Puszta". In the HNP, there are many birds whose habitat is not strict, since they can be found in different zones of the park, like the tawny (*Podargus strigoides*), little and barn owls (*Athene noctua* and *Tyto alba*), swallows (family Hirundinidae), house martins (*Delichon urbicum*), common buzzards (*Buteo buteo*), kestrels (*Falco tinnunculus*), yellowhammers (*Emberiza citrinela*) and many more (Hilbers, 2008).

The masses of migration include, for example, 100.000-300.000 of grey geese (*Anser* sp.) and approximately 100.000 common cranes (*Grus grus*). The number of common cranes at the HNP has been continually increasing since the early 1980s. They usually stage in undisturbed, large drained fishponds and shallow marshes or at the agricultural lands that surround the park from September to late November (Végvári, 2002).

The Mammals taxonomic group is not as rich as the Birds group. However there are some protected species as the European otter (*Lutra lutra*), in the fishponds, where it's also possible to find the common pipistrelle (*Pipistrellus pipistrellus*), the noctule bat (*Nyctalus noctula*), muskrats (*Ondatra zibethicus*), stoats and weasels (*Mustela* spp.), western polecats (*Mustela putorius*), red foxes (*Vulpes vulpes*), wild boars (*Sus scrofa*) and roe deers (*Capreolus capreolus*) often drinking. In the meadows, the red fox is currently common, and a population of Eurasian badgers (*Meles meles*) is increasing. In the "Puszta" and cultivated areas, it is crucial to highlight white-toothed shrews and lesser white-toothed shrews (*Crocidura* spp.), in some years large amounts of common voles (*Microtus arvalis*), red foxes, western polecats, wild boars and roe deers. Golden jackals (*Canis aureus*) could have also been observed in the past few years in the HNP. Finally, the red deer (*Cervus elaphus*) is expanding from the mountains down to the lowlands, so it is observed more often in the grasslands.

Besides its nature conservation role, HNP is also essential for the preservation of ancient shepherd culture and traditional land use. Regarding this, the park got its World Heritage Diploma in the cultural category. Shepherd culture involves pastoral activity using traditional buildings and a deep relation and dependence with the natural environment.

Furthermore, the HNP is also a destination for astrotourists and known as a “dark sky park”, which has a vital role in its conservation, since some of the birds of the park (as geese, cranes or spoonbills) and rare insects are considered light pollution sensitive (Gyarmathy & Kolláth, 2017).

2.2 – Pentezug Wild Horse Reserve

The Pentezug Wild Horse Reserve is a biosphere reserve for “Takhi” (the Mongolian name of the Przewalski’s horse) that consists in 2388 hectares of steppe area located in the core zone of the HNP (Brabender, Zimmermann, & Hampson, 2016; D’Souza-Anjo et al., 2017)

Przewalski’s horses were introduced in the Hortobágy, in 1997, with the primary goal of managing the landscape in the Pentezug area of the HNP, representing an excellent opportunity to study wild horses in a semi-wild habitat, which can directly help the conservation of the “Tahki” and/or populations of *E. ferus* (Zimmermann, Brabender & Kolter, 2009). This population is managed without human interference or parasite routine treatment. Consequently, deworming is only performed on particular occasions, as the translocations of specific individuals.

Like some of the rest HNP steppe areas, narrow mosaics formed by different plant communities also compose Pentezug. In addition to the marsh, which covers about 10% of the area, at least three more different grass communities occur in the drier areas, forming two different sizes of grass in the steppe. In total, almost 45% of the area is covered with short grass steppe, which is rich in fescue species, and 15% is formed by long grass or meadow. Moreover, 30% is eroded area loosely covered with plants tolerant of salt and the climate is semiarid (Brabender et al., 2016). Besides the Przewalski's horse, a herd of domestic cattle (*Bos primigenius taurus*), carefully bred to phenotypically resemble reconstructed aurochs (*Bos primigenius*) use this area for grazing. The reserve is fenced off by electric-fences on all boundaries that keep the horses and cattle contained but, on the other hand, provide a surmountable barrier to the natural inhabitants of the reserve including roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa scrofa*) and the great bustard (*Otis tarda*) (D’Souza-Anjo et al., 2017). The temperature can range between 38ºC, as the maximum in July, and -28ºC, as the minimum in January, with an average of 21ºC in summer and -2,5ºC in winter. The annual rainfall is 500 mm and a 2-10 cm of snow falls on average 40–45 days *per* year (Brabender et al., 2016).

2.3 – Przewalski's horse (*Equus ferus przewalskii)*

The Przewalski's horse (*Equus ferus przewalskii*), also known as "Tahki", in Mongolian, is currently assumed as a subspecies of the extinct *Equus ferus*. It can hybridize with the domestic horse (*Equus ferus caballus)* to produce fertile individuals, however the existence of 2n = 66 chromosomes, compared to the 2n= 64 chromosomes of the domestic horse, proves that the Przewalski's horse is evolutionarily different from the domestic horse, more than any other two breeds of *E. ferus caballus* (King et al., 2017). However, a very recent study suggests that the Przewalski's horse is not a truly wild horse subspecies, but in fact a feral horse descendant from the first archaeological evidence of horse domestication, the Botai (Gaunitz et al., 2018). Nevertheless, since the exact timing and details of the horse domestication remain unknown, the scientific community consider the "Takhi" a subspecies for scientific and conservation purposes. Currently, the International Union for Conservation of Nature (IUCN) considers the *E. przewalskii* as Endangered since 2011, preceded by the classifications Critical Endangered, in 2008, and Extinct in the Wild, in 1996. However, the last observation of this subspecies in the wild happened in the 1960s (King et al., 2017).

In a historical point of view, although the first visual account of "Przewalski-type" wild horses date for more than 20.000 years ago (Wakefield, Knowles, Zimmermann, & van Dierendonck, 2002), this subspecies remained unknown for a long time in western science and it's not even referred in Linnaeus *Systema Naturae*, in 1758. It was first mentioned by John Bell, a Scottish doctor and traveller, in 1763, that located the horses in, what was at that time, the Chinese-Mongolian border (King et al., 2017). At the end of the 19th century, Colonel Nikolai Mikhailovich Przewalski wrote a famous report, after several expeditions to Tibet. While returning from his second expedition in Central Asia, he found some horse body parts in the north of Gutschen, an actual China's territory. These body parts were analysed by Poliakov at the Zoological Museum of the Academy of Science in St. Petersburg. This scientist found that they were from a wild horse and gave the official name to the animal: *Equus ferus przewalskii* (Poliakov, 1881).

Physically, it is possible to define some aspects that distinguish this subspecies from the domestic horse: the Przewalski's horses have a remarkably thick and short neck, a robust body and short limbs. Regarding the hair, the head and neck are usually darker than the body and the flanks are darker than the underparts. The distal segments of the limbs are also dark, even black, and in some cases, they have three to ten dark stripes on the carpus and/or on the tarsus (Groves, 1994) (Fig.3). Additionally, they change the hair from the tail and mane once *per* year (Wakefield et al., 2002).

Figure 3 - Przewalski's horse external aspect (Original)

The social organisation of the Przewalski's horse is similar to other equids. The strong dominance hierarchies are essential for the formation of a harem and the establishment of its space, which is very important to reduce the aggression episodes mainly between harem stallions, grouped stallions and bachelors (Keiper & Receveur, 1992). However, some differences can be found between populations that lead to distinct behaviour. In Australia, a population of feral horses (*E. ferus caballus*) maintain a large physical separation between the harem bands in order to avoid congestion at water holes and unnecessary fighting during the breeding season (Hampson, De Laat, Mills & Pollitt, 2010). Contrarily, Przewalski's horses at the Pentezug population have a different organisation. Even though the harems are considered single-stallion, they are close to each other, forming a big group, which leads to stress and regular aggressive episodes between challenging stallions. This social structure is not commonly described in other populations of the subspecies, but it has also been described in Camargue horses (Brabender et al., 2016).

After dispersing from their natal band, at approximately 2-3 years old, the "Takhi" males can form bachelor groups, which are composed by other sexual matured males and unsuccessful old stallions. When they are 5 years or older, males start forming harems, where the semi-permanent connections are strong all year round. Basically, they adopt already-established harems, steal mares from other males or form groups with dispersed females, becoming harem stallions of those (Boyd & Houpt, 1994).

2.3.1 – Geographic Range

It is assumed that the original distribution of the "Takhi" has covered the whole Eurasian steppe belt, from the Russian Steppes east to Kazakhstan, Mongolia and northern China until the late 18th century (Fig. 4). By 1900, after the rediscovery of the subspecies by Coronel Przewalski and some other European expeditions to Asia, it became clear that the number of individuals had been severely reduced. No consistent reports were published between 1903 and 1947. However, it's assumed that between the 1930s and 1940s, these wild horses must

have been relatively abundant in the Dzungarian Gobi, in southwest Mongolia, although they diminished progressively afterwards. In 1947, Junatov described less than 10 Przewalski's horses, and the Zanciv, a Mongolian author, found 18 horses. From then until 1960, there were sporadic records of hunters and Mongolian Arats sighting small groups of 2 or 3 horses in the limited area between the mountain ridges of the Tachijn-Shar-Nuru and Bajtag-Bogdo (Boyd & Houpt, 1994). As mentioned in the previous chapter, the last recorded individuals in the wild were seen in the desert of Dzungarian Gobi of southwestern Mongolia, in the 1960s (Walzer, Kaczensky, Ganbataar, Enksaikhan, & Stauffer, 2010).

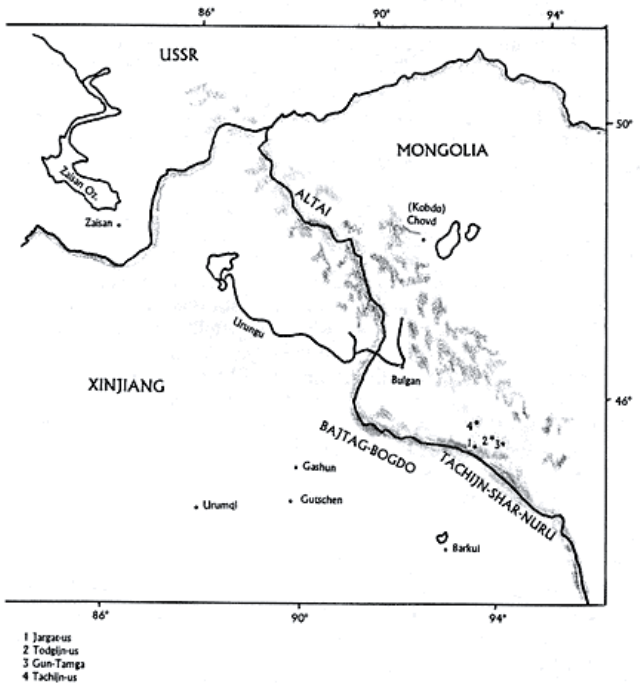


Figure 4 – The known geographical range of Przewalski's horses before the extinction. (Boyd & Houpt, 1994)

While the wild populations were decreasing, some captive populations were increasing in Europe, due to the capture of foals, mainly, since these were easier to capture than the adults were. Even though, most of these foals did not arrive the European countries alive. For instance, four expeditions for capturing foals happened between 1897 and 1902, but only 53 of these foals reached the west alive. Additionally, around the 1930s and the 1940s, only a few Przewalski's horses were caught and mostly died (Boyd & Houpt, 1994). Most of these transported animals are now the ancestors of the *ex situ* populations that exist in zoos, natural reserves and semi-natural reserves worldwide with the main goal of breeding, monitoring genetics and preparing animals for future reintroductions (Wakefield et al., 2002). Nowadays, some of them are located in Pentezug, Askania Nova, Chernobyl Exclusion Zone (CEZ), Cevennes National Park, and others.

The *in situ* populations are associated with the recent reintroduction projects in Mongolia and China, but more details are described in chapter 2.3.3. In summary, until new

publications, this subspecies is currently declared extinct in the wild in Kazakhstan, Russian Federation and Ukraine and extant in Mongolia and China (King et al., 2017).

2.3.2 – Habitat and Ecology

Wild and feral horses can have important effects on the ecosystem by, for example, changing vegetation structure or creating wetland sedimentation. *E. ferus* was already described in a diversity of habitats as steppe, grasslands, shrub, desert, wetlands, marshes, heathlands and woodlands.

Large herbivores can have strong effects on their habitats, depending on the species considered. Comparing with cattle or bison, *E. ferus* is selective, more grazer and less browser and consumes the vegetation much closer to the ground, creating a mosaic of high and low vegetation, which produce a more diverse habitat for herbaceous plants, invertebrates and small vertebrates. However, not only the feeding behaviour affects its ecosystem, but also the movement has ecological effects, by opening the vegetation and promoting the development of some plant species considered disturbance-dependent (Naundrup & Svenning, 2015).

Although the habitats of the Przewalski's horse include steppes and semi-desert zones, during the times, most of the used steppe areas turned into agriculture zones, occupied by livestock, or became degraded, which drove these animals to semi-desert habitats with limited water resources (Boyd & Houpt, 1994).

As mentioned in previous chapters, the last place where these animals were observed was the desert of the Dzungarian Gobi, in Mongolia, however, according to Van Dierendonck & De Wallis Vries (1996), it is questionable whether this area was merely a refuge of the last individuals or representative of the typical range of the "Takhi". Consequently, for the mentioned authors, the *E. ferus* is primarily a steppe herbivore that can survive under arid conditions when there is limited access to waterholes, as what happened with the *E. ferus przewalskii.* Other studies reinforce this idea explaining that, although feral horses can live and reproduce in semi-desert habitats, their survival and reproductive successes are consistently higher in richer grazing areas. Additionally, in Hustai National Park, "Takhi" preferentially select lowland steppe vegetation and seasonal movements are affected by the availability of better-quality vegetation (King et al., 2017).

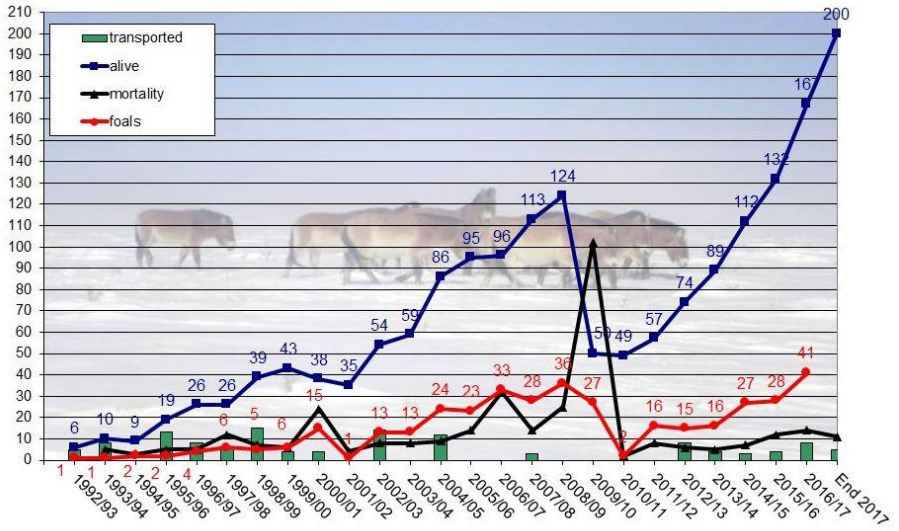
2.3.3 – Conservation

As mentioned, *E. ferus przewalskii* became extinct in the wild during the 1960s, in the Dzungarian Gobi, in south-western Mongolia. The combined effects of pasture competition with livestock, overhunting , significant cultural and political changes, military activities, climatic change, all are mentioned as reasons for the extinction of the natural populations (Walzer, Kaczensky, Ganbataar, Enksaikhan, & Stauffer, 2010; Walzer, Kaczensky, Zimmermann, & Stauffer, 2012).

From this point, the genetics of the subspecies was based in 13 animals and 3 initiatives started: the creation of the Przewalski's horse international studbook at Prague Zoo in 1959, the Species Survival Plan in the 1970s (Walzer, Kaczensky, Zimmermann, & Stauffer, 2012) and a European Endangered Species Programme (EEP) based on Cologne Zoo, in 1986 (King et al., 2017). Many semi-reserves were established worldwide to breed these animals in near natural environments and to prepare some of the individuals for reintroduction. Besides the HNP, it's also important to mention Le Villaret in France, Buchara in Uzbekistan, and CEZ in Ukraine (Wakefield et al., 2002).

In China, the most successful effort started in Xinjiang, where a total of 24 individuals were brought from western zoos to the Jimsar Wild Horse breeding centre in Xinjiang, from 1985 to 2005. In 1988, the first foal was born and the number of animals in the captive population continued to increase since then. The first group of horses was released in the Kalamaili Nature Reserve in 2001, into semi-wild conditions, where supplementary feeding was needed to improve the winter survival and to reduce competition with domestic horses. In 2013, the population was transferred to semi-release, where the first foal was successfully born in the wild. By that date, the reintroduced animals had formed 16 groups in 5 sites, making a total of 127 individuals (Xia et al., 2014). Another recent project was established in 2010, in Gansu Province, with the creation of the Gansu Endangered Species Research Center, where seven captive-born individuals were reintroduced in the Dunhuang Xihu National Nature Reserve, between 2010 and 2012. However supplementary feeding is necessary for this population as well (Liu et al., 2014).

In Mongolia, three distinct projects have been developed during the time. The "Takhiin Tal" project was initiated, and the reintroductions began in the Great Gobi B Strictly Protected Area (GG SPA), in 1992. The first journey was not successful for the captive-born Takhi, selected from various European zoos. A second journey, five years later, in 1998, was performed with a harem in a controlled area and the consequent foals were raised successfully in the wild. A total of 88 animals from different European zoos were introduced in the GG SPA afterwards. The population growth in this area has been suffering several rises and declines (Graph 1).



Graph 1 - Population Growth of the Przewalski's horses in GG SPA, since 1992 (ITG, 2019)

The establishment of the International Takhi Group (ITG), in 1999, as a non--governmental organisation with the main purpose of the total protection of the Gobi habitat and conservation of the GG SPA, allowed the continuous development of the mentioned project in accordance with the reintroduction guidelines established by IUCN (Walzer et al., 2010).

The second established reintroduction site, also in 1992, was the Hustain Nuruu, currently the Hustai National Park. The reintroduction programme is part of the global conservation program of the national park that includes monitoring and research of many biological aspects of the ecosystem. The first group of Przewalski's horses that was formed to have low inbreeding coefficients, arrived in June 1992 and the first foal was born one year later, which became the firstborn in Mongolia since the reintroduction processes started. A second group was sent 1994 and the same method of separation and adaptation before releasing, made in GG SPA and mentioned above, was performed here, which allowed the harems to be fully-integrated before release one year later. In total, 84 horses in five transportations have been shipped to Hustain Nuruu. In 1998, the first surviving second generation of foals was born (Wakefield et al., 2002).

A third reintroduction site, Khomiin Tal in the Great Lakes Depression, was established in 2004 as a controlled zone to the Khar Us Nuur National Park. The responsible Association Takhi introduced 22 Przewalski's horses from Le Villaret in a protected area, between 2004 and 2005. Currently, the primary goals are minimising the risk of hybridisation with domestic horse, guarantying a suitable habitat for wild and domestic herbivores and studying their behaviour before the future definitive reintroduction (TAHK, 2018).

Besides China and Mongolia, other reintroduction sites are planned to be established in Kazakhstan and Russia in the future (King et al., 2017).

There are some threats currently affecting the success of all the reintroduction projects and locations mentioned above. They include the small size of the populations, the limited spatial distribution, the potential hybridisation with *E. ferus caballus* and the competition for resources with domestic horses and other livestock. Furthermore, we can include the transmittable diseases, with special emphasis to agents like *Babesia equi*, *B. caballi* and *Streptococcus equi*, especially important in populations with reintroduced animals from zoos (Tarav et al., 2017). Additionally, illegal mining is considered a recent problem in some of the protected areas. Concretely, in Hustai National Park, the enhanced economic activity in the area was responsible for overgrazing mainly at the beginning of the project. In the GG SPA, there is illegal grazing in some parts of the year, even though outside the core zone. In addition, there also some natural factors that also affect the population growth as the coldest winters, that generally increase the mortality. In the same way, predation by wolves can have an impact on the small populations described, if excessive (King et al., 2017).

As mentioned above, a small number of individuals was maintained in captivity for several generations, leading to a loss of genetic diversity. It is estimated that about 60% of genes of the studbook population have been lost, which is irreversible. Inbreeding depression and hybridisation are two of the most relevant concerns that can be slowed with the correct management of the existing populations (Liu et al., 2014).

2.4 – Gastrointestinal parasitism in equids: general aspects and variations

Equids are known as hosts for a variety of external, including ticks, lice, mites, flies and mosquitoes, and internal parasites, as blood and gastrointestinal agents (GI) (Dissanayake, Rajapakse & Rajakaruna, 2017).

The GI parasites of horses are very diverse. Starting by the less varied group, horses are hosts of two coccidian species, *Cryptosporidium parvum* and *Eimeria leuckarti*, and only three species of cestodes, *Anoplocephala magna*, *Anoplocephala perfoliata* and *Paranoplocephala mamillana*, belonging to the family Anoplocephalidae. Nematodes are responsible for the most diversity of parasites, which includes one ascarid, *Parascaris equorum*, two pinworms, *Oxyuris equi* and *Probstmayria vivipara*, one rhabditoid nematode, *Strongyloides westeri*, three habronematid spirurids, *Habronema muscae*, *Habronema microstoma* and *Draschia megastoma*; and many strongylids that are all members of the superfamily Strongyloidea except one, *Trichostrongylus axei,* which belongs to the Trichostrongyloidea (Bowman, 2014).

From the class Insecta, family Oestridae, species from the genus *Gasterophilus*, namely *G. haemorrhoidalis* and *G. intestinalis,* are cosmopolitan horse parasites in Europe that also has GI important consequences. In fact, when the eggs are hatched, the larvae start

developing within the animal's oral cavity. Then, they mature into the third larvae stage within the stomach or intestine, potentially causing lesions at mucosa and causing GI clinical signs. Then, the larvae pass through the faeces and pupate (Nielsen & Reinemeyer, 2018).

We must take into consideration that GI parasitic infection does not always mean GI disease, since most of the times the host does not show any clinical signs of GI disorder (Nielsen & Reinemeyer, 2018). However, as a biotic relation "parasitism" is always assumed as having a negative effect on the host (Bowman, 2014).

Besides the species of parasite involved, for GI parasitism, the amount of parasites present is very relevant and defining it represents a clinical problem for the individual or a health problem for the population. Domestic equids are more sensitive to parasites than, for example, sheep, goat or cattle, which leads us to consider as more than 500 eggs per gram (EPG) a low infection, 550 to 1000 EPG a moderate infection and more than 1000 EPG a high infection (Madeira de Carvalho, 2001).

The variation in GI parasite load happens due to complex interactions between extrinsic ─ as the exposition level to parasites, population density and habitat's aspects ─ and intrinsic factors ─ as sex, age, immune condition and genetics (Morand, 2015; Debeffe et al., 2016). Talking about the extrinsic aspects, it is clear that a habitat with a large number of parasites leads to a higher parasitism in the populations, however a repeated exposition gradually reinforces the acquired immunity (Duncan, 1973, 1975; Love & Duncan, 1992) and this is an example of the complex interactions between extrinsic and intrinsic factors, mentioned above.

Additionally, there is a relation between the amount and diversity of parasites and the horse population dynamics (Pérez, Meneguz, Dematteis, Rossi, & Serrano, 2006), since large and stable populations tend to have higher numbers and more diverse species of helminths, like what happened in different populations of wild equids studied in Southern African countries (Matthee, Krecek, & McGeoch, 2004). Contrarily, in low density and unstable populations, the diversity and the amount of parasites is lower, like what happens in the Namib desert (Krecek, Louw, & Sneddon, 2011). Furthermore, it is also known that relatively high intensities of parasitism produce high mortality, and on the other hand, at low densities, there is a little parasite-induced mortality. Consequently, we can easily conclude that parasites have a role in the regulation of population growth (Pérez et al., 2006).

Furthermore, the abiotic factors of an ecosystem and their variation have an important role in the parasite life cycle and, consequently, in the level of infection found in a population or individual. In other words, climate and weather influence the microclimates and microhabitats where the eggs and larvae normally develop until infection of the final hosts. Generally, when outside the host, the development of infective stages of a helminth is possible

in the presence of moderate temperatures, adequate humidity, oxygen, and protection from direct sunlight, freezing and desiccation (Kates, 1965).

However, there are some factors and capacities described in some parasites and/or development stages that contribute to the biotic potential of the agent, which is defined as the total capability of helminths to survive inside or outside the hosts, to reproduce and to invade and produce patent infections under optimum conditions (Kates, 1965). Some examples of limiting factors of the biotic potential are: the necessity of an intermediate host and its specific needs like for *Anoplocephala* spp. (Höglund et al., 1998); the high production and resistance of the eggs in the environment, like for *Parascaris equorum* (Kates, 1965); or the capacity of *Strongyloides westeri* maintaining a free-living life cycle for several generations (Nielsen & Reinemeyer, 2018). Analysing the examples mentioned above, *Parascaris equorum* and *Strongyloides westeri* have higher biotic potentials than *Anoplocephala* spp. (Kates, 1965).

Regarding intrinsic factors, it is known that males are generally more parasitized than females (Moore & Wilson, 2002) and, additionally, animals that are dominant or lactating have usually a higher parasite load than subordinate or non-lactating individuals (Moore & Wilson, 2002; Habig & Archie, 2015). Younger individuals have generally more parasites than the adults, although it depends on the species of parasites considered in each study (Debeffe et al., 2016; Kuzmina, Dzeverin & Kharchenko, 2016; Slivinska, Kharchenko, Wróblewski, Gawor, & Kuzmina, 2016). In another perspective, the level of parasitism can be related to the animal condition, to the social status of the individual and its role in the harem, however, this influence is complex and different studies in feral horses suggest distinct relations between the parasitism and the social interactions of the animal. For example, according to Rubenstein & Hohmann (1989), an intestinal parasites infection can have a strong effect on body condition, which influences the reproductive success and, consequently, the social structure, but only with limited influence on behaviour. On the other hand, Habig & Archie (2015) suggest that dominance behaviours, as vigilance and group defence, have energetic costs and are associated with high testosterone and cortisol levels. These hormone levels can have immunosuppressive effects that can explain the higher faecal egg counts (FEC) found in dominant harem stallions comparing to the bachelors of a studied population of feral horses in Sable Island, Canada (Debeffe et al., 2016).

Finally, inside the host, a GI parasite has an optimal microhabitat and is included in an infracommunity, i.e. every single parasite individual from all the parasite species within a unique host or in a considered organ of the host. Even though the interactions between those helminths are not entirely described by ecologists and parasitologists, synergic and competitive interactions are often recognised. Moreover, although those interactions can provoke changes in the intestinal environment or on the parasite community, not all of them

have a clinical impact on the host (Holmes, 1973; Poulin, 2001; Cézilly, Perrot-Minnot, & Rigaud, 2014) .

Different GI parasitological studies regarding Przewalski's horses kept in zoos, semi-reserves or natural reserves have been done in different countries (Kuzmina, Zvegintsova, & Zharkikh, 2017), but not many of them have been done *in situ*. Most of them were based on determining the presence or absence of certain groups of intestinal parasites and calculating nominal levels of infection by the number of parasite eggs in one gram of faeces (EPG), however few publications have been reporting information on the species composition, structure of the intestinal parasite populations or interactions among them (Kuzmina et al., 2017).

2.4.1 – *Parascaris* spp.

Both *Parascaris equorum* and *Parascaris univalens* are two important horse parasites of this taxonomic genus. Molecularly, it is known that *P.univalens* has only one pair of chromosomes, but morphologically these two species are not possible to be distinguished. Although *P. equorum* is more frequently referred in the literature, evidence suggests that, in reality, infections by *P. univalens* are more common than by *P. equorum* (Nielsen & Reinemeyer, 2018). To avoid any nomenclature mistake, we chose to mention it as *Parascaris* sp. in this work.

Unlike other parasites of the superfamily Ascaridoidea that have complex life cycles, *Parascaris* spp. has the simplest cycle of all the members of the mentioned superfamily. When the infective egg of *Parascaris* spp. is swallowed by the definitive host, the larva hatches, pass through the wall of the small intestine, and gets in the blood until it reaches the liver. After the migration in the hepatic parenchyma, the larva enters in the hepatic vein and is carried by the caudal vena cava to the heart, and then, by the pulmonary artery to the lungs, where it reaches the pulmonary parenchyma and completes a moult. After that, the larva ascends in the tracheobronchial tree and returns by way of the lumen of the GI tract until the intestine, where it matures and becomes the largest adult parasite form of the equids, with 50 cm long and three large distinctive lips (Fig.5A), capable of producing 200.000 eggs in one day. Outside the host, a resistant eggshell represents a key for the cosmopolitan distribution and completion of the life-cycle (Bowman, 2014).

According to Kates (1965), due to this direct life-cycle, the number of eggs produced and their resistance in the environment, *Parascaris* spp. is considered as having a maximal biotic potential, when compared to other parasites under an ecology point of view. All of the above-mentioned aspects represent strategies that contribute to high adaptation and the global distribution of this parasite.

Clinically, a massive infection with these adult ascarids can cause interference with digestion and absorption, moderate enteritis and, consequently, subnormal growth, with the main incidence in foals and juveniles (Bowman, 2014). Furthermore, in some geographic regions, the prevalence can reach 100% in foals with less than 12 months (Leathwick, Sauermann, Donecker, & Nielsen, 2016). Although it is mainly associated with young ages, recently Liu, Hu and Li (2018) reported a case of a 6 years-old "Takhi" where a heavy *Parascaris* sp. infection was associated with volvulus, intestinal obstruction and sudden death.

To morphologically diagnose *Parascaris* spp., it is better to observe a microscope slide after using the Willis Floatation technique (described in chapter 2.1.2.2), since this parasite produces light eggs. These eggs are oblong to aspheric with a thick shell and 80-100 micrometres diameter, with one single cell in the middle and sometimes covered by an albuminous coat (Bowman, 2014) (Fig. 5B and C).

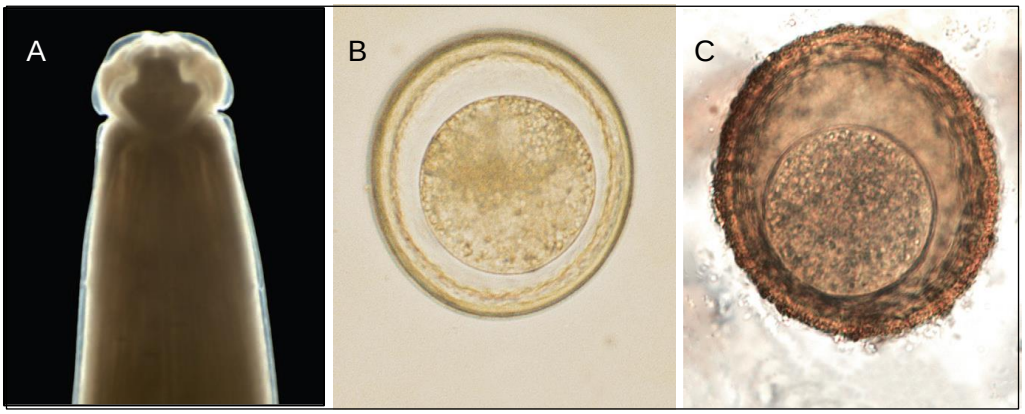


Figure 5 - *Parascaris* spp. adult female head (A), egg (B) and egg with an albuminous coat (C) (Bowman, 2014 - adapted)

2.4.2 – *Oxyuris equi*

Adult oxyuroids are commonly called 'pinworms', because of the long pointed tail of the female, and inhabit the large intestine of their specific hosts (Taylor , Coop, & Wall, 2016). Morphologically, the oxyurid oesophagus has a spherical bulb, often with a valve in its lumen, immediately anterior to its junction with the intestine (Bowman, 2014). The ventrolateral papillae are often absent or, when present, very much reduced (Taylor , Coop, & Wall, 2016).

Besides the long pointed tail, the adult female of *Oxyuris equi* is white, moderate in size (5–8 cm and 3x longer than the male) and may be seen protruding from the anus. However, it can also be observed attached to a palpation sleeve after a rectal examination or in fresh faeces. This female migrates down the colon, rectum and out through the anus to shed her eggs and a cementing fluid in anal or perianal region and dies. After 4 or 5 days, the eggs develop to the infective stage, during which the cementing fluid that involves them dries, cracks, and detaches from the skin in small pieces (Fig. 6), containing large numbers of

infective eggs that can attach to different structures on the environment. An infective egg is ingested and then, in the small intestine, L3 hatches from the egg. It continues its development in the cecum and colon mucosa crypts until L4, which emerge and feed on the mucosa until perform the final moult and becoming an adult stage. Finally, the adult forms reach the dorsal colon, reproduce and the cycle repeats (Bowman, 2014) (Nielsen & Reinemeyer, 2018).

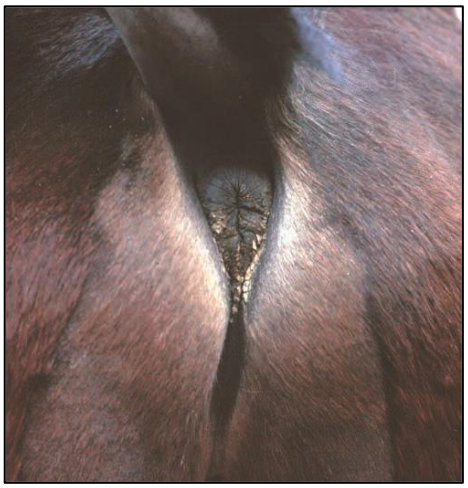


Figure 6 - Clumps due to *Oxyuris equi* infection in the perianal region (Taylor, Coop & Wall, 2016)

Clinically, local mucosal inflammation has been reported during the larval invasion, but the clinical consequences appear to be minimal. In the dorsal colon, secondary irritation from egg shedding can occur. The drying of the cementing fluid seems to damage the host's skin. Consequently, horses try to reduce their discomfort, by rubbing their tails and rumps against fixed objects, causing local damage to the skin, hair coat and tail (Fig. 6). This clinical sign is not considered to be pathognomonic for pinworm infection, however, it is genuinely suggestive (Nielsen & Reinemeyer, 2018).

Studies in wild and feral species use mainly coprological methods and faecal samples, instead of anal scraping that may show false negative results, due to the attachment of the eggs and cementing fluid mentioned above. The eggs should be collected by pressing the adhesive side of a piece of tape against a horse's anus and then by sticking the tape to a microscope slide. These eggs typically have a thick colourless shell, may contain a larva and an operculum in one end (Bowman, 2014)(Fig. 7).

Figure 7 - Oxyuris equi egg (Taylor, Coop & Wall, 2016 - adapted)

2.4.3 – *Trichostrongylus axei*

Even though the term the terms “strongyloid” or “strongylid *sensu latum”* can include this species, this parasite is a member of the superfamily Trichostrongyloidea. Besides *Trichostrongylus* sp., some other important parasite genera in this family include *Haemonchus* sp., *Cooperia* sp., *Ostertagia* sp. and *Teladorsagia* sp., although these last parasite genera are mostly found in ruminants (Taylor, Coop & Wall, 2016).

Trichostrongylus axei is the only gastrointestinal nematode shared between horses and other herbivores, consequently, the cross-infection is possible if equids and ruminants co-graze in the same area and the higher horse infections are associated with this situation.

The adult forms are normally visible but very thin and typically live in the glandular stomach of the host. The male has a very big and strongyloid copulatory bursa (typical from the superfamily Strongyloidea), with unequal spicules and the female produces eggs using double ejectors. The life cycle is similar in all the considered herbivores. During grazing, the susceptible host ingests the L3 infective larvae that invade the gastric glands and develop to the adult stage after two moults, which can copulate and produce eggs in 3 to 4 weeks after the oral infection, which are shed in the faeces.

Clinically, the development of the larvae and the presence of the adults in close contact with the gastric mucosa causes proliferation of these cells, apparently without affecting the gastric pH or the levels of the plasma pepsinogen. However, infections with a big amount of parasites can be responsible or contribute to clinical gastritis (Nielsen & Reinemeyer, 2018).

For the morphological diagnosis, the *T. axei* eggs are a little bit bigger but very similar to strongylid-type eggs, which turns the observation in fresh faeces slides very uncertain. Consequently, in faecal examinations it’s better to diagnose the *T.axei* infections after performing coprocultures, in order to obtain L3-type larvae, which is distinct from the strongylid infective larvae. In summary, the L3 stage of *T.axei* is described as having 16 intestinal cells – organized in a double row, with a triangular or pentagonal shape – and a short sheath in the tail and distal portion (Fig.8) (Madeira de Carvalho, Fazendeiro, & Afonso-Roque, 2007).

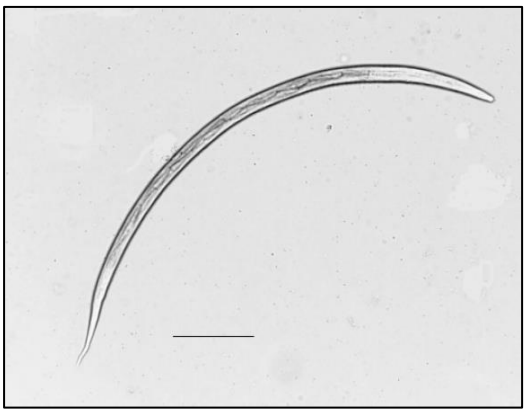


Figure 8 - L3 larva of *Trichostrongylus axei*. (Madeira de Carvalho et. al, 2007)

2.4.4 – Family Strongylidae

When the common term “strongylid” is used, it refers to the members of this taxonomic family, composed by nematodes with a well-developed buccal capsule, a mouth collar with two leaf-crowns, and a strongyloid copulatory bursa. This family can be divided into two subfamilies the Strongylinae, with the common name strongylins, and the Cyathostominae, with the common name cyathostomins, which are described in detail in next chapters (Lichtenfels, Kharchenko, & Dvojnos, 2008).

Of the 65 known species of the family Strongylidae, more than 40 have been reported in domestic horses (Kuzmina et al., 2016).

The eggs produced by the nematodes of this family are very similar and the coproculture to obtain the L3 larvae is always recommended, to have a proper differentiation diagnosis of the genera/species involved in the strongylid infections. Though, cyathostomins tend to have longer eggs, compared to *Strongylus* spp., and *Triodontophorus* spp. have the biggest and asymmetrical eggs of this group of nematodes (Madeira de Carvalho et al., 2007).

2.4.4.1 – Subfamily Strongylinae

The members of this subfamily are usually mentioned as “large strongyles” and their adult stages are mainly parasites of the large intestine of equids.

2.4.4.1.1 – Genus *Strongylus*

Particularly in equids, we should highlight *Strongylus* sp. inside the mentioned subfamily, which adult forms are robust, reddish and easily seen in the intestine mucosa. They also have a well-developed buccal capsule with a *corona radiata*, and, in the male adult form, it is possible to observe a prominent copulatory bursa. The most important species of this genus are *Strongylus vulgaris*, *Strongylus edentatus* and *Strongylus equinus,* whose morphological differentiation of the adult forms is based on the teeth inside the buccal capsule (Fig. 9). Furthermore, the life cycles of those three species have different migrations after the ingestion of the L3 and its penetration in the small or large intestine wall (Taylor, Coop & Wall, 2016). The mentioned differences are described below.

The L3 of *Strongylus vulgaris* turns into L4 in the submucosa of the small intestine. Then, the L4 penetrates in a small artery and migrates under the endothelium reaching the cranial mesenteric artery and its main branches, where it moluts into L5. This form migrates back to the cecum and colon, where it penetrates the submucosa and forms nodules. The rupture of the nodules releases the adults, which are the smallest of the three *Strongylus* spp., with two rounded teeth (Fig. 9A) and produce eggs that are released in the faeces 6-7 months

after the oral ingestion of the L3. Sometimes, it is possible to find some larvae in the aorta or vena cava or in the lungs, heart and liver, due to erratic migrations (Duncan, 1973).

Strongylus edentatus infection has larvae developments and migrations in very different locations comparing to *S. vulgaris*. The L3 turns into L4 in the liver and this form migrates to different abdominal locations as the peritoneum or the hepatic ligament, where it develops until L5 after several months and migrates back to the large intestine. The adult form is released from a purulent nodule and has no teeth in its buccal capsule (Fig.9B). The prepatent period is about 11 months.

Strongylus equinus is also distinct from the above described species. The L3 loses its sheath before penetrating the cecum or colon walls. Here, it forms a nodule in the mucosa or submucosa where it turns into L4. The L4 migrates to the peritoneum and liver, where it develops until L5. This larva migrates to the pancreas and then back to the lumen of the large intestine where it becomes an adult form with 3 conic teeth (Fig. 9C). The adult female produces eggs 9-10 months after the oral infection by the L3 (Bowman, 2014)(Taylor, Coop & Wall, 2016).

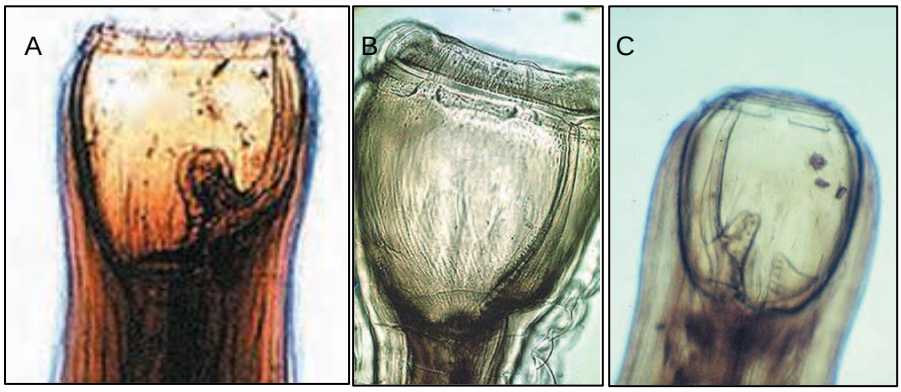


Figure 9 - Morphological differences between *Strongylus vulgaris* (A), *Strongylus edentatus* (B) and *Strongylus equinus* (C) in the buccal capsule of the adult form (Taylor, Coop & Wall, 2016 - adapted)

Besides the morphological differences in the adult form, there are also differences in the L3 larval stages, which are essential if faecal examinations are used instead of necropsies to analyse a population. Even considering that all of them are large strongyles, the L3 of *S. equinus* is very thin, with 16 poorly defined intestinal cells and very light coloured. Contrarily, *S. edentatus* has 18-20 triangular and well-defined intestinal cells, is thin and moves very fast when observed. Finally, *S. vulgaris* is the longest and has 28-32 intestinal cells (Madeira de Carvalho et al., 2007).

Since the migrations are very different, the related clinical signs will be also distinct. Due to its inside-wall migrations in the arteries, *S. vulgaris* can cause arteritis, thrombosis and/or being carried in the bloodstream, may produce partial or total occlusion of important

arteries and compromise the irrigated tissues (Bowman, 2014). On the other hand, *S. edentatus* larvae have been associated to peritonitis and hepatitis and *S. equinus* migrations can cause severe pancreatic dysfunction, which leads to clinical signs of pancreatitis or *diabetes mellitus*, or peritonitis and liver disease. In a clinical point of view, the migration stages are clearly more relevant than the adult stage. However, for feeding, the large buccal capsule of the adult parasite can remove some plugs of mucosa and ingest blood, plasma or mucosa cells. It can cause some focal inflammation and ulceration without significant blood loss or consequent systemic changes (Nielsen & Reinemeyer, 2018).

Additionally, in wild equids, other species of this gender are reported in the literature as *Strongylus asini*, a common internal parasite of zebras and donkeys in Africa.

Finally, in feral/wild horses, as in domestic ones, mixed infections with these three Strongylus species, but also involving all the most important genear/species of Strongylinae and Cyathostominae, can easily occur. Therefore, clinical and pathological consequences regarding horse strongylidosis in the wild will also reflect this phenomenon (Madeira de Carvalho, 2001, 2014).

2.4.4.1.2 – Other members of the subfamily

Triodontophorus, *Craterostomum* and *Oesophagodontus* represent other important genera of equids large strongyles, besides the *Strongylus*.

Their life cycles are not completely recognized in the literature. However, it's known that they do not migrate outside the large intestine wall and they seem to be similar to the life-cycles found in cyathostomins. Instead of migrations, they form large groups of parasites, which are often responsible for causing ulcers in the mucosa of the colon (Madeira de Carvalho, 2014).

Genus *Triodontophorus* includes some important species as *Triodontophorus brevicauda, T. minor, T. nipponicus, T. tenuicollis* and *T. serratus*, all of them with a worldwide distribution and a prepatent period of 2-3 months, which is very short comparing to genus *Strongylus*. Morphologically, they are reddish and have 10–25 mm in length, which make them visible in the colonic mucosa during a surgery or necropsy. The buccal capsule is sub globular and, in its base, it has 3 pairs of large teeth, each one with 2 plates (Fig.10) (Taylor et al., 2016).

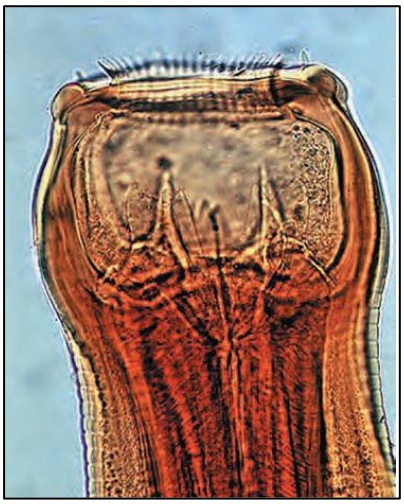


Figure 10 - The buccal capsule of *Triodontophorus* spp. with the evidenced teeth (Taylor, Coop & Wall, 2016)

Craterostomum spp. adult forms are similar to *Triodontophorus* spp., but smaller (6-11 mm), without protruding teeth and, in the female, the position of the vulva is located more anteriorly. One important species of the gender is *Craterostomum acuticaudatum* (Taylor , Coop, & Wall, 2016).

Finally, *Oesophagodontus robustus* is the only species of its gender, which adult form is usually 15-24 mm (Taylor , Coop, & Wall, 2016).

When the diagnostic is based on the coproculture examination, the morphological details of the L3 larvae need to be considered (Fig.11). *Craterostomum* sp. and *Oesophagodontus* sp. have both 16 intestinal cells, which shape in the distinct parts of the larva must be considered for the differentiation. Inside the genus *Triodontophorus*, it's useful to separate *T. serratus* from other species of *Triodontophorus* sp., since the first one has 16 asymmetric intestinal cells and the respective genus has been generally described with 18-20 intestinal cells. As mentioned in the previous chapter, the same number of intestinal cells is found in *S. edentatus*, however *Triodontophorus* sp. is clearly larger (Madeira de Carvalho et al., 2007).

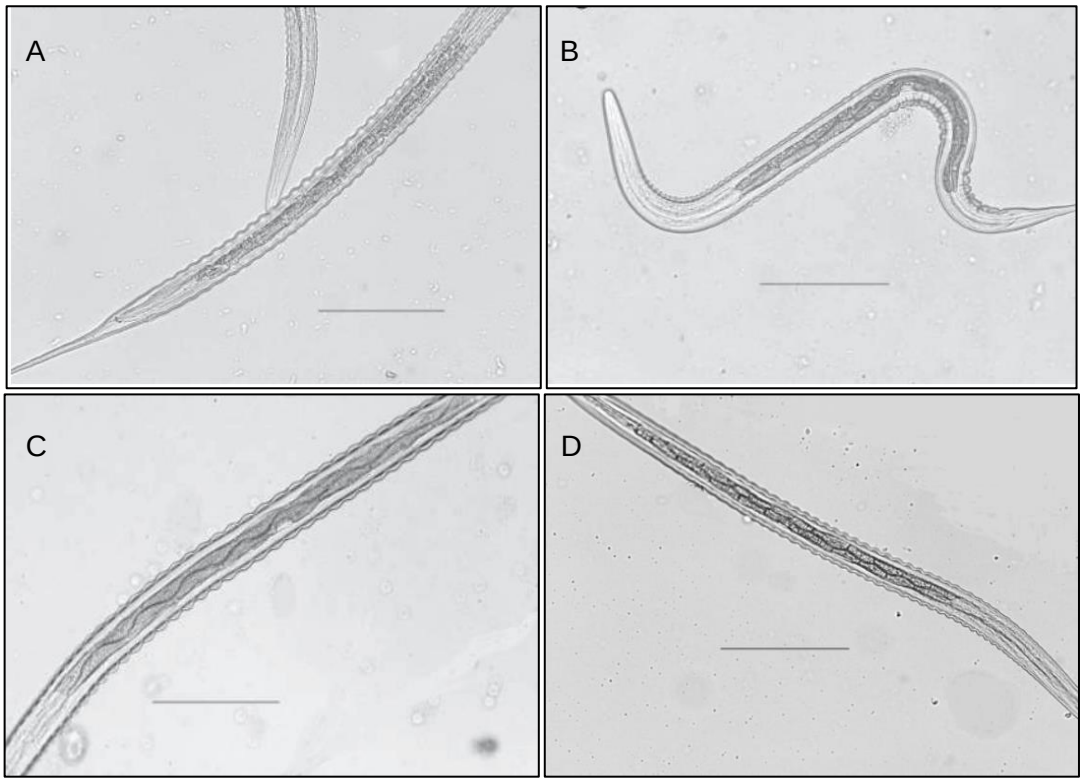


Figure 11 - L3 larvae of different large strongyles. *Craterostomum acuticaudatum* (A), *Triodontophorus serratus* (B), *Triodontophorus* spp. (C) and *Oesophagostomum robustus* (D) (scale: 100 µm) (Madeira de Carvalho et al., 2007)

2.4.4.2 – Subfamily Cyathostominae

This subfamily is composed by an enormous diversity of species of parasites, more than 50, all of them commonly named as small strongyles (5–12 mm long). Morphologically, the members of this subfamily are white to dark red and visible on the mucosa of large intestine, if a necropsy or surgery are performed. The species differentiation, using the adult forms, is based on the buccal capsule and the leaf crowns. However, globally, the buccal capsule is short, cylindrical and does not have any teeth (Taylor, Coop & Wall, 2016).

Some of the most common genera of the subfamily include *Cyathostomum*, *Coronocyclus*, *Cylicocyclus*, *Cylicodontophorus*, *Cylicostephanus*, *Gyalocephalus*, *Poteriostomum*, *Petrovinema* and *Parapoteriostomum* and the adult differentiation is based on the anterior end structures of the parasite (Nielsen & Reinemeyer, 2018) (Fig. 12).

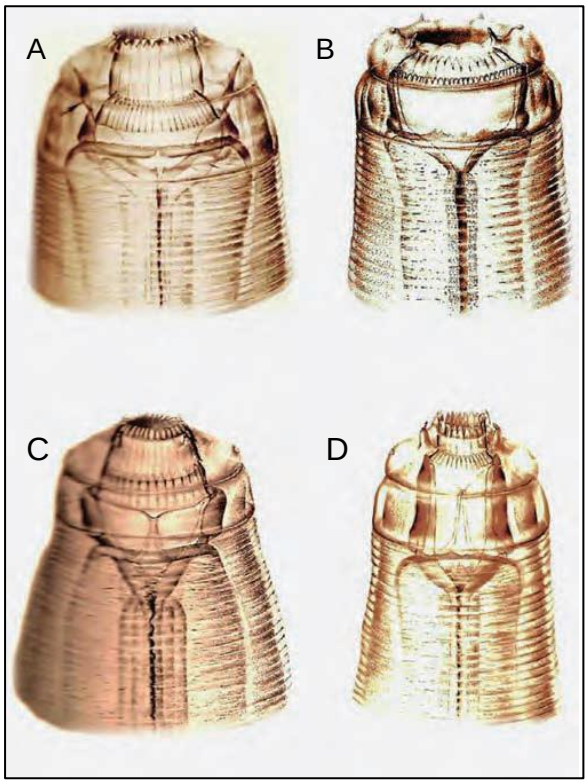


Figure 12 - Characteristic aspect of the anterior ends of *Cyathostomum* spp. (A), *Cylicocyclus* spp. (B), *Cylicodontophorus* spp.(C) and *Cylicostephanus* spp. (D). (Taylor, Coop and Wall, 2016)

The L3 obtained after the coproculture can also be used for the differentiation of the cyathostomin genera and species. In brief, *Gyalocephalus capitatus* is the only type with 12 intestinal cells, *Poteriostomum* spp. has 16 cells but it's usually smaller than the large strongyles larvae, mentioned in previous chapters (Madeira de Carvalho, Fazendeiro, & Afonso-Roque, 2004). Furthermore, Madeira de Carvalho, Fazendeiro, & Afonso-Roque (2008) published a dichotomic key based on the description of the larvae and, for the other genera of cyathostomins, they suggest to group them in morphotypes to simplify their identification. Additionally, Santos, Madeira de Carvalho, & Molento (2018) published some scientific illustrations of the L3 larvae of cyathostomins to help the differentiation between the mentioned morphotypes (Appendices B.1 & B.2).

The life cycle of these parasites does not involve systemic migrations as it has been described for the genus *Strongylus*. After oral ingestion, the L3 invade the wall of the caecum and ventral colon where they develop to L4. Then, they emerge into the lumen and develop until they moult to L5 and become young adult worms. The prepatent periods of cyathostomins are generally between 2 and 3 months. However, these parasites are able to do hypobiosis, which is the partial interruption of the development of larvae inside the host that occurs seasonally, usually at a time when conditions are adverse to the free-living stages. When conditions are optimal for free-living development, the resumption of hypobiosis occur and the contamination of the environment increases (Taylor, Coop, & Wall, 2016).

Clinically, as mentioned, these larvae do not migrate beyond the mucous membrane of the large intestine. Consequently, their pathogenic effects are reduced when compared with those made by the larvae of *Strongylus* spp. However, infection by large numbers of cyathostomins can cause clinical disease, typically in late fall, winter, or early spring. Persistent diarrhoea, progressive emaciation, and marked hypoalbuminemia are the main associated signs, though anasarca can also occur in old and complicated cases. Lesions of granulomatous colitis can also be observed when massive numbers of cyathostomin larvae are embedded in the mucous membrane (Bowman, 2014). Regarding the colic pathology, the strongylids *sensu latum* are firmly considered by many authors as one of the main agents for colics (Taylor, Coop, & Wall, 2016). However, according to Stancampiano, Usai, Marigo, & Rinnovati (2017), cyathostomin infections don't seem to be a risk factor for colic disease.

3 – Material and Methods

3.1 – Objectives of the study

This study is aimed to perform a survey of the gastrointestinal parasitology of the Przewalski's horses from the Pentezug Wild Horse Reserve, at the HNP, in Hungary, through the collection of faecal samples, with the main purpose of contributing to a better understanding of the health status of an *ex situ* population.

3.2 – Studied population and sampling

Most of the studies performed in Przewalski's horses under semi-natural conditions in other geographic regions, are based in smaller populations and using an *in vivo* deworming method before the faecal samples collection (Slivinska, Dvojnos & Kopij, 2006; Kuzmina, Zvegintsova, & Zharkikh, 2009; Kuzmina et al., 2017). However, this methodology was not done in this study, since the Pentezug population is managed without human interference or parasite routine treatment. Consequently, deworming is only performed on particular occasions, as translocations of specific individuals.Due to the collaboration of Dr Viola Kerekes, HNP veterinarian, and Tímea Szabados, Pentezug conservation technician, we were able to identify, collect and transport all the samples and analyzing them at Budapest Zoo and Botanical Garden.

Between 27th September and 7th November 2018, 79 faecal samples were collected in four different two or three-day trips to the HNP. Due to some births and deaths, some small variations on the population size occurred during the collection period. However, we considered 280 animals as our total population number. From the 79 samples collected, 62 are from identified harem members, 2 are from identified bachelors, 15 are from unknown bachelors. We were able to sample individuals from 24 of the 29 harems (Appendix A). The names of the horses are given according to the current year (the first letter corresponds to the birth year) and most of the identified adults have a correspondent number and genetic database developed by University of Davis, in a partnership with the Pentezug.

The animals were observed defecating in a range of approximately 50 to 100 meters and identified by the local veterinary or keeper of the Pentezug (Fig. 13). The majority of the observations were made while animals were eating and resting.

Figure 13 - One of the sampled individuals defecating while identification was performed (Original)

Thereby, the identification was possible for most of the individuals due to the strong harem connections and proximity that defines the wild horses. When an animal started defecating, the animals surrounding it were observed, the harem was determined and then, by using binoculars if necessary, it was possible to sex it and see its body details, identifying the individual. However, most of the bachelors were not likely to be identified, since they don't establish the strong connections that characterise the harems disposition which leads them to have different positions surrounding distinct harems (Rubenstein & Hohmann, 1989).

Consequently, we approached the faeces and verified their freshness by the temperature and visual aspect to be sure we were collecting the newly evacuated faeces that we observed previously. For the collection, plastic bags were used, all of them identified with the number of the sample and animal's name (Fig.14). After each period of collection, they were transported directly from the Pentezug to the Hortobágy Wild Animal Park infrastructures, located right outside the north door of the reserve, and stored inside a fridge in a temperature of 4º C until the end of the fieldwork. At the end of each trip, the samples were transported by train, in a box with iced pack flask, to the Budapest Zoo veterinary clinic, where they were stored in a 4ºC fridge, and analysed at its laboratory.

Figure 14 - Some identified samples being transported after the collection to the Hortobágy Wild Animal Park (Original)

3.3 – Coprological methods

The different coprological techniques used are described below. For all the observations, a Motic® digital microscope was used.

3.3.1 – McMaster Technique – quantitative method

To quantify the number of eggs included in one gram of faeces (EPG) and evaluate the degree of the parasite infection, we used McMaster Technique according to Madeira de Carvalho (2011). In brief, it starts by mixing two grams of the faeces with 28 ml of a saturated sucrose solution in a small cup. Then, the moisture is homogenized using a rod and filtered through a strainer into a new cup. From here, the two compartments of the McMaster slide are filled with the obtained solution. The capacity of the chamber is 0.30 ml distributed in the two mentioned compartments, which the upper slide has a grid that helps the egg counting, usually using a 10x objective, and only the eggs observed inside the limits of both grids are counted. A few minutes later, the eggs become attached to the lower surface of the upper slide because of the floatation induced by the saturated solution. For the extrapolation of the results in McMaster's, chamber we used a conversion factor calculated as follows:

$$\frac{\text{Volume (1g of faeces)}}{\text{Volume (counting chamber)}} = \frac{15\ \text{ml}}{0.30\ \text{ml}}$$

$$\text{Conversion factor} = 50$$

Consequently, to obtain the EPG of the sample, we multiply the total number of the counted eggs inside the grids of both compartments by 50 (Madeira de Carvalho, 2001).

The chamber was from Eggzamin® and it's illustrated in Figure 15.

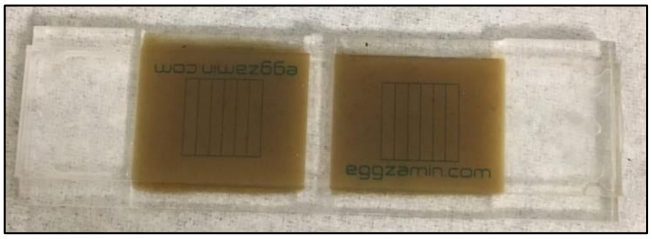


Figure 15 - McMaster chamber filled with the described solution (Original)

3.3.2 – Willis floatation and Natural Sedimentation – qualitative methods

As a qualitative method, the Willis floatation allows us to concentrate and identify the lighter parasite eggs of the samples without counting them and it does not require weighting of the samples, although around 2 grams were usually used and about 25 ml of saturated solution. After mixing, the solution is passed through a strainer into a test tube. Consequently,

the lighter parasite eggs, usually nematode eggs, float and concentrate in the upper part of the tube, due to their low specific gravity compared to the saturated solution. The opposite occurs with the heavier eggs on the bottom of the tube. Then, a cover slide is placed on the top of the tube (Fig.16) for about 15-20 minutes, which is the time required the eggs to attach it. Finally, after placed on a slide, it is observed on a microscope using mainly a 10x objective (Madeira de Carvalho, 2001).

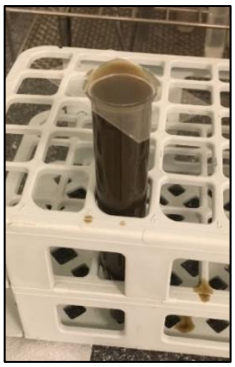


Figure 16 - Willis floatation method (Source: Original)

It is possible to make use of the sediment at the bottom of the tube to perform the natural sedimentation technique after the Willis floatation. Subsequently, the supernatant solution is discarded and 2-3 drops of the remaining liquid are collected by using a Pasteur pipette. Then, the collected material is placed on a slide to be observed on a microscope. To make the observation easier and more precise, a drop of methylene blue is added to the slide in order to have contrast in the microscope image: blue debris with bright gold or brown eggs, since this compound cannot penetrate the intact parasite eggs, namely from Class Trematoda (Fazendeiro, 1989).

3.3.3 – Baermann method

In the literature, many variations of this method can be found, although all of them based on the principle that nematode larvae (usually L1), are not capable of swimming against gravity in a considerable column of water, being the main goal concentrating all of them in the bottom of a tube (Bowman, 2014).

An amount of 5-15 grams of faeces wrapped in gauze creating a spherical form was placed in a conical cup with lukewarm water (Fig.17). This will stimulate the larval mobility and their migration to the bottom of the water column. About 24 hours later, the supernatant was removed and the sediment was transferred to a slide, using a Pasteur pipette, and observed on a microscope as a fresh mount, using the 10x or 40x objectives.

Figure 17 - Baermann apparatus (Original)

3.3.4 – Coproculture - Roberts and O'Sullivan method

For identification of strongylid genera/species involved, it is necessary to obtain their L3 infective larval stages by using a coproculture technique (Bowman, 2014). Thus, the Robert and O'Sullivan method was used with little adaptations according to the available conditions and the methodology described by Madeira de Carvalho (2001).

For each sample, a regular plastic cup was filled with faeces, identified and covered with aluminium foil with several small holes. Each group of samples was stored on a board with 1-2 cm water, in a room with controlled temperature for 14 days (Fig.18) and sprinkled with water every day. After those 14 days, each cup was filled with water and turned upside-down in a Petri dish. Then, a small amount of water was added to the Petri dishes to fill all their surface and the samples were stored like this for 24 hours (Fig.19). After this period, all the water from each dish was collected to an identified test tube and, after 6 hours, natural sedimentation of the larvae occurred in the bottom of the tubes. A controlled amount of pellet was collected with a previously marked Pasteur pipette, transferred to a slide and observed in the microscope, with the 100x and 400x total magnification.

Figure 18 - Coprocultures preparation (Original)

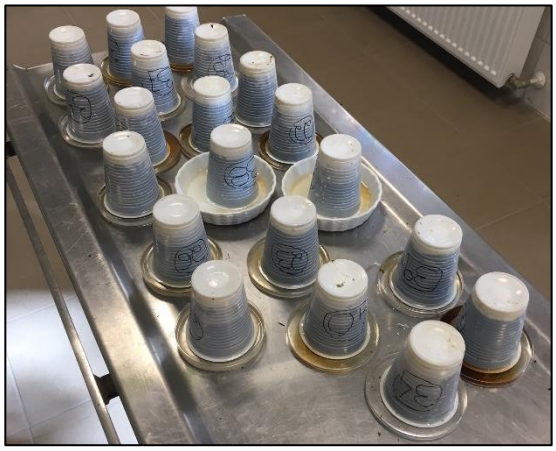


Figure 19 - Cups turned upside-down for 24h (Original)

3.3.4.1 – Identification of the L3 larval stages

The identification of the L3 larvae present in each coproculture was possible due to the attached identification key (Appendix B.1) and also due to cyathostomins illustrated details recently described by Santos, Madeira de Carvalho and Molento (2018) (Appendix B.2). To have an idea of the proportion of infection by Strongylinae, Cyathostominae and Tricostrongylidae, a total of 100 mature L3 larvae were counted and identified in each sample, in order to obtain the proportional abundance percentages of each mentioned subfamilies and family of the order Strongylida.

3.4 – Data analysis

For descriptive statistics, we used Microsoft Excel 365® and IBM SPSS Statistics 25® and, for statistical inference analysis, we used IBM SPSS Statistics 25®.

Regarding the coprocultures, the presence or absence of certain species of parasites was evaluated with the Pearson Chi-square test and Fisher's exact test. When one or more cells in the cross-table have less than 5 cases, only the values of the Fisher's exact test were considered, due to the limitations of the Chi-square test in these conditions.

For all the statistical tests, we considered a confidence level of 95% and a p-value<0.05.

3.5 – Iconographic methods

All the photos presented in the next chapter were taken with a Canon Digital IXUS 95 IS® placed near the ocular of the microscope. For each picture, the magnification of the microscope and camera were registered with the aim of creating a final image scale, using ImageJ® (Ferreira & Rasband, 2012).

4 – Results

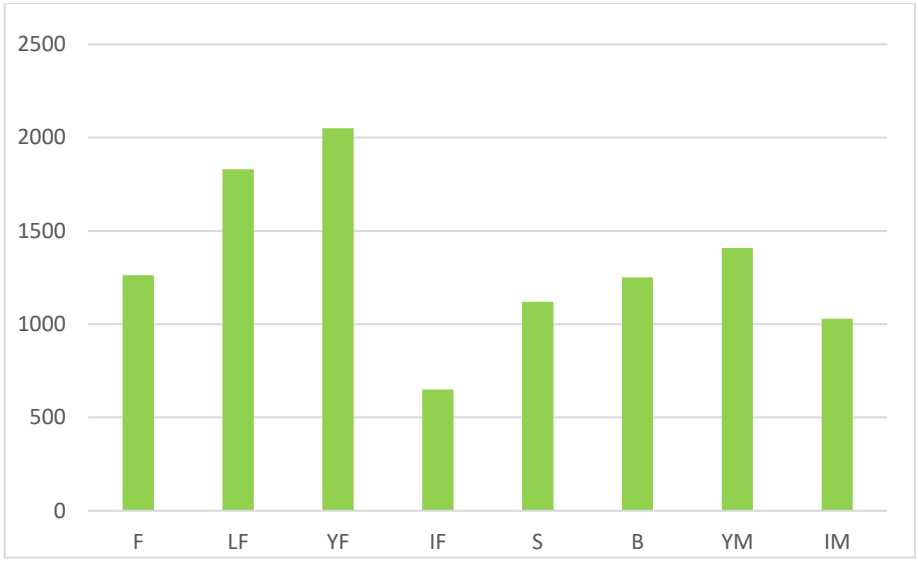
Statistically, the distribution of the results was not normal. In this way, a Kruskal-Wallis analysis of variance test and Mann-Whitney paired samples test were performed for the evaluation of the FEC in different groups and categories that will be mentioned in detail in this chapter. Our observations are independent since each collected sample leads to one result, which corresponds to one observed defecation.

Even though we had a limitation on the identification of the bachelors, when we compare adults (>3 years old) with juveniles (<3 years old), the whole 79 individuals were considered. In this case, the bachelors were considered all adults, mainly because the dispersion from the natal band occurs after the sexual maturity around the 2-3 years old. Consequently, they can be analysed with the remaining adult individuals.

4.1 – Quantitative method

With the McMaster technique, we could evaluate the quantity of parasite eggs in each sample. The average amount of eggs, for the whole 79 samples, was 1286.7 EPG, with a minimum value of 250 and a maximum value of 5050. The median and the standard deviation and standard error mean were, respectively, 1050, 94.2 and 836.9.

Moreover, since the identification of the samples was possible in most of the cases, we could evaluate the level of parasitism according to the social status, age and sex, of the individuals. We divided our population in 8 categories: IM (immature males, with less than 1 year-old), IF (immature females, with less than 1 years old), YM (young males, 1-3 years old), YF (young females, 1-3 years old), F (females, with more than 3 years old), LF (lactating females, with more than 3 years old), S (harem stallions) and B (bachelors) (Graph 2).



Graph 2 - Average level of infection according to faecal egg counts (EPG) considering different ages, sex and social role of the individual. IM (immature males, with less than 1 year old), IF (immature females, with less than 1 years old), YM (young males, 1-3 years old), YF (young females, 1-3 years old), F (females, with more than 3 years old), LF (lactating females, with more than 3 years old), S (harem stallions) and B (bachelors).

Statistically, the Kruskal-Wallis test showed no significant differences between the distinct categories presented in Graph 2 (p-value=0.454). The same happened between sex (p-value=0.784), adults *vs* juveniles (<3 vs >3 years old) (p-value=0.985), lactating *vs* non-lactating females (p-value=0.370), bachelors *vs* stallions (p-value=0.183), bachelors *vs* members of a harem (p-value=0.567) as results of the Mann-Whitney test. In the same way, there are no differences in the EPG value between the different harems of the whole group (p-value=0.238), according to the Kruskal-Wallis test.

4.2 – Willis floatation, natural sedimentation & Baermann methods

The Willis floatation method allowed us to find three different egg types. Globally, all the 79 samples analysed were positive for strongylid-type eggs (79/79) (Fig. 20A), whose species differentiation is not considered in this method. Furthermore, 27.8% were positive to *Parascaris* spp. (22/79) (Fig. 20B) and 2.5% contained *Oxyuris equi* in their expelled faeces (2/79) (Fig. 20C). There are no statistically significant associations between *Parascaris* spp. and age (<3 vs >3 years old) (p-value=0.360), sex (p-value=0.622) or bachelors vs members of a harem (p-value=0.371), according to Fisher's Exact test. By the sedimentation method, we could only evidence a trematode egg (Fig. 20D).

Regarding the Baermann method, we can affirm that 8.9% of the analysed samples (7/79) had L1 strongylid-type larvae in their faeces, which confirms the infection by strongylid *sensu latum*, already evidenced by the results of the Willis floatation method.

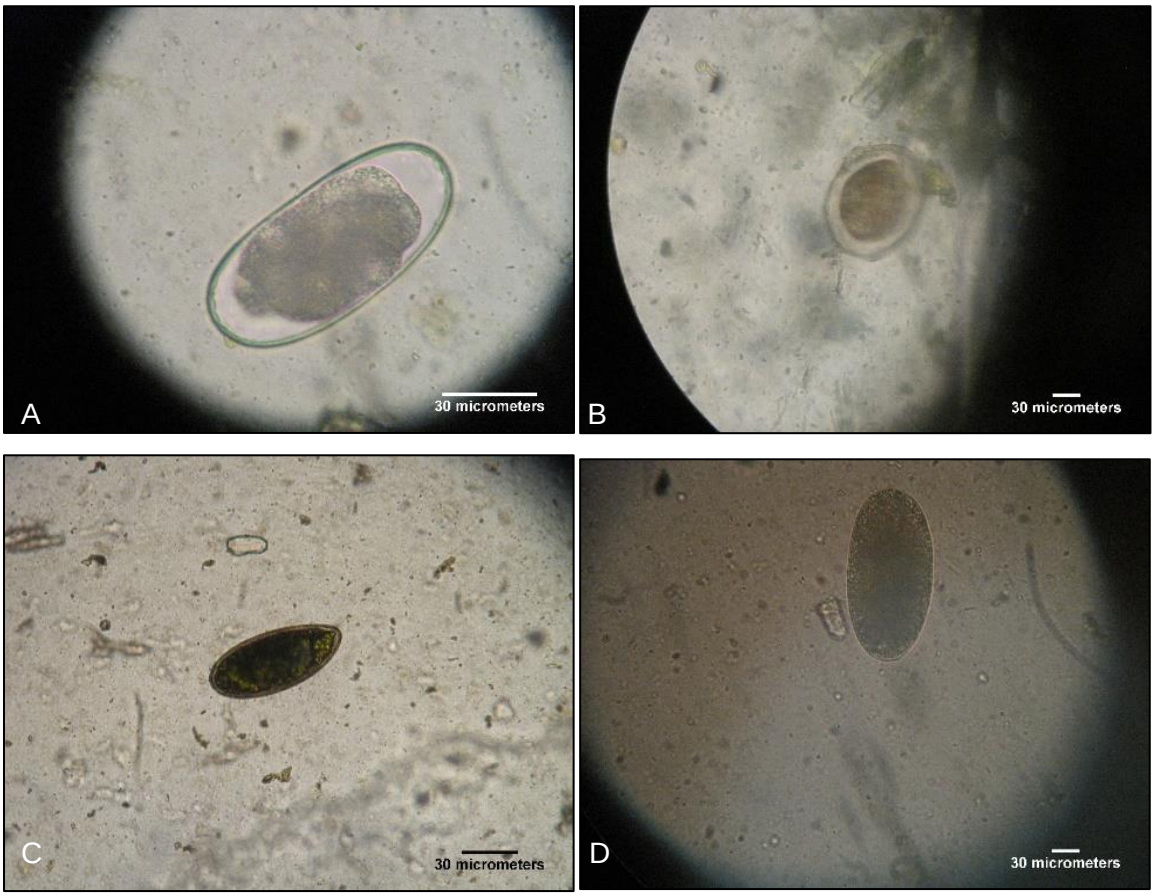


Figure 20 - Eggs obtained after the coprology methods. Strongylid-type egg (A), *Parascaris* spp. egg (B), *Oxyuris equi* egg(C), trematode egg (D) (Originals)

4.3 – Coprocultures

Regarding the coprocultures, we were able to distinguish 15 genera/species of strongyloids. Furthermore, we were able to confirm the 100% strongylid prevalence found in the Willis floatation, since all of the coproculture samples were positive for at least one type of strongylid. Additionally, some of the observed microscopic fields illustrate the high level of infection already described with the quantitative methods (Fig. 21).

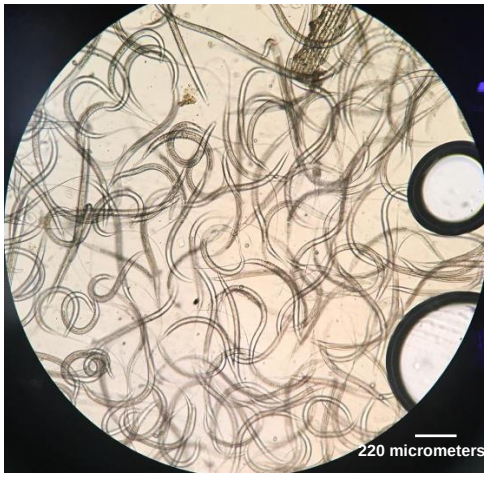


Figure 21 - One of the coprocultures illustrating a high level of infection (Original)

Generally, they revealed that the proportional abundance of strongylid infections are composed by an average of, approximately, 96.3% of Cyathostominae, 2.4 % Strongylinae and 1.3 % Tricostrongylidae.

The presence or absence of certain species of parasites was evaluated with the Pearson Chi-square test and Fisher's exact test. When one or more cells in the cross-table have less than 5 cases, only the values of the Fisher's exact test were considered, due to the limitations of the Chi-square test in these conditions.In Table 1, it is possible to analyse the coprocultures results dividing our population into four categories: B (bachelors), F (adult females, with more than 3 years old), S (harem stallions) and J (juveniles, females or males with less than 3 years). Concretely, it's possible to see that Cyathostomins type A are the most prevalent of the subfamily Cyathostominae (100%), followed by cyathostomins type D (49.4%). In the subfamily Strongylinae, *Strongylus vulgaris* is the most prevalent (40.5%), followed by *Triodontophorus serratus* (12.7%).

Table 1 - Positive cases and prevalence of each identified parasite by categories in the Przewalski's horse population. B (bachelors), F (adult females, with more than 3 years old), S (harem stallions) and J (juveniles, females or males with less than 3 years and members of a harem).

	nr of positive animals					**% of positive animals**				
	B	**J**	**F**	**S**	**Total**	**B**	**J**	**F**	**S**	**Total**
Order ASCARIDA										
Parascaris sp.	3	7	8	4	22	17.6	50	22.2	33.3	27.8
Order OXYURIDA										
Oxyuris equi	0	0	2	0	2	0	0	5.6	0	2.9
Order STRONGYLIDA										
Subfamily CYATHOSTOMINAE										
Cyathostomum s.l. **type A**	17	14	36	12	79	100	100	100	100	100
***Cyathostomum* s.l. type C**	2	6	11	5	24	11.8	42.9	30.6	41.7	30.4
Cyathostomum s.l. **type D**	7	5	18	9	39	41.2	35.7	50.0	75.0	49.4
Cyathostomum s.l. **type E**	0	2	2	0	4	0	14.3	5.6	0	5.1
Cyathostomum s.l. **type F**	4	1	8	4	17	23.5	7.1	22.2	33.3	21.5
***Cyathostomum* s.l. type H**	2	1	2	3	8	11.8	7.1	5.6	25.0	10.1
Gyalocephalus capitatus	0	1	2	1	4	0	7.1	5.6	8.3	5.1
***Poteriostomum* spp.**	4	8	9	2	23	23.5	57.1	25.0	16.7	29.1
Subfamília STRONGYLINAE										
Strongylus vulgaris	9	6	10	7	32	52.9	42.9	27.8	58.3	40.5
S. equinus	1	0	0	0	1	5.9	0	0	0	1.3
S. edentatus	1	0	1	0	2	5.9	0	2.8	0.0	2.5
Triodontophorus spp.	1	0	3	0	4	5.9	0	8.3	0.0	5.1
Craterostomum acuticaudatum	0	2	2	1	5	0	14.3	5.6	8.3	6.3
Triodontophorus serratus	1	5	4	0	10	5.9	35.7	11.1	0.0	12.7
Family TRICHOSTRONGYLIDAE										
Trichostrongylus axei	4	3	14	5	26	23.5	21.4	38.9	41.7	32.9

Some of the identified species are evidenced in Figures 22 and 23.

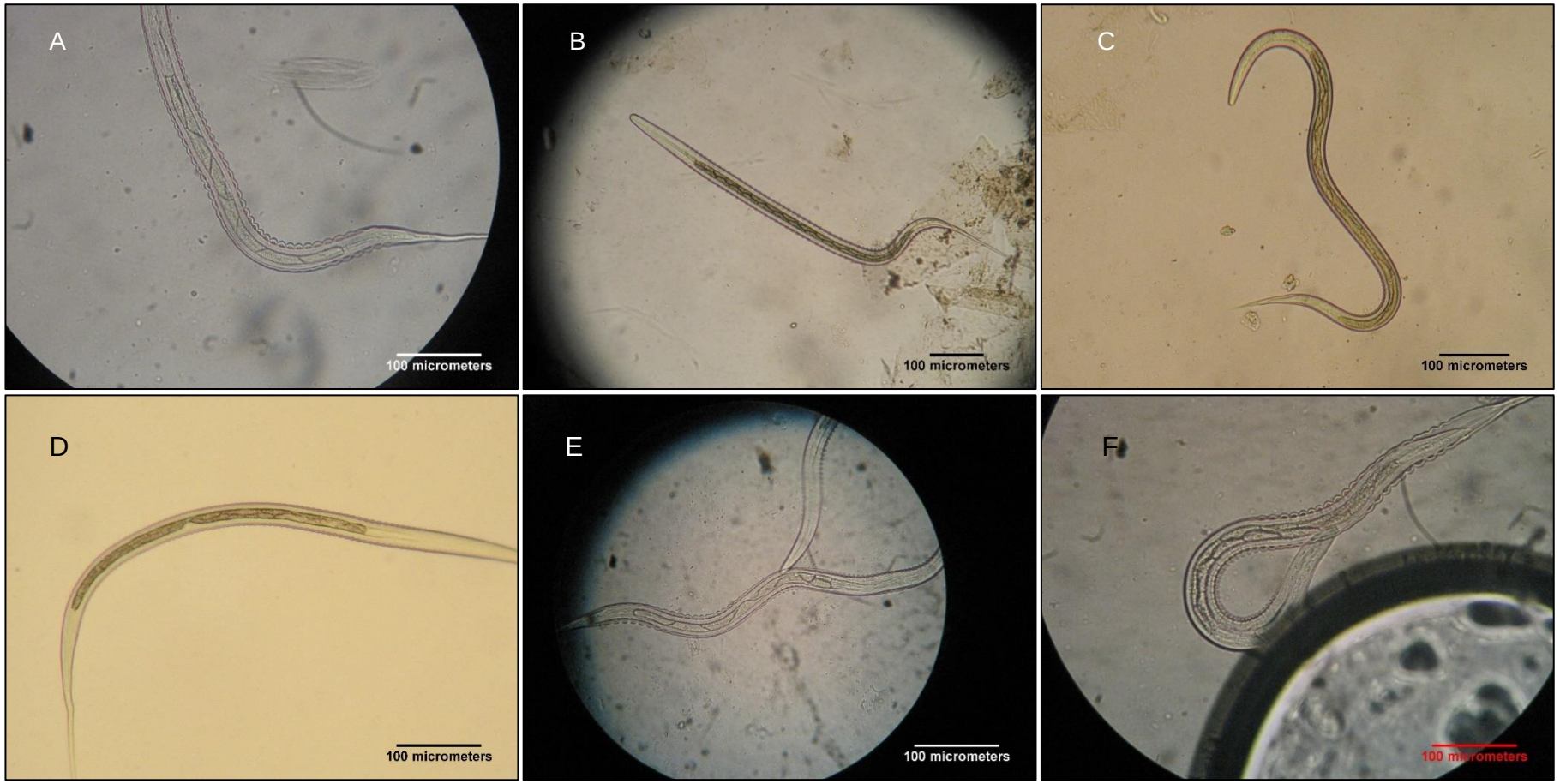
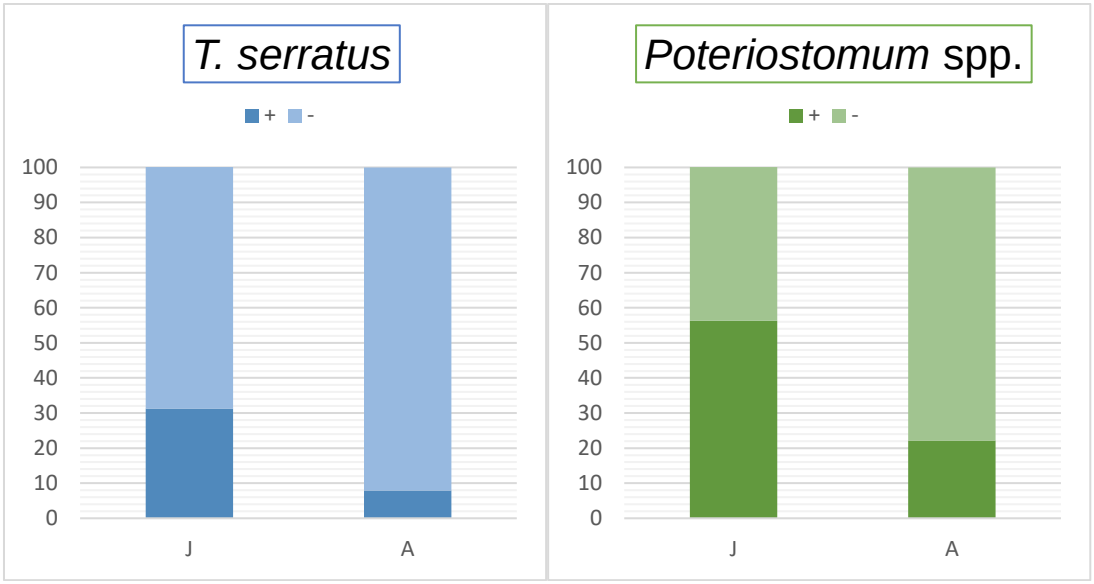


Figure 22 - Coprocultures results (part I). *Cyathostomum* type A (A), *Strongylus vulgaris* (B), *Trichostrongylus axei* (C), *Triodontophorus* spp. (D), *Cyathostomum* type H (E), *Poteriostomum* spp. (F) (Original)

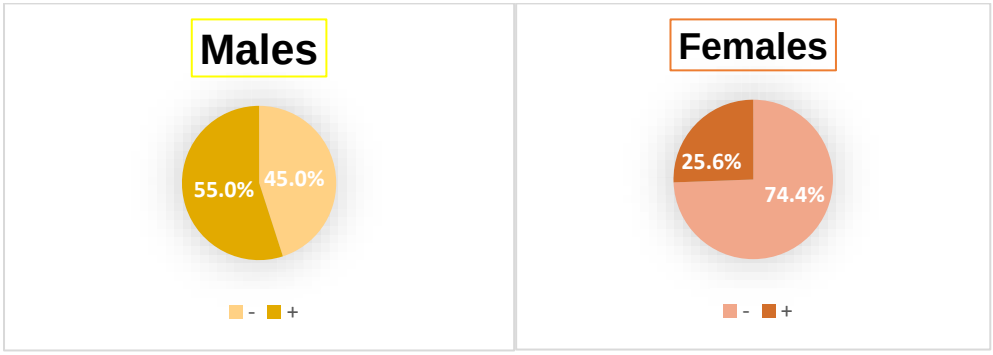
Figure 23 – Coprocultures results (part II). *Cyathostomum* type F (A), *Cyathostomum* type C (B), *Triodontophorus serratus* (C), *Cyathostomum* type D (D), *Gyalocephalus capitatus* (E), *Strongylus edentatus* (F) (Original)

Two parasites showed a significant difference between adults (>3 years old) and juveniles (<3 years old), considering the Fisher's exact test: *Poteriostomum* spp. (p-value=0.013) and *T. serratus* (p-value=0.025). Specifically, 31.3% of the juveniles were positive for *T. serratus*, comparing to 7.9% of adults. In the same way, for *Poteriostomum* spp. had a prevalence of 56.3% in young and 22,2% in adults (Graph 3) (Appendix C). The rest of the species did not show significant differences between the two age groups.



Graph 3 - Proportion of positive (+) and negative (-) animals for *T. serratus* and *Poteriostomum* spp. in juveniles (J) (<3 years old) and adults (A) (>3 years old).

Our population comprises 40 males and 39 females, and a significant difference was found only for *S. vulgaris,* according to the Chi-square test (p-value=0.008) and the Fisher's exact test (p-value=0.012). While 55%, meaning the majority of the males, were positive for this parasite, only 25.6% of the females respected this condition (Appendix D). The remaining parasites did not reveal any significant differences.



Graph 4 - Proportion of positive (+) and negative (-) cases of *S.vulgaris* for males and females (Original)

Furthermore, we tried to search for associations of 2 parasites, i.e. 2 parasites whose infection might be related. Thus, animals that are positive for *Parascaris* spp. tend to be

positive for *Cyathostomum* s.l.l type C, according to Chi-square test (p-value= 0.004) and Fisher's exact test (p-value= 0.006). In the same way, animals positive to *T. serratus* tend to be positive to *Poteriostomum* spp. for the Fisher's exact test (p-value= 0.005) (Appendix E).

In order to correlate the association of *T. serratus* and *Poteriostomum* spp. with the age (adults vs juveniles) already mentioned above, after performing a three-way cross tabulation and a Fisher's exact test, it was concluded that there is a significant association between *T. serratus* and *Poteriostomum* spp. in the juveniles (p-value=0.034). This association exists also, as previously mentioned, when we consider the entire population (p-value=0.005), but not specifically in the adults (p-value=0.307) (Appendix F).

Finally, according to the Mann-Whitney test, the EPG values were significantly different between animals positive and negative for *S. vulgaris* (p value=0.001). In fact, animals that are positive for *S. vulgaris* (n=32) have an average level of 875 EPG and, contrarily, animals that are not infected by *S. vulgaris* (n=47) have a mean value of 1567.021 EPG (Appendix G).

5 – Discussion

5.1 – Quantitative method

With the McMaster technique, the average amount of eggs detected, considering the all group of 79 samples, was 1286.7 EPG (250 to 5050), which is a high level of parasitism, according to the domestic horse values (Madeira de Carvalho, 2006). High infection levels were also reported by Kuzmina et al. (2009, 2017) in populations of the same subspecies.

On the one hand, the density of animals in this population can explain these high levels of parasitism. In the reserve, it is possible to find 0.11 equids *per* hectare. This value does not seem to be high, namely if we consider the recommendation values for domestic grazing horses (1-2 animals/ha) (Nielsen & Reinemeyer, 2018). However, 0.11 is definitely higher than the value found in the current Przewalski's horse natural habitat, Great Gobi B, which is 0.002 equids/ha, or considering also the number of Asian wild asses (ITG, 2019). Furthermore, it is also important to consider that the period of collection corresponded to a time of low quality and quantity of food, mainly at the first part of the autumn, without a significant rainy period before the gathering of faecal samples. It is possible to see in Figure 24 that the size and the colour of the pasture suggest a low quality of food. These horses were grazing too close to the soil and this behaviour increases the ingestion of faecal material and, consequently, the probability of infection (Nielsen & Reinemeyer, 2018).

Figure 24 - Pasture available as a food resource. Taken on 08/10/2018. (Original)

As mentioned in chapter 4.1, there are no significant differences in the distribution of EPG in different social categories. However, we can see that the IM and IF (<1 year old) have the lowest levels of infection. For island feral horses, from North Carolina, USA, Rubenstein & Hohmann (1989) reveal that immatures (IM and IF) are significantly less parasitised than adults, considering the same social groups. However, when they are removed from the analysis, no differences are significant between the other groups. Like in our study, the

differences between stallions and bachelors, between males and females, and also between lactating and non-lactating females, are not statistically significant.

Therefore, these results suggest that the physiological impacts of lactation and breeding hierarchy are not very meaningful in both mentioned populations. In fact, a study performed in this population, revealed no differences before and after foaling in the female body condition score which supports the idea of a low physiological impact of reproduction and lactation in females, maybe lower enough to be not statistically expressed in parasitology studies (Brabender et al., 2016).

As described in chapter 2.3, the Pentezug population usually grazes and moves together, forming a unique group, even though composed by distinct harems with strong connections. Consequently, the pasture area occupied by a harem is frequently confluent with other harems, leading to a cross-contamination of the food and water resources and causing a very similar parasite population between the different harems, as we statistically evidenced.

5.2 - Willis floatation, natural sedimentation & Baermann methods

All the 79 samples analysed were positive for strongylid-type eggs (79/79) in the Willis floatation. The same happened with Slivinska et al. (2006) and Kuzmina et al. (2009) in other Przewalski horse populations in different Ukraine regions.

From the total, 27.8% were positive to *Parascaris* sp. (22/79). Slivinska et al. (2016) present a similar prevalence for Polish primitive horses (27.4%), considering four different regions of Poland. However, considering the Przewalski's horses studies, our prevalence is higher than the one found by Slivinska et al. (2006) and Painer et al. (2011). One possible explanation, already exposed in the previous chapter, can be the higher density of susceptible hosts found in Pentezug when compared to densities revealed in previous both studies, leading to higher pasture contamination, increasing the probability of horses infection. In Pentezug, as mentioned in chapter 5.1, there are approximately 280 "Tahki" in 2388 ha, which is equivalent to an animal density around 0.11 equids/ha. For Slivinska et al. (2006), this value is about 0.0001 equids/ha and for Painer et al. (2011) it's about 0.002, both values considering the number of susceptible hosts mentioned in both publications (Nielsen & Reinemeyer, 2018).

Globally, 2.5% (2/79) of the analysed wild horses contained *Oxyuris equi* in their expelled faeces. It is known that studies using mainly coprological methods and faecal samples, instead of anal scraping may have some false negative results and, consequently, the described prevalence might be lower than in reality, due to the attachment of the eggs and cementing fluid over horse's perianal area. However, even only with faecal examinations, sometimes it is possible to find this parasite (Bowman, 2014). Using faecal samples, Slivinska et al., (2006) reported infection by *O. equi* of 81% analysed Przewalski's horses. On the other

hand, another similar study in Russia only showed negative results for this parasite (Kuzmina et al., 2017). Consequently, in faecal exams, the results for *O. equi* are very variable.

By the sedimentation method, we only could evidence a trematode egg. Since trematode infections in horses are considered to be extremely rare, we strongly suspect to be a case of pseudo-parasitism that mostly occur when the horses share a feeding area with cattle (Nielsen & Reinemeyer, 2018), which, as mentioned above, is what happens in Pentezug. Perhaps, some ruminant faecal material was attached to our samples in the ground, in the moment of the collection, leading to an observation of those when the analysis was performed.

Regarding the Baermann method, the stage of the larvae found in this method did not allow the identification of any larvae. Our main concern was the possibility of identification of lungworms, as *Dictyocaulus arnfieldi*, since it was one of the most prevalent parasites reported by Painer et al. (2011) in the *in situ* population. In fact, the donkey is the major host and most important reservoir for this parasite when these two host species share a grazing area, but many equids can act as reservoirs for this parasite (Nielsen & Reinemeyer, 2018). Painer et. al (2011) mentioned the role of the Asian Wild ass *(Equus hemionus)* as a reservoir of *D. arnfieldi* for the Przewalski's horse in Great Gobi B, in Mongolia. Consequently, it is possible to believe that our population is truly negative for this parasite since there are no other equids in Pentezug. In the same way, the Przewalski's horse populations studied by Kuzmina et al. (2009, 2017) in Ukraine and Russia are also negative for lungworms.

5.3 – Coprocultures

The coprocultures showed that 100% of faecal samples had L3 and the strongylid infections were composed by an average of, approximately, 96.3% of cyathostominae, 2.4 % strongylinae and 1.3 % tricostrongylidae, which is in accordance with the diversity of equid parasites in each one of these taxonomic groups. They also evidence parasite infections by multiple species of strongyles, 15 different strongylids *sensu latum*, which usually happens in populations that are not usually dewormed (Kuzmina et al., 2016), as the considered population. Kuzmina et al. (2017) also revealed a dominance of Cyathostominae compared to Strongylinae, with no detection of Trichostrongylidae in other population of the same subspecies, which might be explained by the reduced size of the population tested by these authors when compared to our study, since our prevalence of trichostrongylids is low, but they were found. Furthermore, Slivinska et al. (2006) didn't report any trichostrongylid infection in the CEZ in both domestic and Przewalski's horses. In this practically abandoned area, there are no other herbivores sharing the grazing area with the horses, contrarily to Pentezug, where we can find bovines. As described in previous chapters, this parasite is shared between different species of herbivores (Bowman, 2014), so this can be the reason for the presence of *T. axei* in Pentezug and absence in CEZ.

In our study, *Cyathostomum* type A were the most frequent larvae, occurring in 100% of the analysed samples, which is in accordance with a *post mortem* study, where three species of this morpho-type (*Cylicostephanus minutus*, *Cyathostomum catinatum* and *Cylicocyclus nassatus*) have more than 90% of prevalence, *C.minutus* with 100%, belonging to the most frequent parasites found (Slivinska et al., 2006).

In the subfamily Strongylinae, *Strongylus vulgaris* was the most common parasite and the same happened with Slivinska et al. (2006) and Kuzmina et al. (2009). It was found in 40.5% of the samples, which is in the middle of the prevalence described in the mentioned studies. This prevalence should be considered as threatening, due to the potentially severe consequences of the larvae migration in the gastrointestinal arterial blood system. In fact, if the faecal samples were positive, at least one migration cycle occurred inside the host. As mentioned in previous chapters, *S. vulgaris* migrations can cause arteritis, thrombosis and/or infarction at the mesenteric arterial system, leading to the sudden and unexpected death of an individual, namely due to the so called thromboembolic colic (Nielsen & Reinemeyer, 2018).

As mentioned in chapter 4.3, *S. vulgaris* has a significant different presence between males and females, according to the Chi-square test and Fisher's exact test. Essentially, males have clearly more infections by this parasite than females. One possible explanation for this result might be the differences between the average ages of both sexes. The males have an average age of 6.46 years, while females have an age of 8.64 years, considering the identified harem members. In fact, in the UK, a study performed with 1221 Thoroughbred horses, reported that strongyle prevalence is strongly influenced by the age, much more than other considered parasite genera or species (Relf, Morgan, Hodgkinson & Matthews, 2013). Furthermore, Duncan (1975) suggested two types of immunity that explain the less frequency of the *S. vulgaris* cases in older animals: the age immunity and the acquired immunity. The last one is particularly explanatory for what possibly happens in the Pentezug population. According to this author, horses exposed to a continuous infection during life develop significant resistance to reinfection with single large amounts of larvae. As mentioned above, the Pentezug population is relatively isolated in a restricted area, where the hosts are continuously eliminating eggs, permanently contaminating the soils with large amounts of parasites and infecting each other mutually. This real situation is very close to the one experimentally referred by Duncan (1975) and might be the reason for the development of an acquired immunity resistance in females, older than males.

The second most prevalent large strongylid was *Triodontophorus serratus*, found in 12.7% of the animals, and there is a statistically significant difference between adults (>3 years old) and juveniles (<3 years old) for the presence of this parasite. Specifically, 31.3% of the juveniles are positive for this parasite, comparing to 7.9% of adults. In fact, a *post mortem* study of 134 horses in Victoria, Australia, reported a prevalence of 26% for *T.serratus* in

juvenile horses (with less than 2 years old). This was the group with the highest prevalence for this parasite in the study and the difference was also statistically significant (Bucknell, Gasser & Beveridge, 1995).

In the same way, *Poteriostomum* spp. has a higher prevalence in juveniles than in adults. A study done by Kuzmina et al. (2016) reported that *Poteriostomum imparidentatum* is more prevalent in animals with less than 4 years old: the group with 1.5-4 years old had the highest value (15.7%), followed by the group of equids with less than 1-year-old (6.9%). According to Love & Duncan (1992), there are three reasons for the development of acquired immunity to cyathostomins, as *Poteriostomum* spp., in older horses. First, the development of the cyathostomins becomes progressively slower, after successive infections. Second, adult hosts produce fewer eggs than juveniles, so the amount of L3 in faeces is lower and the probability of detection is also lower in adults. Finally, the hypobiosis seem to be more frequent in younger animals, leading them to infections that are more frequent by arrested larvae. In this way, we believe that an acquired immunity for *Poteriostomum* spp. can be an explanation for the detected difference between youngers and adults in the studied population.

In fact, the protective immune response described for *S. vulgaris, T. serratus and Poteriostomum* spp. is also very significant in a clinical and therapeutic point of view. After all, for domestic horses, it's being suggested that older grazing animals don't require specific husbandry measures or intensive treatment to avoid parasitic clinical disease, due to their acquired immunity to different nematodes (European Scientific Counsel Companion Animal Parasites [ESCCAP], 2018).

Regarding the parasite associations, we found no other studies that report an association between *Parascaris* sp. and *Cyathostomum* type C or *T. serratus* and *Poteriostomum* spp. in the consulted literature. Even though, the probability of this association being due to coincidence is very low, it exists and can be eventually considered. However, it's been described that interspecific interactions between parasites in a single host can occur and be synergic. For instance, one parasite can manipulate the immune system or modify the intern conditions of the host, turning it favourable to the other parasite (Poulin, 2001; Cézilly et al., 2014) . In this way, we can consider the hypothesis that parasite cooperation exists between *Parascaris* sp. and *Cyathostomum* type C or *T. serratus* and *Poteriostomum* spp., leading to the presence of both in the same hosts. Considering specifically *Poteriostomum* spp. and *T. serratus*, it is possible that the connection found between these two species is related to their separate association with age. In other words, a possible explanation for the association of these two species of parasites can be their common propensity for younger individuals. That is the reason why we performed the three-way cross-tabulation mentioned in chapter 4.3, showing a significant association between *T. serratus* and *Poteriostomum* spp. in the juveniles but not in the adults, consistently supporting the exposed hypothesis. Also regarding age-

differences, according to Cabaço (2014), the equid juveniles can usually have the habit of eating faeces from the adults, which contributes to a more frequent ingestion of parasites eggs and larvae, increasing the chances of infection and parasites prevalence in this age group.

In contrast, we believe that the relation of *S. vulgaris* with the less parasitized individuals (i.e. with lower EPG), can be explained by possible antagonistic relations established by this parasite. These antagonistic relations between two parasites can be intraspecific or interspecific. Therefore, some parasites can induce microenvironmental changes inside the host, turning it unfavourable to other parasites, from the same species or not. These changes can be direct, caused by the parasite, or indirect, with the intervention of the immune system (Cézilly et al., 2014). A study performed by Poulin (2001) describes the competitive interactions between helminths and their consequences; one of them is the numerical response. Numerical responses are essentially the numerical changes in one or more parasite species induced by another one. According to this author, numerical responses can be common and substantial, with a possible reduction of 50% in some parasite species compared to others. Possibly, in our population, *S. vulgaris* is creating a numerical response in some other species of parasites, leading to lower values of EPG in those hosts.

6 – Conclusions

As previously mentioned, this represents the most detailed parasitology survey done in the Pentezug population, with a higher number of samples when compared to similar studies performed in other European populations of Przewalski's horses and consistent with those. In this way, it represents on a real basis the gastrointestinal parasite community of this population.

Comparing with other studies in "Takhi", we believe that the high FECs and the dominance of strongylids globally found in the population are strongly related to the animal density present in the area. In the same way, we also think that the animal density is responsible for the high prevalence of *Parascaris* sp. (27.8%), even though this value is similar to the one found in Polish primitive horses in other studies and areas. Furthermore, the prevalence of *O. equi* (2.5%) can be underestimated due to the collection method. Additionally, the presence of the cattle can influence the results of the horses, mainly increasing the amount of *T. axei* and allowing the pseudo-parasitism by a trematode egg.

We consider particularly important the high prevalence of *S. vulgaris* (40.5%), due to its high pathogenic potential. This parasite is more prevalent in males than in females, possibly due to the age difference between sexes, since females are older and, consequently, more resistant compared to males, due to acquired immunity. *T. serratus* is our second most prevalent large strongyle (12.7%) and it is frequently associated with *Poteriostomum* spp., possibly due to their common affinity with juvenile hosts (<3 years old). Moreover, we found an association between *Parascaris* sp. and *Cyathostomum* type C that we attribute to a possible synergic interaction between both parasites. In contrast, we believe that *S. vulgaris* can cause antagonism with other parasite species.

We hope this study can be an impulse for more parasitology surveys in Przewalski's horses. We firmly believe that the continuous monitoring and further research in this and other *ex situ* populations of Przewalski's horses is vital to understand in detail the impact of the parasitism in this Endangered animal and, consequently, in its conservation.

7 – Study limitations

We cannot exclude the possibility of lapses during the identification of individuals, which could, for instance, result in an incorrect categorisation of an animal in a category. Factually, they can lead to some parasitology results and statistic values that are disconnected with reality. Even though the visual identification is still used on many occasions in domestic equids, it is not as precise as electronic or branding identification, mainly when performed at a distance (Cordes, 2000). Furthermore, even though the harem connections are relatively stable, some changes on the harem structure and composition could have occurred during the period of collection, impairing the identification process.

In some cases, the identification is only possible until the morphologic group or parasite genus. Therefore, the identification key used is based on the domestic horse parasites and their morphology and, sometimes, it is possible to have little parasite differences between distinct equid species or subspecies (Lichtenfels et al., 2008).

8 – Recommendations and Future Perspectives

To understand the dynamics of the parasitism in the studied population, we strongly support the implementation of a monitoring program at Pentezug with some FECs per year, in different seasons, to assess which individuals or social groups are more vulnerable to the GI parasitism. This recommendation was accepted by the local team and we are currently planning it. Globally, this monitoring plan can help in sanitary decisions, as additional parasite control or animal density regulations, and in the selection of the individuals to the reintroduction plan. With the information provided by this monitoring plan, a targeted selective treatment can be implemented and developed. According to ESCCAP Guideline 8 (2018), a selective treatment should be applied in the horses that have relevant FEC, after the monitoring results. Nevertheless, this treatment is especially recommended for adult horses and for small strongyles control. Therefore, we should not forget that this is a wild subspecies and, comparing to domestic horses, wild horses tend to be more stoic and resistant to pathogens, showing clinical signs only in heavy infections (Kuzmina et al., 2009). Furthermore, we think that a frequent anthelminthic treatment performed to all the individuals of a certain group (strategic treatment) can be too expensive and not appropriate for a wild subspecies population.

Furthermore, because of the described pathogenic potential of *S. vulgaris*, we truly recommend the appropriate necropsy of the dead animals, especially those who suffered sudden deaths. This might be helpful not only to understand its real pathogenic potential at Pentezug population in particular, but also to analyse if this parasite is an important threat for this population and, consequently, for the conservation of the subspecies. Additionally, the obtained information might be helpful to plan future treatment strategies.

Even though we could not evidence any susceptibility or resistance for parasitism between the harems, a genetic study might be adequate to study this topic in detail. Almost all the horses are registered in a genetic database, due to the collaboration of the University of Davis. Therefore, it might be interesting for the subspecies preservation to use this genetic data to analyse the GI parasitism according to genetic susceptibilities or resistances. Indeed, it would give valuable information about certain harems or lineages, considering the reintroduction program and the parasite population *in situ*, in Mongolia.

We consider that it might be interesting to create an electronic or branding identification of the individuals, concerning the genetic results. Even though the visual identification is still used in many occasions in domestic equids, it is not as precise as the previously mentioned methods, mainly when performed at a distance, leading to some identification lapses and, consequently, incorrect diagnoses in particular cases.

We believe that it would be relevant to associate the morphological techniques with the molecular methods, to reinforce and detail the presented diagnoses, associations and hypothesis. In some cases, the identification is only possible until the morphologic group or parasite genus. In another way, the identification key used is based on the domestic horse parasites and their morphology and, sometimes, it is possible to have little differences in the morphology of the eggs and larvae of the same parasite in different host species or subspecies.

Finally, we recommend further research in this and other *ex situ* populations of Przewalski's horses in order to know in detail the impact of parasitism in this endangered animal and, consequently, for its conservation. Furthermore, comparing studies between the *in situ* and *ex situ* populations can be helpful to prepare future reintroductions and, for instance, to understand the different health pressures that affect the subspecies in different geographical locations.

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Appendices

Appendix A - Identified collected samples organized by group number

From the 79 samples collected, 62 are from harem members, 2 are from identified bachelors, 15 are from unknown bachelors
Horses names organized by seniority in the harem. Some foals born in 201 (first letters “V” or “W”) were not named yet when the samples were collected.
The bold names correspond to the harem stallion of which group.

1.	3.	5.	7.	10.
F.Soos (♂)	**Huba (♂)**	**Igar (♂)**	Málna (♀)	Ibolya (♀)
2.	Kisasszony (♀)	Gerle (♀)	Thetis(♀)	Kecses (♀)
Farkas (♂)	Tas(♂)	Kikerics (♀)	8.	Uriel(♂)
	Tamás(♂)	Léka (♀)	**Jós(♂)**	11.
	Vanessza(♀)	Mimóza (♀)	Epona (♀)	Onedin (♀)
	4.	Vándor(♂)	Pitypang (♀)	Eper (♀)
	Holló (♂)	Vihar(♂)	9.	12.
	Emese (♀)	6.	**Kerecsen (♂)**	Imola (♀)
		Mályva (♀)	Gréta (♀)	
			Kankalin (♀)	
			Rasputin(♂)	

13.	17.	19.	22.	26.
Piroska (♀)	Gizella (♀)	Thunder(♀)	Ofélia (♀)	unknown (♀)
Teodor(♂)	Hímes (♀)	21	Rubin(♀)	27.
14.	Nóra (♀)	**unknown (♂)**	23.	**unknown (♂)**
Mohar (♂)	Nutella (♀)	Nőci (♀)	**unknown (♂)**	V…(♀)
Olga (♀)	Ronni(♀)	Rumba(♀)	25	
Pemzli (♀)	18.	Titán(♂)	Juhar (♀)	
16	**unknown (♂)**	Tacco (♀)		
Pajzsika (♀)	Panda (♀)	Vas(♂)		
Ujjongo(♂)	Panka (♀)			
	Varázs(♂)			

List of identified bachelors

Orix

Luxus

Appendix B.1 – Dichotomic key used to identify the L3 larvae obtained after the coproculture

(adapted and translated from Madeira de Carvalho et al., 2008)

1. Larvae without sheath..... **2**
Larvae with sheath.....**3**

2. Presence of visible mouth, rhabditiform esophagus (with bulb), presence of males, females and eggs in the same microscopic field, become hyper-stained by the lugol solution ..... **Free living nematodes**
Filariform esophagus occupying more than 1/3 of the body length, larva tail ends in small v-shape …….....***Strongyloides westeri***

3. Very short sheath tail, 80 to 115 µm from the anus to the posterior end of the sheath, not whip-shaped, with an overall mean length of 738 µm…….. ***Trichostrongylus axei***
Long sheath tail (≥ 175 µm) and whip-shaped **4**

4. Medium-sized larvae with 6 to 9 intestinal cells, with average total lengths from 773 to 886 µm. ……..... **5**
Small to large larvae with more than 9 intestinal cells, average total lengths from 731 to 992 µm **8**

5. Medium-sized larvae with 8 well-organized intestinal cells and shape, average total lengths from 812 to 848 µm ..... **6** (*Cyathostomum sensu latum* types A, B, C e D).
Medium to large-sized larvae with 6 to 9 intestinal cells without organization and shape, average total length from 773 to 886 µm ... **7** (*Cyathostomum sensu latum* types E, F, G e H).

6. Medium-sized larvae with 8 intestinal cells, where the first two (triangular or rectangular) form a double row and the remaining six (trapezoidal or rectangular) form a single row, average total length of 812 µm……..***Cyathostomum* spp. type A**
Medium-sized larvae with 8 double-row triangular or pentagonal intestinal cells, mean total length of 828 µm ..... ***Cyathostomum* spp. type B**
Medium to large larvae with 8 intestinal cells, in which the first four form a double row (pentagonal, triangular or rectangular) and the remaining four (trapezoidal) are arranged in a single row, average total length of 848 µm. .....…… ***Cyathostomum* spp. type C**
Medium to large larvae with 8 single-row intestinal cells with trapezoidal or triangular shape, average total length of 843 µm …….. ***Cyathostomum* spp. type D**

7. Small larvae with 6 triangular and / or trapezoidal intestinal cells, arranged differently, in double or single row, lower average total length of this group, 773 µm……..... ***Cyathostomum* spp. type E**
Medium-sized larvae with 7 differently arranged triangular and trapezoidal intestinal cells, 2-4 cells in double row and the remaining in single row or mixed arrangement. Average total length of 824 µm ..... ***Cyathostomum* spp. type F**

Medium to large larvae with 8 triangular and / or rectangular (elongated and narrow) intestinal cells, trapezoidal (distal portion), miscellaneous arrangement, average total length of 848 µm ..... ***Cyathostomum* spp. type G**

Large larvae with 9 elongated triangular intestinal cells, the first 6 in a double row and the remaining in a single row,> mean total length of this group 886 µm ……..... ***Cyathostomum* spp. type H**

8. Larvae with 12 intestinal cells arranged in double row (rectangular and pentagonal shaped cells), or 6-10 paired cells and the remaining single row (trapezoidal and triangular), with an average total length of 731 µm. ..... ***Gyalocephalus capitatus***

Larvae with more than 12 intestinal cells **9**

9. Larvae with 16 intestinal cells …… ....… **10**

Larvae with more than 16 intestinal cells **12**

10. Medium size larvae (average length 786 µm and average width 28 µm), with rectangular and pentagonal intestinal cells, with a larval body / distal portion (lb / dp) ratio = 2.1: 1 ... . ***Poteriostomum* spp.**

Large size larvae **11**

11. Large larvae (average length 992 µm and average width 35 µm), long intestine (415 µm) and large, triangular, sometimes elongated pentagonal cells, lb / dp ratio = 2; 4: 1 ..... ……..……***Oesophagodontus robustus***

Large larvae (average length 862 µm and average width 29 µm) with rectangular (proximal, double-row cells), pentagonal and triangular cells (distal cells, intermediate position or single terminal cell), w / d ratio = 1.8: 1 ..... ***Craterostomum acuticaudatum***

Large larvae (average length 907 µm and average width 30 µm), with elongated rectangular proximal cells and the remaining pentagonal cells. The two distal cells are asymmetrical, one is half the length of the other but they end at the same level, proportion lb / dp = 1.7: 1 ..... ***Triodontophorus serratus***

Large and thin larvae (average length 901 µm and average width 18 µm), poorly differentiated intestinal cells, poorly distinguished transition between oesophagus and intestine, tail of the larva with a lobe at end, short sheath tail, proportion lb / dp = 4.1: 1 ……..…….. ***Strongylus equinus***

12. Larvae with 18 to 20 intestinal cells..... **13**

Larvae with more than 20 intestinal cells..... **14**

13. Small to medium-sized larvae, thin (average length 789 µm and average width 23 µm), with narrow and elongated triangular intestinal cells, poorly defined, short oesophagus, w / w ratio = 2.2: 1. ..... ***Strongylus edentatus***

Large to medium-sized larvae, thick (average length 834 µm and average width 28 µm), with pentagonal (most frequent), rectangular and triangular intestinal cells (distal, juxtaposed or in

intermediate position), long oesophagus (about of 1/3 of the larva's body length), lb / dp ratio = 2.1: 1… ……..... ***Triodontophorus* spp.**

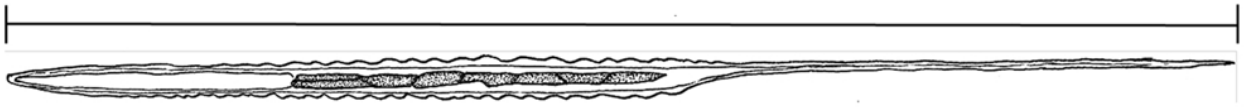
(Except Triodontophorus serratus)

14. Large and thick larvae (average length 936 µm and average width 32 µm), with a well-defined pentagonal and triangular intestinal cells, very dark, short oesophagus, lb / dp ratio = 2.8: 1 ..... ***Strongylus vulgaris***.

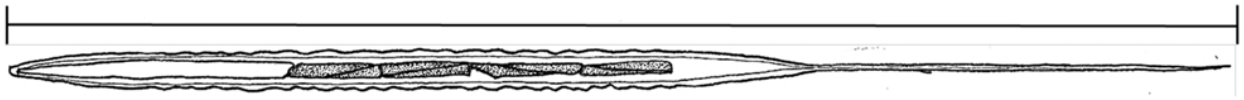
Appendix B.2 – Illustrated key for the identification third stage cyathostomin larvae

(adapted from Santos et al., 2018)

1. Eight IC, (2+6 IC); the two first are triangular or rectangular arranged in a double row, and the six last are trapezoidal or rectangular in a single line. *Cylicocyclus insigne*, *Cc. nassatus*, *Cc. radiatus*, *Cylicostephanus minutus*, *Cyathostomum catinatum*, *Cy. pateratum*, *Petrovinema poculatum* .... Type A

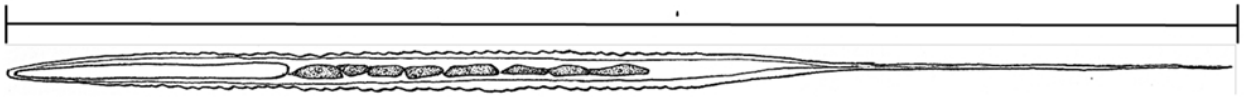


2. Eight IC, (4+4 IC); triangular or pentagonal shape organized in a double line of four cells in each side. *Cc. brevicapsulatus*, *Cc. ultrajectinus*, *Cylicodontophorus bicoronatus*..... Type B

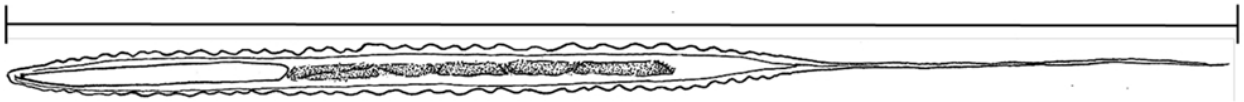


3. First four IC in a pentagonal triangular or rectangular shape, settled in a double line, and the four last are trapezoidal in a single line (2+2+4 IC). *Cyl. calicatus*, *Cyl. hybridus*..... Type C

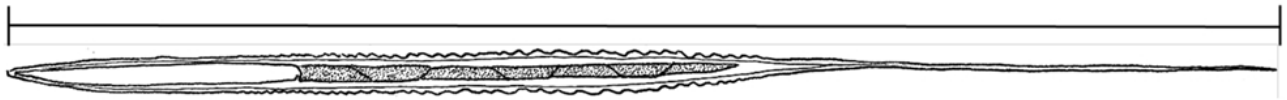
4. Eight IC of trapezoidal or triangular shape, arranged in a single line ..... Type D



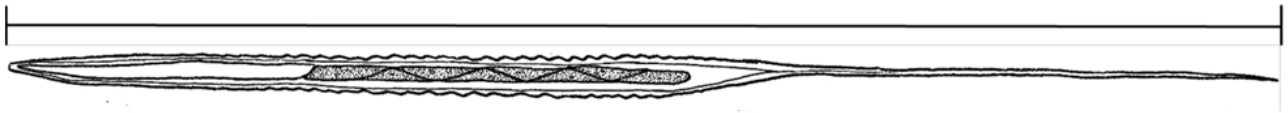
5. Six triangular and/or trapezoidal IC in different arrangements distributed as a single or double line (6 IC) ..... Type E



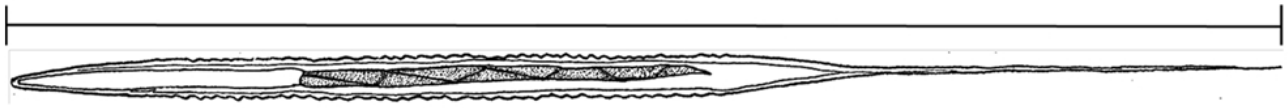
6. Seven elongated triangular or trapezoidal IC in a variety of arrangements; 2-4 in a double line, and 2-3 as a single line (7 IC) ..... Type F



7. Eight IC distributed in an uncharacterized arrangement with triangular and/or rectangular (elongated) shape (8 IC type with uncharacterized arrangement) ..... Type G



8. Nine elongated triangular IC, the six first in a double row and the three last in a single line (9 IC) ..... Type H



9. 12 IC arranged in a double line, in a rectangular and pentagonal shape, or 6 to 10 IC in a double row, and the others in a single line in trapezoidal and triangular format..... *Gyalocephalus capitatus*

10. 16 rectangular or trapezoidal IC, arranged in a double line ..... *Posteriostomum* spp.

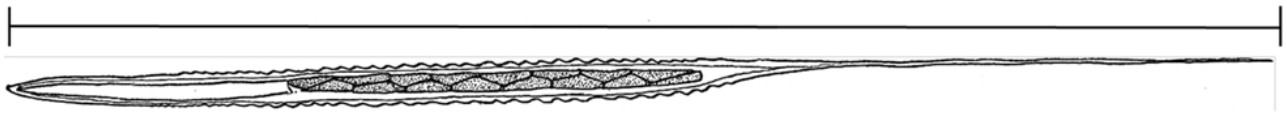


Figure 25 - Key for the identification third stage Cyathostomin larvae of equids using numbers and shapes of intestinal cells (IC) (Santos et al., 2018)

Appendix C - Chi-square test and Fisher's exact test for *T.serratus* in adults and juveniles and for *Poteriostomum* spp. in adults and juveniles

T.serratus

Crosstab

			Tserratus		
			0	1	Total
adultjuv	0 (<3yo)	Count	11	5	16
		% within adultjuv	68,8%	31,3%	100,0%
	1 (>3yo)	Count	58	5	63
		% within adultjuv	92,1%	7,9%	100,0%
Total		Count	69	10	79
		% within adultjuv	87,3%	12,7%	100,0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6,273[a]	1	,012		
Continuity Correction[b]	4,341	1	,037		
Likelihood Ratio	5,210	1	,022		
Fisher's Exact Test				**,025**	,025
N of Valid Cases	79				

a. 1 cells (25,0%) have expected count less than 5. The minimum expected count is 2,03.

b. Computed only for a 2x2 table

Poteriostomum spp.

Crosstab

			Poteriostomum		
			0	1	Total
adultjuv	0 (<3yo)	Count	7	9	16
		% within adultjuv	43,8%	56,3%	100,0%
	1 (>3yo)	Count	49	14	63
		% within adultjuv	77,8%	22,2%	100,0%
Total		Count	56	23	79
		% within adultjuv	70,9%	29,1%	100,0%

Chi-Square Tests

	Value	Df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7,159[a]	1	,007		
Continuity Correction[b]	5,605	1	,018		
Likelihood Ratio	6,628	1	,010		
Fisher's Exact Test				**,013**	,011
N of Valid Cases	79				

a. 1 cells (25,0%) have expected count less than 5. The minimum expected count is 4,66.

b. Computed only for a 2x2 table

Appendix D - Chi-square test and Fisher's exact test for *S.vulgaris* between genders

Género * *S vulgaris* Cross tabulation

			Svulgaris		
			-	+	Total
Gender	F	Count	29	10	39
		% within Género	74,4%	25,6%	100,0%
	M	Count	18	22	40
		% within Género	45,0%	55,0%	100,0%
Total		Count	47	32	79
		% within Género	59,5%	40,5%	100,0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7,063[a]	1	,008		
Continuity Correction[b]	5,897	1	,015		
Likelihood Ratio	7,198	1	,007		
Fisher's Exact Test				,012	,007
N of Valid Cases	79				

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 15,80.

b. Computed only for a 2x2 table

Appendix E - Associations between parasites: *Parascaris* sp.*Cyathostomins Type C and *T.serratus** *Poteriostomum* spp.

Parascaris * CyaTipC Crosstabulation

			CyaTipC		
			0	1	Total
Paras	0	Count	45	12	57
		% within Paras	78,9%	21,1%	100,0%
	1	Count	10	12	22
		% within Paras	45,5%	54,5%	100,0%
Total		Count	55	24	79
		% within Paras	69,6%	30,4%	100,0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	8,419[a]	1	**,004**		
Continuity Correction[b]	6,910	1	,009		
Likelihood Ratio	8,033	1	,005		
Fisher's Exact Test				**,006**	,005
N of Valid Cases	79				

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 6,68.

b. Computed only for a 2x2 table

Tserratus * *Poteriostomum* Cross tabulation

			Poteriostomum		
			0	1	Total
Tserratus	0	Count	53	16	69
		% within Tserratus	76,8%	23,2%	100,0%
	1	Count	3	7	10
		% within Tserratus	30,0%	70,0%	100,0%
Total		Count	56	23	79
		% within Tserratus	70,9%	29,1%	100,0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	9,274[a]	1	,002		
Continuity Correction[b]	7,144	1	,008		
Likelihood Ratio	8,350	1	,004		
Fisher's Exact Test				**,005**	,005
N of Valid Cases	79				

a. 1 cells (25,0%) have expected count less than 5. The minimum expected count is 2,91.

b. Computed only for a 2x2 table

Appendix F - Three-way cross tabulation and Fisher's exact test for *T.serratus*Poteriostomum spp.* in adults and juveniles

Tserratus * *Poteriostomum* * adultjuv Crosstabulation

adultjuv				Poterium 0	Poterium 1	Total
0	Tserratus	0	Count	7	4	11
			% within Tserratus	63,6%	36,4%	100,0%
		1	Count	0	5	5
			% within Tserratus	0,0%	100,0%	100,0%
	Total		Count	7	9	16
			% within Tserratus	43,8%	56,3%	100,0%
1	Tserratus	0	Count	46	12	58
			% within Tserratus	79,3%	20,7%	100,0%
		1	Count	3	2	5
			% within Tserratus	60,0%	40,0%	100,0%
	Total		Count	49	14	63
			% within Tserratus	77,8%	22,2%	100,0%
Total	Tserratus	0	Count	53	16	69
			% within Tserratus	76,8%	23,2%	100,0%
		1	Count	3	7	10
			% within Tserratus	30,0%	70,0%	100,0%
	Total		Count	56	23	79
			% within Tserratus	70,9%	29,1%	100,0%

Chi-Square Tests

adultjuv		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
0	Pearson Chi-Square	5,657[c]	1	,017		
	Continuity Correction[b]	3,366	1	,067		
	Likelihood Ratio	7,509	1	,006		
	Fisher's Exact Test				**,034**	,029
	N of Valid Cases	16				
1	Pearson Chi-Square	,993[d]	1	,319		
	Continuity Correction[b]	,190	1	,663		
	Likelihood Ratio	,874	1	,350		
	Fisher's Exact Test				**,307**	,307
	N of Valid Cases	63				
Total	Pearson Chi-Square	9,274[a]	1	,002		
	Continuity Correction[b]	7,144	1	,008		
	Likelihood Ratio	8,350	1	,004		
	Fisher's Exact Test				**,005**	,005
	N of Valid Cases	79				

a. 1 cells (25,0%) have expected count less than 5. The minimum expected count is 2,91.

b. Computed only for a 2x2 table

c. 3 cells (75,0%) have expected count less than 5. The minimum expected count is 2,19.

d. 2 cells (50,0%) have expected count less than 5. The minimum expected count is 1,11.

Appendix G - Mann-Whitney test for EPG in presence or absence of *S.vulgaris*

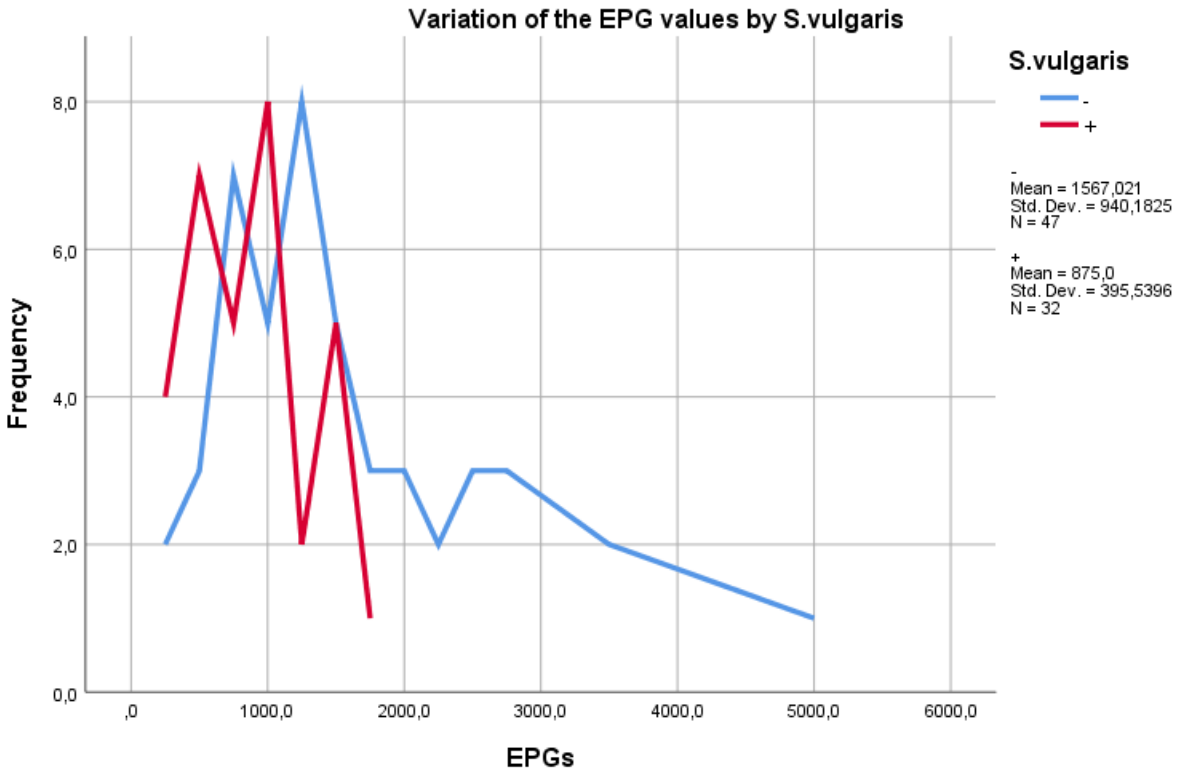
Ranks

	S.vulgaris	N	Mean Rank	Sum of Ranks
EPGsMcMaster	0	47	48,04	2258,00
	1	32	28,19	902,00
	Total	79		

Test Statistics[a]

	EPGsMcMaster
Mann-Whitney U	374,000
Wilcoxon W	902,000
Z	-3,778
Asymp. Sig. (2-tailed)	**,000**

a. Grouping Variable: Svulgaris



Appendix H – Full Text submitted for the IZW/EAZWV/ECZM Conference Proceedings 2019 as a Poster communication

GASTROINTESTINAL PARASITES IN PRZEWALSKI HORSES (*EQUUS FERUS PRZEWALSKII*) AT HORTOBÁGY NATIONAL PARK, HUNGARY – PRELIMINARY RESULTS

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Summary

A collection of 79 faecal samples was performed between September and November 2018 from a population with approximately 280 Przewalski horses (*Equus ferus przewalskii)* from the Pentezug Wild Horse Reserve*.* The coprological methods (McMaster, Willis flotation, natural sedimentation, Baermann and coproculture) and microscopical identification were performed in all the samples. Results show an average level of 1287 Eggs per Gram (EPG), which is considered a high level of parasitism. All the 79 analysed samples were positive for strongylid-type eggs (100% prevalence). Cyasthostominae were dominant, when compared to strongylinae and tricostrongylidae and with a total of 15 different morphological L3 types and/or species identified of the order Strongylida. Additionally, 27.8% were positive to *Parascaris* sp. and 2.5% contained *Oxyuris equi* in their expelled faeces. By the sedimentation method, we could only evidence a Trematoda egg. These results are consistent with the other studies performed in the same subspecies and represent the first survey of gastrointestinal parasites performed with this level of detail in the Pentezug Przewalski horse population.

Introduction

Przewalski horses (*Equus ferus przewalskii*) were introduced in the Hortobágy National Park (HNP), in 1997, with the main goal of managing the landscape in the Pentezug area of the HNP. It represented a good opportunity to study wild horses in a semiwild habitat, directly helping the conservation of this subspecies and other populations of *E. ferus* spp. Nowadays, the number of individuals is about 280, and besides the Przewaslki horse, a herd of domestic cattle (*Bos primigenius taurus*), carefully bred to phenotypically resemble reconstructed aurochs (*Bos primigenius*), use this area for grazing (ZIMMERMAN et al., 2009). In equids, nematodes, when compared to cestodes or trematodes, are the group responsible for the greater diversity of parasites, which includes ascarids, as *Parascaris* sp. , pinworms, as *Oxyuris equi* and many strongylids, all members of the superfamily Strongyloidea, except *Trichostrongylus axei*, from the Trichostrongyloidea (BOWMAN, 2009). Besides the species of parasites involved, the amount of parasites present is very relevant and defines if it represents an animal health and clinical problem for the individual or population. Equids, as a group of species, are more sensitive to parasites than, for example, sheep, goat or cattle, which leads us to consider faecal egg counts as lower than 500 Eggs per Gram (EPG) a low infection, 550 to 1000 EPG a moderate infection and more than 1000 EPG a high infection (SOULSBY, 1986; MADEIRA DE CARVALHO, 2006). Most of the studies performed in Przewalski horses under semi-natural conditions in other geographic regions are based in

smaller populations and using an *in vivo* deworming method before the collection (SLIVINKSA et al., 2006; KUZMINA et al., 2009, 2017), which was not done in this study, since the Pentezug population is managed without human interference or routine parasite control. Consequently, deworming is only performed in particular occasions, as the translocations of specific individuals. Regarding the described situation, the main goal of this study was performing a general but detailed survey of the gastrointestinal parasitology of the Pentezug population, as a tool for future parasite monitoring plans of these Przewalski horses.

Material and methods

Individual identification and sample collection

The animals were observed and identified, while defecating in a range of 50-100 meters. The majority of the observations were performed at the resting moments, while animals were eating and resting. Thereby, the identification was possible for most of the individuals due to the strong harem connections and proximity that defines the wild horses (RUBENSTEIN & HOHMANN, 1989). When an animal started defecating, the animals surrounding it were observed and the harem was determined and then, by using binoculars if necessary, it was possible to sex it and observe its body details, identifying the individual. However, most of the bachelors were not possible to be identified, since they do not establish the strong connections that we find inside the harem, which leads them to have irregular positions surrounding different harems. Afterwards, a small amount of fresh faeces was collected from the ground, using individual identified plastic bags and preserved at 4-5ºC until reaching the laboratory. From the 79 samples collected, 62 were from identified harem members, 2 were from identified bachelors and 15 were from unknown bachelors.

Coprological methods

To quantify the EPG and evaluate the degree of parasite infection, we used the McMaster Technique. By mixing two grams of faeces with 28 ml of a saturated sucrose solution and filling a 0,30 ml Eggzamin® chamber, the total number of counted eggs inside the grids of both compartments is multiplied by 50 as a conversion factor, in order to obtain the EPG for each sample (MADEIRA DE CARVALHO, 2001). The Willis floatation and natural sedimentation methods, were performed afterwards in order to identify the light and heavy parasite eggs, respectively, present in the samples (LAJAS *et al*., 2015). The Baermann method allows the migration and concentration of the L1 respiratory larvae present in a piece of faeces in the bottom of a conical cup full of water (LAJAS *et al.* (2015). Coprocultures were performed in order to obtain L3 infective strongylid larvae, which allowed the differentiation of parasites of this group, namely due to a recent dichotomic key and to the cyathostomins visual details recently described by SANTOS et. al, (2018). To have an idea of the proportion of infection by Strongylinae, Cyathostominae and Tricostrongylidae, a total of 100 matured L3 larvae were counted and identified in each sample, in order to obtain the percentages of each mentioned family and subfamilies of the Order Strongylida.

Results and discussion

The average EPG for the whole 79 samples, was 1287 EPG (ranging from 250 to 5050), which is a high level of parasitism, according to the domestic horse values (SOULSBY, 1986; MADEIRA DE CARVALHO, 2006). KUZMINA et al. (2009, 2017) also reported high infection levels in other Przewalski horse populations. In reality, more than a half of the population (43/79) revealed a high infection level. In fact, 11 samples had less than 500 EPG, revealing a lower level of infection, 25 samples had between 550 and 1000 EPG, a medium level of infection and, finally, 43 samples showed more than 1000 EPG, which is considered a high level of infection, according to the domestic horse evaluation levels (SOULSBY, 1986; MADEIRA DE CARVALHO, 2006).

The different diagnoses found with coprology methods are illustrated in Fig.1. All the 79 analysed samples were positive for strongylid-type eggs (79/79) in the Willis floatation, the same happened with SLIVINSKA et al. (2006) and KUZMINA et al. (2009) in other Przewalski horse populations in different Ukraine regions.

From the total, 27.8% were positive to *Parascaris* sp. (22/79), which is higher than the one found by SLIVINSKA et al. (2006) and PAINER et al. (2011). In fact, Pentezug has a higher density of susceptible hosts when compared to densities presented in both previously mentioned studies, leading to a higher pasture contamination and increasing the probability of horse infection.

Globally, 2.5% (2/79) of the analysed wild horses contained *Oxyuris equi* in their expelled faeces. It is known that studies using mainly coprological methods and faecal samples, instead of anal scraping, may have some false negative results. Consequently, the described prevalence might be lower than the real one, due to the attachment of eggs and cementing fluid over the horse perianal area. However, with only faecal examinations, sometimes it is possible to find this parasite (BOWMAN, 2009). Using faecal samples, SLIVINSKA et al., (2006) reports infection by *O. equi* of 81% analysed Przewalski horses. On the other hand, other similar study in Russia only showed negative results for this parasite (KUZMINA et al., 2017). Consequently, with faecal exams, the results for *Oxyuris equi* are very variable.

By the sedimentation method, we only could evidence a trematode egg. Since trematode infections in horses are considered to be extremely rare, we strongly suspect to be a case of pseudo-parasitism. This phenomenon mostly occur when the horses share a feeding area with cattle (NIELSEN & REINEMEYER, 2018), which, as mentioned above, is what happens in Pentezug.

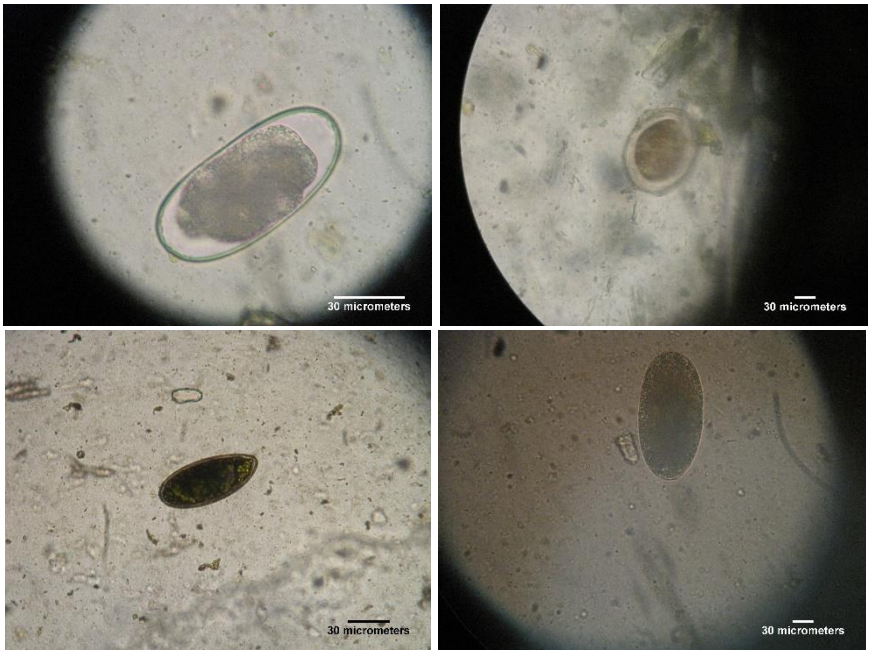

Fig. 1 – Left to right, top row: strongyle-type egg, *Parascaris* sp. egg,
Bottom row: *Oxyuris equi* egg, trematode egg. Originals.

The coprocultures showed that 100% of faecal samples had L3 and the strongylid infections were composed by an average of, approximately, 96.3% of Cyathostominae, 2.4 % Strongylinae and 1.3 % Tricostrongylidae, which is in accordance with the diversity of equid parasites in each one of these taxonomic groups. They also evidence parasite infections by multiple species, 15 different strongylids *sensu latum*, which usually happens in populations that are not usually dewormed (KUZMINA et al., 2016), as the considered population. KUZMINA et al. (2017) also revealed a dominance of Cyathostominae compared to Strongylinae, with no detection of Tricostrongylidae, which might be explained by the reduced size of the population tested by these authors when compared to this study, since our prevalence is low, but we found it. In our study, L3 of *Cyathostomum s.l.* type A (Fig.2-left) were the

most frequent, occurring in 100% of the analysed samples. This agrees with a *post mortem* study, where three species producing this morpho-type (*Cylicostephanus minutus, Cyathostomum catinatum* and *Cylicocyclus nassatus)* had more than 90% prevalence, *C. minutus* with 100%, being the most frequent parasites found (SLIVINSKA et al., 2006). In the subfamily Strongylinae, *Strongylus vulgaris* (Fig.2-right) was the most common parasite of this group and the same happened with SLIVINSKA et al. (2006) and KUZMINA et al. (2009). This parasite species was found in 40.5% of the samples of our research, which is in the middle of the prevalence described in the mentioned studies. This prevalence should be considered as threatening, due to the potentially severe consequences of the larvae migration in the gastrointestinal arterial system, well described for the domestic horse. In fact, in the faecal samples found positive, at least one migration cycle occurred inside the host. *S.vulgaris* migrations can cause arteritis, thrombosis and/or infarction, leading to the sudden and unexpected death of an individual (NIELSEN & REINEMEYER, 2018).

Fig. 2 – Two of the 15 different strongylid *sensu latum* found in our study: *Cyathostomum sensu lato* morpho-type A (left) and *Strongylus vulgaris* (right). Originals.

Even though some sampled individuals might be counted more than once, particularly the unknown bachelors group, the results are analysed and interpreted as populational results in the considered period of collection. As previously mentioned, this represents the most detailed parasitology survey performed at the Pentezug population, with a higher number of samples, when compared to similar studies performed in other European populations of Przewalski horses and consistent with those. In this way, it represents an idea of the parasite community of this population. However, a similar continuous monitoring, during the different seasons, can be a significative aspect for its management and possible reintroductions, considering the average 1287 EPG and maximal values around 5000 EPG. This future regular parasitological surveillance will enhance if there is a real influence of the parasitism in the body condition, reproduction or immune response of the host and in last instance, repercussions at the population level and survival rates, at both Pentezug and in future reintroduction sites.

Acknowledgements

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