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Population processes in a Przewalski's horse herd in the Hortobágy National Park

Thesis for the Degree of Doctor of Philosophy (PhD)

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Hereby I declare that I prepared this thesis within the Doctoral Council of Natural Sciences and Information Technology, Doctoral School of Juhász-Nagy Pál, University of Debrecen in order to obtain a PhD Degree in Natural Sciences at Debrecen University.
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Population processes in a Przewalski's horse herd in the Hortobágy National Park

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Written by Viola Kerekes certified biologist

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PART I

1. Introduction

Protecting and restoring species and ecosystems are crucial tasks (Svenning et al., 2024). The Przewalski's horse (*Equus ferus przewalskii*) is the only surviving subspecies of ancient wild horses that has never been domesticated. These animals were once part of the large grazers of the Eurasian steppe ecosystem (Van Dierendock and de Vries, 1996). According to the International Union for Conservation of Nature (IUCN) Red List, Przewalski's horse is currently classified as endangered (King, 2005). By protecting wild horses, we also help to conserve grasslands, which are among the least protected habitats due to human impacts such as agriculture, overgrazing, and mining (Wesche et al., 2016). There are only 2,500–3,000 individuals of this species, and most are found in zoos and semi-reserves. In China and Mongolia, some 1,000 horses are living in 4 separate subpopulations in semi-wild or wild grassland ecosystems (Turghan et al., 2022), but these populations are prone to fluctuate due to adverse weather like extreme cold winter called "dzud".

1.1. The Przewalski's horses

Przewalski's horses differ in appearance from domestic horses. They stand about 130 cm tall at the withers and weigh between 250–350 kg. They have a very muscular and compact body structure, and their coat is typically dune-colored, with variations ranging from darker to lighter shades. Many individuals have stripes on their legs or a shoulder cross at the withers. Their mane is always short and erect, and their tails feature short, lighter-colored hairs, along with longer ones (Wakefield et al., 2002). The horses are primarily grazers with a hindgut digestive system and live in harem groups similar to feral horses and zebras (Rubenstein, 1978). Przewalski's horses and domestic horses have 64 and 62 chromosomes, respectively (Benirschke et al., 1965), but they can interbreed, and their offspring are fertile (Short et al, 1974).

1.2. Przewalski's horses in Hortobágy, Hungary

In the following chapter I will briefly describe the different aspects of Pentezug Project and for more details you can see Kerekes et al. 2019 and 2021.

1.2.1. The Pentezug project

The Pentezug project was launched in 1997 by Hortobagy National Park (HNP) and Cologne Zoo. The aim of the project was to restore the grassland ecosystem in the heart of the national park with large grazers and minimal human intervention. For that purpose, Przewalski's horses and reconstructed aurochs were introduced to a 2500 ha fenced area in 1997 and 1999, respectively. More than 70% of the area is typical grassland with grass species such as fescue. The area also has marshes, reed beds, and a canal, which provides abundant water all year round. In 2017, another 600 ha were added to the area, with 300 ha of reed beds and 300 ha of grassland. No apex predators exist in the area. In the early years, human activities and interventions were mostly prohibited in the area, ensuring that the horses' social structure remained unaltered by humans. However, as the population grew, human intervention became necessary. Some of these interventions affected population density and various aspects of the social structure (see below).

1.2.2. Founder population in Pentezug Reserve

The transportation of Przewalski's horses within Europe is determined by the European Ex situ Program (EEP) of Association of Zoos and Aquaria (EAZA) for Przewalski's horses. The EEP holds all the pedigree information of the whole European zoo population, enabling the selection of a founder population based on genetic diversity. At the beginning of the Pentezug project, in 1997, only a few animals lived in wild or semi-wild conditions, hence members of the founder population of Hortobagy arrived from different European Zoos, such as Winterthur and Langenberg in Switzerland, Ahaus and Leipzig in Germany, Whipsnade in England, Dolni Dobrejov in Czech Republic and Neusiedel, Mautern and Wien in Austria. Altogether, 31 horses arrived at Hortobágy. HNP, with the Pentezug Przewalski's horse project, became the member of EEP as non-EAZA institution.

1.2.3. Population management in Pentezug Reserve

Due to international and national regulations, HNP is required to send annual reports to different organizations. Identifying individuals is the basis of these reports. Individual recognition is non-invasive and based on three pillars: individual characteristics, position in the harem, and DNA records.

In Pentezug Reserve, about 90% of the horses are unmarked, making photo catalogues and group composition lists essential for identifying individuals and managing the population. Staff from the Pentezug Reserve visit the area twice a week to monitor the horses, using cameras and binoculars to update the photo catalogue, which includes photos in different coats, studbook numbers, and unique traits like coloring and hair whorls. The catalogue is updated biannually, while group lists are revised monthly.

The horse identification process involves several steps: (1) Identifying the harem using distinctive individuals or group characteristics. (2) Cross-referencing the harem members with the latest composition list and photo catalogue. (3) If discrepancies arise, such as missing or new individuals, the entire population's lists are reviewed to maintain accuracy. (4) Missing horses are removed from the harem list, and surplus horses are documented and photographed. (5) To confirm an "extra" horse's identity, staff compare it with other harem groups and, if necessary, collect a DNA sample for verification.

Building on the challenges of individual recognition in harems, identifying bachelor stallions also became difficult as their numbers grew. Since about 20% of the horses in the reserve are bachelors, their population has only been estimated since 2008. However, in 2019, repeated DNA sampling allowed us to accurately identify these individuals again. By comparing deaths, studbook records, and genetic data, we determined that the estimated number of bachelors differed from the actual count by no more than 9%.

Continuing from the challenges in identifying bachelor stallions, DNA analysis plays a crucial role in accurately tracking the horse population. Biopsy and hair samples are collected for this purpose. Biopsy samples are taken using DanInject and PneuDart needles shot from pneumatic rifles. These special needles quickly fall off, carrying small tissue samples. Each horse is first sampled at around one year of age to record their DNA "fingerprint" and determine genetic parentage, often while foals are still with their mothers.

Hair samples are collected from dead or tranquilized animals to confirm their identity. Repeated DNA sampling is particularly useful when mares change groups, stallions form new harems, or when an individual's identity is unclear due to death or other circumstances. The genetic analyses are conducted by the Veterinary Laboratory at the University of California, Davis, following CITES regulations for endangered species.

After the first few years in the Pentezug Reserve, horses started to breed, and their number grew rapidly. The population required human management due to the lack of apex predators in the area. Three control interactions were made in the Pentezug Reserve: transports, immunocontraception treatment (Pig Zona Pellucida, PZP), and culling. Every action concerning Przewalski's horses is determined by the HNP staff with the help of EEP. Local authorities also must give their consent to any action proposed by HNP staff.

Przewalski's horses from Hortobagy have been transported to several locations (Russia, Spain, Mongolia, Czech Republic). We transported 30 horses to Orenburg Reserve (Russia) in

2016–2017 (Bakirova and Zharkikh, 2019), and several individuals went to the transport center in Prague and later to Mongolia. Rewilding initiatives maintain interest in Przewalski's horses in Europe. Last year, 16 horses were transported from Pentezug to the Iberian highland. Moreover, Kazakhstan became interested in the horses. According to the international plans, 4 horses will be transported from Hungary in 2025. But these transports are scarce, and management cannot rely on them in the long run.

Preventing animals from reproducing is often necessary, especially in smaller enclosures, like zoos. Contraceptives can be hormone-based, but these methods alter behavior and the mating rhythms of animals (Fedorka and Troedsson (2021). In the last few decades, advanced contraception methods have been developed, including immunocontraception, an immune response-based method (Kirkpatrick et al., 2011). Immunocontraception includes gonadotropin-releasing hormone (GnRH) and porcine zona pellucida (PZP). Both immunocontraceptive methods are reversible, which is advantageous if the population dynamics change and treated individuals need to breed again. However, reversibility depends on several factors, such as dosage, frequency of use, and timing of vaccination. Additionally, empirical studies on reversibility are lacking in many species. The GnRH vaccine stimulates antibody production against GnRH to reduce the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in females and testosterone production in males (Massei et al., 2020). The GnRH vaccine can be used in both sexes but can affect non-reproductive tissues and elicit broader effects (Miller et al., 2013). PZP directly affects the oocytes; it stimulates antibody production against the zona pellucida glycoproteins to prevent the sperm from crossing the egg layer (Sacco, 1978.). PZP can only be used on females but only elicit direct effects; PZP only affects the receptors on the oocyte (Kirkpatrick et al., 2011). PZP, which was developed by the Science and Conservation Center Billings, USA (Kirkpatrick and Turner, 1990), was successfully used on equids, deer, and elephants (Kirkpatrick et al, 2011). We used PZP on our female Przewalski's horses. Currently most of the older females have been treated, leaving only the young ones (2-5 years old) with the opportunity to foal. In total, 90 out of 140 females have been treated with PZP in 2024.

Another method of population control is culling, which involves euthanizing horses via gunshot. This euthanasia method is only performed in the field by a professional hunter. This approach targets horses that are extremely weak, seriously injured, or suffering from chronic or genetic issues and has rarely been employed in the reserve. Each instance of culling requires individual permission from local authorities (Environmental Office of Hajdú-Bihar County, Hungary), where all our horses are registered.

1.3. Aims of the dissertation

In my doctoral dissertation, I examined various social and demographic changes within the Przewalski horse population, based on a database collected over several decades. We hoped that the comprehensive studies would enable a better understanding of the processes affecting the population and provide guidance for managing the herd not only here in Hortobágy but also in other parts of the world. In the four studies, my specific objectives were as follows:

1. We aimed to investigate social and demographic changes in the Pentezug population as density increases, and to examine how human and non-human-induced phenomena affect population growth.
2. We wanted to measure changes in habitat use, activity, and condition of Przewalski's horses during two distinct periods with significantly different densities of large grazers.
3. We planned to examine the foaling rate and body condition of females in relation to age, population density and changing weather patterns (precipitation and temperature).
4. We aimed to study the collective movements of Przewalski's horses and explore the relationships between society structure and motion patterns.

2. Theoretical background

2.1. Evolution of Przewalski's horses

The first representative of the *Equus* genus evolved in North America 52 million years ago (Orlando, 2015). The ancestors of currently living Equids then crossed the Beringia and reached Europe, Africa, and Asia. Ancestors of present-day asses and zebras and the ancestors of horses arrived in Eurasia 2 million and 7 thousand years ago, respectively (Orlando, 2015). In the following centuries, the distribution of horse populations significantly changed in Eurasia. Horses flourished in the Pleistocene (Orlando et al., 2013), but collapsed during the Last Glacial Maximum when conditions were very harsh. By this time, horses disappeared from North America (Orlando, 2015). During the late Pleistocene and Holocene, wild Equids gradually disappeared from Europe due to natural and human factors (Pushkina, 2008) and were replaced by their domestic representatives. Horse domestication most likely happened in approximately 3500 BC in the Eurasian Steppe, the area of present Ukraine and Kazakhstan (Vila et al., 2001, Kavar and Dovc, 2008). The populations of domestic horses (*Equus ferus caballus*) and Przewalski's horses diverged approximately 45 thousand years ago, but some gene flow between the two subspecies occurred (Goto et al. 2011, Sarkissian, et al., 2015).

2.2. Modern history of Przewalski's horses (from 1881 to the present)

The last wild descendants of Pleistocene wild horses became extinct in Europe and only managed to persist in greatly reduced numbers in the very remote border area between of China and Mongolia. As a result, the wild horses were not included in Linneau's nomenclature system and remained unknown to the western world. The first mention of Przewalski's horses came from John Bell, who traveled from St Petersburg to Peking (Mohr, 1971). Przewalski's horses were not scientifically described until 1881 when a Polish soldier, Nikolai Michailovich Przewalski obtained some skins during his expeditions to Mongolia, and a scientist from the Zoological Museum of the Academy of Science in St. Petersburg, Ivan Semjonovich Poliakov, described them based on these samples (Bouman, 1986, Wakefield et al., 2002). Between 1887 and 1902 several capturing expeditions were organized by European animal collectors, including Baron von Falz-Fein and Carl Hagenbeck (Wakefield et al., 2002). Adult horses were impossible to catch at that time; hence adults were shot, and young foals were live-captured and put together with domestic stepmothers. Later, between the 1930's and 1950's, only a few horse groups were spotted and attempts to capture them were largely unsuccessful (Wakefield et. al., 2002). The last wild female horse (“Orlitza III”) was captured in 1947 and transported to Askania Nova (Bouman 1986). After that time, no more transports occurred, and Przewalski’s horses became extinct in the wild by 1969 (Bouman and Bouman, 1994).

There were other, more severe factors that contributed to the extinction of Przewalski's horse in the wild beyond capturing expeditions (Van Dierendock and de Vries, 1996). Their population was already very small in the 20th century and a combination of severe weather and human activities accelerated their decline. A series of harsh winters in the 1940s and 1950s caused massive losses of both Przewalski's horses and local domestic animals (Bouman and Bouman, 1994). Additionally, advances in weaponry made hunting more effective, and the lands bordering China and Mongolia were increasingly used for domestic livestock (Van Dierendonck and de Vries, 1996, King, 2005).

Fortunately, the species survived in captivity. Altogether, 54 young foals were captured and transported to Askania Nova (Southern Russia at that time), Hamburg and Halle (Germany), Edinburgh (Scotland), London (England), Paris (France), Goolist (Netherlands), New York, and Cincinnati (USA) (Bouman, 1986). Many of the foals died soon after their arrival, and only 12 of them reproduced. During this time one of the stepmothers reproduced with them. Before World War II, 40–50 horses lived in 15–20 zoos (Bouman, 1986). During the war, many horses died; only 31 individuals survived, with 12 reproducing from this group (Wakefield et al., 2002). Altogether, 12 wild horse genomes contribute to today's population, but these were mixed with 4 domestic horse genomes (Sarkassian et al., 2015).

After the loss of the last horse in the wild, different breeding programs were established to avoid inbreeding and preserve genetic diversity as much as possible. In 1960, the first studbook (official record of the pedigree of animals) was published by Prague Zoo (Volf, 1994). This studbook collected data, including parentage, birth date, sex, date of death, and other vital statistics for each individual, from the founders to the current population. This studbook is still in use today, allowing for informed decisions regarding the transportation and breeding of horses in zoos to increase and maintain genetic diversity and minimize inbreeding at the population level. In 1979, a Species Survival Plan was set up for Przewalski’s horses in the USA, and in 1986, a similar program, the European Endangered Species Program, was established in Europe (Bouman and Bouman 1994, King, 2005). This program was later renamed the European Ex-situ Program (EEP). Due to these two programs, horses with known pedigrees were exchanged between zoos, to increase genetic diversity. As a result of these breeding programs, the number of Przewalski's horses increased significantly. Hence, the reintroduction of the species to the wild became possible (Wakefield et al., 2002).

In 1992, two reintroduction projects were started in parallel in Mongolia. One was initiated by a Dutch couple, Inge and Ian Bouman, and supported by the Dutch Ministry of Development Aid (Wakefield et al., 2002, Turghan et al., 2022). Between 1992 and 2000 they transported 84 Przewalski's horses to the 500 km^2 Hustai Nuruu National Park (Hustai) in the mountain steppe of central Mongolia from Dutch semi-reserves (Dorj and Namkhai, 2013). This grassland area proved to be highly suitable for the horses, who have reproduced and reached approximately 420 horses by 2023. (Florian Drouard, project coordinator of Takh Association, personal communication, 2024).

The second reintroduction project was started in the 9,000 km2 Great Gobi B Strictly Protected Area in the Dzungarian Gobi in SW Mongolia (Gobi) by the Mongolian government. This project was initially supported by the Mongolian government and Christian Oswald Foundation and since 1999 by the International Takhi Group (ITG), a Swiss-Mongolian NGO. Altogether, 131 horses were transported from different zoos to the area between 1992 and 2019 (Bernátková et al., 2022). Between 1992 and 2007, 92 individuals were transported from different zoological gardens and the population slowly grew to 146 by the end of July 2009. However, the combination of a summer drought and an extreme winter ("dzud") in 2009/2010 destroyed 60% of the population (Kaczensky et al., 2011). New transports were organized by Prague Zoo and ITG and an additional 39 captive-born horses were transported to the Gobi between 2021–2019 (Bernátková et al., 2022). The number of horses peaked at 420 by the end of July 2022 (Ganbaatar Oyunsaikhan, the former director of the Gobi Strictly Protected Area, personal communication, 2024).

A third reintroduction in Mongolia occurred in Komin Tal National Park in 2004 (Enkhtur, 2007). This project was supported by Takh (Association pour le cheval de Przewalski), a French organization. Altogether 26 individuals were transported there between 2004 and 2011. This is the only place in Mongolia where horses live in semi-wild conditions in a 140 km^2 fenced area, in contrast to the other Mongolian populations where they are free-ranging. Currently, 144 horses live in this area (Jean-Louis Perrin, director of the Takh Association, personal communication, 2024).

In China reintroduction projects began with the transfer of 24 horses from Europe and USA to three breeding centers between 1985 and 2005 (Wakefield et al., 2002, Zhigang et al., 2019, Turghan et al., 2022). These centers were the Xinjiang Wild Horse Center, the Wuwei Endangered Species Breeding Center and the Beijing Nanhaizi Milu Park (Zhigang et al., 2019).

At the turn of the millennium, an attempt was made to translocate horses from these breeding center into the wild. Four protected areas were selected for the reintroduction of Przewalski's horses: Kalamaili National Park (KNP) in Xinjiang, Anxi Extreme Drought Desert National Nature Reserve (ANR) and Dunhuang Xihu National Nature Reserve (DXNR) in Gansu, and Daqingshan National Nature Reserve (DNR) in Inner Mongolia. The most successful was the 14700 km^2 Kalamaili National Park. Between 2001 and 2013, a total of 89 horses were translocated from Xinjiang Breeding Center to Kalamaili (Xia et al., 2014). By the end of 2018, there were 221 horses roaming freely, though they need supplementary feeding in winter (Turghan et al., 2022). By the end of 2023, the population in Kalamaili National Park had increased to 340 Przewalski's horses (Wenxuan Xu, personal communication, 2024).

Due to all these combined reintroduction efforts, it was possible to downlist the IUCN Red List status Przewalski's horses from Extinct in the Wild in 1996 to Critically Endangered in 2008, to Endangered in 2012 (Walzer et al, 2012, King et al. 2015).

Besides reintroduction sites in Asia several semi-reserves were established in Europe in the 1990s where Przewalski's horses were introduced. The largest of these was the 3,000-hectare Pentezug Reserve in Hungary, which began in 1997.

In the 2010s, more large reserves were established. In 2015, a 16,000-hectare semi-reserve was established for Przewalski's horses in Orenburg, Russia. Between 2015 and 2017, 36 horses were transferred there from European semi-reserves (Bakirova et al., 2019). The current population stands at 107 horses (Rafilya Bakirova, director, personal communication, 2024). The newest introduction site is the Iberian Highland, Spain, where 25 Przewalski's horses arrived in 2023 from France and Hungary. The goal here is to reintroduce wild large grazers to the area. In the recent years, there has been an ongoing effort to reintroduce the Przewalski's horse to Kazakhstan, and thanks to international cooperation, the first seven horses arrived at an acclimatization center in the Altyn Dala steppe in 2024.

2.3. Demographic traits of Przewalski's horses

The population growth of large grazers is influenced by various factors, including reproductive parameters such as reproductive rate and fecundity and environmental factors like food availability and weather conditions (Bronson, 1985). In areas where grazers are abundant, high population density can cause low birth rates, increased juvenile mortality, and declining body condition due to overgrazing (Grange et al., 2009, Richard et al., 2014). Weather conditions play a crucial role in food availability and significantly impact body condition, and extreme weather events affect birth rates and mortality (Coulson et al. 2001).

Equus genus includes only seven extant species, all of which are non-ruminant grazers primarily inhabiting semi-arid or arid open grasslands. Their feeding patterns, digestive system, reproductive physiology and general behavior are very similar (Rubenstein et al., 2016).

Horses are highly adaptive, and feral population can be found across the world on open grasslands. Extensive research has been concluded on their demographic traits. Various environmental factors, such as harsh winters, extreme weather conditions, and population density affect different age groups in distinct ways. Middle-aged individuals and females without foals are less vulnerable to these challenges and their fecundity remains relatively unaffected by high density compared to younger horses (Richard, 2014). Extreme weather can reduce overall annual survival rate (Garrott et al., 1990).

Przewalski's horses went extinct in the wild before any studies could be conducted on their demographic parameters (Slotta-Bachmayr et al., 2004). However, observations during their time in zoos revealed reproductive behavior similar to that of domestic and feral horses (Duncan, 2012). Initial observations of reintroduced Przewalski's horses suggested that first foaling of females typically occurs between the ages of 2 to 5 years (Bouman, 1998) and that mares are most productive until the age of 15. However, population dynamics differed between Hustai and the Gobi. Enkhtur (2007) observed that at the beginning of the projects the reproductive rate of females and the survival of their foals were about 2 to 3 times higher in Hustai as compared to Gobi. Possible explanations included different management regimes and sub-optimal ecological condition in the mountain steppes of Hustai versus the semi-desert in the Gobi (Enkhtur, 2007, van Dierendonck and de Vries 1996).

Central Asian drylands are prone to extreme weather events, with particularly extreme winters ("Dzuds") known to result in massive losses of domestic and wild ungulates (Bekenov et al. 1998, Kaczensky et al. 2011). In the Gobi, extreme winter events have negatively impacted the reintroduced Przewalski's population from the beginning and were identified as the biggest threat to small and spatially confined populations (Slotta-Bachmayr et al. 2004). The validity of this prediction was proven correct in 2010 when a population crash occurred in Gobi, documented by Kaczensky et al. (2011). Several snowstorms in 2009–2010 resulted in the loss of 60% of the Przewalski's horse population alongside 67% of the livestock. But even in in Hustai, Dorj and Namkhai (2013) demonstrated that severe winters caused 31,1% of the total deaths between 1993 and 2011. Tatin et al. (2011) examined mortality and female fecundity in a predator-free, closed population of Przewalski's horses in La Villaret, France between 1994 and 2003. The population was small and increased from 11 to 55 during the study. Adult mortality was lower than juvenile mortality and did not correlate with density. However, density significantly affected subadult female fecundity. Decreasing space may have limited population growth, even when food availability was not a constraining factor (Tatin et al., 2011).

2.4. Foraging and activity of Przewalski's horses

Equids are primarily grazers, with grass species comprising the bulk of their diet, supplemented occasionally with shoots, berries, and branches (Berger et al., 1999, Mutillod et al., 2024). Their cecal digestion system necessitates extensive grazing to maintain their nutritional needs (Janis, 1976, Duncan 2012). The last population of Przewalski's horses inhabited a semi-desert region on the border of Mongolia and China, which was initially believed to be their natural habitat. Subsequent debates among scientists suggested that the Gobi more likely served as a refugium rather than their preferred habitat (Ryder, 1993, Van Dierendonck and Wallis de Vries, 1996, Turghan et al., 2022). This suggestion is also in line with findings that Przewalski's horses were likely forced to exist on a mixed diet of grass and scrubs prior to extinction to avoid persecution by humans (Kaczensky et al. 2017).

Early studies on reintroduced Przewalski's horses indicated a preference for rich grasses and fescues, displaying seasonal selectivity in habitat choice to avoid harsh weather conditions and particularly favoring low or mid altitudes (King and Grunnel, 2005,). Despite daily fluctuations in activity during summer, horses tend to graze less as temperatures rise, often migrating to higher altitudes (Boyd and Bandi, 2002, King and Grunnel, 2005, Souris et al. 2007). Their habitat preference typically aligns with areas boasting nutritious vegetation, resembling the behavior of feral horses (Duncan, 1983, King, 2002). While the horses are presumed to spend more time grazing during daylight hours in winter compared to summer, studies have reported conflicting findings, with some reports suggesting the opposite trend (Van Dierendonck et al., 1996, Berger et al., 1999, Kuntz et al., 2006). The reason behind this could be the differences in climate and food quantity and quality in the various study areas (Bernatkova et al,, 2022).

2.5. Social structure of Przewalski's horse populations

Przewalski's horses live in polygynous societies. The reproduction units are harems which normally included one male (harem stallion) and several not closely related females with their offspring (Klimov, 1998, Bouman, 1986), similar to feral horses (Rubenstein, 1986, Duncan, 2012, Linklater and Cameron, 2000) and plain zebras (Klingel, 1969, 1974). Non-harem stallions form bachelor groups consisting of young males and/or former harem stallions. The bachelor groups are very flexible, while the harem groups are usually much more stable and stay together for years. Offspring, both males and females, always leave the natal group and join another group. Males usually spend 3–5 years in bachelor groups before becoming mature enough to have a chance to claim their own harem. Przewalski's horses usually have only one stallion in a harem, but on rare occasions, there can be more than one adult male, similar to feral horses (Klimov, 1998, Linklater and Cameron, 2000). The initial phase of the reintroduction harem and bachelor groups usually have their own home ranges at reintroduction sites that can overlap slightly (King, 2002). These home ranges usually contain food and water sources and shelter (King, 2002, Ganbaatar, 2004). However, the Przewalski's horses in Hungary ten years after introduction, started to move in one large herd, similar to plains zebras in Africa (Rubenstein, 1986) or feral horses (Duncan, 2012). The same phenomenon was observed in the Gobi B (Ganbataar Oyunsaikhan, personal communication, 2024). The most frequently accepted reason for large grazers uniting in herds is for protection from predators (Rubenstein, 1978). In this large unit, lactating females usually decide where to go (Fischhoff et al., 2006).

3. Objectives, methods, and results of our studies

This Ph.D. thesis is based on four studies, which were published in four scientific articles (Kerekes et al., 2019, Kerekes et al., 2021, Heiko et al., 2023, Ozogány et al., 2023). A short summary of the objectives, methods, and results of the four articles is presented in this chapter, while the articles themselves can be found in the appendix.

3.1. Study 1

Viola Kerekes, István Sándor, Dorina Nagy, Katalin Ozogány, Loránd Göczi, Benjamin Ibler, Lajos Széles, Zoltán Barta (2021) Trends in demography, genetics, and social structure of Przewalski's horses in the Hortobagy National Park, Hungary over the last 22 years. Global Ecology and Conservation, *25*, e01407, Impact factor: 3.969, Q1 (Ecology).

3.1.1. Objectives of the Study

In 1997, when this project was initiated, the demographic parameters and social life of Przewalski's horses were largely unexplored. Some experts were skeptical about whether Przewalski's horses could survive in the Hungarian grassland for two reasons. Firstly, Przewalski's horses are not identical genetically to the prehistoric wild horses originally living in the area. Secondly, the founders (and their ancestries) lived in captivity. Moreover, introducing large grazers to an adequate area without predators can be expected to lead to unlimited population growth until food sources become scarce and the population crashes. We also know that the entire global Przewalski's horse population is based on only 12 wild founder horses. Thus, monitoring the genetic diversity and degree of inbreeding is important to maintain a viable population. In this study, we investigated social and demographic changes in the Pentezug population, examined how human and non-human-induced phenomena affect population growth, and determined how basic genetic characteristics changed on a population level. It is well known that species within the Equus genus are grazers and inhabit open grasslands (Rubenstein, 1986). We expected that this area, with its abundant grass and water sources, would be suitable for Przewalski's horses, and that their population would grow until it reached the area's carrying capacity.

3.1.2. Methods of the Study

HNP has been gathering data on horses, including information on births, deaths, imports, exports, parentage, group composition changes, and effects of PZP (immunocontraception) treatment on females, since the start of the project. Data collection was based on an individual identification system we developed, comprising three key components: group structure composition, individual photographic identification, and DNA examination of parentage. For this article, we utilized data collected between 1997 and 2018. Based on these comprehensive databases, we were able to calculate population growth rate, foaling rate, and mortality rate over time and explore the underlying reasons behind the observed changes. Continuous feedback from DNA parentage examinations enabled us to assess gene diversity and the inbreeding coefficient on the population level. Additionally, we analyzed changes in group size and number over the years. In this study, we focused on horses, but we also used data on the population size of the cattle to calculate large grazer density.

3.1.3. Results of the Study

Both horse and cattle numbers increased during the study period, reaching a peak in 2017 with 329 horses and 580 cattle. However, in 2018, the horse population experienced a significant decline. Factors influencing horse numbers included both non-human (births and deaths) and human-induced (imports, exports, immunocontraception treatment, and culling) factors. The net effects of these factors can be observed in the population growth rate, which peaked in 2001 and was always above zero, except in 2018. The foaling rate was notably impacted by PZP treatment after 2013. Non-human-induced phenomena included an increased number of large grazers, which resulted in a decrease in foaling rate among non-treated females and a shift in the age of first foaling from 2–3 years old to 3–5 years old.

Mortality rates were age and sex dependent. Mortality rates were higher in younger and older individuals and older males compared with females in the same age group. The only year when the mortality rate exceeded the birth rate was 2018, when almost 100 horses perished, likely due to overpopulation, insufficient food caused by a drought, and severely cold spring conditions.

These demographic changes also had implications for genetic parameters. Gene diversity remained relatively stable around 0.8 while the inbreeding coefficient exhibited a slight increase. A notable change in the social structure of horses was observed; individual home ranges transitioned to the collective movement of the entire herd (comprising all harem and bachelor groups) approximately 8–10 years after their initial arrival.

3.2. Study 2

Viola Kerekes, Katalin Ozogány, István Sándor, Zsolt Végvári, Csilla Czető, Bettina Nyírő, Timea Szabados, Lajos Széles, Zoltán Barta (2019) Analysis of habitat use, activity and body condition scores of Przewalski's horse in Hortobagy National Park, Hungary. Nature Conservation Research, *4*(Suppl. 2) 31-39. Q2 (Nature and Landscape Conservation).

3.2.1. Objectives of the Study

The Pentezug Project was established to maintain grassland with large grazers and minimal human intervention. Thus, Przewalski's horses and reconstructed aurochs were introduced to the area. Regular data collection, including the number of horses and cattle, and changes were calculated typically at the end of each year. However, limited data were collected on other aspects of large grazers, such as condition scores, location and behavior, and their environment. These data were typically collected by staff members or students at random points throughout the project's duration. Upon reviewing datasets collected from horse observations, it became apparent that a comparison of data from the initial and later phases of the project was possible. The initial and later phases of the project were characterized by lower (typically under 100 or slightly more) and higher (typically more than 250) horses in the area, respectively. Additionally, a habitat map of the Pentezug Reserve was created in 2003. We hypothesized that these findings could provide insights into how a growing population affects food availability and the consequent impact on horse viability. In this study, we aimed to determine the difference in habitat use, activity, and condition of Przewalski's horses in two distinct periods when the density of large grazers was significantly different. Our hypothesis was that as the number of horses increased, food would become limited, leading to a decline in their condition. This might cause them to alter their activity patterns and habitat preferences.

3.2.2. Methods of the Study

We compared three types of data pertaining to horses during two distinct periods of the project: GPS data, activity observations, and condition scores. GPS data on the location of horses were collected from 2004 to 2006 and 2013 to 2014, when there were 51–76 and 229–268 horses, respectively. The GPS data were processed into a shapefile using ArcMap 10.0 and integrated with the existing habitat map shapefile. To assess the habitat preference during the two periods, we compared the occupied proportion of different habitats relative to the entire area and the proportion of time horses spent in each habitat. We then compared the three most frequently used habitat types in different seasons between the two time periods. Activity data, including resting, grazing, moving, alertness, interaction, and comfort behaviors, were collected using scan sampling methods from 2008 to 2009 and 2013 to 2014. The data were collected on 10 and 90 females, respectively, during the two periods. During these periods, 111–132 and 229–268 horses, respectively, were in the area. We calculated the ratio of each activity type to all recorded activities and compared activity percentages between the two periods. Condition scores, based on visual estimation, were collected in 2005 and 2018 from 50 and 90 horses, respectively. During these periods there were 68 and 267 horses in the reserve, respectively. Our condition scoring system was adapted from Rudman and Keiper (1991) and utilized hind-quarter scores ranging from 0 to 5. The data was grouped into bachelor, adult females, and young males, and condition scores were compared across these groups between the two time periods.

3.2.3. Results of the Study

The number of large grazers steadily increased from the inception of the project and peaked in 2017 at approximately 930 individuals, including cattle and horses. The population density at that time was 38.8 animals/km^2. Several aspects of habitat use and body condition of Przewalski's horses were significantly different in the two distinct periods, but the activity percentage did not change. Grazing emerged as a predominant activity throughout the year. However, in summer, horses spent much more time resting than in winter. Habitat use changed significantly in the winters of 2004–2006 and 2013–2014. During both periods, horses predominantly spent time grazing in three habitats - alkali meadow, alkali steppe, and open alkali grassland - accounting for 85% of the area. However, the proportion of use varied; horses utilized the alkali meadow and salt marsh less than expected based on habitat availability. Notably, during the initial phase of the project, horses exhibited a strong preference for weed vegetation, which diminished over time. This shift may be attributed to the initial search for high nitrogen and phosphorus content or was simply due to these horses coming from zoos and, consequently searching for shelter, which was provided by little houses surrounded by weed habitat. Seasonal habitat preference of the three most used habitats showed that in the initial phase of the project, the horses used these three habitats equally in each season, but, in the later phase of the project, the open alkali grassland was not used and the alkali meadow was used in a much higher proportion in winter. This change probably occurred because grass was available in the alkali meadow while the rest of the area was overgrazed. The body condition of females and young males significantly decreased with growing density, but the harem stallion's body condition did not change. Female condition scores ranged from 3–4.5 and 2.5–4.5 in 2005 and 2018, respectively. Young male body condition scores were 3.5–4.0 and 2.5–4.0 in 2005 and 2018, respectively. The growing animal density may have contributed to these observed differences between the two phases of the project.

3.3. Study 3

Heiko G. Rödel, Benjamin Ibler, Katalin Ozogány, Viola Kerekes (2023) Age-specific effects of density and weather on body condition and birth rates in a large herbivore, the Przewalski's horse, Oecologia, *203*(3), 435-451, Impact factor: 2.3, Q1 (Ecology, evolution, behavior and systematics).

3.3.1. Objectives of the Study

In the first two articles, we collected general data about Przewalski's horses and made simple observations about the effects of the growing number of large grazers on body condition and habitat use due to decreased food availability. Food availability may also affect demographic traits like foaling rate and age of first foaling in primiparous females. In this study, we aimed to further examine the foaling rate and body condition of females in connection with changing weather and density. We expected that harsh weather conditions and limited rainfall would negatively impact the foaling rate and overall condition of the horses.

3.3.2. Methods of the Study

We used the same database as in Study 1 plus weather data in the analysis. We examined the parturition of females born in the reserve and not treated with PZP (contraception). We used foaling data of females ≥ 2 years old. Data were collected between 2000 and 2019. Foaling data were collected by HNP staff. DNA data analyses were conducted to confirm identity and parentage. During the analyses, we considered the reproductive effort of mothers (if they had foaled in the previous year). We examined if the late summer precipitation affected pregnancy and if the weather gives an advantage to females in the first year of their lives. We also examined the effects of late winter temperatures on conception, and, a year later, on foaling probability. We examined if the weather conditions affected the body condition of females.

3.3.3. Results of the Study

Most females gave birth at the age of 3 years during the examined period. After a middle age (5-13 years old) plateau, the foaling rate decreased with mothers' age. Previous reproductive efforts only affected younger and older females. In Study 1, we demonstrated that density negatively impacted foaling rates. We refined this observation by demonstrating that younger females are more sensitive to population density than older ones. Weather effects, such as temperature in winter or precipitation in late summer-early autumn, typically only altered the foaling rate if they were combined. For example, the lack of late summer precipitation in the first year of the females' lives combined with a cold late winter before conception caused decreased foaling rates. The combination of lack of late summer precipitation both in the first year and the year of potential pregnancy also caused decreased foaling rates, especially among young females (2-4 years old). We observed similar density and weather effects on body condition. Increasing population density was accompanied by decreasing body condition in young females. The lack of late summer precipitation in the first year of life also significantly decreased body condition in young females.

3.4. Study 4

Katalin Ozogány, Viola Kerekes, Attila Fülöp, Zoltan Barta and Máté Nagy (2023) Fine-scale collective movements reveal present, past and future dynamics of multilevel society in Przewalski's horses, Nature Communications, *14*(1), 5096, Impact factor: 14,7 Q1 (Biochemistry, Genetics and Molecular Biology).

3.4.1. Objectives of the Study

In Study 1, we demonstrated basic changes in the social structure of Przewlaski's horses in the Pentezug Reserve. Specifically, we observed that harems and bachelor groups no longer used separate home ranges. This observation led us to investigate how the herd in Hortobágy synchronizes its movement and to identify the underlying reasons of the herd staying together. With a 23-year database about parentage (supported by DNA data) and group composition structure of individuals, we had a unique opportunity to investigate how fine scale movement reflects social structure in this population of Przewalski's horses. At the start of the research, we knew that horses maintained harem formation even within the large herd. We hypothesized that this would be reflected in movement similarities and physical distances between horses. We also thought that friendships among females (how long they were in the same harem) might influence how closely they stay to one another, potentially affecting the entire herd.

3.4.2. Methods of the Study

We made high-resolution video records of the whole herd of horses in Pentezug Reserve. Additionally, another drone made video records of individuals and groups. In this later video, horses were individually identified, and their positions in the first video record were determined. This method allowed us to follow the movement of most of the individuals in the herd. Five-minute videos were recorded on five different days in 2018. We examined the distance and the movement similarity of each pair of horses and compared the movements to long-term demographic and social structure data

3.4.3. Results of the Study

The first important result of this study was the determination of social levels of the Przewalski's horse herd based on fine scale movement patterns. Due to the distance and movement similarity of pairs of horses, we distinguished two levels: harem and herd. Even inside the harem, we were also able to identify families (female and her offspring) based on movement similarity. We observed that the synchronization between bachelor group members was lower than between harem members, indicating that bachelor groups were not as stable as harem groups. Familiarity (how much time females spend with each other in any harem) affected females' distance from each other within the harem. On the harem level, older harems with more experienced males had more members and a more central position in the herd's social network. When we examined harem social distances, we found that harems with closely related males were closer to each other, and the exchange of females between these harems was higher. Interestingly, a female whose movement was more similar to another harem was more likely to move to this new harem in the next few years.

4. General discussion and future perspectives

We investigated the Przewalski's horse population in Hortobágy. Our findings have not only deepened our understanding of equine population dynamics in general but have also led to more effective population management strategies, a paramount challenge in recent years. Our investigations covered diverse facets of horse behavior and ecology, and we made key observations that have broader implications for similar conservation projects. These three key observations and the future perspectives are discussed in the following paragraphs.

4.1. Management of large grazing projects in the absence of large predators requires adaptive human control

In Europe, there is a growing interest in rewilding abandoned areas and reintroducing wild or feral large grazers and large predators to restore lost trophic chains (Svenning et al., 2016). While domestic grazers were traditionally used in nature conservation areas to manage grasslands (Fraser et al., 2022), recent studies suggest that wild grazers may have different effects on vegetation (Mutillod et al., 2024). However, when introducing wild animals that have been missing in the area for decades or even for thousands of years, the natural question of whether the habitat is still adequate for these animals arises. Our research in HNP demonstrated that the grassland habitat is suitable for Przewalski's horses and reconstructed aurochs. Over a span of 15 years, the population of large grazers increased to the carrying capacity of the area (Study 1 and Study 2). However, we observed natural limitations on population growth, such as reduced foaling rates with increasing density, particularly among primiparous females (Study 3). Human intervention, including immunocontraception treatment (PZP) and occasional culling (Study 1 and Study 2), are necessary to maintain the balance between births and deaths. We started using culling and PZP (the later only on females) from 2011 and 2013, respectively (Study 1 and Study 2). PZP was used on overrepresented females and young females, following the protocol used on feral horses in the USA. Despite these efforts, a population high number of deaths occurred in 2018, prompting us to implement measures based on our observations, including reducing the number of cattle and horses. We catch cattle every year in a corridor system to easily reduce their number. Reducing the number of horses required long-term planning based on the results of Studies 1-3. We began to use culling more systematically, mostly in early spring, to eliminate weak, old, and injured individuals (Study 1). We started to use PZP on middle-aged and older individuals and let primiparous females foal to reduce the number of females who can give birth (instead of 50–60 females, only 20–30 could foal) and the actual number of surviving foals was reduced because young females are more sensitive to environmental effects (Study 3). Even if the young females give birth, they are often inexperienced in protecting the foals. In 2022 and 2023, we managed to balance the number of births and deaths. Ultimately, our findings suggest that managing wild grazers in the absence of large predators is feasible but requires adaptive management approaches to prevent adverse events.

4.2. Characteristic features of the population and environment have multilateral effects on the social behavior of horses and the demographic traits of the population

Through continuous monitoring of social and demographic data, we observed significant changes over the project's lifespan. Social structure shifted from separate home ranges to entirely overlapping ranges (Study 1 and Study 4). Furthermore, horses, including harem and bachelor groups, synchronized their movement, though harem and bachelor groups and even families within harems can be distinguished based on the level of movement synchronization (Study 4). We highlighted some aspects of how horses synchronize their movement and how kinship and friendship can shape the structure of harems and the herd (Study 4). We also showed that average harem size is the same in Hungary and Mongolia (Study 1). Thoroughly and simultaneously observing all individuals in these large groups and collecting data on individual behavior is very challenging. In Study 4, we have shown that 280 individuals can be simultaneously followed but processing the data and gaining meaningful results took years.

With growing density, we observed reduced fecundity and foaling rates (Study 1 and Study 3) similar to other Przewalski's (Tatin et al., 2009) and feral horse populations (Duncan, 2012). We also observed changes in habitat choice (Study 2) and reduced condition scores (Study 2 and Study 3), probably due to less available food. However, we could not measure vegetation changes along with the growing number of horses to support this hypothesis.

Creating automated or semi-automated systems for identifying individual animals, tracking various behaviors, and assessing their condition based on video recordings would represent a highly efficient tool for obtaining real-time insights. Monitoring deteriorating conditions among horses or vegetation destruction through drone footage could serve as an early warning sign, prompting timely intervention to avert potential crises. This approach holds promise not only for managing Przewalski's horses but also for rewilding initiatives involving other newly introduced large grazers in environments with limited or absent predator populations.

4.3. Individual identification is a very effective and useful tool for management and scientific studies

All reintroduced areas and semi-reserves use individual identification of Przewalski's horses based on individual traits and group position (Study 1). We have DNA parentage data of all our individuals that reach the age of one year, which is unique to the HNP. This dataset was very useful when we had to intervene with culling, transports, and PZP treatment (Study 1 and Study 2). We could plan events, especially PZP treatment and transport, while taking into account the genetic diversity at the population level. When transporting horses to new places, we can choose a highly diverse founder population. When treating horses with PZP, we can allow genetically valuable horses to reproduce while reducing the reproduction of horses that are overrepresented in the population. In this way, we can keep our genetic diversity high (Study 1). For these reasons, the population of Przewalski's horses in the HNP is a very valuable gene pool reserve for the whole species.

Individual recognition proved to be useful not just for management decisions but for more accurate and valuable scientific research. Current movement data can be compared to 20-plus-year-old information on age, sex, and position in harems (Study 4). Following the lifelong fecundity of each female and determining the effects of weather on foaling was also possible (Study 3). However, the time-consuming nature of individual recognition presents a challenge, requiring frequent checks by staff members to maintain identification accuracy, especially in large populations.

In conclusion, our studies offer insights into the dynamics of Przewalski's horse populations and provide valuable lessons for managing similar rewilding projects. By combining scientific research with adaptive management practices, we effectively conserved wild grazers and their habitats, contributing to broader conservation efforts and biodiversity conservation.

4.4. Future perspectives

In the future, we plan to investigate other aspects of demography, social structure, and population management of Przewalski's horses in the Pentezug Reserve.

First, we would like to continue to analyze the relationship between demographic traits and the environment to have a better understanding of how to best manage our population.

Currently, we are working on a manuscript focused on foal survival, and we are preparing a report on the long-term effects of PZP on fecundity and reversibility in Przewalski's horses.

Second, our goal is to deepen our understanding of the intricate relationship between horses and their environment. Planned research initiatives include comparative studies across continents to elucidate how environmental shifts impact demographic and social traits in diverse Przewalski's horse populations. We believe that existing demographic and weather data collected on semi-wild and wild Przewalski's horses could be restructured to facilitate comparisons among all sites. Additionally, we aim to synchronize our data collection methods with other Przewalski's horse sites to enable standardized real-time comparisons.

Third, we are focusing on developing automated identification systems to streamline fieldwork. Collaborating with Stuttgart University, Debrecen University and Eötvös Lóránt Universtiy, we have been working on automatic horse identification using drones for the past three years. Stuttgart University aims to develop drones that automatically follow horses and record 3D pose data. We plan to utilize this technology to create an automatic condition-scoring system for estimating the well-being of our population, particularly during the winter when daylight hours are limited.

Last, ongoing efforts to gather comprehensive data on vegetation, field use, parasitology, water quality, and weather aim to uncover reciprocal effects between horses, cattle, and their environment. Through these initiatives, we endeavor to advance our comprehension of the intricate interplay between equines and their surroundings.

Collectively, our completed, ongoing, and planned studies, presented in this dissertation, contribute to a better understanding of the demography and social life of an endangered species, the Przewalski's horses, and may help to develop more effective population management strategies for large grazers.

5. Summaries

5.1. Összefoglaló (Summary in Hungarian)

Jelen doktori disszertációban a Hortobágyon élő Przewalski lovakkal (*Equus ferus przewalskii*) kapcsolatos tanulmányaimat ismertetem. A faj őse a prehisztorikus vadló, mely az ember előretörésével fokozatosan eltűnt Európából majd Ázsia nagy részéről is. 1881-ben Nyikolaj Przewalski kapitány Mongólia és Kína határvidékén megfigyelt és leírt egy vadló populációt. A faj a zord időjárási körülmények, a katonai tevékenységek és az elfogó expedíciók hatására eltűnt a vadonból, az utolsó egyedet 1969-ben látták. Az állatkertnek és a nemzetközi összefogásnak köszönhetően a faj fennmaradt, és szaporodásnak indult, majd lehetővé vált visszatelepítésük az eredeti élőhelyükre. 1985-ben Kínába, majd 1992 Mongóliába vittek egyedeket állatkertekből. Később európai területekre (Ukrajna, Magyarország, Franciaország) is telepítettek lovakat. Jelenleg mintegy 2500-3000 példány él a világon, ezek összesen 12 egyed leszármazottjai.

A Hortobágyi Nemzeti Park vezetősége 1997-ben döntött úgy, hogy egy olyan területet alakít ki, ahol nagytestű legelő állatok élnek „félvad" körülmények között minimális ember beavatkozás mellett. Ezért Przewalski lovakat és rekonstruált őstulkokat telepítettek be a 3000 hektáros, villanypásztorral körbekerített, un. Pentezug területre. A Hortobágyi Nemzeti Parkban (más területekhez hasonlóan) a lovak hárem csapatokban élnek, ahol egy hárem csődör védelmezi a csapatot és hozzá évekig hűséges kancák és csikóik élnek együtt. Az ún. agglegény csapatban fiatal mének vagy kiöregedett hárem csődörök verődnek össze. A fiatal egyedek mindig elhagyják a csapatot: a kancák egy másik, nem közel rokon mén által vezetett csoporthoz csatlakoznak, a mének pedig az agglegényekhez. A lovakat egyedileg ismerjük fel csoportbeli hovatartozásuk és egyedi bélyegeik alapján Ezen felül a lovakból vett biopszia minta genetikai elemzésével a szülők személye biztosan beazonosítható.

A doktori értekezésemben a Przewalski ló populációjának különböző szociális és demográfiai változásait vizsgáltam, a több évtizeden át gyűjtött adatbázis alapján. Abban bíztunk, hogy az átfogó tanulmányok elehetővé teszik a populációra ható folyamatok jobb megértését és támpontot adhatnak az állomány kezelésében nemcsak itt Hortobágyon, hanem a világ más tájain is. A négy tanulmányban konkrét céljaim a következők voltak:

1. Az első tanulmányban a pentezugi populáció szociális és demográfiai változásait követtük nyomon. Megvizsgáltuk, hogy az emberi és nem emberi eredetű jelenségek hogyan

befolyásolják a populáció növekedését, és meghatároztuk, hogyan változtak az alapvető genetikai jellemzők populációs szinten.

2. A második a tanulmányban célunk az volt, hogy meghatározzuk a Przewalski lovak élőhelyhasználatának, aktivitásának és kondíciójának különbségét két különböző időszakban, amikor a nagytestű fűevők egyedsűrűsége jelentősen eltérő volt.

3. A harmadik a tanulmányban célunk az volt, hogy megvizsgáljuk a csikózás arányát és a kancák kondícióját a változó időjárás és az egyedsűrűség összefüggésében.

4. A negyedik tanulmányban megvizsgáljuk, hogyan tükrözi a finom léptékű mozgás a szociális struktúrát ebben a Przewalski ló populációban.

A tanulmányokból származó eredmények alapján megállapítottuk, hogy a pentezugi vadló populációra mind a szociális mind a környezeti faktorok hatással voltak. A lovak száma dinamikusan növekedett 1997 után és az eleinte elkülönült hárem csapatok egy nagy ménest alkotva mozogtak. A mozgási mintázatok tükrözték az állatok szociális viszonyait és a ménesen belül ez alapján jól elkülöníthetőek voltak a hárem és agglegény csapatok. Az állomány növekedésének megfékezésére 2013-ban kezdődött a területen a mesterséges termékenység-szabályozás a Porcine zona pellucida (PZP) alkalmazásával. Az egyedszám növekedésével párhuzamosan csökkent a PZP-vel nem kezelt kancák ellési rátája és az első ellés 3 éves korról 4-5 évesre tolódott. A nagyobb állatlétszám hatására megváltozott az lovak területhasználata és kondíciójuk is csökkent. Bizonyos időjárási jelenségek kombinálódása (például hideg tél, kevés őszi csapadék a kancák első évében vagy ellés előtt), az tovább csökkenthette az ellési rátát. 2018-ban az elhullás jelentősen megnövekedett a korábbi évekhez képest az extrém hideg tavasz, az előző aszályos évek, és a nagy állatlétszám (lovak és marhák) miatt. Azóta a lovak populációja újra növekedésnek indult, de igyekszünk az eddigi tudást felhasználni az állomány növekedésének visszafogására.

A kutatómunkák során a lovak demográfiával, szociális viselkedésével kapcsolatban három általános megfigyelést tettünk. (1) A vadon tartott nagytestű fűevők állományának szabályozása adaptív emberi beavatkozást igényel. (2) A lovak szociális viselkedését és az állomány demográfiai jellemzőit számos belső és külső tényező együttesen határozza meg. (3) A lovak egyedi felismerése nagyon hatékony és hasznos eszköz mind az állomány szabályozásban mind a kutatásban. Összegzésképpen elmondhatjuk, hogy a kutatásaink hozzájárultak a Przewalski lovak demográfiájának és szociális viszonyainak jobb megértéséhez, továbbá a nagytestű fűevőekkel történő területkezelés hatékonyságának növeléséhez.

5.2. Summary in English

In this doctoral dissertation, I present my studies related to the Przewalski's horses (Equus ferus przewalskii) living in the Hortobágy. The species' ancestor is the prehistoric wild horse, which gradually disappeared from Europe and most of Asia due to human expansion. In 1881, Captain Nikolai Przewalski observed and described a wild horse population along the border of Mongolia and China. Due to harsh weather conditions, military activities, and capture expeditions, the species vanished from the wild, with the last sighting occurring in 1969. Thanks to zoos and international cooperation, the species survived and began to reproduce, allowing for its reintroduction into its original habitat. In 1985, individuals were taken to China, and in 1992 to Mongolia from zoos. Later, horses were also introduced to European regions (Ukraine, Hungary and France). Currently, about 2,500–3,000 individuals exist worldwide, all descendants of 13 individuals.

In 1997, the management of the Hortobágy National Park decided to create an area where large grazing animals could live under "semi-wild" conditions with minimal human intervention. As a result, Przewalski's horses and reconstructed aurochs were introduced to the 3,000-hectare, electric-fenced area called Pentezug. In the Hortobágy National Park (as in other areas), the horses live in harem groups, where a harem stallion protects the group, and mares and their foals live together, remaining loyal to the stallion for years. In the so-called bachelor groups, young stallions or former harem stallions band together. Young individuals always leave the group: mares join another group led by an unrelated stallion, and stallions join the bachelors. We recognize the horses individually based on their group affiliation and unique markings. Additionally, by performing genetic analysis on biopsy samples taken from the horses, the identity of the parents can be reliably determined.

In my doctoral dissertation, I examined various social and demographic changes within the Przewalski horse population, based on a database collected over several decades. We hoped that the comprehensive studies would enable a better understanding of the processes affecting the population and provide guidance for managing the herd not only here in Hortobágy but also in other parts of the world. In the four studies, my specific objectives were as follows:

1. In the first study, we tracked the social and demographic changes of the Pentezug population. We examined how human and non-human factors influenced population growth and identified how basic genetic characteristics changed at the population level.

2. In the second study, our goal was to determine the differences in habitat use, activity, and condition of the Przewalski horses during two different periods when the density of large grazers varied significantly.
3. In the third study, our aim was to investigate the foaling rate and the condition of the mares in relation to changing weather patterns and population density.
4. In the fourth study, we examined how fine-scale movements reflect the social structure in this Przewalski horse population.

Based on the results of the studies, we determined that both social and environmental factors affected the Pentezug wild horse population. The number of horses increased dynamically after 1997, and initially distinct harem groups began to move as one large herd. Movement patterns reflected the social relationships among the animals, and within the herd, the harem and bachelor groups were clearly distinguishable. To control the population's growth, artificial fertility regulation began in 2013 with the application of Porcine zona pellucida (PZP). As the population grew, the foaling rate of mares not treated with PZP decreased, and the age of first foaling shifted from 3 years to 4–5 years. The increase in the number of animals also affected the horses' land use, and their condition declined. Certain weather phenomena (such as a cold winter or low autumn rainfall during a mare's first year or before foaling) further reduced the foaling rate. In 2018, mortality increased significantly compared to previous years due to an exceptionally cold spring, previous drought years, and the high animal density (horses and cattle). Since then, the horse population has started to grow again, but we are striving to use the knowledge gained to curb the population's growth.

During our research, we made three general observations regarding the demography and the social behavior of the horses. (1) The regulation of large grazing herbivore populations in the absence of predators requires adaptive human intervention. (2) The social behavior of the horses and the demographic characteristics of the population are influenced by a combination of internal and external factors. (3) Individual recognition of the horses is an effective and useful tool for both population management and research. In conclusion, our research has contributed to a better understanding of the demography and social relationships of Przewalski’s horses and has enhanced the effectiveness of managing areas with large grazing herbivores.

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PART II

8. List of Publications related to the dissertation

1. Kerekes, V., Sándor, I., Nagy, D., Ozogány, K., Göczi, L., Ibler, B.,Szeles, L. & Barta, Z. (2021). Trends in demography, genetics, and social structure of Przewalski's horses in the Hortobagy National Park, Hungary over the last 22 years. Global Ecology and Conservation, 25, e01407.

2. Kerekes,V., Ozogany, K., Sandor, I., Vegvari, Zs., Czeto, Cs., Nyiro, B., Szeles, L. & Barta, Z. (2019). Analyses of habitat use, activity and body condition scores of Przewalski's horses in Hortobagy National Park, Hungary. Nature Conservation Research. 4(Suppl. 2) 31-39.

3. Rödel, H. G., Ibler, B., Ozogány, K., & Kerekes, V. (2023). Age-specific effects of density and weather on body condition and birth rates in a large herbivore, the Przewalski's horse. Oecologia, 203(3), 435-451.

4. Ozogány, K., Kerekes, V., Fülöp, A., Barta, Z., & Nagy, M. (2023). Fine-scale collective movements reveal present, past and future dynamics of a multilevel society in Przewalski's horses. Nature Communications, 14(1), 5096.

9. The author’s contribution to the studies

	Study 1	Study 2	Study 3	Study 4
Original idea	x	x	x	
Study design and data collection	x	x	x	x
Data analysis	x	x		
Manuscript writing	x	x	x	

10. Appendices

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Trends in demography, genetics, and social structure of Przewalski's horses in the Hortobagy National Park, Hungary over the last 22 years

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ABSTRACT

The world population of Przewalski's horses has recovered from very few survivors, and is still categorized as "Endangered" in the IUCN Red List of Threatened Species. For this reason, the maintenance of genetically valuable populations is highly important. The 3000 ha Pentezug Reserve in Hortobagy National Park, Hungary, was home to 270 Przewalski's horses at the end of 2018, approximately 30% of the total European population. In this study, we show the main changes in demographic, genetic, and social characteristics of the population since the establishment of the reserve in 1997. The first years clearly demonstrated that the steppe ecosystem and the wetlands in this area were ideal for the population. We observed that the growing number of individuals affected the total number of harems, but not the average size of the harems. Remarkably, a new phenomenon, herd formation, also appeared. The number of foals per year increased for 17 years (the zenith was in 2014, N = 60 per year) then started to decrease due to both non-human factors (e.g. delay in female fecundity and decreasing foaling rate) and human intervention (e.g. immunocontraception treatment). The total number of horses peaked in 2017 (N = 328) and in 2018 decreased (N = 276) due to decreasing foaling rate, exports, and a population crash. The inbreeding coefficient increased slightly after 2012, while gene diversity stabilized at a relatively high value. Today many individuals from this well-monitored population can be found in Russia and Mongolia. Collectively, understanding of the social structure and mechanisms of population self-control in Przewalski's horses is improved by our observations. From a population management point of view, our study highlights the importance of human interventions for birth-control and interactions between Przewalski's horse projects in different countries.

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1. Introduction

Przewalski's horses (*Equus ferus przewalskii*) were only discovered at the end of the 19th century and were extinct in the wild, just 70 years after their discovery. Fortunately, several individuals were captured and transported to various zoological gardens, making the survival of the taxon possible (Bouman and Bouman, 1994). All Przewalski's horses alive today are descendants of only 12 wild-caught horses and another 2–4 domestic horses (*Equus ferus caballus*) mixed with the wild ones in captivity (Der Sarkissian et al., 2015). After a significant increase in the number of captive animals, reintroduction projects started in 1985 and 1992 in China and Mongolia, respectively (Wakefield et al., 2002; King, 2005; Walzer et al., 2012; Xia et al., 2014).

Today the majority of Przewalski's horses live in Europe and Asia. There are 900 horses in European zoological gardens and semi-wild populations (Ukraine, Russia, France, Hungary) (International Studbook of Przewalski's horses; Bakirova and Zharkikh, 2019). Approximately 1360 horses live in breeding centers, under semi-wild conditions and in the wild in Asia (Mongolia, China) (Jiang and Zong, 2019 regarding Chinese population and personal communications with Jaroslav Simek and Dashpurev Tserendeleg regarding Mongolian population in 2019). In the United States, 120 horses live in zoological gardens (personal communications with Amanda Faliano and Lee Boyd in 2019). The populations living in monitored semi-wild or wild conditions are important not only for the conservation of the subspecies, but also for studying the social structure, demography, and behavior of Przewalski's horses, which is essential for maintaining their successful reintroduction into the wild.

Early studies on populations of Przewalski's horses in Mongolia and China revealed that their social structure resembles feral horses (*Equus ferus caballus*) and plains zebras (*Equus quagga*) in many aspects (Klingel, 1969, 1974; Wakefield et al., 2002; King 2005; Duncan, 1992). Przewalski's horses live in two types of social groups: harem groups and bachelor groups (Klimov, 1988). Harem groups, the breeding units of horses, consist of a harem stallion, mares, and their predispersal offspring. The single-sex bachelor groups consist of young males and former harem stallions. Przewalski's horses exhibit female defense polygyny; harem stallions defend females from other stallions (Klimov, 1988). Youngsters always leave their natal groups; young females join other harems around the age of 2–3 years and young males join a bachelor group. At the age of 5–7 years, males may become strong enough to gain females and protect a harem from other stallions. Home ranges of harems are reported to be slightly overlapping, but separated in Hustai National Park (King, 2002).

Populations of large grazers, which have been (re)established by humans in closed but otherwise adequate areas lacking predators, require close monitoring and human interventions because they usually start to increase rapidly in numbers (Caughley, 1981; Coulson et al., 2001; Forsyth and Caley, 2006). The growing number of individuals affects the environment due to grazing pressure (Mysterud, 2006), resulting in decreasing vital rates (Gaillard et al., 1998, 2000). In some documented cases of sheep and feral horses, the population size reached the area's maximum carrying capacity and the population collapsed, with a very high number of deaths, especially during extreme weather conditions (Milner et al., 1999; Scorolli et al., 2006). To avoid overpopulation, and hence, possible massive losses, different methods of population management can be introduced like culling, removals, or contraception (Nuñez et al., 2016). When population growth is artificially limited, it is important to plan which individuals are treated or removed from the population, to avoid inbreeding depression, to which small isolated populations are particularly vulnerable (Keller and Waller, 2002).

In this article, we focus on population, demographic and behavioral changes, over the last 22 years, in the population of Przewalski's horses at Pentezug Reserve, Hortobagy National Park (HNP), Hungary. The Pentezug Reserve was established in 1997 by HNP and Cologne Zoo to preserve open grasslands using semi-wild grazers with minimal human interference. To reach this goal, reconstructed aurochs (a hardy breed of domestic cattle) and Przewalski's horses were introduced to create a relatively diverse grazing community (Kerekes et al., 2019). Since the establishment of the reserve, both the Przewalski's horse population and the cattle population successfully adapted to the new environment and grew rapidly. Currently, the reserve is home to around 300 horses and 250 cattle. We describe the long-term (>20 years) demographic changes of the horse population and the major changes in age distribution. We also show our methods and results on horse population management and the possible effects on genetic diversity. Based on individual recognition, we show that while the harem size seemed to be independent of the population size, changes in the harems' home ranges were observed as the population grew. We also report the annual changes of the cattle population kept in this area.

2. Materials and methods

2.1. Study area

The Pentezug Reserve (3000 ha) belongs to the core area of the HNP. The reserve is located in Eastern Hungary at 47°31′03.3″N 21°05′34.1″E. Based on pollen analysis and paleo-ecological research, the reserve and the surrounding areas were grasslands well before human husbandry (app. 30,000 years ago). The area has a semiarid-continental four-season climate; the average annual temperature and precipitation are 10 °C and 550 mm, respectively. The area is typical alkali grassland with characteristic fescue grass species, such as *Festuca pseudovina* (Török et al., 2012). The reserve is bordered by a 24.8 km long, 1.4 m high, 3 lined, New Zealand type electric fence. Notably, the fence inhibits the migration of large herbivores, but does not isolate the area in other aspects. For instance, wild animals (e.g. rabbits, foxes, and deer) can easily cross the fences. In the reserve, most human activities are prohibited to protect the open grassland habitat. Przewalski's horses are not given any surplus food or water and they are not medically treated. Their social structure is naturally formed; humans do not interfere in mate choices. In 2004, a Wild Animal Park was established in Hortobagy-Malomhaza, with a special breeding group of Przewalski's horses consisting of individuals genetically less represented in the Pentezug population.

2.2. Founder population

The originating herd at HNP consisted of 31 horses from different zoological gardens. Twenty-two of them were transported to Pentezug Reserve, 20 of them during the first 4 years of the project (1997–2001) and two others in 2007 (Appendix Table A1 and A3). One horse died in the quarantine area in 1997 and never reached Pentezug. Eight horses were transported to Malomhaza Wild Animal Park between 2004 and 2017 (Appendix Table A2 and A4). The founder individuals were selected by the EEP (European Endangered Species Program) after a comprehensive genetic analysis to maximize population diversity.

2.3. DNA database and analyses

For DNA analysis, biopsy and hair samples are collected. Biopsy samples are taken using DanInject and PneuDart needles and pneumatic rifles. The special biopsy needles fall off the animals soon after the shot and contain small tissue samples. Each individual is first sampled at the age of one year to record DNA "fingerprints" and determine genetic parentage. At this time foals are often close to their mothers and still in the natal group. Hair samples are taken from dead or tranquilized animals to confirm identity. When mares change groups, stallions recruit new harems, or individuals die and their identities are ambiguous, repeated DNA sampling can be used to determine the identity, using the DNA fingerprint database. The genetic analyses are done by the Veterinary Laboratory at the University of California, Davis. The sampling and analyses are carried out according to the rules and regulations of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

The inbreeding coefficient and the gene diversity based on kinship matrix are calculated by PMX 1.0 software using the pedigree database (Lacy et al., 2012). Individuals are considered inbred when their parents shared ancestors (Keller and Waller, 2002).

2.4. Individual recognition

Individual recognition of horses in Pentezug Reserve is managed by a combination of recording horse characteristics, group membership, and DNA records. A large majority (app. 98% in 2018) of the horses are not branded or marked in any way. Hence, group composition lists and a photo catalogue contribute to individual recognition and are critical for population management. The area is usually visited two times a week by the staff of the Pentezug Reserve (1–2 people), who carefully monitor the horse population. Horses are accustomed to people and one can approach the herd within 20–50 m. Binoculars and cameras are used to collect data and update the photo catalogue, which contains key information about individuals (photos from different sides in both summer and winter coats, name, studbook number, parents, and characteristics, including coloring, shoulder cross, leg stripes, and hair whorl position on the forehead). The photo catalogue is updated at least twice a year. The group composition list contains the members of each harem group and is updated at least once a month. Foals born in the same year are named with the same first letter, progressing yearly in alphabetic order. Thus, horse names indicate the year of birth (horses born in 1998 were named with "A", the ones born in 1999 with "B", and so on).

The horse identification routine for updating the group composition list and photo catalogue of harem groups typically contains the following steps. (1) We identify the harem group with the help of the latest group composition list and characteristic individuals in the harem. There are approximately 20–30 individuals in the entire population (10%) that are unique enough to be easily identified (due to features such as a white spot on the forehead or body, missing ear tips or other old injuries that can be seen for a lifetime). Other features of the harem, such as the number of animals and foals, the ratio of females and youngsters, the ratio of darker/lighter animals are helpful in harem identification. The characteristic individual could change harems. In this case, we determine the identity of the new harem based on the previously mentioned characteristics. (2) Once we have identified the harem, we start to identify the members. For this we use the latest group composition list, containing information about the presumed members of the harem, and the photo catalogue. (3) The observed versus the latest recorded member list of the given harem can be identical, or different (individuals left, joined). The second case may indicate that the structure of other harem(s) has been changed, as well. Therefore, updating the group composition lists of all harem groups within a short time is important to maintain consistency. (4) If a horse is missing from the harem compared to the latest record, we remove its name from the list of members of the given harem and note that its position is unknown on the day of observation. If there are surplus individuals relative to the last record, we take pictures and record as much data as possible (sex, the position of hair whorl, stripes on the legs, etc.). In this way, we can distinguish the individual(s) from other members of the harem. (5) For identifying an "extra" horse in a harem, we try to check all other harem groups for a "missing" horse. If an "extra" and a "missing" horse match in all known features (e.g. gender, age, whorl, shoulder cross, etc.), we modify the group composition list accordingly. If still uncertainty remains, we take a biopsy sample and use the DNA fingerprint database to find out the identity of the given horse.

The routine horse identification for PZP treatment or transport typically contains the following steps. (1) We check which harem the given horse belongs to with the help of the group composition list. (2) We determine the key features, which are useful in identifying the harem in the field (such as characteristic individuals, number of horses, etc.). (3) We screen horses and harems in the field until we find the given harem. (4) We check whether the given individual is still a member of the harem. If we cannot find the given horse in the harem, we update the entire group composition list. (5) We shoot the identified individual with PZP or tranquilizer.

2.5. Temporary infertility using the PZP vaccine

As part of population control, we treat females in Pentezug Reserve with an immunocontraception vaccine from porcine zona pellucida (PZP), which causes reversible infertility by preventing fertilization of the egg and/or affecting ovarian function (Kirkpatrick and Turner, 1990, 2002; Turner et al., 2002). Vaccines are purchased from The Science and Conservation Center, Billings, USA, since 2013. The vaccine consists of two parts: PZP and two types of adjuvant. Freud's Modified adjuvant (FMA) is used for first inoculations (primer) and Freund's Incomplete Adjuvant (FIA) is used for subsequent inoculations (booster). PZP arrives in a powder form. After arrival, the PZP is dissolved in 0.5 ml distilled water and frozen immediately until use. The adjuvant arrives in 10 ml bottles and is kept at 4 °C until use. When treating a horse, the PZP is mixed with 0.5 ml adjuvant and shot into the horse. According to the recommendation of The Science and Conservation Center, we use 3 inoculations to reach 4–5 years of infertility. The first inoculation (primer) is shot in February–March (before the breeding season). The first booster is given (in most cases, still before the breeding season) 4–6 weeks after the primer. In the next year (app. 13–15 months after the primer), a second booster is given.

The treatment does not affect the estrous cycle (Liu et al., 1989), therefore, mares show normal sexual behavior. However, the treatment can extend the breeding season and females who receive contraception may leave their original harem, affecting the stability of harem structures (Nuñez, 2009). The selection of individuals for treatment by the project manager is based on recommendations from the EEP coordinator, according to genotypes, phenotypes, fitness, such as the number of surviving foals, inbreeding coefficient, and mean kinship, which is calculated from the pedigree database. In the first 2 years after introducing PZP-treatment, females with high values for all three categories were selected. Beginning with the third year, predominantly 2-year-old individuals were treated to delay the first foaling. Young females with low kinship or inbreeding values were left untreated.

2.6. Data collection in the field

Every event and related information concerning individuals are recorded, including birth dates, paternal and maternal data (later on these data are confirmed by genetics), details of exports and imports, time of PZP treatment, and time and cause of deaths, which can be non-human induced or human-induced (culling). Animals for PZP treatment and culling are selected by the EEP coordinator and HNP staff together, taking into consideration genetics and population management. Culling (death by gunshot) is performed in the field by a professional hunter. This method eliminates horses that are extremely weak or have a serious or chronic injury or genetic problems, but culling is only rarely used in the reserve. Before culling, individual permission for each culled horse is required from local authorities (Environmental Office of Hajdu-Bihar County, Hungary), where all of our horses are registered. The body is always transported out of the reserve and, after medical examination, the remains are fed to wolves and vultures at the Malomhaza Wild Animal Park. Later, the bones are collected by the staff at Malomhaza and transported by ATEV Zrt, a company responsible for eliminating animal products (such as carcasses).

The harem group composition list is usually updated monthly but the individual recognition of bachelors became challenging with their growing number. Consequently, the number of bachelors, which represents approximately 20% of the horses in the reserve, is an estimated number since 2008. In 2019, we managed to identify bachelors again with the help of repeated DNA sampling. Based on the deaths, studbook records, and genetic data, we concluded that the difference between the estimated number and the actual number of bachelors is not more than 9%.

Location sites (latitude and longitude) of harem groups were collected all year round in 2001 and from October 2013 to July 2014 to compare home ranges of harems in two distinct periods. The observer marked the approximate location of the group on the vegetation map of Pentezug and the coordinates were later collected from Google Maps. In 2001, three harem groups and one bachelor group existed in total, but one of these harem groups was only formed in October 2001. Coordinates were collected 3–5 times per month. Between October 2013 and July 2014, a total of 27 harem groups and a few bachelor groups existed. Seventeen harem groups existed continuously through the whole observation period (2013–2014) and 8 harem groups existed only for a shorter time range (Appendix Table A5). Coordinates were collected 1–2 times per month. In the later period, the harem groups generally were very close to each other (50–300 m). Thus, we recorded the same coordinates for the harems closer than 300 m from each other.

2.7. Cattle population

The presence and especially the size of the cattle population in the reserve is an important factor affecting the environment (e.g. food availability) and, thus, the horse population, although other aspects (e.g. social structure of cattle) are out of the scope of this study. Cattle are marked with ear tags at 2–5 days of age. All individuals are captured once a year for veterinary examination. At this time, the cattle are individually recognized with the help of the ear tag, their numbers are recorded, and the missing animals are noted as dead. If necessary, captured cattle are transported outside the reserve to reduce their number. The demographic data of the cattle were analyzed similar to the demographic data of the horses (see section 2.9).

2.8. Analysis of foaling rates and the effect of PZP treatment

Females were considered as "PZP-treated" only from the year following the start of the treatment, because the treatment is not effective in the first year, as the female may be already pregnant or the breeding season has passed. We investigated the effect of PZP treatment on foaling rate by fitting a generalized linear mixed-effect model (GLMM) with binary error distribution (lme4 package of R, Bates et al., 2015). In the model the dependent variable was whether a given female has a foal in a given year or not, the fixed effects were the year, the age of the female and whether she was treated with PZP or not. As exploratory analyses suggested non-linear effect of age, we also included squared age into the analyses as fixed effect. Interactions between treatment and year and between treatment and age (both linear and squared) were also included. We entered horse IDs as random effect. After fitting the model specified above we sequentially removed non-significant interactions. We compared treated and untreated females by using the emmeans package of R (Lenth, 2020). We further investigated the effects of PZP treatment on post-treatment fertility in treated mares by fitting a GLMM with binary error distribution. Here, the dependent variable was if a given mare has a foal in a given year or not. The fixed effects were the zero centered year, zero centered age, the time elapsed from finishing PZP treatment (year after treatment), and how many years the mare received treatment (length of treatment). We entered mare IDs as a random effect in the model. Foaling rates in different years were expressed as the ratio of the number of foals vs. adult females in the given category (e.g. non-treated and PZP-treated).

To investigate the non-human induced effects on foaling rate, we examined the age at first foaling in non-treated females over time. Given the non-normal distribution of the data, we used quasi-Poisson generalized linear model (GLM) tests to evaluate whether the age of females at first foaling differs significantly over time. To determine the relationships between foaling rate of non-treated females and population size, we fitted a GLMM with binomial error distribution. The response variable was whether a given female foaled or not in a given year, while the explanatory variable was the population size in the given year. As exploratory analysis indicated a nonlinear relationship between foaling rate and population size, we also included the quadratic of population size into the model (poly function in R). Females' ID was included as random effect. Similar analyses were run between foaling rate and year and foaling rate and age of females.

2.9. Analysis of demographic data

For population demography analyses, we counted the total number of individuals, events of birth and death and its causes, and exports and imports at the end of each year. The growth rate of the population was calculated as follows: (number of foals plus imported animals in the given year) minus (number of dead and exported animals in the given year) divided by the total number of individuals in the previous year. The age distribution was calculated separately for males and females using one-year intervals based on the collected data at the end of the year. Females that were 2 or more years old were considered adults, because they are sexually mature and able to produce foals (Tatin et al., 2009). One-year-old horses were considered juveniles and younger horses were considered foals (Tatin et al., 2009).

To analyze mortality during the examined period (1997−2018), we collected the number of horses alive at the beginning of a given year and the number that died during that year for each sex and in each year. Because the year 2018 had an extreme mortality level across all age classes, we excluded this year's data from certain analyses. We fitted a GLM with binomial error distribution to these numbers. All of our candidate explanatory variables (i.e. year, number of horses, age) were highly intercorrelated (all $r > 0.7$). Thus, to avoid problems of collinearity, we analyzed their effects separately, with sex, the given candidate variable, and their interaction entered as explanatory terms in these models. In the case of mortality, exploratory analyses indicated that the effect of these candidate variables might be curvilinear. Therefore, we also entered their squared terms into the models (poly-function in R). In the case of juvenile mortality, neither the interactions (all $p > 0.49$) nor the sex (all $p > 0.15$) had any significant effect in these models; thus, we only report the results on the candidate variables. We analyzed whether PZP treatment had any effect on mortality in adult females in 2018 with GLM.

We collected archive temperature and precipitation data (between 2013 and 2018) from the closest meteorological station (Debrecen, located 40 km from the reserve) from the web page of the Hungarian Meteorological Services (OMSZ) (https://www.met.hu/eghajlat/magyarorszag_eghajlata/eghajlati_adatsorok/Debrecen/adatok/napi_adatok/index.php).

2.10. Analyses of harem sizes and home ranges

To investigate changes in the number and the size of harems over the years in Pentezug, we counted the number of horses (all individuals, adults, juveniles, foals) in each harem in each year and calculated the average harem size by year. We fitted a GLMM in R with Poisson error distribution to these counts with year (centered to zero) as a fixed effect and harem ID as a random effect with random intercept and random slope with year (centered to zero). Data visualization was carried out using GraphPad Prism software.

Home ranges were calculated using 100% Minimum Convex Polygons (MCPs) and visualized together with the locations on the Pentezug Reserve map using Q-GIS and its Concave Hull plugin (https://planet.qgis.org/plugins/concavehull/). The home range overlap of harem i with the harem j was calculated as Oi,j = Ii,j/Ai, where Ii,j is the area of the intersection between the two home ranges and Ai is the area of harem i's home range, resulting in an asymmetrical overlap matrix. Overlaps were calculated only for coexisting harems; harems existing only for a short period (one sample day) were excluded from analyses.

2.11. *Aerial photo*

The aerial photo was taken with a GoPro Hero 3 Black camera mounted on a flying helium-filled balloon and stabilized with remote-controllable gimbal, on the morning of July 6, 2014 in the Pentezug Reserve. The observers were standing cca. 50 m from the edge of the herd. The camera recorded video at 3840 × 2160 pixel resolution and 12.5 fps, and the image was exported using ffmpeg (https://ffmpeg.org). Positions of horses were marked by ImageJ (https://imagej.nih.gov/ij/). Harems and bachelor groups were determined based on the coherent movement of the group members over several minutes, and were identified with the help of group composition lists by matching the list of group members with the visible features (body size and colour) of horses in the groups.

3. Results

3.1. *Population size and age distribution*

Since the initiation of the project, the Przewalski's horse population in Pentezug grew rapidly from 22 founder horses (arrived between 1997 and 2007) to 329 individuals in 2017. By the end of 2018, the number decreased to 267 (Fig. 1A) due to various reasons (for details see section 3.4.). Forty-seven cattle founders continuously arrived at Pentezug Reserve between 1999 and 2015. Similar to the horses, the number of cattle peaked in 2017, when there were 580 individuals (Fig. 1A).

Although the vast majority of horses in the population were born in Pentezug, some horses originated from various zoological gardens or the Malomhaza Wild Animal Park. Founder horses born in captivity formed the majority of the Pentezug population from 1997 to 2000. In 2000, horses born in Pentezug outnumbered the founders. In 2018, there was only one horse born abroad. Malomhaza, which is located 2 km from Pentezug, is home to a smaller horse population (20 individuals). Since 2004, the horses imported from abroad are released in Malomhaza, not in Pentezug, to increase the chance of reproduction and survival of foals.

The age distribution in Pentezug Reserve was biased to adults in the first years because predominantly adult horses were imported to the area (Fig. 1B). For many years, a prominent "gap" in the age distribution existed between the adult horses imported from zoological gardens and their foals. As founder horses aged out and the population enlarged, the "gap" almost completely closed. The current distribution is shaped like a pyramid, which is typical for young and growing populations (Fig. 1B). Regarding the gender distribution of the founders, there was a bias towards females (9 males vs. 14 females), because the founder population consisted of mainly harem groups. With the growing population, the difference between the number of males and females quickly disappeared. The birth-sex ratio of males to females was 1:0.99 (274 males vs. 272 females; statistically not different from 1:1, $\chi^2_1 = 0.007$, $p = 0.932$) in horses born between 1998 and 2018. At the end of 2018, the ratio of males to females was 1:1.05 (130 males vs. 137 females; statistically not different from 1:1, $\chi^2_1 = 0.184$, $p = 0.668$).

3.2. *Population dynamics*

In the 22 years of the project, there were large differences in the effects of non-human induced (births and deaths) and human-induced (imports, exports, and culling) factors on the number of animals. To facilitate the presentation of our results, we divided the study period into four intervals. The first period (1997–2001) was dominated by imports (n = 20) and relatively few foals were born (mean number of newborn foals ± SD per year: 3.20 ± 3.11). Deaths were predominantly imported animals (6 of the 7 death cases were of imported horses) in this period. In the second period (2002–2010), the number of births increased substantially (mean number of newborn foals ± SD per year: 18.56 ± 8.37), because more females reached maturity. The number of deaths was minimal (mean number of total deaths ± SD per year: 4.44 ± 2.60 vs. mean number of non-human induced deaths ±SD per year: 4.33 ± 2.45 individuals per year) and the number of transported animals was negligible in this second period. In the third period (2011–2015), the number of foals peaked (mean number of newborn foals ± SD per year: 52.80 ± 6.61). The number of deaths was much higher than in previous years, partly because of increased culling (mean number of total deaths ± SD per year: 19.40 ± 4.39 vs. the mean number of non-human induced deaths ±SD per year: 15.80 ± 2.59). In this period, we also started to export horses (n = 20) and introduced contraception treatment. In the fourth period (2016–2018), three factors affected the population. First, a large number of individuals (n = 36) were exported to a reintroduction area located in the Orenburg Reserve, Russia. Second, due to the PZP treatment, which was introduced in 2013, the number of foals started to decrease (for details see section 3.3). Last, there was a massive loss in 2018, when 90 individuals died and another 8 were culled (Fig. 1C and D).

The net effects of these non-human induced and human-induced events can be seen in the population growth rate (Fig. 1E). After peaking in 2001 (0.47), the population growth shows a moderate decreasing trend, which was still above 0 until 2017. The only year when the population decreased was in 2018 (growth rate: 0.18). The negative population growth rate was predominantly the consequence of the massive loss (see section 3.4).

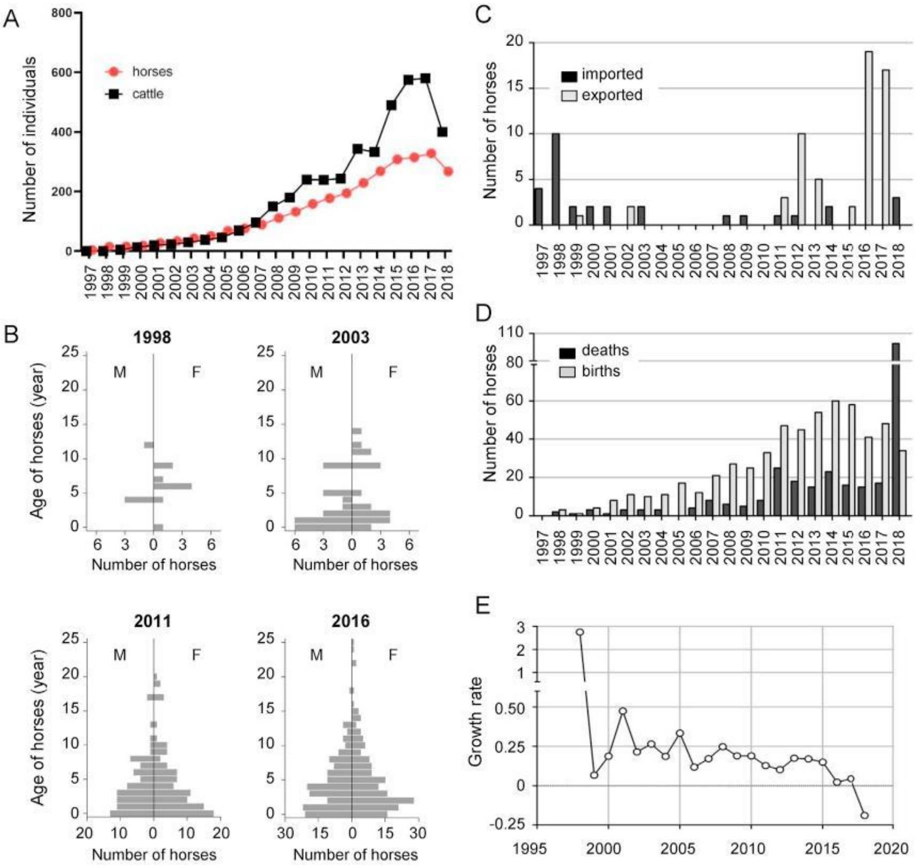

Fig. 1. Przewalski's horse population in Pentezug Reserve, Hortobagy National Park (HNP), Hungary between 1997 and 2018. (A) The number of Przewalski's horses and cattle in Pentezug Reserve from the initiation of the project. (B) Age-trees of horses in the reserve in the indicated years, separately for males (M) and females (F). (C) The number of imported and exported Przewalski's horses in the reserve. (D) The number of individuals born or died in Pentezug Reserve. (E) Population growth rate.

3.3. Foaling rate and PZP treatment

The number of adult females (2 or more years old) constantly increased since the establishment of the project (Fig. 2A). The number of foals also grew, roughly following the number of females in the first 15 years. This continued growth rate would have been unsustainable for the resources at Pentezug, therefore HNP and EEP decided in 2013 that reversible contraception (PZP) should be implemented. We treated 77 animals between 2013 and 2017 in different age groups (Table A9) and the number of treated animals grew every year (Fig. 2B). The foaling rate of non-treated females was always higher than that of the treated ones between 2014 and 2018 (Table A6). The most successful year of the treatment was 2016 when only 8% of treated horses gave birth (Fig. 2C). PZP treatment significantly decreased foaling rate (GLMM: 0.469 vs. 0.232, $z = 4.189$, $p < 0.0001$). As a result, the number of foals started to decrease in 2015 (Fig. 2A). Neither the time elapsed from finishing PZP treatment (GLMM: $\beta = 0.077$, $z = 0.178$, $p = 0.859$) nor the length of treatment (GLMM: $\beta = -0.045$, $z = -0.078$, $p = 0.938$) affected foaling rate significantly among treated females. Age did not have an effect on foaling rates in treated females (GLMM: linear term, $\beta = -0.015$, $z = -0.210$, $p = 0.8340$; quadratic term, $\beta = 0.006$, $z = 0.498$, $p = 0.6185$).

An analysis of various attributes of reproduction revealed that non-human induced phenomena, such as a delay in female fecundity or decreased foaling rate in non-treated females, may have also contributed to the decreased number of foals (Fig. 2D and E). We observed the first foaling age in 95 females between 2001 and 2018. We found that the age at first foaling increased significantly over time (quasipoisson GLM, $\beta = 0.057$, $t = 4.315$, $p < 0.001$). Between 2001 and 2009, females

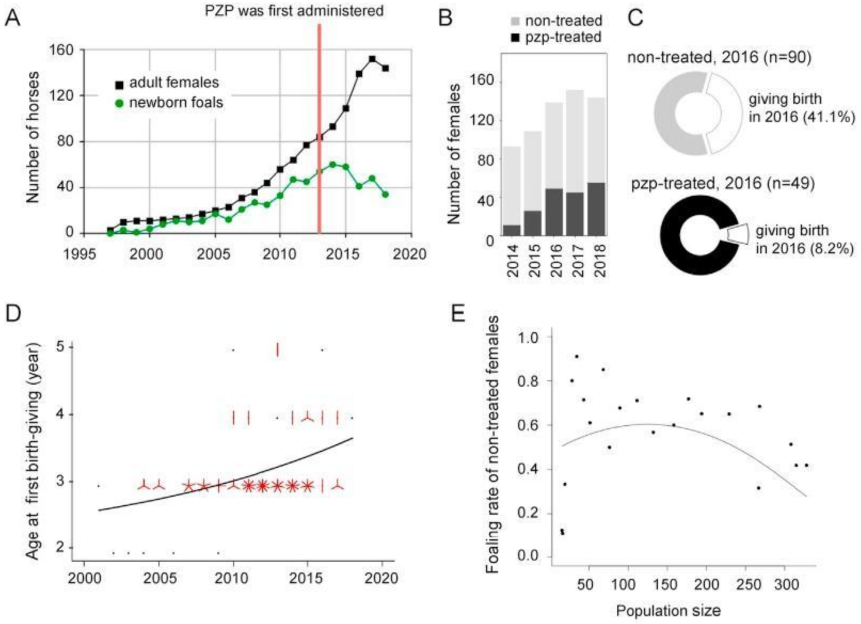

Fig. 2. Reproduction, birth control and foaling rates of Przewalski's horse population in Pentezug Reserve. (A) The number of adult females (2-year-old or older) and newborn foals in the reserve by years. (B) The numbers of PZP-treated (black) and non-treated females (grey) from 2014 (the first year of effective treatment). (C) Foaling rate of non-treated and PZP-treated adult (2-year-old or older) females in 2016. (D) Sunflower plot showing how the age at their first foaling of non-treated females grew over time. In each year the number of first foaling females at a given age are symbolized by 'sunflowers' instead of simple dots. The number of petals in these cases indicates the number of individuals falling in that combination. The solid black line was obtained by fitting a GLM with quasi-Poisson error distribution to the data (see main text for the details). (E) Foaling rate of adult non-treated females versus the population size. The solid line shows the fitted GLMM (see main text).

typically produced foals for the first time at the age of 2–3 years. However, between 2010 and 2018, the age at first foaling was delayed to 3–5 years (Figs. 2D) and 67.5% of females produced foals for the first time at the age of 3 during this period.

Another phenomenon that contributed to the decreased number of foals was the reduced foaling rate among non-treated females when the population size was high (Fig. 2E). Population size has a clear nonlinear effect on foaling rate (GLMM with binomial error, quadratic term: $\beta = -7.207$, $z = -3.138$, $p < 0.002$). The linear term was non-significant (GLMM with binomial error, linear term: $\beta = -4.952$, $z = -1.890$, $p = 0.059$). This suggests that foaling rate is density dependent above a certain density. The finding that the linear term was not significant may suggest that an Alee effect may also work here. Note, however, that we obtained a similar relationship between foaling rate and year (GLMM with binomial error, quadratic term: $\beta = -12.218$, $z = -5.279$, $p < 0.001$; linear term: $\beta = -5.628$, $z = -2.245$, $p = 0.025$). Because of the strong correlation between population size and year (as we have a growing population) we cannot clearly separate the effect of population size from that of years.

In non-treated females, foaling rate first increased then decreased with age (GLMM: linear term, $\beta = 0.426$, $z = 8.406$, $p < 0.0001$; quadratic term, $\beta = -0.045$, $z = -7.839$, $p < 0.0001$). Interestingly foaling rate decreased significantly with year at the same rate in treated and untreated females (GLMM: $\beta = -0.566$, $z = -6.359$, $p < 0.0001$).

3.4. *Mortality rate*

The total number of deaths was 272 between 1997 and 2018 (Fig. 3A). In 45% of all deaths, the carcasses were found and the cause of death was determined by the staff or veterinarian. The carcass was found but the cause of death could not be determined in 13% of the cases, mainly because sampling was not feasible from the carcass. In other cases (42%), the carcasses were not found, but these horses had not been seen for a long time and, therefore, were noted as dead. In cases where the cause of death could be determined, the most frequent causes were accident/injury, shot, and weakness (Fig. 3A). When we examined the overall mortality rate in Pentezug Reserve (excluding the year 2018), we found that neither year (GLM, $\chi^2 = 1.377$, $df = 2$, $p = 0.502$) nor number of horses (GLM, $\chi^2 = 2.364$, $df = 2$, $p = 0.307$) had a significant effect on the death rate. On the other hand, age had a curved relationship with mortality dependent on sex (GLM, age × sex interaction,

$\chi^2 = 21.998$, df = 2, $p < 0.0001$). Mortality first decreased with age then increased. In most age classes, mortality in males was higher than in females (Fig. 3B). We related the mortality rate of juveniles to year, population growth, and increasing inbreeding coefficient. As the fits of separate models indicate, juvenile mortality was significantly affected by all of our candidate explanatory variables (GLM, year: $\beta = 0.131$, $\chi^2 = 4.228$, df = 1, $p = 0.040$; number of horses: $\beta = 0.006$, $\chi^2 = 4.973$, df = 1, $p = 0.026$; level of inbreeding: $\beta = 194.34$, $\chi^2 = 6.460$, df = 1, $p = 0.011$). Note, however, that their effects, because of the high level of collinearity among them, cannot be separated. So, during the years in Pentezug, both the number of horses and the level of inbreeding tended to increase, which covary with juvenile mortality.

In 2018, the mortality rate was much higher compared to previous years and 26.6% of the population died. If we analyze mortality for 2018, we find that the probability of mortality decreased then increased with age and the non-linear effect was highly significant (GLMM: $\beta = 14.319$, $z = 4.118$, $p < 0.0001$), similar to the phenomena in previous years. However, sex did not have an effect on mortality in 2018 (GLMM: $\beta = -0.070$, $z = -0.269$, $p = 0.788$); there were 50 males and 47 females (and one unknown). The largest mortality rate was observed among juveniles (50% of this group died) and adult horses older than 20 years (100% died) (Table A8). When we compared the mortality of PZP treated and non-treated females, we found that PZP treatment tended to decrease mortality (GLMM: $\beta = -0.792$, $z = -1.794$, $p = 0.0728$).

The most obvious reason behind the massive loss in 2018 was the extreme cold weather in March coupled with heavy snowfall (Fig. 3C). Consequently, the whole area was covered with snow for several days and the horses had limited access to grass. Moreover, in the previous years, there was a drought and the population of horses and cattle had grown (Fig. 1A, Table A7) leading to poorer quality and quantity of food. We assume that the combination of these factors together caused the massive loss in 2018.

3.5. Population genetics

The mean inbreeding coefficient of all horses in Pentezug Reserve shows a slightly increasing trend in parallel with the growing number of animals, reaching its highest value in 2018 ($F = 0.176$) (Fig. 4A). Based on studbook data, the gene diversity values increased from 0.746 to 0.795, in the period 1998–2005 when the majority of imported animals arrived. The gene diversity reached the highest value in 2011 (0.804). Since then, the gene diversity values stabilized at approximately 0.800 (Fig. 4B). These data collectively suggest that, although the inbreeding coefficient is increasing, gene diversity has stabilized at a relatively high value.

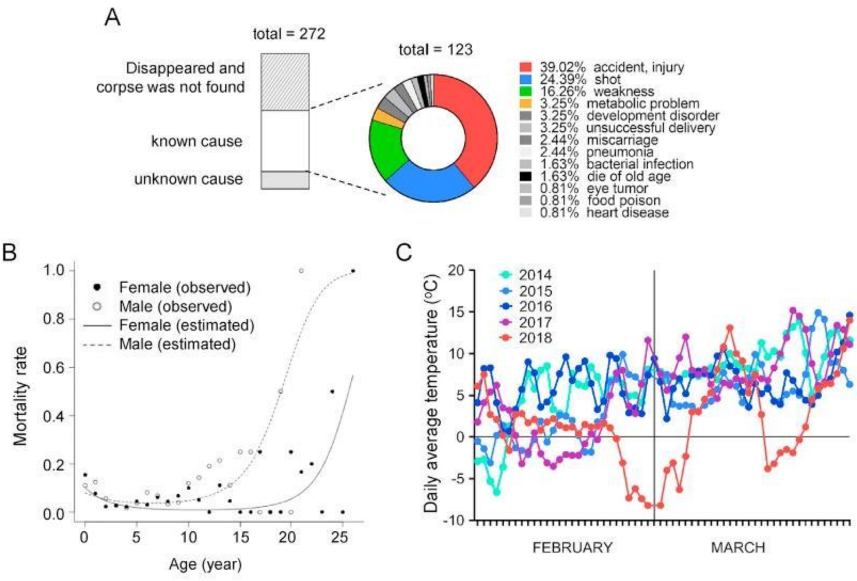

Fig. 3. Mortality in the Przewalski's horse population in Pentezug Reserve. (A) The percentages of various causes of death between 1997 and 2018 (n = 272). (B) Mortality rate versus age of horses between 1997 and 2017. White and black dots indicate the death rate of males and females, respectively. (C) Daily average temperatures (°C) in February and March between 2014 and 2018. Meteorological data was obtained from the Hungarian Meteorological Services (OMSZ) web page.

3.6. Changes in the social structure

Most of the horses imported in 1997 and 1998 arrived as harem groups (in total 3 harems with 12 horses). Ten horses arrived in pairs or alone between 1998 and 2007. The imported young males formed a bachelor group (Klimov, 1988). The analysis of harems revealed that the number of harems increased together with the number of individuals in Pentezug Reserve (Pearson correlation $r = 0.981$, $p < 0.001$, $n = 22$; Fig. 5A). Over the years, the number of harems continuously grew until 2010 and there was an increasing trend since 2010. The peak of both the number of harems and the entire population was in 2017, when 30 harems and 329 individuals were observed in the area. The average harem size did not change significantly with the growing population (GLMM with Poisson error: $\beta = 0.01372$, $z = 1.146$, $p = 0.252$; Fig. 5B).

We found that the ratio of population size and the harem number was 10.0 ± 0.8 in Pentezug Reserve, which was very close to the ratios in other areas (data from Xia et al., 2014 and oral communications by Jaroslav Simek regarding Gobi B and Dashpurev Tserendeleg regarding Hustai Nuruu in 2019) (Table 1). Of note, the population size includes bachelors, while the harem groups do not. Therefore, the average number of horses in the harems is lower than the ratio of the population size and the number of harems.

Harems of Przewalski's horses have been shown to have separate home ranges (King, 2002). We observed the same phenomenon in Pentezug at the beginning of the project (in 2001), when harem group ($n = 3$) home ranges were not overlapping (Fig. 5C). In 2014, the average overlap of home ranges of all harems ($n = 25$) was very high, 69% (Fig. 5C). Horses used 29% and 81% of the available area in 2001 and 2014, respectively. Interestingly, in 2001 the bachelor group also used a separate home range, which did not overlap with the harem groups. In 2014, bachelors were not recognized individually; thus, their home ranges were not recorded. However, bachelor groups were generally observed on the periphery but close to the harem groups (Fig. 5D).

4. Discussion

The HNP successfully established a semi-reserve for Przewalski's horses more than 20 years ago. The steppe ecosystem and the wetlands have provided sufficient food and water for the growing population and the fenced area prohibited unwanted hybridization with local domestic horses (Kerekes et al., 2019). The continuous monitoring of these horses and analysis of the data revealed interesting changes in demographic, genetic, and social characteristics of this population.

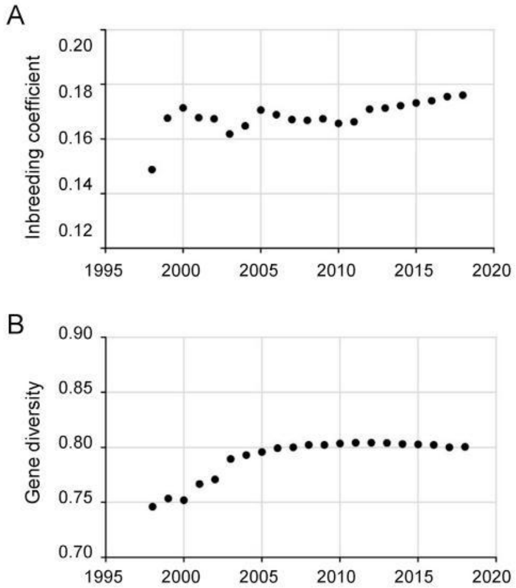

Fig. 4. Genetic characteristics of the Przewalski's horse population in Pentezug Reserve. (A) Pedigree mean inbreeding coefficients for the whole population by year. (B) Gene diversity based on kinship matrix by year.

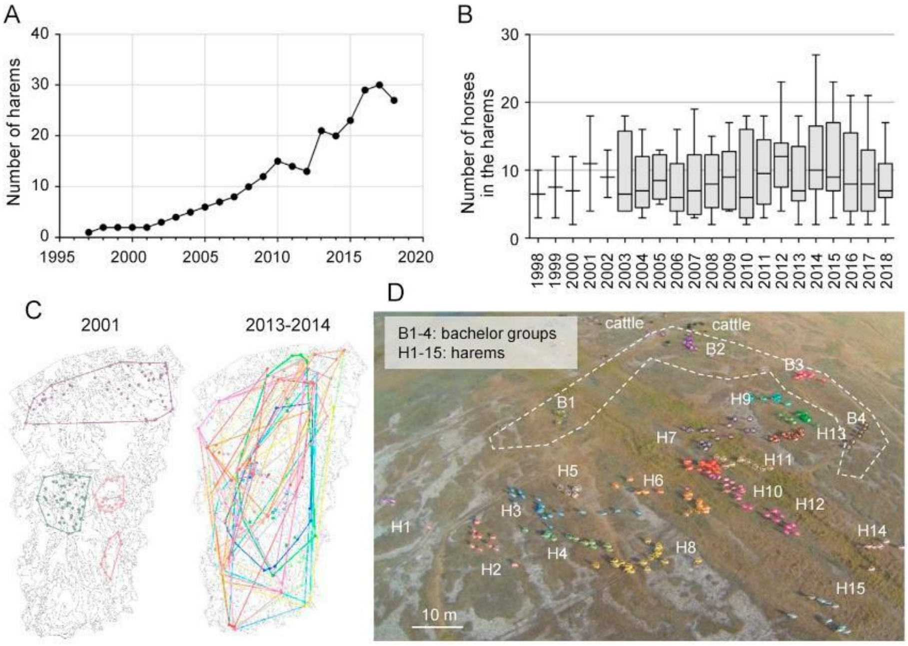

Fig. 5. Przewalski's horse harems in Pentezug Reserve. (A) The number of harems in the reserve by year. (B) The number of horses in harems as box-and-whisker plot. Median, first, and third quartile values are shown with boxes, while whiskers indicate the minimum and maximum values. (C) Home ranges (100% minimum convex polygons) of harems in two time periods (solid lines) and the positions of harems in different observation days (dots) on the map of Pentezug Reserve; different colors denote different harems. (D) Aerial photo taken with a camera mounted on a flying helium-filled balloon in the summer of 2014 in Pentezug Reserve. Different colors denote horses belonging to different harem (H) and bachelor (B) groups as indicated. The scale shows dimension of the foreground. Dotted line shows bachelors groups at the edge of the herd of harem groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.1. Demographic changes

After the first few years of the project, the population grew rapidly, similar to other equid populations introduced to closed areas without apex predators (Grange et al., 2009; Tatin et al., 2009; Scorolli and Cazorla, 2010). To control this rapid growth, the HNP and EEP decided to use three different methods, including exporting, culling, and contraception treatment. Each method has advantages and limitations (Nuñez et al., 2016). Exports are the most reasonable solution for an endangered species and very useful to decrease population size. Thus, whenever export was an available option, we exported animals to various zoological gardens and reintroduction areas. However, transports are very costly and can be dangerous for animals. In addition, finding places that can accept new individuals is difficult. Culling is used to eliminate injured or weak animals or animals with genetic disorders (such as fox gene carrier or born with abnormality). Culling is also used to eliminate animals with chronic injuries (such as lameness, teeth problem, etc.), especially if it already had many offspring. Culling helps reduce the number of horses inexpensively and effectively. However, using culling as a tool to limit population size, results in the elimination of healthy animals and raises serious ethical and animal welfare concerns. The third possibility for population control is immunocontraception with PZP. The advantages of PZP treatment are the reversibility and the ability to treat many individuals simultaneously. Our observation suggests that PZP had minimal or no effect on the health of the treated females and all adult age groups can be treated effectively. Thus, PZP-treatment is an efficient way to control the reproduction of females and in this way the size of the entire population. We observed that some of the treated horses returned to fertility after treatment but the majority of the treated horses are still on the period of induced infertility or could not give birth because of other reasons. Since we started birth control treatment in 2013 and the infertility period is 4–5 years, we cannot fully evaluate the percentage of treated females that returned to fertility at this time. We also cannot address how the circumstances of the treatment (the length or the number of repetitions) affect the return of fertility. In the future, after the accumulation of more data, these questions can be addressed. Immunocontraception, however, also has several limitations. PZP treatment is labor-intensive and requires individual recognition, which can be challenging in large populations (Hobbs et al., 2000). Another drawback is that PZP can alter behavior. Observations suggest that effectively treated females tend to change harem groups more often than non-treated ones, however, we did not perform systematic analyses on this aspect of

Table 1
Population size, number of harem groups, and their ratios for different populations of Przewalski's horses.

	Hustai National Park	Gobi-B	Kalamaili National Park	Pentezug Reserve 2016	Pentezug Reserve 2017	Pentezug Reserve 2018
Population size	382	277	127	315	329	267
Number of harems	35	25	13	29	30	29
Ratio	10.9	11.1	9.8	10.9	10.9	9.2

PZP treated versus non-treated females. Therefore, we cannot claim a significant difference. Nevertheless, our anecdotal observations are consistent with previous studies on feral horses. Feral horse mares receiving PZP change harem groups more often than non-treated ones, which can affect the harem structure, suggesting that treatment should be used carefully (Nuñez, 2009; Nuñez et al., 2009).

In parallel with human-induced population control, we observed that self-controlling processes also affect population growth. Delayed first reproduction, decreased fecundity, and increased juvenile mortality were observed in the last 5 years as population size was at its maximum. Our results suggest that these processes can be density-dependent, similar to other studies in Przewalski's horses (Tatin et al., 2009), other equids (Grange et al., 2009) and large mammals (Fowler, 1987). Note, however, that because of the strong correlations among years, population size and age of horses we cannot eliminate the possibility that the detected density dependence is not confounded by these other factors. In our study, the mortality rate of the whole population was not density-dependent, but it was affected by sex and age. Similar to feral horses on other areas (Garrott and Taylor, 1990) males usually do not live longer than 15 years in the reserve, probably due to the continuous stress of fighting of harem stallions. Younger age groups can be more sensitive to environmental changes, juveniles (one-year-old) having much less fat deposits can be more sensitive to long winters or poor food resources.

The only massive loss (26.6%) in Pentezug Reserve was observed in 2018. Similar massive losses of Przewalski's horses and feral horses have been documented in other areas. Kaczensky et al. (2011) observed population crash among Przewalski's horses in Gobi B National Park in the winter of 2009/2010, when a severe winter ("dzud") caused the death of 64% of the population of 138 horses. Scorolli et al. (2006) observed a similar phenomenon in Argentina in 2002, when 29.7% of the 650 feral horse population died within 2 days due to a heavy rainstorm. Stochastic environmental conditions and population density have a major effect on population dynamics in ungulates when living in predator-free circumstances (Saether, 1997). In our case, the combination of a very cold spring (snow and 15 °C below zero) in March 2018, limited food resources, caused by the drought in 2016–2017 and the large number of both cattle and horses (Appendix Table A7) led to this massive loss. After 2018 our population increased to 290 individuals (July 2020). Therefore, population control is still among the most important task.

4.2. Genetic characteristics

The careful selection of Przewalski's horses for breeding has been and is a critical issue (Der Sarkissian et al., 2015). The founders of the HNP population were selected by considering their genetic background and pedigree. Furthermore, the different genetic characteristics of this population in HNP were monitored carefully from the beginning of the project. In our study, we focused on two features of population genetics: gene diversity and inbreeding. Analyses of annual changes revealed that the gene diversity stabilized at a relatively high level, meaning that many individuals more or less equally contribute to the genome of the population. The reasons behind this high gene diversity may be the natal dispersal and active changes in the positions of harem stallions. This phenomenon was enforced by artificial interference, when we temporarily excluded females with more offspring from breeding, with the help of immunocontraception. In contrast, the growing value of the inbreeding coefficient might suggest that the population is endangered. Among inbred animals, juvenile mortality can be high (Ralls et al., 1988) and harmful genes or gene combinations may accumulate (Lacy, 1993). These processes can be critical in the case of small populations. To our knowledge, mean inbreeding coefficients have not been published for the Mongolian and Chinese Przewalski's horse populations and there has been little evidence of harmful inbreeding in Przewalski's horses. However, a negative correlation between fecundity and inbreeding coefficient has been demonstrated in the captive Prague population: two groups with different inbreeding coefficient values (up to 0.07 and 0.59) had different fecundities: 0.8 and 0.4 foals/mare, respectively (Bouman, 1977). Notably, our latest inbreeding coefficient value (0.176) is substantially lower than in the above highly inbred population with problematic infertility. However, we intend to slow down the growing inbreeding coefficient. One possible way to do this is to introduce new, unrelated individuals, possibly from rarer bloodlines compared to Pentezug. Our current plan is to keep a special breeding group at Malomhaza Wild Animal Park and transport young offspring to Pentezug Reserve. We may also obtain imports from abroad in the near future. These observations highlight the importance of close cooperation with other holders of Przewalski's horses and the necessity of exchanging animals.

4.3. Harem structure

During the last 22 years, we observed remarkable changes in the demography of the Przewalski's horses in the area. We were curious whether certain characteristics of social structure also changed in parallel with increases in the size and density of the population. Remarkably, we observed that the growing number of individuals affected the total number of harems, but not their average size. Horses are highly social individuals, their social bonds inside the harems are very strong and their behavior and activity are highly synchronized within a harem (Boyd et al., 1988). However, the number of these possible bonds and the number of females a harem stallion can protect are limited. The multi-male harems structure, which is common among feral horses (Rubenstein, 1981), is extremely rare in Pentezug Reserve; they were observed only twice between 1997 and 2018. Based on our observations and international data on Przewalski's horse populations concerning harem numbers and sizes, we assume there is an optimal range of harem size, which seems to be independent of area and population size.

The previously described separated home range structure (King, 2002) was only observed in the early years of the project. Later, both harem and bachelor groups synchronized their movement and activity and separate home ranges disappeared. The whole population eventually formed a large herd moving together all year round, using almost the entire available area. Of note, some harems, mainly the newly formed ones, sometimes stayed isolated, keeping a large distance from the united herd. The reason for the large herd phenomenon and the manner in which animals interact is unknown, but is likely due to environmental (limited area, abundant food, and water supply) and social factors (long term social relationships and genetic relatedness). Interestingly, bachelor groups seemed to stay on the periphery of the large herd. Thus, the large herd formation may be a good defense strategy against bachelors. Very similar social structures can be observed in feral horses in different countries (Duncan, 1992; Berger, 1986) and plain zebras in Africa (Rubenstein and Hack, 2004; Klingel, 1969).

5. Conclusions

We demonstrated that the Pentezug Reserve is an adequate area for Przewalski's horses. To avoid overpopulation in the area, human interventions (birth control, transport, etc.) are essential. Monitoring the genetic values at the population level is also important, because changes in these characteristics indicate the necessity for human interventions. Our results show that exchanging individuals among projects is also very important, even if the project is successful in terms of increasing numbers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gecco.2020.e01407.

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ANALYSIS OF HABITAT USE, ACTIVITY, AND BODY CONDITION SCORES OF PRZEWALSKI'S HORSES IN HORTOBAGY NATIONAL PARK, HUNGARY

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A founder population of Przewalski's horses (*Equus ferus przewalskii*) was set free in a semi-reserve called Pentezug in the Hortobagy National Park (Hungary) in 1997. Beside the Przewalski's horses reconstructed aurochs (*Bos taurus taurus*) were bred as well in the 24.5-km² large area. Both species reproduced successfully in this steppe reserve, which was once the homeland of their distant ancestors. The number of large grazers has grown so quickly that different strategies of population control had to be implemented for reconstructed aurochs and horses in 2007 and 2013, respectively. The activity, habitat use, and body condition of Przewalski's horses were monitored regularly throughout the 22 years. We detected seasonal changes in the activity of the horses but the proportions of the main activity types were similar in two distant time periods. In contrast, habitat use and body condition scores for the horses showed remarkable differences between early vs. late years of the project, implying that horses were forced to use non-preferable areas and their condition was worsened in parallel with the increased population density of the large grazers. During the cold spring in 2018, there was a massive loss of both horses and cattle. These observations suggest that limited food sources could lead to changes in habitat use and/or worse body condition and a greater death rate in extreme weather conditions. In summary, strict birth control measures must be implemented in dense Przewalski's horse populations, and the habitat use and body conditions must be monitored to balance the population size and carrying capacity of the areas.

Key words: *Bos taurus taurus*, carrying capacity, *Equus ferus przewalskii*, large grazers, population control, Pentezug Reserve, reconstructed aurochs, semi-reserve

Introduction

In the Holocene period, wild horses (*Equus ferus* Boddaert, 1785) inhabited both Europe and Asia (Vörös, 1987). Their descendant subspecies disappeared from the wild, first in Europe and then in Asia in the 1960s (Sommer et al., 2011). Fortunately, Przewalski's horses (*Equus ferus przewalskii* Poliakov, 1881), a Mongolian subspecies, survived in captivity and their descendants can be found in zoos and in the wild again due to various projects (Bouman & Bouman, 1994). Reintroduction projects were started in China (Xinjiang Wild Horse Breeding Centre and Kalamaili Mountain National Park) and Mongolia (Khustain Nuruu National Park and Great Gobi B Strictly Protected Area) in 1985 and 1991, respectively (Wakefield et al., 2002; King, 2005; Walzer et al., 2012; Xia et al., 2014).

Besides the reintroduction projects in Mongolia and China, many closed natural conservation areas are home to Przewalski's horses all over the world. The herd in the Pentezug Reserve (Hortobágyi National Park, Hungary) represents the largest population of Przewalski's horses in Europe. The project, which was started in 1997, was coordinated, supervised, and financed by the Hortobágy National Park (hereinafter HNP) and the Cologne Zoo (Germany). Horses and a hardy breed of domestic cattle (*Bos taurus* Linnaeus, 1758), called Heck cattle or reconstructed aurochs, were kept together in the area surrounded by an electric fence. The Heck cattle, which have been used for landscape management in several other regions in Europe (Lorimer & Driessen, 2013) are phenotypically similar to the extinct aurochs (*Bos taurus primigenius* Bojanus, 1827), which once inhabited the Hungarian Steppe. The aim of this project was to create an alternative grazing ecosystem with minimal human intervention compared to grazing livestock. The Pentezug Reserve is part of the steppe vegetation of HNP and provides adequate grass species and water supply (marshes and canals) for large grazers. However, apex predators of large herbivores are missing.

Equidae have a cecal digestion system (Janis, 1976), which means that the food goes through their digestion system quickly and they can live on vegetation with low nutritional content. As a consequence, horses choose habitats based on a green plant content (Duncan, 1983; King, 2002) and have to graze more in winter when the nutritional content of vegetation is lower (Boyd & Bandi, 2002; Souris et al., 2007). With a growing density of large grazers, horses might have to use areas that are not optimal (Beest et al., 2014), affecting their body condition.

In this study, we describe our observations on habitat use, activity, and body condition over several years. Seasonal changes and changes occurring over larger time intervals, parallel to the population growth, can be distinguished. The results, accumulated during more than 20 years, contribute to our understanding of the activity and habitat use of Przewalski's horses and may facilitate herd management or novel project designs.

Material and Methods

Study area

The HNP, established in 1972 as the first national park in Hungary, is located in the Eastern part of the country, 150 km from Budapest. The research area, called Pentezug Reserve (24.5 km^2), can be found in the middle of the HNP at 47.5175 N, 21.092778 E. The climate is dry continental with 550 mm annual precipitation. Four seasons can be differentiated, where the winter is cold and dry with a minimum temperature of -15°C, 20–40 day snow cover (2–15 cm) and the summer is hot with a maximum temperature of +35°C. There are moderate temperatures and more rainfall in autumn and spring. The reserve is part of the continental alkali grassland region that is characterised by salt and water content of the soil and the natural floods. The groundwater, which is rich in different salts and close to the surface, easily evaporates when the weather is hot. As a consequence, these salts accumulate in the upper soil level (Török et al., 2012). Due to these phenomena, these grasslands are not suitable for agricultural utilisation and have been used as pastures for centuries (Molnár, 2014). We can differentiate eight habitat types in the Pentezug Reserve with different plant associations showed in Table 1 (Deák et al., 2014a,b,c).

Table 1. Habitat types in Pentezug Reserve (Hungary)

Habitat Type	Association	Characteristics	Characteristic plant species	Habitat area in Pentezug (km^2)
Forest	Planted oak forest	along the river	*Quercus robur* L.	0.315887
Alkali marsh	*Bolboschoenetum maritimi*	shallow water cover, considerable salt content both in the soil and water	*Bolboschoenus maritimus* (L.) Palla	2.207703
Reed bed	*Phragmittetum communis*	deep water cover	*Phragmites australis* (Cav.) Trin. ex Steud.	0.712105
Weed	*Onoprodetum acanthi, Hordaetum hystricis*	on former livestock farms, high soil N and P content	*Onopordium acanthium* L., *Hordeum hystrix* Roth.	0.277978
Alkali steppe	*Artemisio santonici – Festucetum pseudovinae*	short grass on solonetz soil, moderate humus content, low to moderate salt content in the deeper soil layers	*Festuca pseudovina* Hack ex Wiesb., *Artemisia santonicum* L., *Achillea setacea* Waldst. & Kit.	7.398344
Open alkali grassland	*Camphorosmetum annuae, Puccinellietum limosae*	high salt content close to or even on the soil surface, halophyte species	*Camphorosma annua* Pall., *Puccinella limosa* (Schur) Holmb.	2.620432
Alkali meadow	*Agrostio stoloniferae – Alopecuretum pratensis, Beckmannietum*	tall grass, high ground water level, low to moderate salt content in the soil, surface water from spring to early summer	*Alopecurus pratensis* L., *Agropyron repens* (L.) Gould, *Beckmannia eruciformis* (L.) Host	10.154303
Loess steppe	*Salvio nemorosae – Festucetum rupicolae*	high fertility humus rich soil, monocot and forb species	*Salvia pratensis* L., *S. nemorosa* L., *Festuca rupicola* Heuff.	0.156431

In 2000, with the help of aerial photographs and field observations, these different habitat types and areas (i.e. polygons) were recorded and shape files were created using the ArcMap 10.1 program. Each polygon of the attributed table contains one identification code for each of the eight habitats. We calculated the area of each habitat with the help of the ArcMap 10.1 program. The Pentezug Reserve is surrounded by a three-lined electric fence. Inside the area, horses and cattle can move freely between habitat types. Other wild animals, such as red fox (*Vulpes vulpes* Linnaeus, 1758), roe deer (*Capreolus capreolus* Linnaeus, 1758) and wild boar (*Sus scrofa* Linnaeus, 1758) can cross the fence. Surplus food or water was not offered except occasionally in winter and any human intervention (such as herding and mowing) was banned.

Przewalski's horse and cattle populations in the Pentezug Reserve

The founders of the horse population were selected by the coordinator of the *European Endangered Species Programmes* (*EEP*) to create the widest genetic base. The coordinator uses the studbook data (which contains parentage, location, birth, and other important data on the horses) to calculate the mean kinship and genetic diversity of the horses and create a viable population with the help of PMX 0.1 software. According to the advice of the EEP coordinator, we imported 31 horses and exported 59 horses between 1997 and 2018. Przewalski horses are not marked by branding or by chips. However, most individuals are recognised by the HNP staff based on their gender, age, special marks/characters, and the harem they belong to. The marks/characters include coat colour, stripes on the legs, shoulder crosses, cowlick positions, and other special features, such as double shoulder crosses or missing eartips. The horses were identified and counted regularly (every month, if possible) in the field. Members of bachelor groups are counted regularly, but they have not been recognised individually since 2008. Cattle founders arrived from different Hungarian, German, and Austrian zoological gardens and private gardens. The first cattle arrived at the reserve in 1999 and a total of 47 cattle arrived up until 2015. Cattle were marked with eartags since 2007 and counted once a year. However, before 2007 they were recognised with special characteristics individually and counted monthly just like the horses. We recorded births, deaths, imports, and exports for both the horses and cattle. In this way, we could calculate the number of horses and cattle at the end of every year and use this number to illustrate the yearly changes in the Pentezug Reserve.

Recognition and registration of horses in the Pentezug Reserve

Horses are registered in three databases, (1) a photo catalogue, (2) a group composition list, and (3) a DNA database. The photo catalogue consists of winter and summer portraits, summer and winter coats on both sides, and the back side of the animal. The group composition list consists of the positions of horses in different groups. The positions are recorded monthly from the beginning of the project with the exception of the years between 2008 and 2011, when there were only 1–4 observations annually. The genetic database of our horses is in the Davis Laboratory (Veterinary Genetics Laboratory, California, USA). We sampled each imported horse and all foals at the age of 1–2 year using a pneumatic rifle (operating with CO_2) and special biopsy needles (PneuDart and DanInject). In this way, small amounts of skin or biopsy samples can be taken without tranquilising animals. If we cannot identify a horse, we take a repeat sample and the Davis Laboratory can identify the horse in question.

Analysis of habitat use data

We recorded 258 and 291 GPS data in 2004–2006 and in 2013–2014, respectively. We collected data once a day at the most and transferred data to two Excel databases. We made shape files from the GPS Excel files in the ArcMap 10.1 program for both periods. We integrated these GPS shape files and a habitat map shape file using the ArcMap 10.1 program. In this manner, every GPS point had a habitat identification number. We calculated how many times horses stayed in different habitats in both periods and seasonally. Habitat preference was evaluated by comparing the proportion of habitat (relative to the whole reserve area) and the proportion of time horses spent in the given habitat (compared to all observation). We checked the seasonal changes in the usage of the three largest habitats by comparing the proportion of time the animal spent in one habitat in one season and the time they spent in all habitats in the given season.

Monitoring and analysis of activity data

We differentiated eight activity types (Table 2) during our study based on the work of Boyd & Houpt (1994). We recorded the activity of ten females in two harem groups every 2 min. in 2008–2009 and 90 females in 28 harem groups every 4 min. in 2013–2014 using a scan sampling method during daylight hours. The sampling lasted for 16 minutes and for 60 min. in 2008–2009 and in 2013–2014, respectively. We compared the activity percentage by calculating the ratio of the activity type to all recorded activities in the two periods. We examined the seasonal differences among activity types in 2013–2014 by calculating the proportion of time animals spent at a given activity compared to all activities in a season. We compared the three most remarkable activity types seasonally. Data visualisation and Kruskal-Wallis statistical tests were carried out using GraphPad Prism software.

Body condition scores

The body condition records were based on visual estimation (Rudman & Keiper, 1991). The body condition was scored by checking the hind quarter of the horse and giving a score between 0 (very bad condition) and 5 (very good condition). We collected body condition scores of 50 horses and 90 horses in 2005 and 2018, respectively. We used binoculars and went as close as possible (20–40 m) to the given animal and waited till we could see the bottom symmetrically. Each animal had one score between July and October in each year. Five groups of horses were classified based on age, sex, and position in the social structure. (1) Bachelors: members of the bachelor group, either males who left natal groups and do not have a harem yet or former harem stallions. (2) Harem stallions: owners of a harem at the time of scoring. (3) Adult females: females who left natal groups. (4) Young males: still in natal groups. (5) Young females: still in natal groups. We compared the data of each group for the two time periods. We only used data where a minimum of five records were collected in both years. In this way, we could compare the data of the harem stallion group (n = 5, n = 16), the adult female group (n = 21, n = 60), and the young male group (n = 9, n = 26). We used Mann-Whitney tests to examine whether the groups were different in the two time periods. Data visualisation and statistical tests were carried out using GraphPad Prism software.

Results

Population changes

Since 1997, cattle and horse populations have grown rapidly in the area (Fig. 1). The total number of animals was highest in 2017 (n = 950) and the population density reached 38.8 animals per km^2. Within one year the total number of animals dropped by 33% when the intensive decrease in the number involved both cattle and horses. The number of cattle was 400 at the end of 2018. The total number of calves was 1135 and 738 cattle were sold or died in the history of the Pentezug Reserve. The cattle population grew constantly until 2010 and stagnated for three periods (2010–2012, 2013–2014, 2016–2017). The robust decrease in the cattle population after 2017 was due to human intervention (transportation to other areas or to slaughter-houses). The cattle population consisted of mostly adult cows and their offspring, only two breeding bulls were temporarily transported to the area since 2007. At the end of 2018, the horse population consisted of 280 animals. Approximately 259 animals died between 1997 and 2018 and 567 foals were born. The horse population was increasing until 2015. Between 2015 and 2017, the population grew slower due to human interventions (transports and contraception treatment). In 2018 there was a massive loss because the cold spring devastated many weak individuals.

Table 2. Description of activity types

Activity type	Description
Grazing	When an animal eats grass, its head lowered down to the ground. Either standing or slowly walking in the meantime.
Resting	An animal is standing, ears are usually lateral, or lying on the ground.
Moving	An animal is walking without grazing, can be short (few steps) or long (wandering). An animal is running.
Alert	An animal is standing straight, looking to one direction, its ears are turned to the direction.
Interaction	An animal has friendly or not friendly contact with another one.
Comfort	An animal is scratching itself on something, scratching itself with its teeth, rolling on the ground.
Other	Special activity types that could not fit in the previous six, such as drinking, urinating, defecating, chewing, etc. Usually, a small number of events compared to the whole time budget.
Undetermined	When an animal was not seen during the sampling period

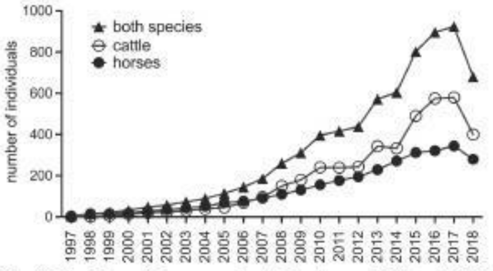

Fig. 1. Number of horses and cattle between 1997 and 2018 in the Pentezug Reserve, Hortobágyi National Park, Hungary.

Habitat use of Przewalski horses

The three most typical habitats in Pentezug Reserve are the alkali grassland, open alkali steppe, and alkali meadow (Fig. 2A,B). These habitats together cover approximately 85% of the total area. Horses spent most of their time in these habitats irrespective of the year of observation (either 2004–2006 or 2013–2014). Forest and loess steppe patches were hardly used in both periods (Fig. 2B). A comparison of the proportion of a given habitat and the proportion of time horses spent in the habitat revealed their habitat preference (Fig. 2B). When the proportion of habitat was higher than the proportion of time spent there, then the habitat was not preferred. Horses did not prefer the alkali meadow and salt marsh, while they preferred the alkali steppe, alkali grassland, and weed. Interestingly, the weed habitat was highly preferred in 2004–2006, partly because this habitat could be found around small houses or former stables that could provide shelter for horses against the sun and wind, but they were broken down by 2013. Also, a high nitrogen and phosphorous content can be attractive for horses.

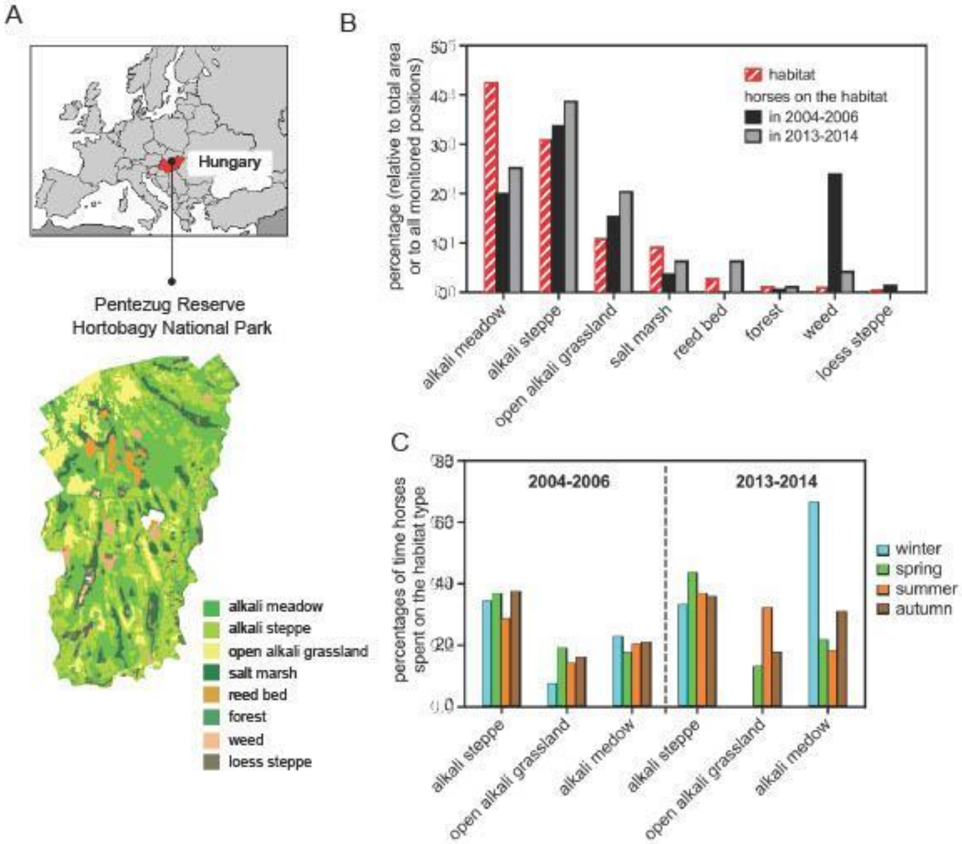

Fig. 2. Habitats of Pentezug Reserve and habitat use of Przewalski's horses. (A) Habitats of the reserve as determined by botanists of the Hortobágyi National Park in 2000. The areas are colour coded as indicated. (B) The proportion of habitats and time horses spent on the given habitat are shown together. The proportion of time horses spent on the habitats was determined in two periods based on GPS data. (C) The proportions of time horses spent in the habitats seasonally in two periods. Results are shown only for the three largest habitats.

We also investigated whether habitat use exhibited seasonal changes in the two periods. Most of the habitats were used similarly in 2004–2006. Only open alkali grassland was used less frequently in winter. In 2013–2014, we detected seasonal differences in the case of two habitats. Animals spent more time on open alkali grassland in summer than in spring or autumn and they did not use the open alkali grassland in winter. Also, horses could be found on alkali meadows much more in the winter than in the other two habitat types (Fig. 2C). These data suggest that, in 2013–2014, the open alkali grassland was not used.

Activity of horses

We recorded 2482 and 5995 activity events in 2008–2009 and in 2013–2014, respectively. In both monitored years, we found that three activity types, namely grazing, resting, and moving, covered 90% of the yearly activity (Fig. 3A). The ratio of these activities did not change from 2008 to 2013. The only remarkable changes were in the undetected activities, which increased in 2013 compared to 2008 (Fig. 3A) because monitoring groups with more members resulted in more undetected horses. Seasonal activity ratios in 2013–2014 demonstrate that grazing is the most common activity in all seasons. However, horses spent almost the same amount of time resting as grazing in spring and summer (Fig. 3B). Similar seasonal changes can be found in 2008–2009 (data not shown). Grazing, resting, and moving were significantly different seasonally (Fig. 3C). Horses spent more time grazing in autumn and winter than in spring and summer. On the other hand, they rested much more in warmer seasons than in colder ones. Horses spent almost the same amount of time moving in the spring, summer, and autumn, but much less in the winter.

Changes in body condition

The condition scores of adult females and young males decreased from 2005 to 2018 (Mann-Whitney test $p < 0.0001$). Female scores varied between 3–4.5 and 2.5–4.5 in 2005 and in 2018, respectively. Scores of young males varied between 3.5–4.0 and 2.5–4.0 in 2005 and 2018, respectively. The scores for harem stallions did not differ significantly between the two time periods (Mann-Whitney test $p = 0.87$) and the more animals we could score, the more variable results we got. The scores of bachelors and young females could not be compared, because of inadequate data ($n = 3$ for bachelors in 2018 and $n = 3$ for young females in 2005).

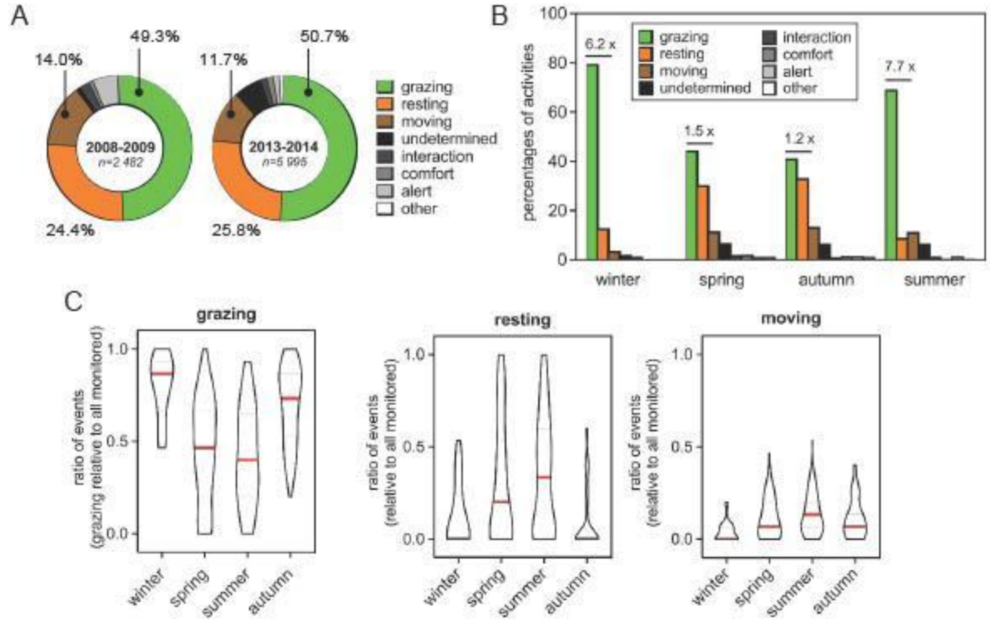

Fig. 3. Seasonal changes in the activity of Przewalski's horses in Pentezug Reserve. (A) The proportion of activities in two periods of time (2008–2009 and 2013–2014), both covering 12 months, are shown. (B) The proportion of the activities for four seasons is shown separately. The ratio of the two most frequent activities (grazing and resting) is shown for each season. (C) The density of the data at different values for four seasons and three activities is shown on violin plots. The medians are indicated in red. The comparison of four data sets revealed significant changes ($p < 0.001$) according to Kruskal-Wallis tests in all three activities.

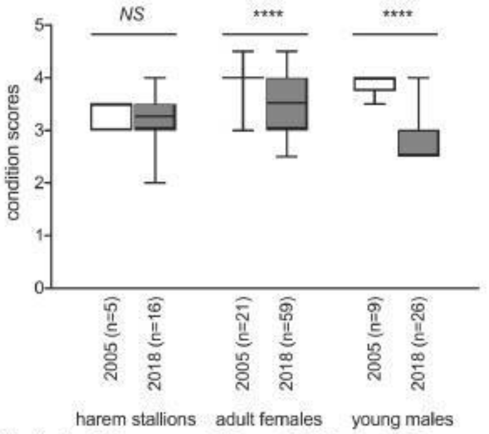

Fig. 4. Condition scores of Przewalski's horses in Pentezug Reserve. The number of monitored individuals is shown in brackets. The condition scores obtained from two years were compared separately for each group using Mann-Whitney statistical tests (NS – not significant; **** – $p < 0.0001$).

Discussion

The number of Przewalski's horses and reconstructed aurochs has remarkably increased in the Pentezug Reserve since launching the project. We can see similar phenomena in other areas where grazers were introduced and the former apex predator was missing (Caughley, 1981; Coulson et al., 2001). Since these two species compete for grasslands to a certain degree (Duncan et al., 1990; Menard et al., 2002), it is important to consider the cattle population when examining the horses' behaviour or activities. In 2007, the number of cattle started to grow dramatically. Two different strategies were selected to control the population growth of the two species. Horses were treated as wild animals but cattle were caught once a year (in winter) for the purpose of population regulation. The thresholds in the cattle population graph show all the years when we managed to sell more animals compared to how many were born and could control the population growth. Horses, on the other hand, were not caught easily and the only way to treat them is when tranquilised one by one. Furthermore, the Przewalski's horse is a rare species and categorised as endangered in the IUCN Red List (Walzer et al., 2012). For this reason, controlling the population growth is a delicate question. We could cull animals with fatal injuries or the ones suffering on the edge of death. However, we have to ask individual permission from the local authorities, where all our animals are registered. Immunocontraception is an option for management of large horse populations (Kirkpatrick et al., 1990). In 2013, following the recommendation of the EEP coordinator of Przewalski's horses, we decided to use Pig Zona Pellucida (PZP) immunocontraception (Turner et al., 2002) to slow down the population growth. In 2015, the population growth did slow down as an effect of this treatment. Nevertheless, we had an enormous loss of horses (40–50 horses) and cattle (20–30 cows) in 2018, most likely due to a combination of a very cold spring (snow and 15 degrees below zero) in March, a massive drought in 2016–2017, and a large number of both cattle and horses. This phenomenon can also be observed in areas occupied by feral horses (Scorolli et al., 2006). We decided to reduce the number of both species as soon as possible. We sold more cattle compared to the number born, resulting in a fast reduction in the animal population. We are planning to increase the number of PZP-treated female horses.

We assume that the changes in number will not only alter the vegetation, but also the behaviour of the Przewalski's horse population. Horses prefer a habitat with grass species dominated by *Festuca pseudovina* Hack. ex Wiesb. This species can grow even if the temperature is below zero providing small but continuous amounts of green plant parts that are important for horses when choosing habitat (Duncan, 1983; King, 2002). Open alkali grasslands are attractive in summer in higher animal density, when horses destroy its vegetation by trampling and create dust baths to avoid insects. As a consequence, these habitats cannot provide food in winter. On the other hand, grass species on alkali meadow grow and dry quickly but provide quite a large amount of food in winter, when other habitats cannot offer enough food because of the growing animal density (Beest et al., 2014). As a consequence of their preference for weed habitat, horses prove to be a good tool for maintaining the natural grassland vegetation and they can eliminate small amounts of weed vegetation by trampling or feeding on it.

Activity types for Przewalski's horses in Hortobagy are similar to Przewalski's horses or feral horses in other areas. Grazing takes up the largest part of their time and they rest more and graze less in the summer than in winter (Boyd et al., 1988; Duncan, 1992; Boyd & Bandi, 2002;

Souris et al., 2007). Berger at al. (1999) found a similar pattern, although they recorded horse movement even during the night. Horses have to increase food intake during winter, when the food is less nutritious (Berger et al., 1999). We expected that the growing population and decreasing amount of food in the area would alter the grazing-rest ratio. This ratio, however, did not change in the two periods, which suggests the population number did not alter the activity percentage in Pentezug.

If we compare the body condition in 2005 and in 2018, we can conclude that the condition of most horses greatly decreased probably because of the larger density and less available food (Rudman & Keiper, 1991). Environmental effects have more impact on young horses while they are still growing and cannot collect fat deposits. The harem stallions' physical condition did not change over the years, but the data show a high variability for this group. Harem stallions go through different stages when owning a harem. In the beginning, they have to put a lot of effort into protecting the harem and keeping the new members together. Also, when they are old or injured their physical condition quickly decreases and they often lose the harem.

Conclusions

We demonstrate that the Pentezug Reserve has been an adequate area for both Przewalski's horses and reconstructed aurochs for the last 22 years. To avoid overpopulation of the area, human interventions (birth control, transport, etc.) are essential. Monitoring the habitat use and body conditions of Przewalski's horses are also important because changes in these characteristics could indicate the necessity for human interventions.

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АНАЛИЗ ПАРАМЕТРОВ ИСПОЛЬЗОВАНИЯ ТЕРРИТОРИИ, АКТИВНОСТИ И ФИЗИЧЕСКОГО СОСТОЯНИЯ ЛОШАДЕЙ ПРЖЕВАЛЬСКОГО В НАЦИОНАЛЬНОМ ПАРКЕ ХОРТОБАДЬ, ВЕНГРИЯ

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Популяция лошади Пржевальского (*Equus ferus przewalskii*) был основана в полурезервате Пентезуг в Национальном парке Хортобадь в 1997 г. Кроме лошадей Пржевальского, там также разводят восстановленных туров (*Bos taurus taurus*) на огороженной территории площадью 24.5 км². Оба вида успешно размножились в степном заповеднике, некогда бывшим родиной их далеких предков. Количество крупных травоядных росло так быстро, что для восстановленных туров и лошадей пришлось применять разные стратегии контроля популяции в 2007 и 2013 гг., соответственно. На протяжении 22 лет проводился постоянный мониторинг активности, использования территории и физического состояния лошадей Пржевальского. Мы обнаружили сезонные изменения в активности лошадей, но соотношение основных типов активности были схожи в два разных периода времени. Напротив, были выявлены значительные различия в использовании территории и физическом состоянием лошадей в ранние и поздние годы проекта. Имеется в виду, что лошади были вынуждены использовать непривлекательные районы, и их состояние ухудшалось по мере увеличения плотности популяций крупных травоядных. Во время холодной весны 2018 г. произошла массовая гибель как лошадей, так и туров. Исследования показали, что ограниченные запасы кормов могут привести к изменениям в использовании среды обитания и/или ухудшению физического состояния, увеличению смертности при экстремальных погодных условиях. Таким образом, строгие меры контроля над рождаемостью лошадей Пржевальского должны применяться в популяциях с высокой численностью, и необходимо контролировать использование территории и физическое состояние животных, чтобы находить баланс между численностью популяции и емкостью угодий.

Ключевые слова: *Bos taurus taurus*, *Equus ferus przewalskii*, восстановленный тур, емкость угодий, заповедник Пентезуг, контроль численности, крупные травоядные, полурезерват

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ORIGINAL RESEARCH

Age-specific effects of density and weather on body condition and birth rates in a large herbivore, the Przewalski's horse

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Abstract

Reproduction in young females can show a particularly sensitive response to environmental challenges, although empirical support from individual-based long-term studies is scarce. Based on a 20-year data set from a free-roaming Przewalski's horse population (*Equus ferus przewalskii*), we studied effects of large-herbivore density (horses + cattle) and weather conditions experienced during different life stages on females' annual birth rates. Foaling probability was very low in 2-year-olds, reaching maximum values in 5 to 10-year-olds, followed by a decrease in older females indicating reproductive senescence. Mother's previous reproductive investment affected her current reproduction; young and old mothers (as opposed to middle-aged ones), which had nursed a foal for at least 60 days during the previous year, reproduced with a lower probability. Foaling probability and body condition of young females were lower when large-herbivore density was high. Reproduction was also influenced by interactive weather effects during different life stages. Low late-summer precipitation during the females' year of birth was associated with a pronounced decrease in foaling probability in response to harsh late-winter temperatures prior to the mating season. In turn, increased amounts of late-summer rain during this early age together with more late-summer rain during the females' current pregnancy led to an increased reproductive probability in 2–3-year-olds. These results were corroborated by the ameliorating effects of late-summer rain on body condition in such females. In conclusion, our findings highlight the interactive importance of weather conditions experienced during early life, and of density and weather during current pregnancy on foaling probability, particularly in young females.

Keywords Age-dependence · *Equus ferus przewalskii* · Foaling · Parturition · Precipitation · Reproduction · Senescence · Winter temperature

Introduction

Exploring the environmental drivers of age-dependent reproduction is an important step in the study of the dynamics of age-structured populations (Emlen and Pikitch 1989; Coulson et al. 2005; Frederiksen et al. 2014). Typically, high population densities and adverse weather conditions can negatively affect reproductive parameters (Bronson 1985; Fowler 1987). For example, various mammal species show lower birth rates when densities are high (small mammals: Frylestam 1980b; Rödel et al. 2004a; large mammals: Albon et al. 2000; Coulson et al. 2000; Focardi et al. 2000; Stewart et al. 2004; Richard et al. 2014). In particular in herbivores, a key mechanism frequently discussed to underlie such negative effects on fertility parameters is the increased competition for and depletion of food resources at high population densities, negatively impacting the animals' body condition (cf. Helle and Kauhala 1995; Bonardi et al. 2017). In

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addition, multiple weather variables have the potential to even concomitantly affect vital rates including reproduction (Rödel et al. 2004b; Louthan et al. 2021), predominantly via their impact on food availability or quality. Studies in different species of ungulates, including feral domestic horses (*Equus ferus caballus*), support negative effects of harsh winter weather on annual birth rates (Coulson et al. 2000; Richard et al. 2014). Such effects can be enhanced when population density and different weather variables operate interactively (Stewart et al. 2004; Rodriguez-Hidalgo et al. 2010; Richard et al. 2014; Gamelon et al. 2017).

At the individual level, reproductive performance frequently changes with age. There is increasing support for reproductive senescence in old females compared to prime-aged (usually middle-aged) mothers from wild or free-ranging populations, for example in terms of reduced litter sizes or lower birth rates in the former (Gaillard et al. 2000; Rödel et al. 2004a; Turbill and Ruf 2010). Furthermore, young or primiparous females often show a comparatively lower (Garrott et al. 1991; Helle and Kauhala 1995; DelGiudice et al. 2007) or at least a more variable reproductive performance than older mothers (review in: Gaillard et al. 2000). Studies in ungulates revealed that such high variation in young females may be attributed to their relatively high sensitivity to challenging environmental conditions, as exemplified by the comparatively stronger decrease in young females' birth rates in response to high density (bighorn sheep *Ovis canadensis*: Festa-Bianchet et al. 1995, Soay sheep *O. aries* and red deer *Cervus elaphus*: Coulson et al. 2000) or harsh winter weather (moose *Alces alces*: Markgren 1969, red deer: Coulson et al. 2000). In turn, the lower sensitivity of vital rates to environmental variation in prime-aged compared to young females may contribute to buffer the effects of fluctuating environmental conditions on changes in population growth (Morris and Doak 2004; Hilde et al. 2020).

The individual reproductive history is a further parameter potentially influencing the reproductive probability or performance of females. As reproduction, and in particular lactation is energetically costly (McNab 2002; Speakman 2008), a relatively high current reproductive effort can be expected to alter the reproduction or survival of an individual in the future (Stearns 1992). In long-lived species such as in ungulates, which typically follow a slow life history strategy, the costs of reproduction can be expected to mainly be apparent in terms of a lower future reproduction rather than by a reduced future survival of the mother (Hamel et al. 2010a). Even if the exact physiological mechanisms driving these life-history trade-offs are still poorly understood, it has been suggested that the costs of reproduction are especially evident when females are exposed to unexpected and unfavorable environmental conditions (Zhang and Hood 2016). Furthermore, some studies in ungulates indicate that reproductive trade-offs can be particularly pronounced in young, primiparous mothers, as evident by their reduced reproductive probability during the following year (feral horse *Equus caballus*: Garrott and Taylor 1990; Soay sheep *Ovies aris*: Regan et al. 2022).

Furthermore, challenging conditions experienced during early life can have long-lasting effects on individual life histories including on reproductive traits (Descamps et al. 2008; Rödel et al. 2009). For example, female Soay sheep experiencing high population densities during the summer after birth showed a lower probability of reproduction as yearlings (Forchhammer et al. 2001), and a similar delayed effect of early-life population density on the probability of pregnancy later in life was found in a Mediterranean red deer population (Rodriguez-Hidalgo et al. 2010).

In summary, different environmental interactions can affect parameters of reproduction on the short-term as well as on the long-term, and features such as age can alter an individuals' susceptibility to such effects. However, integrative studies taking into account the interplay of these various parameters are scarce, as the necessary individual-based long-term data are difficult to collect and thus rarely available (review in: Schradin and Hayes 2017).

We analyzed such a long-term data set collected from a population of a large mammal, the Przewalski's horse (*Equus ferus przewalskii*), living under natural conditions in a 3000-hectare area at the Hortobágy National Park in Hungary (Kerekes et al. 2019, 2021). Our main goal was to investigate the interactive effects of different weather parameters and density as well as the impact of the individual reproductive effort during the previous year on the females' age-specific foaling probability during the annual breeding season. The Przewalski's horse is a particularly interesting model for the study of harsh weather conditions, as its natural semi-arid steppe habitat can provide challenging environmental conditions by hot summers with poor vegetational quality as well as by cold winters (Janssen et al. 2016). Przewalski's horses, which are considered as the last truly wild horses, diverged from ancestors of the domestic horse (*E. f. caballus*) around 45,000 years ago (Der Sarkissian et al. 2015), and are listed as 'Endangered' in the international IUCN Red List of Threatened Species (King et al. 2015). Przewalski's horses are seasonal breeders and the majority of the foals is usually born in May and June (Chen et al. 2008). Similar to the domestic horse (Heck et al. 2017), the gestation period of the Przewalski's horse is around 330–340 days (Monfort et al. 1991; Maltzan et al. 2007), and mothers usually give birth to a single foal (Chen et al. 2008).

The study area in the national park included, next to the Przewalski's horses, a herd of semi-wild cattle (reconstructed aurochs, *Bos taurus taurus*; Kerekes et al. 2019). In years with increased densities of these two large grazers, the ground vegetation, i.e., the horses' only food source, was increasingly depleted (Kerekes et al. 2019). Thus, we (i) predicted that following such high-density years, possibly either due to a decreased probability of successful conceptions or by increased (early) pregnancy losses (Satué and Gardon 2016), the probability of giving birth during the next foaling season will be lower. Furthermore, (ii) low winter temperatures, in particular during the late winter season, which have the potential to delay the green-up of the grass vegetation (cf. Mech et al. 1987; Rödel et al. 2005), might lead to a decreased rate of successful conceptions during the following months. As a consequence, low winter temperatures may negatively affect females' foaling probability with a delay of 1 year (corresponding to Przewalski's horses' gestation time of 11–12 months). In contrast, (iii) high amounts of precipitation during late summer, thus counteracting the negative effects of the usual summer droughts and leading to the regrowth of green pasture prior to the winter season, might increase the animals' body condition and thus may decrease the probability of pregnancy losses (Satué and Gardon 2016). Therefore, we predicted that rains in late summer may have increased the probability that females will give birth during the following year. Most importantly, (iv) we focused on age-specific responses to such environmental challenges, as young horses (see Gaillard et al. 2000 for a review on other ungulates) may be particularly sensitive to such effects. That is, we predicted that birth rates in young females would be prone to more pronounced negative density effects and show stronger responses to harsh winter conditions. Younger females, supposedly in a lower body condition, may also respond particularly sensitively to the absence of late-summer rains, and thus such conditions may lower the foaling probability in this age class during the following season. We (v) also explored possible long-term consequences of late-summer rains to which foals were exposed to during their first year of life on their later reproductive performance. We predicted that foals experiencing more advantageous early life conditions in terms of more late-summer rain, thus potentially benefitting from higher food quality, would show higher birth rates during early adult stage. Finally, we (vi) predicted that possible direct (year-to-year) costs of reproduction in terms of reduced future reproduction, i.e., a lowered foaling probability of females which had already reproduced during the previous year, should be particularly pronounced in young females. Based on a smaller sample size from 6 years, we (vii) also explored potential effects of these different environmental challenges on female body condition, as such effects may provide some insights into the mechanisms linking weather and density to female reproductive performance (Frylestam 1980a; Rödel et al. 2005; Flajšman et al. 2017).

Materials and methods

Study population

The study was conducted on animals from a Przewalski's horse population living in a fenced area of 3000 ha (2400 ha before 2018) in the Pentezug reserve of the Hortobágy National Park in Hungary. The area is an alkali grassland with marshes and few interspersed groups of trees along a river (Kerekes et al. 2019). The natural grass pasture was the animals' only food source. The first horses were introduced to the Hortobágy National Park in 1997, and since then the population size increased (Kerekes et al. 2021). The Pentezug reserve was (and still is, in the year of publication) also inhabited by another large grazer, a herd of semi-wild cattle (reconstructed aurochs), which were also reproducing and growing in population size since their introduction in 1999 (Kerekes et al. 2021). There were no large predators in the area. No visitors were allowed in this part of the national park, although the horses were approached and observed by staff of the national park for population survey at an almost daily basis.

Furthermore, for a necessary population control, several of the female horses were injected with an immunocontraceptive (porcine zona pellucida, PZP) during the years 2013–2015 and 2017–2019, with around 15 females treated per year (more details in: Kerekes et al. 2021). The treatment related to the PZP injection procedure was minimum invasive, since animals were not captured, but the injections were administered from a distance using a blowgun. During the different years of treatment, different selection criteria were applied, as either younger or older females were preferably chosen. Most importantly, starting with the year a female was subjected to this PZP treatment, its data were excluded from the analysis presented in this paper (see details in Table A in Suppl. Materials). Nevertheless, (a) for five of our focal females for which reproductive probabilities were assessed, mothers were treated with PZP when these females were still at the foal stage. (b) For another five of our focal females, mothers were treated with PZP prior to their pregnancy with those females, i.e., the PZP treatment was obviously not always efficient in preventing pregnancies. However, our analyses revealed that the age-dependent reproductive probabilities of these females with PZP-treated mothers of conditions (a) and (b) did not differ significantly from females of untreated mothers (LMM: (a) $\chi_1^2 = 0.001$, $p = 0.988$; (b) $\chi_1^2 = 0.011$, $p = 0.916$), and thus these 10 females were kept in our data set for further analyses.

Reproductive activity of females

Study period and sample size

We explored the females' annual probability of parturition (events at the individual level; foaling: yes/no) starting at an age of 2 years, when Przewalski's horse females potentially start to reproduce (Kerekes et al. 2021). We only considered females born inside the Pentezug reserve. In total, data for this analysis were collected during 20 years (2000–2019), from a total of 146 females, born between 1998 and 2017. Females were between 2 and 16 years old when their reproductive activity was surveyed. This resulted in a sample size of $n=712$ observations of presence (57.4%, 409/712) or absence (42.6%, 303/712) of annual parturitions.

Surveys of reproductive activity

The population was surveyed for the occurrence of new foals in all harem groups (i.e., one stallion with up to ten adult females including their offspring; see: Klimov 1988; Kerekes et al. 2021; Ozogány et al. 2023), around 5–6 times a week during the main foaling season (late April to late May), and around 2–3 times a week during the rest of the year, when parturitions were less frequent (Volf 1996). This was done by two to three-hour checks by car. Harem groups with present foals, which could be easily spotted from the distance by the aid of binoculars, were approached closely to around 50 m. Mothers were assigned by the nursing of their foal. Horses of this population were not individually marked, although all adult females—including the mothers—could be identified by the trained personnel based on the combination of different characteristics and patterns such as differences in coloration, stripes on the legs, shoulder crosses etc. (see a detailed description of individual identification in: Kerekes et al. 2021). Assignments of mothers based on observational data were re-confirmed by genetic maternity assessments in around 90% of cases (Kerekes et al. 2021). These genetic analyses revealed that only 2.5% of observational assignments of mothers were inaccurate, and these were corrected later on in our data base.

Mothers' previous reproductive effort

We assessed mothers' reproductive effort during the previous year (***R***), which was used as a factor with two levels in our multifactorial analysis (see details below and in Fig. 1a). Therefore, by our regular surveys (see above), we determined the survival of foals until postpartum day 60, until the time when in the domestic horse approximately the peak in lactation is reached (Oftedal et al. 1983). Note that in our population, the peak foal mortality during the first year occurred during early postnatal life (around postnatal day 17, median). The vast majority, more than 90% of the foals surviving until postnatal day 60 were still with their mother during the following spring.

Lactation imposes considerable energetic costs, as in larger mammals the energy expenditure of lactating mothers is at least 1.5 times higher than of non-lactating ones (McNab 2002). Compared to the high energetic costs of lactation, gestation is usually considered to be less costly, as exemplified by studies showing that the differences in energy demands between non-breeding and pregnant females can be rather low (Gittleman and Thompson 1988; Speakman 2008; Rödel et al. 2016). Thus, for later analysis, and in accordance to a study in Soay sheep (Regan et al. 2022), we distinguished between females which (a) had not reproduced during the previous year or whose foal had died shortly after parturition, within the first 60 days, and (b) females which had reproduced during the previous year and whose foal was alive at least until postnatal day 60 (see more details below and in results). Note that in our study in the Pentezug reserve, mares exclusively gave birth to singletons.

Body condition scores of females

We also analyzed weather and density effects on females' body condition based on a data set collected during 6 years; see below for details on sample sizes and a schema in Fig. 1b.

Following the method developed by Rudman and Keiper (1991) for feral ponies (*E. ferus caballus*), we used a body condition score from 0 (very low body condition, although animals with such a low score were never observed during our study) to 5 (very high body condition), in steps of 0.5. As the basis of this score, we assessed the shape of the horses' hind quarters from an observer position behind the animal, by the aid of binoculars, from a distance of around 20 to 40 m. Detailed drawings describing the method of quantification can be found in Rudman and Keiper (1991), and more details on the application of this method to Przewalski's horses are in Kerekes et al. (2019).

Density of large herbivores (horses and cattle)

The number of cattle in the Pentezug reserve was counted once per year, in November or December when all cattle were locked into a small and closed area for veterinary inspection. The number of horses was known due to regular surveys at the individual level; that is, individual compositions of all harem groups were known (see above in "Surveys of reproductive activity"). Foal mortalities and their survival times were known due to surveys at the daily to weekly basis (more details in: Kerekes et al. 2021). For consistency with density data available from cattle, we used the density of horses assessed in November/December of each

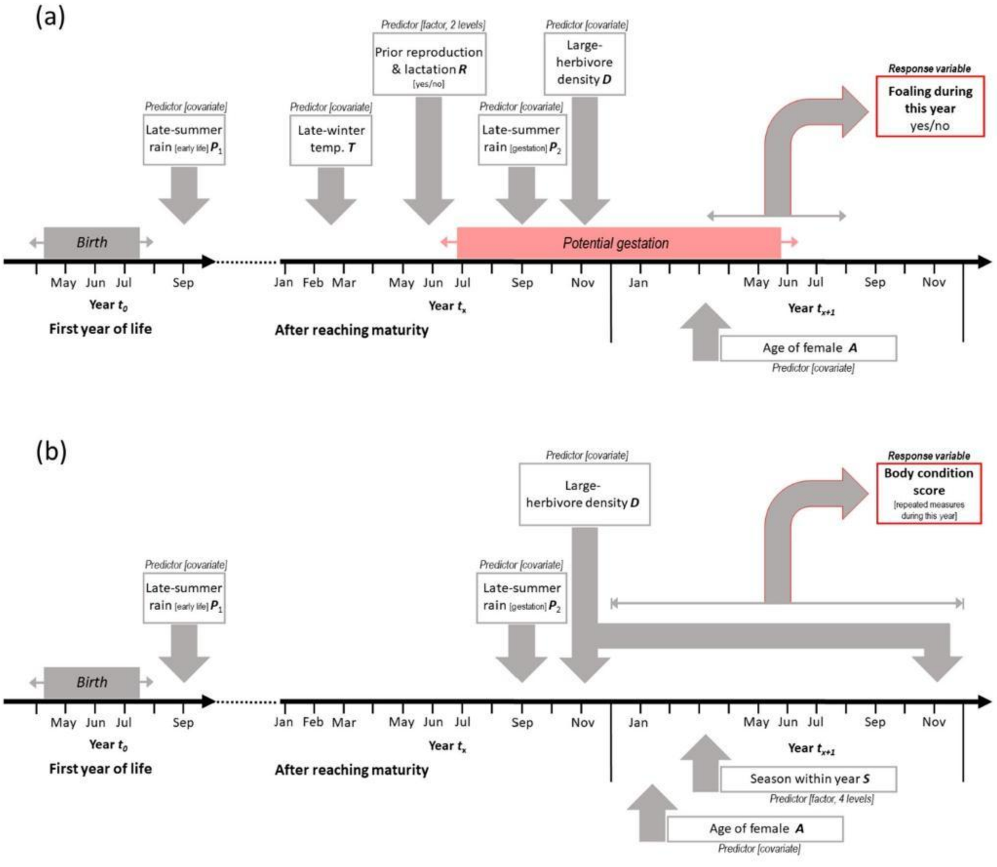


Fig. 1 Schemas of the different variables used for multifactorial statistical analyses. **a** Outline of analyses related to foaling probabilities (Table 1 and Table A in Suppl. Materials). The effects of prior reproductive investment (*R*) were explored by a separate analysis, only including females of at least 3 years. **b** Outline of the analysis related to body condition scores (Table 2). For analysis, large-herbivore density (*D*) in association with body condition scores collected in winter (January–April) was quantified in November/December of the previous year (t_x) whilst D in association with scores collected during the remaining year was quantified in November/December of the current year (t_{x+1})

year. During the study period, November/December densities of horses varied from 0.08 to 1.37 individuals per 10 ha, and densities of cattle varied from 0.05 to 2.41 individuals per 10 ha. In the beginning of the study period (in 2000), the densities of horses and cattle were 0.08 and 0.05 individuals per 10 ha, and in the end of the study period (in 2019), the densities were 0.93 and 0.64 individuals per 10 ha, respectively. Annual densities of horses and cattle were strongly and positively correlated ($R^2 = 0.913$, $\beta = 0.956 \pm 0.069$ SE, $p < 0.001$). Changes in densities of horses and cattle were due to variation in reproduction and survival. Furthermore, each year, several cattle were transported into or out of the study area. To a minor extent, this was also true for the horses, as some individuals were transferred from or to other enclosure populations (e.g., to zoological parks). However, note that all Przewalski's horse females, which were not born in our study population were excluded from the analysis of foaling probabilities.

D: For statistical analysis, we used the total density of cattle and horses (all age classes of both species per area size), hereafter referred to as ‘large herbivore density’ (see Fig. 1a, b).

Weather data

Data on precipitation (daily amounts) and on ambient temperatures (daily averages) were obtained from a close-by meteorological station (Debrecen, 38 km away from the center of the Pentezug reserve), situated at the same altitude of around 120 m a.s.l.. Based on these daily values, we calculated different weather variables, used as predictor variables for our statistical analyses.

P_1: The summed-up amount of precipitation in late summer (September) during the females' year of birth (see Fig. 1a, b). Observations (by VK and colleagues, unpublished) during the last decades had revealed that rainy weather in late summer leads to a notable regrowth of green pasture, thus increasing the quality and quantity of the horses' only food source. As stated upfront, we predicted that such a boost in food availability during early life may positively affect the body condition of the foals with potential long-term effects on their reproductive performance (Lummaa and Clutton-Brock 2002). In few cases, females were either born during September (7 out of 146 = 4.8%) or were born shortly after, in October (6 out of 146 = 4.1%). We did not exclude these cases from our analysis of reproductive probabilities, as such late-born foals might have at least benefitted indirectly from higher amounts of late summer precipitation, possibly via mother's higher energy intake and thus increased lactational performance. However, when excluding these cases of late-born foals ($n = 13$ females) from statistical analyses, we obtained the same results.

P_2: The summed-up amount of precipitation in late summer (September) during the females' potential pregnancy (see Fig. 1a, b). We predicted that the regrowth of green pasture related to higher amounts of rain during this period will improve the chance that females will keep their (potential) pregnancy during the critical early period of gestation (cf. domestic horse: Satué and Gardon 2016).

T: The average ambient temperature during late winter (February/March) for each year of the study period. For analysis of females' foaling probability, this variable was calculated for late winter prior to the female's breeding season, i.e., 1 year prior to the potential parturition in focus. This time-delayed effect of winter temperature was taken into account since we predicted that harsh winter conditions may have possibly decreased the chance of a successful conception thereafter (see Fig. 1a).

Statistical analysis

Effects of different predictors on foaling probability

Statistical analyses were done with the program R, version 4.3.0 (R Core Team 2023). We analyzed the effects of different predictor variables on females' probability of foaling (binary response variable) by generalized linear mixed-effects models (GLMM) for binomial data with a logit link, fitted by the Laplacian maximum likelihood approximation, using the R package *lme4* (Bates et al. 2015). *P*-values were calculated by type-3 Wald chi-square tests (Bolker et al. 2009).

This analysis was based on a total of 712 observations (occurrence of annual foaling: yes/no) from 146 females (2–16 years old) over a period of 20 years. We included female identity as a random (intercept) factor, since the data set included repeated measurements of individual females across consecutive years. The current year of reproduction was included as a further random factor to adjust for year-to-year variation which remained unexplained by the environmental predictors considered.

These environmental predictor variables (all covariates), which are defined above, were D, P_1, P_2 and T (details in Fig. 1). We also considered the females' age in years (A, covariate), since in various species of mammals and birds, young adult females often show a lower reproductive performance compared to older age classes (Clutton-Brock 1988; Rödel et al. 2004a; Monclús et al. 2014). In addition, we tested for polynomial (quadratic) effects of age on foaling probability, predicting an initial increase in reproductive performance with a maximum in middle-aged females, followed by a decrease in old females, as it has been described in several studies on small and large mammals (Rödel et al. 2004a; Hayward et al. 2013; Nussey et al. 2013). All covariates were scaled for analysis, i.e., they were centered to the mean which was set to zero and the standard deviation to 1.

As a further predictor variable (2-level factor), we considered the females' previous reproductive effort (R); see details above. As Przewalski's horses do not give birth before reaching at least an age of 2 years (Kerekes et al. 2021, and details in "Results" section), the inclusion of this age class would lead to a strong collinearity between age and previous reproductive effort. Thus, we ran the analysis including this factor on a reduced data set by removing all data from 2-year-old females, leading to a sample size of $n = 566$ observations from 107 females. However, we still analyzed the effects of previous reproductive effort by a multifactorial model, including the same set of predictors as described above (see Table B in Suppl. Materials).

We tested all 2-way interactions between the predictors considered (see Table 1 and Table B in Suppl Materials). Non-significant interactions ($p > 0.05$) were stepwise removed and models were re-calculated (Engqvist 2005). We visually checked for temporal autocorrelations by plotting the years of study versus the model residuals, revealing a random pattern without any indications for autocorrelations. We also checked for the occurrence of (multi)collinearities using variance inflation factors (VIF). This was done for all models, including all interactions. As VIF were always lower

Table 1 Effects of different predictor variables on the age-specific foaling probability of female Przewalski's horses

Source of variation	χ^2	*df*	*Estimates* ± *SE*	*p*
Female age (2nd order polynomial effect) A	117.528	2	-25.999 ± 3.740	**<0.001**
Large grazer density after potential conception D	22.269	1	-0.918 ± 0.195	**<0.001**
Precipitation in late summer during 1st year of life P_1	1.911	1	0.160 ± 0.116	0.167
Precipitation in late summer after potential conception P_2	1.281	1	0.212 ± 0.187	0.258
Temperature in late winter prior to potential conception T	0.376	1	0.118 ± 0.193	0.540
$D \times A$	4.239	1	0.315 ± 0.153	**0.034**
$P_1 \times A$	0.006	1	-0.011 ± 0.147	0.941
$P_2 \times A$	0.096	1	0.046 ± 0.148	0.756
$T \times A$	0.103	1	-0.054 ± 0.168	0.749
$P_1 \times P_2$	8.011	1	0.440 ± 0.155	**0.005**
$P_1 \times T$	6.128	1	-0.292 ± 0.118	**0.013**
$P_2 \times T$	0.240	1	0.138 ± 0.281	0.624
$P_1 \times D$	1.532	1	-0.151 ± 0.122	0.216
$P_2 \times D$	1.820	1	-0.392 ± 0.201	0.177
$T \times D$	0.242	1	0.093 ± 0.188	0.623

Analysis by GLMM for binomial data based on $n = 712$ observations from 146 females over 20 years. Non-significant interactions ($p > 0.05$) were stepwise removed and the models were re-calculated. The proportional variance explained by the final model excluding all non-significant interactions was $_{\text{marginal}} R^2 = 0.532$

Significant effects are highlighted in bold

Female identity and current year were used as random intercept factors

than 3.5, there were no indications of interfering (multi)collinearities (Faraway 2006).

Effects of different predictors on body condition

The effects of the environmental variables D, P_1, and P_2 on female body condition scores (response variable) were tested by multifactorial linear mixed effects models (LMM) using the R package *lme4* (Bates et al. 2015) (Fig. 1b). As the distribution of these equidistant body condition scores (Rudman and Keiper 1991) was unknown, we calculated *p*-values by parametric bootstrapping (1000 iterations) using the package *afex* (Singmann et al. 2022).

The available data set included $n = 393$ measurements of score values from 64 females (2–17 years old) collected all year round during 6 years (2004–2007, 2018, 2019). Thus, the analysis was based on 6 different measurements of P_2 stemming from 6 years, although from 19 different measurements of P_1 (late-summer rain during first year of life), since the females included in this analysis were part of 19 annual birth cohorts. As for each year, only one census of large herbivore density taken in Nov/Dec was available, body condition scores measured during the early season (Jan–Mar) were set in association with the density from the previous year (i.e., with the value of the density census done shortly before), whereas scores measured from April to December were set in association with the density census of the current year, which included the summed-up numbers of foals and calves that survived during that year. In this way, eight different large-herbivore density measurements were included in our analysis. Further predictors considered in this analysis were females' age in years (A, covariate) and the season (S) during which the measurements were taken (factor with 4 levels; Jan–Mar; Apr–Jun, Jul–Sep, Oct–Dec) (Fig. 1b). Covariates were scaled for analysis (details above), and female identity and year were used as random (intercept) factors.

We did not include the effects of late winter temperature (T) in this analysis, as body condition scores from April on, i.e., after the end of this late-winter period, were only available from 4 years. Thus, the sample size ($n = 4$ years) was too low to allow such a correlative analysis between T and body condition scores.

When analyzing the effects of P_1 on body condition, we did not only test for linear but also for (second-order) polynomial effects. Even if increasing precipitation levels during late-summer rain may improve the animals' body condition via the green-up of the ground vegetation, in particular during foal stage, extremely high amounts of rain may have negative consequences, leading to a non-linear (inverted U-shaped) association. Such potentially negative effects on body condition and survival may be mediated via a higher persistence of infective stages of endoparasites outside the host's body when the ground vegetation is consistently wet (e.g., Rödel and Starkloff 2014; Bond et al. 2023).

We tested all 2-way interactions between female age and the environmental variables D, P_1, and P_2. Further interactions among the environmental variables were not considered due to the rather moderate number of different years available for this analysis. Variance inflation factors were always lower than 3.6, thus models showed no indications of interfering (multi)collinearities (Faraway 2006).

Results

Reproduction

Out of the 146 females included in our study, 85 (58.2%) gave birth at least one time during the 20 years considered for analysis (2000–2019). Parturitions occurred from early February to late November. The vast majority, 80% of these took place in spring/summer (*10th percentile*: April 29th, *90th percentile*: July 17th), with an average parturition date on May 30th (median parturition date: May 19th).

Age effects

The onset of reproductive activity was age-dependent, with the majority of females starting to give birth when 3 years old. In detail, 3.4% of mothers (5 out of 146) started to give birth at an age of 2 years, 56.1% (60 out of 107) at an age of 3 years, 17.4% (16 out of 92) at an age of 4 years, and 5.1% (4 out of 78) of the females started when 5 years old. None of the females included in our study reproduced for the first time when older than 5 years.

By our analysis, we explored age-dependent changes in the probability of foaling. Therefore, in our logistic model (Table 1), we fitted female age effects by a second-order (quadratic) polynomial, which explained the associated changes in reproductive probability significantly better than a function with a simple sigmoidal shape (model comparison by likelihood ratio test: $\chi^2_1 = 59.499$, $p < 0.001$). This model (see significant age effect in Table 1) predicted a steep increase in the probability of reproduction in 2 to 4-year-old females, then reaching a plateau in middle-aged ones, and finally leading to a slight decrease, although with high 95% confidence interval, in old females (Fig. 2a).

Prior reproductive effort

As 2-year-old females inevitably had a previous reproductive investment of zero, this age class was removed from this analysis, resulting in a reduced sample size of $n = 566$ observations from 107 females. This analysis (details in Table B of Suppl. Materials) predicted a significantly lower current reproductive probability (0.710, $CI_{95\%}$ [0.567, 0.820]) in females which had a foal during the previous year which survived at least until postnatal day 60 (53.0% of cases), compared to females (reproductive probability: 0.870, $CI_{95\%}$ [0.759, 0.935]) which either did not reproduce (38.5% of cases) or had experienced an early loss of their foal during the previous season (8.5% of cases; foal mortality on average on postnatal day 6.9 ± 1.7 SE) (GLMM for binomial data: $\chi^2_1 = 11.276$, $\beta = -1.050 \pm 0.313$ SE, $p < 0.001$). All predictors and interactions among them, which were significant in the previous analysis (Table 1) were again significant (see Table B in Suppl. Materials). However, we did not find any significant interactions between the environmental variables P_1, P_2 and T and the females' previous reproductive effort (all $p > 0.10$; Table B in Suppl. Materials). A further analysis confirmed that, as expected, the reproductive probabilities of mothers which either did not reproduce or had experienced an early loss (< postnatal day 60) of their foal during the previous season did not differ significantly ($\chi^2_1 = 0.204$, $\beta = 0.277 \pm 0.613$ SE, $p = 0.651$).

An additional, age-specific analysis, using age as a factor with three levels instead of as a covariate, revealed that such a negative effect of previous reproductive effort on current reproduction was only significant in the young age class of 3 to 4-year-olds ($\chi^2_1 = 3.955$, $\beta = -1.887 \pm 0.948$ SE, $p = 0.047$; Fig. 2b.i) as well as in old females of 11 to 16 years ($\chi^2_1 = 3.999$, $\beta = -3.493 \pm 1.747$ SE, $p = 0.045$; Fig. 2b.iii). In contrast, there was no significant effect of the previous reproductive effort in middle-aged females of 5 to 10 years ($\chi^2_1 = 0.219$, $\beta = -0.190 \pm 0.407$ SE, $p = 0.640$; Fig. 2b.ii).

Density of large herbivores

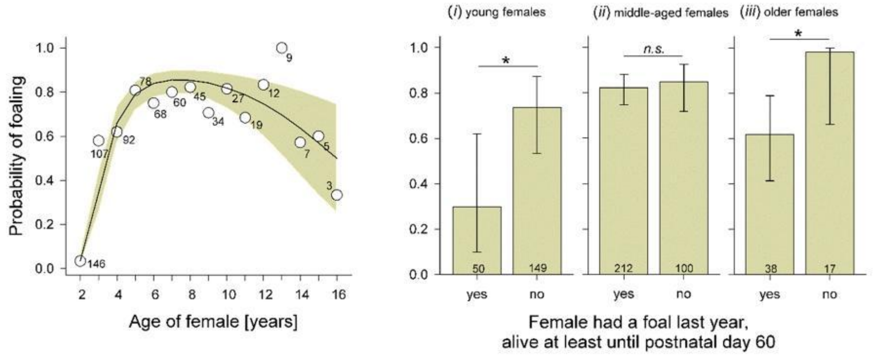
The foaling probability of individual females depended on the density of large herbivores in an age-specific way, as evident by the statistically significant interaction between female age and density (Table 1). That is, the foaling probability showed a clear negative density dependence in young age classes, particularly visible in 3 and 4-year-olds (Fig. 2c). The slope of the negative association in 2-year-olds was comparatively lower, since females of this age class overall showed a very low probability of foaling (see also Fig. 2a). In females older than 4 years, the negative density dependence gradually disappeared with increasing age (Fig. 2c for females until an age of 11 years). More details in Fig. A in Suppl. Materials.

Interactive weather effects

Weather conditions significantly affected the females' probability of parturition at multiple levels. First, this was apparent by the significant interaction between the amount of precipitation in late summer (September) during the females' first year of life (i.e., at the foal stage, P_1) and the average ambient temperature (T) in late winter (February/March) prior to the onset of the mating period (Table 1). Females, which experienced low amounts of P_1 during their first year

(a) Age effect

(b) Age-specific effect of prior reproductive effort



(c) Age-specific density effects

(d) Late-summer precipitation and winter temperature interaction

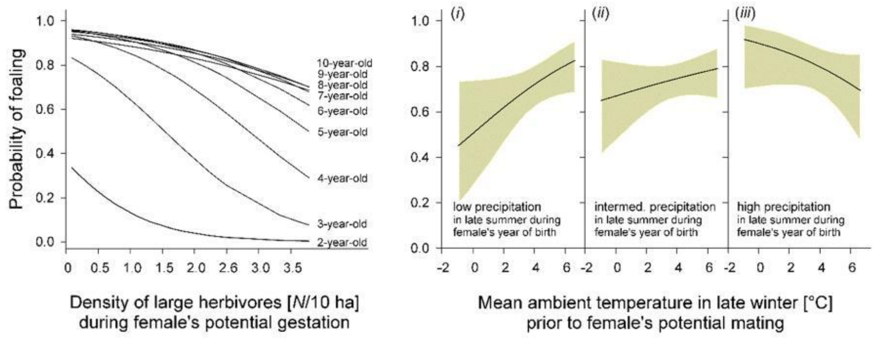


Fig. 2 Effects of different predictors on the foaling probability of female Przewalski's horses, aged between 2 and 16 years. Regression lines (**a**, **c**, **d**) and the bar charts (**b**) show predicted values including 95% confidence intervals (gray shading: **a**, **d**), based on estimates provided by the multifactorial model (Table 1). Data points in **a** are the average probabilities per age class; sample sizes are given beneath the circles. In **b**, model results for (i) young females (3–4 years old), (ii) middle-aged females (5–10 years old) and (iii) older females (11–16 years old) are given. The conditions in the interactive graphs (**d**) of (i) low, (ii) intermediate, and (iii) high precipitation are exemplary cases (10th, 50th and 90th percentiles) of the continuous variable P_1 (see Table 1). All analyses were done with multifactorial GLMM for binomial data. Details on statistics for **a**, **c**, **d** are given in Table 1 (based on $n = 712$ observations from 146 individuals). For **b**, 2-year-old females were excluded from the analysis ($n = 566$ observations from 107 individuals; see Table B in Suppl. Materials)

of life were particularly affected by negative effects of low T on their probability of foaling (Fig. 2d.i).

Second, there was a significant interaction between P_1 and the amount of precipitation experienced in late summer during adulthood (P_2), during the period of potential gestation (Table 1). Particularly in females which experienced more P_1 during early life, the exposure to more P_2 increased the probability of giving birth during the following year (Fig. 3a.iii).

An additional analysis revealed that the above-described interactive effect of $P_1 \times P_2$ was also statistically significant when only considering young females of 2 and 3 years (GLMM for binomial data: $\chi^2_1 = 9.636$, $\beta = 1.304 \pm 0.420$ SE, $p = 0.002$), and even showed a notably steeper slope (Fig. 3b.iii). That is, only females which experienced higher precipitation in late summer during their year of birth had a notably high probability of foaling during their 2nd and 3rd year, when late-summer precipitation during their current gestation was high. In contrast, the interaction $P_1 \times P_2$ was not significant when pooling together females older than 3 years ($\chi^2_1 = 1.946$, $\beta = 0.224 \pm 0.161$ SE, $p = 0.163$).

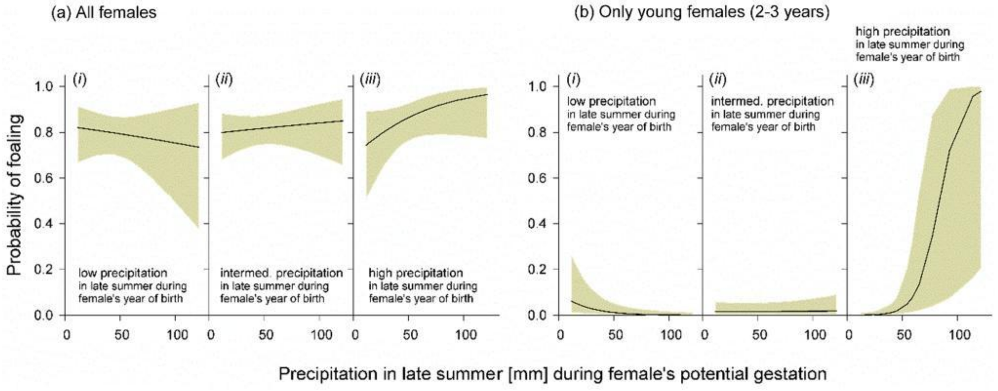


Fig. 3 Interactive effects of late-summer precipitation (September) experienced by females' during their first year of life and during their current (putative) gestation on the annual foaling probability of female Przewalski's horses. Regression lines show predicted values including 95% confidence intervals (gray shading), based on estimates provided by the multifactorial model (see Table 1 and text). The conditions in the interactive graphs of (i) low, (ii) intermediate, and (iii) high precipitation are exemplary cases (10th, 50th and 90th percentiles) of the continuous variable P_1. Analysis by GLMM for binomial data, based on data from **a** all females ($n = 712$ observations from 146 individuals) and from **b** 2-year-old and 3-year-old females ($n = 253$ observations from 146 individuals)

Body condition score

Age-specific effects of large herbivore density

The body condition of adult females decreased significantly with increasing annual large herbivore density, although in an age-dependent way as evident by the significant interaction of $D \times A$ (Table 2). That is, this negative density-dependence was particularly pronounced in young females and decreased gradually with increasing age (Fig. 4a).

Weather effects

We found age-specific long-term effects of the amount of precipitation that females experienced in late summer during

Table 2 Effects of different predictor variables on body condition scores (after Rudman and Keiper 1991) of female Przewalski's horses (2–17 years old)

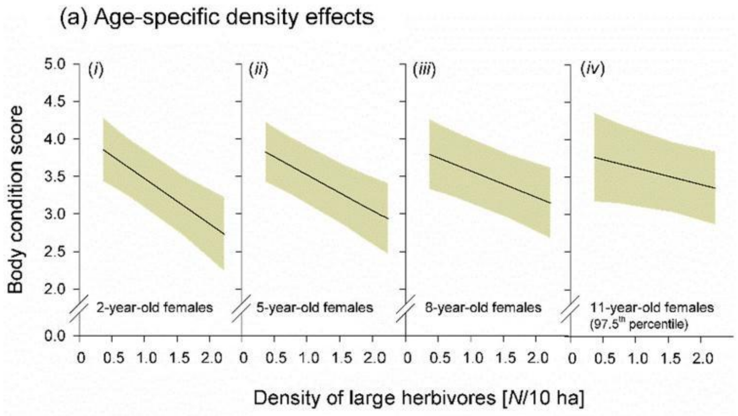
Source of variation	χ^2	*df*	*Estimates* ± *SE*	*p*
Female age *A*	0.06	1	0.027 ± 0.110	0.859
Annual large grazer density (census in late autumn) *D*	17.61	1	−0.579 ± 0.124	**<0.001**
Season	1.45	3		0.724
[2]			0.063 ± 0.074	
[3]			0.085 ± 0.077	
[4]			0.011 ± 0.109	
Precipitation in late summer during 1st year of life P_1	3.59	1	0.212 ± 0.111	0.083
Precipitation in late summer last year P_2	0.01	1	0.021 ± 0.249	0.952
$D \times A$	5.15	1	0.140 ± 0.059	**0.039**
$P_1 \times A$	8.08	1	−0.203 ± 0.070	**0.005**
$P_2 \times A$	0.86	1	0.041 ± 0.044	0.401

Analysis by LMM with parametric bootstrapping (1000 iterations), based on $n = 393$ score values from 64 females collected during 6 years (2004–2007, 2018, 2019). All 2-way interactions between female age and the different environmental parameters were tested. Non-significant interactions ($p > 0.05$) were stepwise removed and the models were re-calculated. The proportional variance explained by the final model excluding all non-significant interactions was $_{\text{marginal}} R^2 = 0.196$

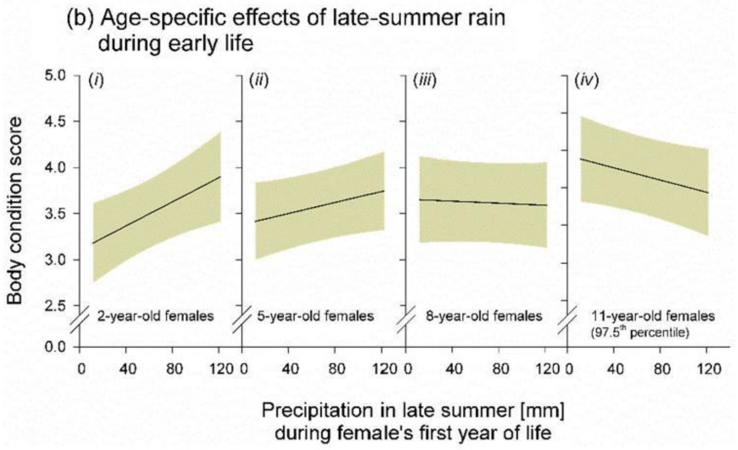
Significant effects are highlighted in bold

Female identity and current year were used as random intercept factors. Season was a factor with 4 levels ([1] Jan–Mar; [2] Apr–Jun; [3] Jul–Sep; [4] Oct–Dec)

Fig. 4 Effects of different predictors on body condition scores (after Rudman and Keiper 1991) of female Przewalski's horses, aged between 2 and 17 years. Regression lines show predicted values including 95% confidence intervals (gray shading), based on estimates provided by the multifactorial model (Table 2). The conditions in the interactive graphs (**a**, **b**) of (i) two, (ii) five, (iii) eight and (iv) 11-year-old females are exemplary cases of the continuous variable age (*A*) (see Table 2). Analysis by LMMs with parametric bootstrapping based on body condition scores collected during from 6 years ($n = 393$ observations from 64 individuals)



(a) Age-specific density effects



(b) Age-specific effects of late-summer rain during early life

their first year of life (P_1) on their body condition during later life, as evident by the significant interaction of $P_1 \times A$ (Table 2). Young adult females of 2 to around 3–4 years which experienced higher amounts of P_1 showed a notably increased body condition compared to females of the same age experiencing lower amounts of late-summer precipitation early in life (Fig. 4b). Although, this positive weather effect disappeared with increasing age.

Alternatively, we also tested the non-linear (second-order polynomial) effect of late-summer precipitation during early life (P_1) in interaction with age. This interaction between the polynomial effect of P_1 and age was also statistically significant (LMM: $\chi^2_2 = 8.77$, $p = 0.018$; not shown in Table 2). However, it did not explain the data significantly better than the model including the interaction $P_1 \times A$ based on the linear effect of P_1 (Likelihood ratio test: $\chi^2_2 = 1.01$, $p = 0.604$).

We did not detect any significant effects, including an age-specific interaction, of late-summer precipitation during the previous year (P_2) on the body condition during the current year (Table 2).

Discussion

Age-specific reproductive activity

Our long-term study confirms and extends published information on reproductive parameters in the Przewalski's horse. Parturitions peaked in mid to late May, which corresponds well to findings from other studies on free-ranging Przewalski's horse populations (Chen et al. 2008; Dorj and Namkhai 2013). Some females of our study population had their first foal during their second year of life (more details in: Kerekes et al. 2021), although this happened only in few cases (3.4%, 5 out of 146 females included in our study). Another study on Przewalski's horses, from a Mongolian population, found first foaling at an age of 3 years, reporting that 24.7% of females already reproduced at this age (Dorj and Namkhai 2013). Whilst such quantitative information from free-ranging Przewalski's horses (*E. f. przewalskii*) is scarce, data on the well-studied feral horse (*E. f. caballus*) suggest a notable variation in early reproductive activity among populations and among years. Some studies reported that feral horse mares do not reproduce before reaching an age of 3 years (Seal and Plotka 1983; Garrott and Taylor 1990; Goodloe et al. 2000), whilst others found foaling in 2-year-olds with low to intermediate (Garrott et al. 1991; Linklater et al. 2004; analysis based on pregnancy rates in Grant et al. 2021), or even high rates of up to 37% (Berger 1986). Furthermore, our current study provides indications for female reproductive senescence (see reviews on mammals in: Gaillard et al. 2000; Turbill and Ruf 2010), in terms of a decreasing reproductive probability in Przewalski's horse mares older than around 14 years, which is in accordance with studies in feral horses (Garrott et al. 1991) and in the closely related Asiatic and African wild asses (*E. hemionus*, *E. africanus*: Ibler and Fischer 2017).

Age-dependent effects of density and weather

The prime interest of this study was to investigate possible age differences in the females' foaling probability in response to an increased large-herbivore density and to harsh (or beneficial) weather conditions. We found consistent support that young females, as compared to middle-aged or older ones, were particularly sensitive to such environmental effects, which is in line with other studies on ungulates (review in: Gaillard et al. 2000). Extending these studies, we also found interactive environmental effects, suggesting that the interplay of weather conditions experienced during different life stages can shape female reproduction.

Large-herbivore density

Negative effects of population density on body condition and reproductive rates have been found in several large herbivores (e.g., Fowler 1987; Stewart et al. 2004; Bonardi et al. 2017) including in the feral horse (Garrott and Taylor 1990; Richard et al. 2014). Our study revealed pronounced negative effects of high large-herbivore density on female body condition, particularly on the body condition of the youngest female age-class considered (2-year-olds), with a gradually decreasing magnitude until the females reached an age of around 5 years (see Fig. 4a). Accordingly, negative density effects on foaling probabilities were strongest in 3- and 4-year-old females, whilst such effects were virtually absent in females older than 5 years (see Fig. 2c). Two-year-old females generally showed very low foaling rates (Garrott et al. 1991; Linklater et al. 2004), and thus negative density effects on reproduction were hardly detectable in this age class. Even though we could not directly test for a statistical association between body condition scores and foaling probability due to sample size restrictions in the former variable, we strongly suggest that the density-dependent decline in body condition of young females was the main driver of their low foaling rates under high large-herbivore densities (cf. Scorolli and López Cazorla 2000). This is further supported by a study in roe deer (*Capreolus capreolus*), showing that particularly in young females, body condition is a strong predictor of reproductive performance (Flajšman et al. 2017).

Interactive weather effects

Body condition scoring revealed that young females were particularly sensitive to rainy weather conditions experienced in late summer (September) during their first year of life (foal stage). Such late-summer rains typically lead to the green-up of the ground vegetation. Foals may have benefitted directly from such an increase in food availability and quality, but also indirectly via potentially positive effects of the available green pasture on mother's lactational performance (see Morand-Fehr and Sauvant 1980 for a study in goats *Capra hircus*). Thus, females experiencing higher amounts of late-summer precipitation during early life were in a better body condition, at least during the following 1–2 years (see Fig. 4b). Such beneficial environmental conditions were also associated with the females' foaling probability, although in an interactive manner across different life stages. Only in females, which experienced higher amounts of late-summer rain during their first year of life, a positive effect of late-summer rain during their current year of potential pregnancy was apparent. That is, only such females, at an age of 2–3 years, showed a majorly increased foaling probability during the following season when exposed to higher amounts of late-summer rain during their (potential) pregnancy (see Fig. 3b). We suggest that the increased availability of green pasture growing in late-summer and autumn may have decreased the probability that these 2- to 3-year-old females experienced pregnancy losses (Satué and Gardon 2016).

Moreover, females exposed to low amounts of late-summer rain during their first year (i.e., as foals) showed, after reaching maturity, a low probability of reproduction when experiencing harsh late-winter temperature conditions prior to the mating season. That is, in females experiencing such early-life conditions, harsh winter weather possibly limited female's receptivity or decreased the probability of successful conceptions during the subsequent mating season. This is supported by the finding of Scheibe and Streich (2003) on another Przewalski's horse population, who have shown that harsh winter conditions, possibly via a delayed green-up of pastures, decreased the body condition of the females during the following season. Furthermore, our finding of interactive, longer-term effect of late-winter temperature on the foaling probability with a delay of 1 year extends the results of a study on feral horses in Canada, reporting that mild winters during gestation had direct, positive effects on reproductive probability and foal survival (Richard et al. 2014).

Potential mechanisms

What drives such a higher sensitivity to environmental conditions in young females? Adults of younger age classes, even if already reproductively active, are often not fully grown (e.g., mountain goat *Oreamnos americanus*: Houston et al. 1989; European rabbit *Oryctolagus cuniculus*: Rödel et al. 2004a), which may restrict their energy allocation to reproductive processes, in particular when food resources are limited. This might also apply to the Przewalski's horse, as for example females of the closely related feral horse (e.g., in Australia: Csurhes et al. 2016) usually do not reach their maximum adult size until an age of around 4 years. Similar results have been obtained by studies in different breeds of domestic horses, in which females are reaching adult body size and mass at an age of around 4–5 years (Fernandes et al. 2020; Lopes Teixeira et al. 2021). A further key mechanism leading to such an age-specific sensitivity in female Przewalski's horses, and possibly also in other group-living ungulates, could be based on the association between the females' age and their social rank position. Female rank hierarchies are often positively correlated with age, as older females frequently occupy higher ranks whilst younger females start with subordinate positions when recruited into a social group (e.g., Rutberg 1983; Thompson 1993; Rödel et al. 2004a). Such a largely age-specific structuring of the social rank hierarchy of females, including the occurrence of agonistic behavior mainly initiated by higher ranking females towards younger, subordinate ones, has also been shown for different feral horse populations (Clutton-Brock et al. 1976; Keiper and Sambraus 1986; Heitor et al. 2006) and has been also described for two harem groups of Przewalski's horses (Keiper and Receveur 1992). Females holding a lower social rank position frequently experience higher and even enduring levels of social stress, which can lead to an increased activation of the hypothalamic–pituitary–adrenal (HPA) axis, thus to high levels of circulating glucocorticoids (von Holst 1998; Abbott et al. 2003). Such chronically increased stress hormone concentrations can lead to immunosuppression, exert negative effects on reproductive functions and may generally make an animal more susceptible to environmental challenges (Sapolsky 1992; von Holst 1998).

In our study, an additional mechanism may have contributed to exert comparatively stronger, negative effects of large herbivore density on young females. During our 20-year study period, the population of Przewalski's horses as well as the herd of wild cattle showed a tendency of a general increase in numbers, even though there were periods of stagnating or negative population growth (Kerekes et al. 2021). Consequently, a large proportion of younger females, which were inevitably born during the later part of the study period, had experienced higher density conditions during all their life. In turn, this was not the case for many older females, which tended to experience comparatively lower densities predominantly during their early years of life. Even though such conditions of persistent population growth over longer periods are not unusual for the dynamics of natural populations, we cannot fully exclude that the observed age-specific density effects on foaling probability may have been less pronounced in periods of a more stable or declining population density.

Age-dependent effect of prior reproductive effort

Reproduction frequently reduces the probability that a female will reproduce during the following season (large mammals: Hamel et al. 2010b). Such a negative association indicative of fitness costs of reproduction was also apparent in our study, although in an age-specific manner. Excluding 2-year-old females from the analysis for methodological reasons (see "Methods" section), we found that young (i.e., 3- to 4-year-old) females as well as old females (> 10 years) showed a significantly lower foaling probability when they had nursed a foal for at least 60 days during the previous year (see Fig. 2b). However, there clearly were no such effects in middle-aged (5 to 10-year old, 'prime-aged') females, which also showed the highest foaling rates in our study (see Fig. 2a). Generally, such a pattern of higher costs of reproduction during young and/or old ages appears typical for different species of mammalian herbivores including ungulates (Clutton-Brock 1984; Proaktor et al. 2007). Age-specific reproductive costs were also apparent in a study on a feral horse population in Montana, USA, in which primiparous females, but not multiparous ones were less likely to reproduce during the following season (Garrott and Taylor 1990). A study on Soay sheep also found pronounced negative effects of previous reproductive effort on lambing probability in young (yearling) females (Regan et al. 2022). In our study, we had chosen foal survival until postnatal day 60 (i.e., at around peak lactation in domestic horses; Oftedal et al. 1983) as a proxy of mother's prior energetic investment. Although, the vast majority, more than 90% of foals, which survived until day 60 also survived at least until the following spring. This may have potentially imposed further energetic/lactational costs to mothers during the winter, thus reinforcing the effects of previous reproduction on foaling probability during the following breeding season.

As discussed above, young female horses still need to allocate energy into their own growth until an age of around 4 years (Csurhes et al. 2016), and thus the energetic investment of young mothers into reproduction can be proportionally higher compared to older and fully grown females, possibly leading to higher reproductive fitness costs in young mothers. This hypothesis finds further support by a study on a feral horse population on an island in Nova Scotia, Canada, showing that low quality females carried the highest costs of reproduction in terms of a notably reduced foaling probability during the subsequent season (Debeffe et al. 2017). Horses, as capital breeders, can hardly compensate for a lack of energy during current reproduction via an increased food intake, which can typically lead to such delayed effects of low body condition on reproductive performance (Jönsson 1997).

At an older age, female horses in our study might have increased their relative energy allocation into current reproduction, as it can be predicted by the terminal investment hypothesis (e.g., Isaak and Johnson 2005). Such a disproportionally high reproductive investment in older mothers may have led to the observed negative effects of nursing a foal on such mothers' subsequent foaling probability (see Fig. 2b. iii). At the proximate level, the apparently high reproductive costs in old, multiparous mothers may also be driven or reinforced by accumulating negative effects of consecutive reproductive events on females' bioenergetic processes, constraining their reproductive abilities (cf. Zhang and Hood 2016).

Conclusions

In summary, our study highlights a higher susceptibility of young female Przewalski's horses to environmental challenges and to previous reproductive effort. Even though many studies on mammals have described effects of density and weather conditions on fitness-related traits such as on body condition and seasonal reproductive probability, age-dependent interactive effects of weather conditions experienced during different life stages, as we show in our study, are still rarely explored (Scorolli and López Cazorla 2000; Rödel and Dekker 2012; Richard et al. 2014). Our finding on the long-term effects of late-summer precipitation experienced by the females during their year of birth may be particularly noteworthy. Such early-life environmental effects can lead to consistent differences in traits such as body condition and reproductive performance among cohorts of individuals born during different years (Forchhammer et al. 2001; Gaillard et al. 2003), and as shown by the interactive effects in our study, may consistently affect the responses of females born during the same year to current environmental challenges, at least during younger age classes. Cohort effects may have stabilizing or even destabilizing effects on population fluctuations (Lindström and Kokko 2002), and thus knowledge of the mechanisms leading to such cohort differences may provide valuable information for the study of the dynamics and for the management of feral and Przewalski's horse populations (Collins and Kasbohm 2017; Kerekes et al. 2021).

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Author contribution statement HGR, BI, KO and VK conceived the study. VK supervised the field work, collected the data and created and maintained the data base. KO assisted in the maintenance of the data base. HGR statistically analyzed the data, and wrote the first draft of the manuscript. All authors contributed to the data analysis through constructive discussions, revised the manuscript, and gave final approval for publication.

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Data availability Data used in the study are available from the corresponding authors upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Permission to keep Przewalski's horses in the Pentezug reserve of the Hortobágy National Park has been granted by the Hungarian Ministry of Rural Development in National Parks (regulation number 134/2013.XII. 29, §1, 9). The data collection related to this study, which exclusively consisted of non-invasive surveys and counts of the animals, was done by the trained personnel of the national park.

Consent to participate Not applicable.

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Fine-scale collective movements reveal present, past and future dynamics of a multilevel society in Przewalski's horses

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Studying animal societies needs detailed observation of many individuals, but technological advances offer new opportunities in this field. Here, we present a state-of-the-art drone observation of a multilevel herd of Przewalski's horses, consisting of harems (one-male, multifemale groups). We track, in high spatio-temporal resolution, the movements of 238 individually identified horses on drone videos, and combine movement analyses with demographic data from two decades of population monitoring. Analysis of collective movements reveals how the structure of the herd's social network is related to kinship and familiarity of individuals. The network centrality of harems is related to their age and how long the harem stallions have kept harems previously. Harems of genetically related stallions are closer to each other in the network, and female exchange is more frequent between closer harems. High movement similarity of females from different harems predicts becoming harem mates in the future. Our results show that only a few minutes of fine-scale movement tracking combined with high throughput data driven analysis can reveal the structure of a society, reconstruct past group dynamics and predict future ones.

Understanding social structure and dynamics of animal societies is an important task in which utilising emerging technologies and high-throughput methods could be the key. Multilevel societies are arguably among the most complex forms of social organisation in nature[1,2]. Individuals in these societies aggregate through multiple nested levels[2]: the core units (the lowest social level) are usually breeding units (e.g., one-male, multifemale groups), and higher levels of social organisation are formed by the aggregation of lower-level units. They are best known from primates[3–7], but are also found in cetaceans[8,9], elephants[10], equids[11–14], and birds[15]. Moreover, the vast majority of human social systems show multilevel structure as well[16–20], thus studying multilevel societies can fundamentally contribute to our understanding of the evolution of sociality[2,21–23].

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One such taxon where multilevel social organisation can occur is the Przewalski's horse (*Equus ferus przewalskii*)[14,24], which is the last extant subspecies of wild horses (*Equus ferus*). The mating system of Przewalski's horses is female defence polygyny, where year-round stable harems are the core units of the society, and the harem's single breeding male (the harem stallion) protects the adult females and their juvenile offspring belonging to the harem[11,25–27]. Another type of social groups are the single-sex bachelor groups formed by non-breeding adult males[27,28]. Previous observations show variation in the occurrence of higher-level social units in Przewalski's horses: harems are observed to live isolated in almost exclusive home ranges in Hustai National Park[29], while in other populations they aggregate and form multilevel herds[14,24,30]. In Hortobágy National Park, Hungary, where the largest captive population of Przewalski's horses lives in a 3000-ha fenced but otherwise natural habitat, both cases were observed: in the first years after introduction harems had non-overlapping home ranges, while recently the population forms a massive multilevel herd[14]. However, the detailed structure of this society (e.g., the bonds between harems leading to herd formation) remains unclear.

A commonly used approach in the study of social structures is the analysis of social networks, where the links between individuals are traditionally quantified by numerous direct observations of social interactions over a long period of time[31,32]. However, uncovering the structure of a multilevel societies' social network would require the observation of many, possibly as much as a few hundreds of individuals. Recent advances in bio-logging and remote monitoring enables the collection of large amounts of behavioural data over short periods and may involve the majority or even all individuals in a social group, hence affording the quick and reliable study of social structures[33]. New technologies thus offer the possibility of a more detailed analysis of complex societies than ever before, however, a deeper understanding is required on how these detailed "snapshots" of the system reflect relationships among individuals that have developed over longer timescales[34,35].

In this work, we present the results of drone observations to track movements of Przewalski's horses in Hortobágy National Park ($n = 278$ individuals) and combine the high-resolution movement data of several minutes with long-term demographic data collected over 23 years of continuous population monitoring (Fig. 1). We aimed to study the collective movements in this multilevel herd of Przewalski's horses and explore relationships between society structure and motion patterns. We show that the structure of the society (i.e., associations of individuals to harems) can be determined from movements, which we expected from our previous studies[30]. Moreover, by characterising the society with proximity networks during movements, we uncover novel relationships between individuals and harems based on kinship and familiarity, we reveal that network centrality is related to harem traits, and that the network's structure is related to past and future social dynamics, i.e., member exchanges between harems, which exchanges for the future—as an unexpected finding—we can predict from the movements of individuals.

Results

Data acquisition techniques

Our aerial observations consisted of 5-min long video sessions captured on five different days. We recorded 4k videos of the herd's movements with two drones simultaneously to get global motion patterns and enough details for individual recognition (Fig. 1a; see Methods). During these observations the herd followed their natural daily routine, moving undisturbed in the reserve. We tracked each individual's movement on the footage (pixel coordinates) and referenced the locations on the images to the background for earth-fixed metric coordinates and thus reconstructed movement trajectories in high temporal (12.5 position/s) and spatial (+/− 0.2 m) resolution (Fig. 1b, c; see Methods). All horses, except bachelor males, were individually identified ($n = 238$) in the footage and their identities were matched across recording sessions on different days (Fig. 1c; Supplementary Table 1).

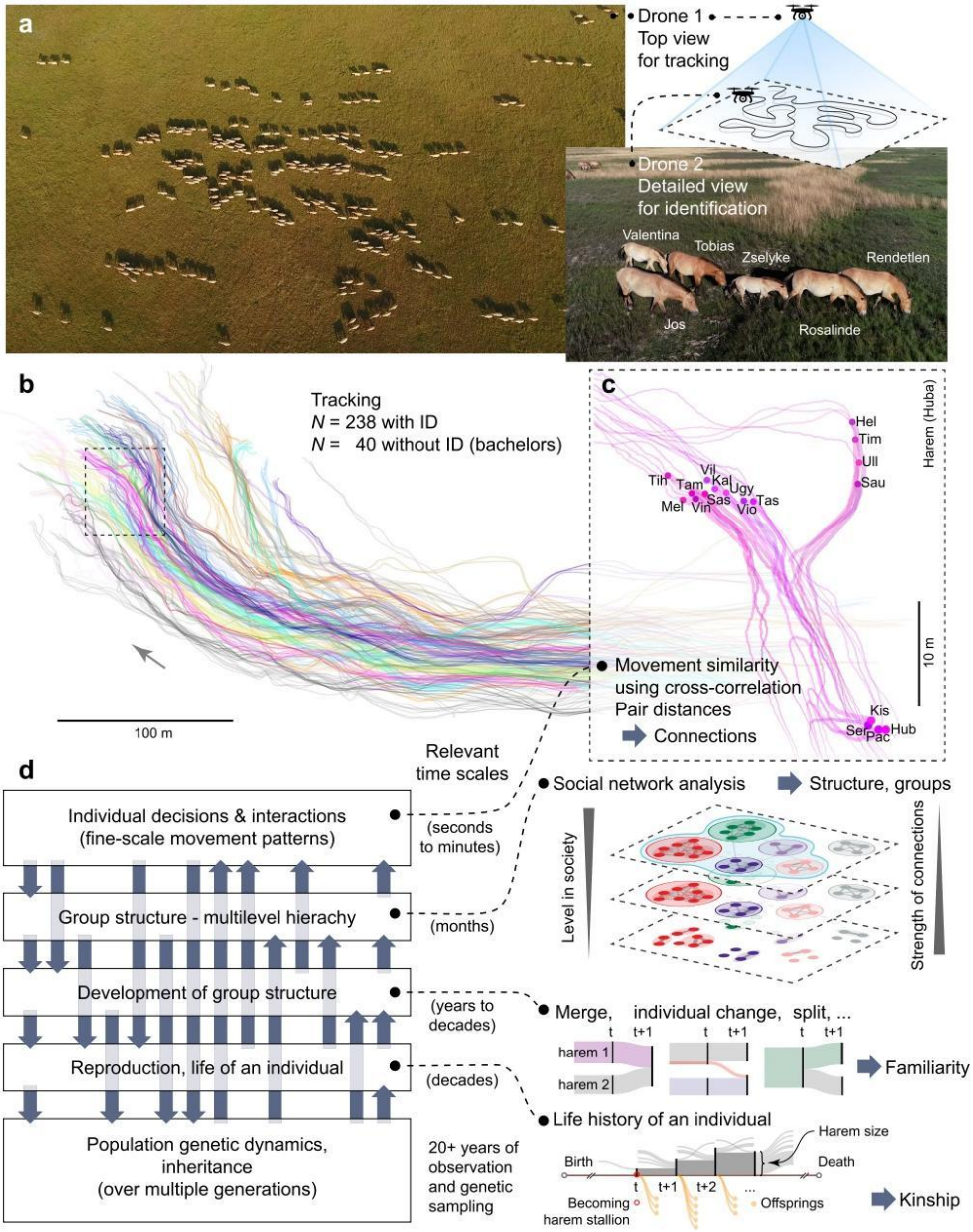
Przewalski's horses living in the Pentezug reserve of the Hortobágy National Park typically formed a single compact herd and moved in a very coordinated way during all recording sessions (Fig. 1a, Supplementary Movies 1 and 2). To quantify local pair interactions between individuals during movements, we calculated two variables for each horse pair, namely (i) pairwise distance d, and (ii) movement similarity C, i.e., directional correlation between trajectories of horse pairs[36,37]. Both variables were assessed over each 5-min observation session and averaged over the five 5-min observation sessions (see Methods).

From its founding in 1997, all individuals of the Hortobágy population have been individually identified and monitored by the reserve staff. Monitoring includes recording individuals' life-histories (e.g., births, deaths), parentage confirmed by genetic sampling, associations of individuals to sub-units of the multilevel social system (i.e., harems) and changes in harem memberships[14]. These population monitoring data have typically spanned for timescales of years to decades but are sparse and less detailed (updated monthly), in contrast to the detailed and fine-scale but short-term drone observations (Fig. 1d; see Methods). We investigated how these two data with different timescales relate to each other, and whether the latter can provide a robust measurement for the multilevel social structure.

Due to the long-term population monitoring, group dynamics in the population, including temporal development of harems, dispersal of individuals between harems, and associations of individuals in the same harem was known, not only at the time of movement tracking, but also for the previous 21 years prior to the drone observations as well as for the subsequent 2 years (Fig. 2). Some harems existed for more than a decade, and their composition typically changed slowly over time (Fig. 2). Individuals may have spent several years in the same harem, during which time they may have developed familiarity with each other, which may influence their behaviour also after leaving their harem and already belonging to different harems. To quantify familiarity, we used t_{past}, the time a pair of individuals has been together in the same harem in the 2 years prior to the movement observations (see Methods). From the genetic sampling the population's genealogy could be reconstructed, and thus the kinship between each pair of individuals was determined (Fig. 3).

Levels of the society

We first assessed the relationship between motion patterns and the multilevel social structure of the herd (associations of individuals to sub-units was known from long-term population monitoring). Social levels, as expected[2], were associated with different levels of cohesion between their members: pairwise distance was lower ($p < 0.0001$) and movement similarity was higher within harems than among harems within the whole herd ($p < 0.0001$, $n_1 = 711$, $n_2 = 21374$, randomisation tests, see Methods for details; see Supplementary Table 2; Fig. 4b, c, Supplementary Fig. 1). Since juveniles stay in the parental harem for several years[27,28], we considered an adult female and its subadult, not yet dispersed offspring as a sub-unit within harems and called it "family" (note that foals still dependent on their mothers were not included in "families"). We found that pairwise distance within "families" was typically lower ($p < 0.001$) and movement similarity was higher than in harems ($p < 0.0001$, $n_1 = 70$, $n_2 = 711$, randomisation tests, Fig. 4b, c, Supplementary Fig. 1). Although this sub-unit in equids (i.e., a female and its juvenile offspring) is usually not considered as a separate social level[2], its high cohesion shows similarity to a social level, nested within harems. We investigated the behaviour of bachelor males as well, but in this case only pairs of a bachelor male and a harem member individual could be considered, because bachelor males could not be individually identified and thus it was not possible to



distinguish intra-group and inter-group bachelor pairs. We found lower cohesion between a bachelor group and a harem group than between two harem groups, as pairwise distances were higher ($p < 0.0001$) and movement similarities were lower ($p < 0.0001$, $n_1 = 8778$, $n_2 = 20959$, randomisation tests, Fig. 4b, c) between a bachelor and harem member individual than between two harem-living individuals belonging to different harems.

Considering the features of collective movement, we found that the herd's multilevel structure was obvious in the d–C plots, both for a single 5-min and for averaged sessions, as individual pairs belonging to the same harem were separated from the pairs belonging to different harems, primarily along the axis d (Supplementary Fig. 2a, b). Accordingly, the distribution of pairwise distances clearly showed two peaks, where the first peak corresponded to the harem level, while the

Fig. 1 | Overview of the main concept and the data acquisition technique. **a** Sample images and a sketch of the setup for 4k filming of Przewalski's horses at Pentezug reserve, Hortobágy National Park, Hungary, in 2018 with two drones. The higher drone provides a large-scale top view for tracking individuals and the background to get coordinates and movement in an earth-fixed coordinate system (**b**). The lower drone scans the area with horses to get a detailed view for individual recognition. **b** Example trajectories of all horses belonging to the population ($n = 278$) from a 5-min long drone recording. Arrow shows the main direction of motion of the herd. Individuals (known identification, $n = 238$) are colour-coded based on the group they belong to (out of 31 harems), or shown as grey in the case of bachelor males (i.e., males that are not part of a harem, $n = 40$). **c** Detailed view of trajectories of a single harem, with all individuals shown with dots at a given point in time. Three letter codes show their identities. **d** Diagram of the main concept (on the left) showing important aspects of collectively living animals, and the complex interplays between these components. The components may have a relevant temporal scale (shown in the middle) that spans through several orders of magnitudes (from seconds and minutes to several decades). A detailed schematic explanation of each component is provided on the right.

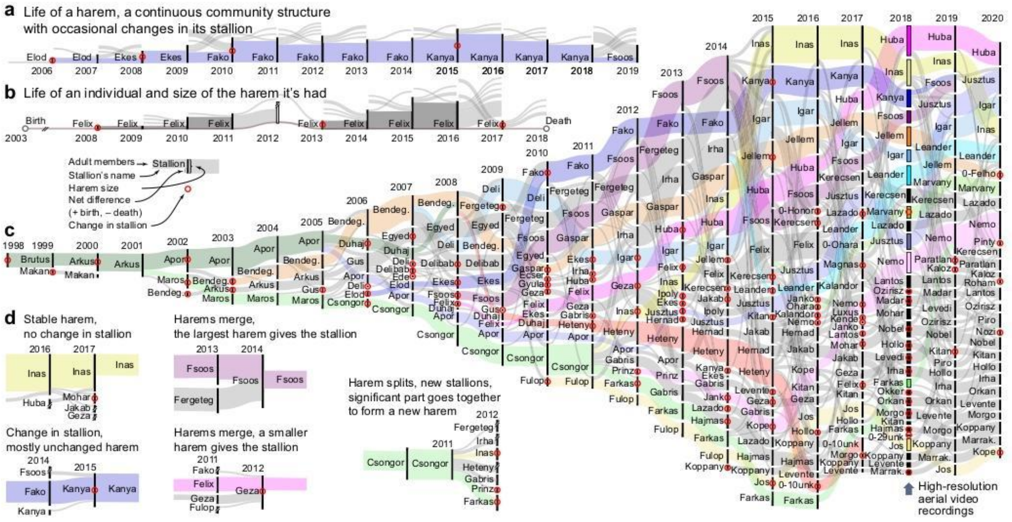


Fig. 2 | Group dynamics and the temporal development of harem structure of Przewalski's horses at Hortobágy National Park (Hungary) between 1997 and 2020. **a**, **b** Sample timeline from the point of view of a harem (**a**) and of an individual harem stallion (**b**). For each year, the length of the vertical heavy black line indicates the total number of members, with in-coming (and out-going) edges pointing from (and towards) other harems. All adult members are presented except bachelors. Names indicate the harem stallion, red circles indicate stallion changes. **c** Data visualisation from the long-term population monitoring from 1998 to 2020. For 2018, when the high-resolution aerial observations were recorded, colour indicates several harems with matching colour in Fig. 4d. The longest lasting harems are indicated by coloured lines. **d** Examples of important events related to changes in harem structure or to emergence of a new harem stallion.

second peak to the herd level (Fig. 4a). Although the two peaks were more obvious for averaged sessions, the two peaks were noticeable even in a single 5-min session (Supplementary Fig. 2c–e). Therefore, pairwise distances during collective movements averaged over several minutes are enough to detect multilevel structure of horse herds, and to classify individuals into sub-units based solely on their movement without any prior knowledge on their social relationships (Supplementary Fig. 3, see Supplementary Note 1 for details).

Society, kinship and familiarity

As pair distances during collective movements were clearly related to the social structure, we built proximity networks of harems and individuals to characterise the herd's multilevel society. These networks were based on averaged distances between individuals during movements, i.e., network edges represent typical distances less than a given threshold (see Methods; Fig. 4d, Supplementary Fig. 4). We investigated how this social network relates to kinship (Fig. 3) and familiarity of individuals (i.e., duration of common past membership for a pair of individuals, t_{past}; Fig. 2).

First, we studied the bonds between adult females within harems. Here, kinship did not seem to influence the structure of the social network, as we found no significant differences in network distances if we compared close kin, i.e., full or half-sibling ($p = 0.079$, $n_1 = 15$, $n_2 = 199$) and parent-offspring, adult female pairs to more distant relatives ($p = 0.334$, $n_1 = 9$, $n_2 = 199$, randomisation tests; see Supplementary Note 2 for additional info). Furthermore, the harem choice of females seemed not to be affected by kinship, as sibling female pairs were not found more frequently in the same harem than in the whole population ($p = 0.107$, $n = 31$, randomisation test). Familiarity had, however, an effect on network distances within harems, as adult female pairs were closer to each other in the network if they spent more time in the same harem in the previous 2 years (Pearson's $r = -0.175$, $p = 0.006$, $n = 199$, using randomisation; excluding close kin).

Next, we studied the bonds between the harems. The network distance between harems was shaped by stallion kinship, as harems of sibling stallions were located closer in the network than harems of more distantly related stallions ($p < 0.001$, $n_1 = 53$, $n_2 = 411$, randomisation test, Fig. 5a, b; note that shorter network distances mean shorter spatial distances and usually also higher movement similarities). The harems of full sibling stallions were even closer than the half-siblings' harems ($p = 0.046$, $n_1 = 9$, $n_2 = 44$, randomisation test, Fig. 5a, b). Sibling relations of stallions could not be separated from familiarity, as group membership of stallions while being bachelors was not known. However, common past membership of stallions in the parental harem at young age did not seem to affect proximity of their harems, as

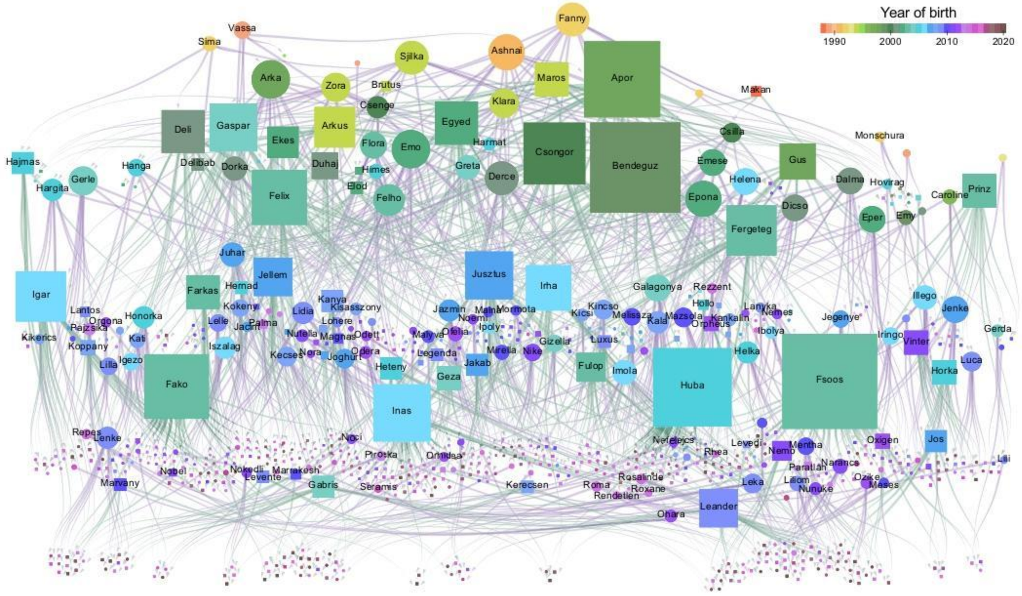


Fig. 3 | Genealogy of the Przewalski's horse population at Hortobágy National Park between 1997 and 2020. Nodes correspond to individuals living in the reserve (circles for females and squares for males) from the establishment of the population in 1997 until the end of 2020. The node size is proportional to the total number of their offspring, node colour indicates the year of birth. Edges point from parent to offspring (purple for mother, green for father). Due to the parentage database kinship relation between each pair of horses is known.

harems of non-related stallions were not closer in the network if the stallions lived longer in the same parental harem in the past (Pearson's $r = -0.035$, $p = 0.260$, $n = 381$, randomisation test). Network distances of harems were associated with female kinship too, as harems containing full or half-sibling adult female pairs were usually located closer to each other than the harems, which contain only more distantly related females ($p = 0.004$, $n_1 = 65$, $n_2 = 206$, randomisation test; excluding harems with familiar females). The network distance between harems with parent-offspring adult female pairs did not, however, differ significantly from harems not containing close kin females ($p = 0.342$, $n_1 = 14$, $n_2 = 206$, randomisation test; excluding harems with familiar females).

Group dynamics

We investigated whether the network among harems is related to group dynamics. First, we asked whether the females' harem changes can be predicted on the basis of this network, i.e., changes happen more frequently between neighbouring harems or not. The network distance of harems that had exchanged females in the 2 years prior to the movement observations was lower than the distance between other harems ($p = 0.021$, $n_1 = 27$, $n_2 = 438$), and the same was true for harems that exchanged females in 2 years following the observations (i.e., in the "future", $p = 0.017$, $n_1 = 24$, $n_2 = 441$, randomisation tests, Fig. 5c, d). Next, we focused on the individuals and asked whether we can predict which females are going to change harem. We quantified the time a pair of females spent in the same harem in the subsequent 2 years following the movement observations (t_{future}). When investigating the future of females that were currently harem-mates, we found that if they were closer to each other in the network then they typically spent more time in the same harem in the subsequent 2 years following the observed movements (Pearson's $r = -0.160$, $p = 0.012$, $n = 199$, randomisation test; excluding close kin). When investigating the future of females from different harems, we assessed movement similarities, since pairwise distances are primarily determined by the location of their harems. Interestingly, the adult female pairs from different harems, which later became harem-mates (for at least 3 months) in the subsequent 2 years following the movement observations, already had more similar movement paths than their female harem-mates' average ($p = 0.007$, $n = 109$, for non-related females with $t_{\mathrm{past}} = 0$, $t_{\mathrm{future}} > 90$ days vs. average movement similarity of females from their harems with $t_{\mathrm{future}} = 0$, randomisation test).

Network centrality

To reveal further details of the harem network, and hence of the possible origins of herd formation, we studied how different harem traits were related to network centrality—a network metric characterising importance of nodes (Supplementary Fig. 5). We found that a harem's closeness centrality (i.e., the reciprocal of the mean shortest path distance from all other reachable nodes) was positively associated with the harem's age (Pearson's $r = 0.600$, $p < 0.001$, $n = 31$) and the harem stallion's experience in harem keeping (i.e., the number of years the stallion has had a harem, in total; Pearson's $r = 0.663$, $p < 0.0001$, $n = 31$, randomisation tests; Fig. 5e, Supplementary Fig. 6a, c). These latter two variables were also related to each other, suggesting that older harems typically belong to more experienced stallions (Pearson's $r = 0.724$, $p < 0.0001$, $n = 31$, randomisation test; Supplementary Fig. 7a; see Supplementary Note 3 for additional info). Unsurprisingly, we found a connection between the harem's closeness centrality and the stallion's age as well (Pearson's $r = 0.631$, $p < 0.001$, $n = 30$; Supplementary Fig. 6e), because a stallion's harem keeping experience is in strong correlation with its age (Pearson's $r = 0.849$, $p < 0.0001$, $n = 30$, randomisation tests). The size of a harem including adult and subadult

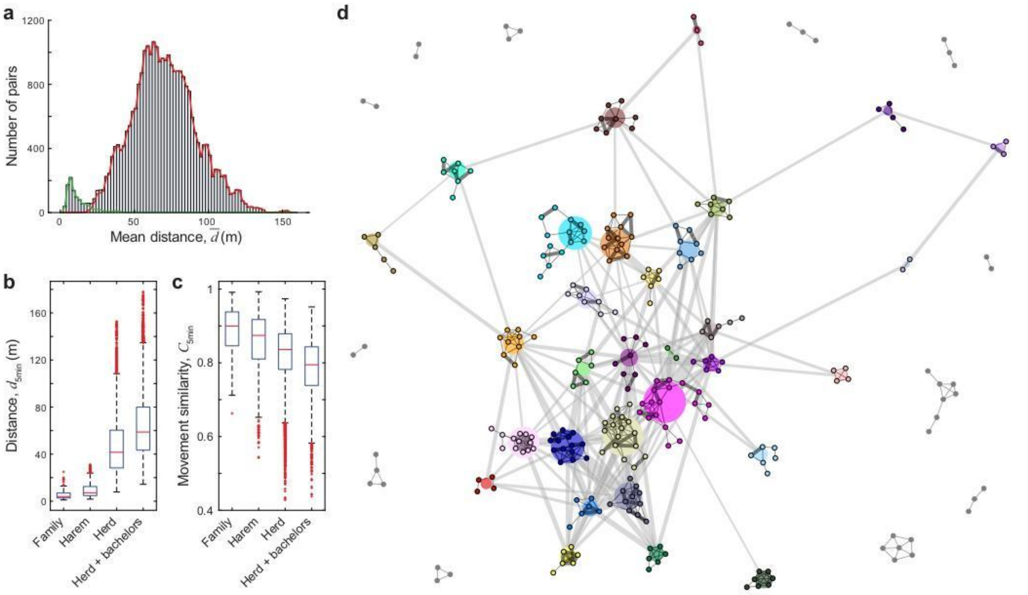


Fig. 4 | Social network of the Przewalski's horse herd based on local pair interactions during collective movements. **a** Histogram of pair distances between horses during collective movements averaged over five 5-min observation sessions (bars), for intra-harem (green line) and inter-harem pairs of individuals (red line); the two peaks correspond to the two social levels of the multilevel society: harem and herd. **b**, **c** Pairwise distance and movement similarity of horse pairs belonging to the (i) same family (i.e., a female and its 1–5 years old pre-dispersal offspring, $n = 70$ horse pairs), (ii) different family but same harem ($n = 707$), (iii) different harem (same herd, $n = 20959$); and (iv) of a bachelor and a harem-living individual pair ($n = 8778$), in a single 5-min observation session. The pairs' distance increases while movement similarity decreases from family to herd including bachelors. Boxes range from the 25th to 75th percentile, while central marks denote medians, whiskers extend to extreme data points not considered outliers (marked in red). **d** Multilevel social network of the herd, presented as two networks overlaid, based on average proximity during observed collective movements. *Top layer:* Nodes (small circles) correspond to individuals, while colour denotes the harem they belong to or shown with grey for bachelors. Bonds (black lines) are based on averaged pairwise distances between individuals: an edge is drawn between two individuals if their averaged distance $\bar{d} < d_{th}$ (for further details, see main text). Heavy connections depict close family ties. *Bottom layer:* Social network of harems, nodes (larger circles) correspond to harems (with matching colours of the individuals and size proportional to harem size), and edges (grey lines) show spatial proximity between harems.

members was also related to the harem's closeness centrality (Pearson's $r = 0.468$, $p = 0.005$, $n = 31$; Supplementary Fig. 6g), although this was not the case if counting only the adult members (Pearson's $r = 0.286$, $p = 0.061$, $n = 31$, randomisation tests). On the other hand, we found that average distance of the closest bachelor male to harems was not related to the closeness centrality (Pearson's $r = -0.133$, $p = 0.241$, $n = 31$), although the harems with more adult members (i.e., containing more adult females) were typically further from the bachelors (Pearson's $r = 0.430$, $p = 0.010$, $n = 31$, randomisation tests; Fig. 5f).

Discussion

In this study, we simultaneously tracked all individuals of a large herd of Przewalski's horses and showed that analysing their collective movements for a few minutes is sufficient to determine the current harem membership of the individuals and to infer past and future social dynamics of the population.

Despite our analyses being based on a few minutes long recordings, fine-scale tracking and high-throughput data driven analysis of movements uncovered novel social relationships among the horses. Our results on intra-harem bonds primarily provided by familiarity are consistent with known sociality of polygynous equids[38]. Inter-harem bonds, and thus the formation of large herds, however, is not fully understood in equids[12–14]. Therefore, more research is needed to determine the possible preconditions and factors that may lead to massive herds from independent harems. Our results suggest that aggregation of harems is associated with kinship in this Przewalski's horse population, and male-male and female-female sibling relations are both important. Similarly, in plains zebras (*Equus quagga*), a closely related species, aggregation of harems is driven by kinship, but only by female-female kin relations[39]. In other systems, such as human and other primate societies, association of groups can be based on male-male bonds, which may or may not be associated with genetic relatedness[4,40,41]. We expected association between kinship and aggregation of harems, since both the relatedness among harem stallions and adult females may strengthen inter-harem tolerance and thus reinforce herd formation[42]. Note, however, that stallion kinship could not be separated from familiarity in this study, because there is a possibility that genetically related males were formerly group-mates while being bachelors, and developed familiarity with each other. Female familiarity probably also contributes to harem aggregation, due to the member transfers between harems[42]. On the other hand, female transfers were more frequent between nearby harems, which suggests a reinforcing effect between female exchange and proximity of harems.

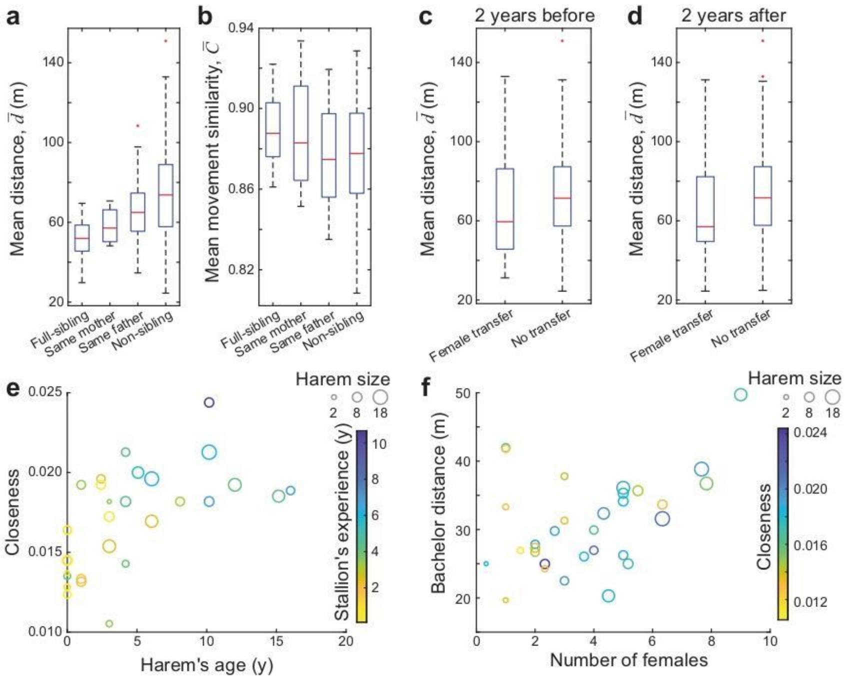


Fig. 5 | Social and group dynamic processes in connection with the social network. a, **b** Distance and movement similarity of harems, averaged over five 5-min observation sessions, for different kinship relationships between their stallions: for full sibling ($n = 9$ harem pairs), maternal ($n = 4$) and paternal half-sibling ($n = 40$), and more distantly related stallions ($n = 411$). **c**, **d** Harems between which female transfer occurred in the 2 years prior (**c**) or following (**d**) the movement observations were closer (and had more similar movement) than harems without female transfer ($n_{\text{prior,transfer}} = 27$, $n_{\text{prior,no transfer}} = 438$, $n_{\text{following,transfer}} = 24$, $n_{\text{following,no transfer}} = 441$ harem pairs). Boxes range from the 25th to 75th percentile, while central marks denote medians, whiskers extend to extreme data points not considered outliers (marked in red). **e** Closeness centrality of harems in the network against the harem's age in years. Node size denotes the harem size, while node colour the stallion's experience of harem keeping (i.e., how long the stallion kept a harem in years). **f** Average distance of the closest bachelor male to harems against the number of adult female members in the harem. Node size denotes the harem size, while node colour the closeness centrality of harems.

Relationship between network centrality and harem traits, namely that older and larger harems with older and more experienced stallions occupy more central locations in the network, may suggest benefits of central positions. Network centrality implies vicinity of more harems and their stallions, which, due to communal defence, may promote protection from bachelor males. In this way, stallions of central harems may reduce the risk of takeovers and thus prolong their tenure, while females may reduce male harassment and the risk of infanticide[43,44], which were both observed in the studied population (V.K., pers. obs.). Moreover, centrality might influence exposure to horseflies and other biting insects. On the other hand, centrality of older harems may be an emergent property as well, since newly formed harems are often temporarily isolated and join the herd later[14]. In some primates multilevel group formation is similarly driven by conspecific social threats, e.g., bachelor threat and infanticide[42,45,46]. Also, herd size of plains zebras is shown to depend on environmental and social factors, such as the number of bachelor males[12]. Accordingly, this suggests that herd formation in Hortobágy might be the result of an interplay between several factors, such as kinship, familiarity and coalitions of harem stallions against bachelor threat, and may also be driven by females, however, this suggestion needs further investigation.

Familiarity (i.e., past associations in the same harem) affecting movements of individuals was expected, as social bonds develop over time. On the other hand, the relation between current movement and future associations, namely that based on movement it can be predicted which female pairs will become harem-mates later or which ones will stay in the same harem, years after the observations, is surprising.

Our findings can be useful in population monitoring, e.g., to determine the number and size of harems in a herd, which would probably work for other Przewalski's horse or feral horse populations as well. Applicability in other species, however, strongly depends on the dynamics of the animals' movement and group stability (e.g., in fission-fusion societies longer observations and/or more occasions would be necessary to detect the multilevel structure). Although we only studied herd movements here, tracking individuals while foraging or resting would likely lead to interesting insights into what kind of social interactions hold individuals together or space them apart, e.g., how exactly the affiliative or agonistic interactions influence the spatial organisation in a herd.

Drones provide a precise and low-disturbance method for behavioural and movement tracking of animals in the wild, however their application has been rare to date[47–51], possibly due to technological difficulties of simultaneous tracking. Automatic tracking methods, like machine learning, would offer new perspectives in the study of complex social systems and their collective behaviour[51]. Our study highlights how relatively short observations of movements can reveal detailed social dynamics of a population not just in the present, but in the past and future, and moreover may provide a unique tool to expand our knowledge on development and function of complex societies.

Methods

This study was approved by the Government Office for Hajdú-Bihar County (Hungary) under the reference number HB-03/KTF/00779-24/2017, and by the Hortobágy National Park Directorate under the reference number 3482-2/2017.

Study area and population

We studied Przewalski's horses in Hortobágy National Park, located in eastern Hungary (47°31'3.3"N 21°5'34.1"E). In 1997, Przewalski's horses were introduced to the Pentezug reserve, a 3000 ha steppe area in Hortobágy National Park, as part of an ecological habitat management scheme by large grazers: wild horses and cattle. The area is surrounded by an electric fence that prevents migration of large grazers but does not limit the native fauna. The conditions are close to wild, human activities are restricted, and the horses are not fed or watered. The total number of large grazers was around 680 during our observation period (273–278 Przewalski's horses and cca. 400 Heck cattle). The Przewalski's horse population originated from 31 founder individuals which arrived in the reserve between 1997 and 2017 from different European zoological gardens, though during the observations only two founder individuals were still alive. The formation of social groups and mate choice are natural, without any human intervention. Since the population is closed, birth control was used from 2013 treating females with the immunocontraception vaccine porcine zona pellucida[52]. The inbreeding coefficient in the population is relatively high (0.176 during the study year)[14], due to a strong bottleneck effect caused by the extinction in the wild of this species in the 1960s[53].

From the beginning, horses have lived in year-round stable harems, consisting of a single male, several adult females and their young. Female offspring leave the natal harem at the age of 2–3 years and join other harems; males leave at the age of 2–4 years and join with other non-breeding but already dispersed males and former harem stallions to form single-sex bachelor groups. In the first years, harems and bachelor groups stayed isolated, and used non-overlapping home ranges; then later, and also at the time of our observations, all harem and bachelor groups united into a massive herd with a common home range[14].

Population monitoring

The Przewalski's horse population in the Pentezug reserve has been monitored regularly since its founding in 1997. All individuals except young males while bachelors are individually identified on the basis of natural pelage colouring and characteristic features and injuries, supported by a photo catalogue, harem composition lists and DNA-fingerprint database[14]. Data collection includes records on life history events, like birth date, death date, identity of parents and changes in harem membership. Biopsy samples are taken from each individual at the age of 1 year, which serves for determining parentage and updating the DNA-fingerprint database. Parentage records based on observations are supported by DNA-tests in the majority of cases (74%). Males are individually known until leaving the parental harem, then again when they acquire a harem or when they die, but membership information is lacking while in bachelor groups, which have a less consistent membership. Harem composition lists are updated with a temporal resolution of 12 +/− 8 (mean +/− SD) observations per year. Individual traits known from population monitoring include age, sex, role in the social system (adult female, subadult individual in parental harem, harem stallion, bachelor), relatedness to other individuals, and monthly updated records on harem membership.

Drone observations

We performed aerial video recordings of the herd on the move with two DJI Phantom 4 drones simultaneously at 4k resolution and 25 fps (frames-per-second). Prior to the study we tested the disturbance of drones with decreasing flying altitudes and observed that horses started to avoid drones at around 3–4 m flying altitudes. A drone flying high (100–300 m from the ground) recorded the moving herd and provided the image for movement tracking, while a drone flying low (10–30 m from the ground) scanned through the whole herd and provided a detailed image for individual identification (Fig. 1a). The recordings of the two drones were synchronised, and thus identities of individuals could be matched with the tracked trajectories. The top-view drone's video was processed with the Motion Tracking function of Blender v2.79b[54] and the global position of each individual was determined in every second frame, i.e., with 12.5 fps temporal resolution. By tracking several fixed points of the background, we solved the camera motion and reconstructed the tracking scene, then projected the horse movement tracks to the 3D view of the background. We set a ground level based on three background-fixed points, a local origin and x-axis, and scaled the background with two distinctive landscape items (e.g., a well, a solitary tree, etc.) by measuring the distance between them on Google Maps. This way we obtained horse coordinates in metres in a background-fixed coordinate system. The x–y coordinates of a horse were defined on its withers (the ridge between the shoulder blades) at $z = 1.25$ m altitude (the average height of Przewalski's horses). Coordinates of horses in each frame were exported from Blender to 'csv' files with a custom-written script in Python 2.7. The accuracy of horse positions in the background-fixed system was +/− 0.2 m (the average noise when tracking a stationary point). Identities of individuals were determined on the lower drone's videos by one observer based on natural colouring and characteristic marks.

We recorded 320 s of continuous movements (called as observation sessions) of the herd when most of the individuals were moving continuously through the whole session between feeding and drinking places or towards a dust bath (300–600 m travel distance). The study contains five observation sessions, recorded on five different days, 1–2 weeks apart (August 17, August 24, September 5, September 13, and October 2 in 2018), during daylight hours (between 8 a.m. and 3 p.m.). Average travelling velocity was around 0.85 m/s, the horses were moving at a pace from a walk to a gallop. The population size on the first observation day was 278 individuals, in total, of which 238 were individuals belonging to harems and 40 were bachelor males. We aimed to track the whole population and to identify all harem-living individuals, but in some cases a few individuals were not possible to recognise or did not appear on the video (Supplementary Table 1). As the bachelor males could not be identified individually, we denoted their tracks as "unidentified bachelor". The size of the population slightly decreased during our aerial observations due to a few deaths, and was 273 on the last observation day (Supplementary Table 1). Identification reliability, i.e., the ratio of individuals that got the same ID during repeated identification attempts, was 93%.

Data analyses

Movement variables. We calculated pairwise movement variables, pairwise distance (d) and movement similarity (C) over two timescales: (i) a "5-min" (i.e., 320 s) observation session, where a high-resolution drone video recording was used for tracking at 12.5 fps (resulting in 4000 "frames", timestamped locations for each individual visible during a session), (ii) averaged sessions, i.e., the averaged data over five 5-min observation sessions recorded on different days. We defined the 5-min distance ($d_{5\mathrm{min}}$) between two individuals as the distance of the pair in metres averaged over a 5-min recording session. Averaged distance $\bar{d}$ is the average over the five 5-min sessions. We calculated movement similarity ($C_{5\mathrm{min}}$) between a pair of individuals as the directional correlation with a time delay between their trajectories[36,55], for a 5-min session. Trajectories were smoothed using Gaussian smoothing ($\sigma = 1.2$ s). For every pair the entire duration (320 s; 4000 frames) of the trail was used (as a single time window) and the highest correlation was chosen using all possible time delays in the range of [−16 s, 16 s] with a step of 1 frame (0.08 s). Pairwise correlation values were omitted, where the correlation was negative. For all other aspects, the steps of this movement analysis were identical to previous studies[56]. Robustness against the values of the parameters were analysed in the previous studies, but we rechecked it here as well. Averaged movement similarity $\bar{C}$ was obtained as the average over the five sessions. Bachelor males were considered only in the analyses of 5-min sessions and excluded from the analyses of averaged sessions, since their identity could not be matched through different observation days.

Society. The single breeding male in a harem is called the "harem stallion", aged between 6 and 15 years ($n = 31$). Females that have already dispersed from their natal harem or are at least 2 years old and their harem stallion is not their father due to a stallion change, are considered "adult females" ($n = 115$). A "bachelor" is a non-breeding male, including young males, which have dispersed from their natal harem and have not gained a harem yet, and old males, which previously had a harem ($n = 40$). A family is defined as an adult female and her pre-dispersal offspring except foals born in the year of observation, where the offspring may be 1–5 years old, and the father of the offspring may be one or multiple stallions. A harem consists of a harem stallion, several adult usually non-related females associating with the stallion, and their pre-dispersal offspring. Individuals in the study were assigned to a harem based on the group composition records at the first observation day. Harem size denotes the number of all individuals belonging to the harem, both adult and subadult, while adult harem size counts the adult females and the harem stallion (there were no multi-male harems). In the pairwise analyses bachelor-bachelor pairs were excluded, as bachelors could not be identified individually, and hence we could not classify them as same-group or different-group pairs.

Social network. To study the multilevel social structure, we constructed a proximity network of harems and proximity networks of individuals within each harem, based on pairwise distances of individuals. In the network of harems an edge connected two harems if the averaged distance between any of their members was less than a threshold ($\bar{d} < d_{\text{th}}$; Supplementary Fig. 4). In the analyses we used the harem network with the smallest d_{th} that ensures a single connected component ($d_{\text{th}} = 53$ m; Fig. 4d). Also within each harem, we defined a network of individuals in a similar manner, where two individuals were connected with an edge if their averaged distance was smaller than a threshold, $\bar{d} < d_{\text{th,harem}}$. Since typical distances varied among harems, $d_{\text{th,harem}}$ was determined separately for each harem as the smallest threshold, where all members of the harem were connected to the network (Fig. 4d). When determining $d_{\text{th,harem}}$ we excluded females that changed harem during the observation period, and hence, were members of multiple harems on different observation days. Distance in the social network of individuals and harems was calculated as the length of the shortest path between the nodes.

In our study, we chose an arbitrary d_{th} distance threshold when calculating the social network. To test the robustness of the results on the $d_{\text{th}} = 53$ m harem network (association between network centrality and different harem traits), we also investigated networks with 10% higher and lower d_{th} and obtained similar results (see Supplementary Note 4 for details).

Kinship and familiarity. For the association of the social network with kinship, we tested whether close relatives (i.e., offspring-parent, full and half-sibling pairs) behave differently than individuals, which are more distantly related. We defined the following kin relationships: full siblings (both parents are common), half-siblings with same mother, half-siblings with same father, parent-offspring (mother and daughter), and more distantly related (i.e., all other individuals). For quantifying familiarity, we used the group composition lists between 1997 and 2020 to calculate shared membership of horse pairs (i.e., both individuals present in the same harem). For adult females, familiarity (t_{past}) was defined as the number of days in the same harem by a pair in the 2 years prior to the movement observations (between 2016 and 2018). Similarly, t_{future} was the number of days a female pair spent in the same harem in the 2 years following the movement observations (between 2018 and 2020). Note that for t_{past} and t_{future} shared time in the same harem can refer to multiple harems (i.e., association with several different stallions), in this case the number of days in the same harem is summed up for all common harems. For harem stallions, $t_{\text{past*}}$ was the number of days the stallion pair spent in the same harem during young age while being subadults. Note that since bachelor group compositions were not recorded during population monitoring, time spent in the same bachelor group could not be quantified for stallions.

When investigating the effect of kinship and familiarity, we tried to separate these effects, as far as possible, by analysing certain subsets of the data. Harem stallions and adult females were analysed separately, and adult females were further divided into females from the same harem, and females from different harems. The effect of kinship was studied in subsets where individual pairs did not share past membership in the previous 2 years ($t_{\text{past}} = 0$), except in case of stallions; and the effect of familiarity was studied in subsets where closely related individual pairs (parent-offspring, full sibling, half-sibling) were excluded.

We considered female kinship between two harems in the following harem pair subsets: (i) harem pairs containing full and/or half-sibling inter-harem adult female pairs but not containing parent-offspring inter-harem adult female pairs, (ii) harem pairs containing parent-offspring inter-harem adult female pairs but not containing full or half-sibling inter-harem adult female pairs, and (iii) harem pairs containing only more distantly related inter-harem adult female pairs. To quantify the familiarity of adult females between two harems, we calculated $t_{\text{past\_af\_harem}}$, as the sum of t_{past} for all possible pairs of adult females from the two harems. When studying the association between female kinship and harem distances, we compared two of the above subsets; to exclude the effect of familiarity, only harem pairs were considered where $t_{\text{past\_af\_harem}} = 0$.

Group dynamics. We considered only adult females changing their breeding harems, i.e., excluded harem changes of young females from their natal harem to the first breeding harem, in the 2 years prior (2016–2018) and after (2018–2020) the movement observations. By comparing the membership data of 2 years (e.g., memberships in 2016 and 2018), we counted the number of female transfer between each harem pair, regardless of direction (i.e., if one female moved from harem A to B and one female from B to A that means two changes happened between harems A and B).

Harem traits. We approached harems as dynamically changing "communities" and considered them as the same "community" unless a given amount of change in the membership occurred. When studying the development of harems, we used one observation per year from the group composition records of Hortobágy National Park, the one prior to 1st September in each year. In 1997 the first record defined the initial harems. Then, in each subsequent year i, we calculated the Jaccard index between the adult members (2-year-old and older females and the stallion) of all harems in year $i{-}1$ and year i. The ancestor of a harem in year i is the harem in year $i{-}1$ with the maximal Jaccard index, but with at least two common adult individuals. If a harem in year *i–1* has more descendant harems in year *i*, the one with the most common individuals will be the descendant, or the oldest one if the common part is equal in size. If a harem in year *i* does not have an ancestor in year *i–1*, then it is assigned as a new harem with a starting date of the first observation; if a harem in year *i–1* does not have a descendant in year *i*, then the harem is assigned as ended. Harem ages were calculated from the harems' starting dates.

A male's harem keeping experience is defined as the time in years during which the male kept the position of being a harem stallion. If a stallion had more than one harem throughout its life, the harem keeping time is summed up for all harems.

Statistical tests. Randomisation tests were performed as follows: (i) a test statistics is calculated, i.e., (a) a mean difference between two samples of a variable, (b) a mean difference between paired samples of a variable, (c) a correlation coefficient between two variables; (ii) the empirical dataset is rearranged randomly, i.e., (a) the two samples are pooled then divided again randomly to two samples according to the original sample sizes, (b) and (c) paired data points in one of the variables are permutated randomly, while the other variable is unchanged; then the test statistics is calculated on the permutated data; this point is repeated (number of iterations was $n = 10{,}000$ in all tests) to estimate the test statistics' probability distribution; (iii) *p*-value is obtained from the cumulative distribution function of the test statistics (i.e., at the empirical value of test statistics). All randomisation tests were one-sided.

Calculations and statistical analyses were performed in MATLAB R2021a[57] and CUDA 11.5. For data organisation and storing MATLAB R2021a and Microsoft Excel for Mac 16.54 were used.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Data generated during the analyses that support the findings of this study have been deposited on Github at https://github.com/katalinozogany/wildhorse_mls. The raw data are available under restricted access for nature conservation reasons, access can be obtained from the Hortobágy National Park Directorate and the first author on reasonable request.

Code availability

Custom codes used in the analyses have been deposited on Github at https://github.com/katalinozogany/wildhorse_mls.

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Author contributions

K.O., M.N. and Z.B. designed the main concepts of the study; K.O. designed and performed the movement observations and tracking; V.K. collected the population monitoring data; K.O. and V.K. curated the data; M.N. designed and performed the trajectory analyses; K.O. and M.N. designed and performed the data analyses and the visualisations; all authors contributed to the interpretation of the results; K.O. wrote the initial manuscript, A.F., Z.B. and M.N. edited the final manuscript.

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Competing interests

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