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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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A B S T R A C T

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 $O_{i,j} = I_{i,j}/A_i$, where $I_{i,j}$ is the area of the intersection between the two home ranges and A_i 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

? how are they all founders then

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. why

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, $\chi^2_1 = 0.184$, $p = 0.668$).

what about individual years?
Are bachelors counted?
Make something in R - which

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).

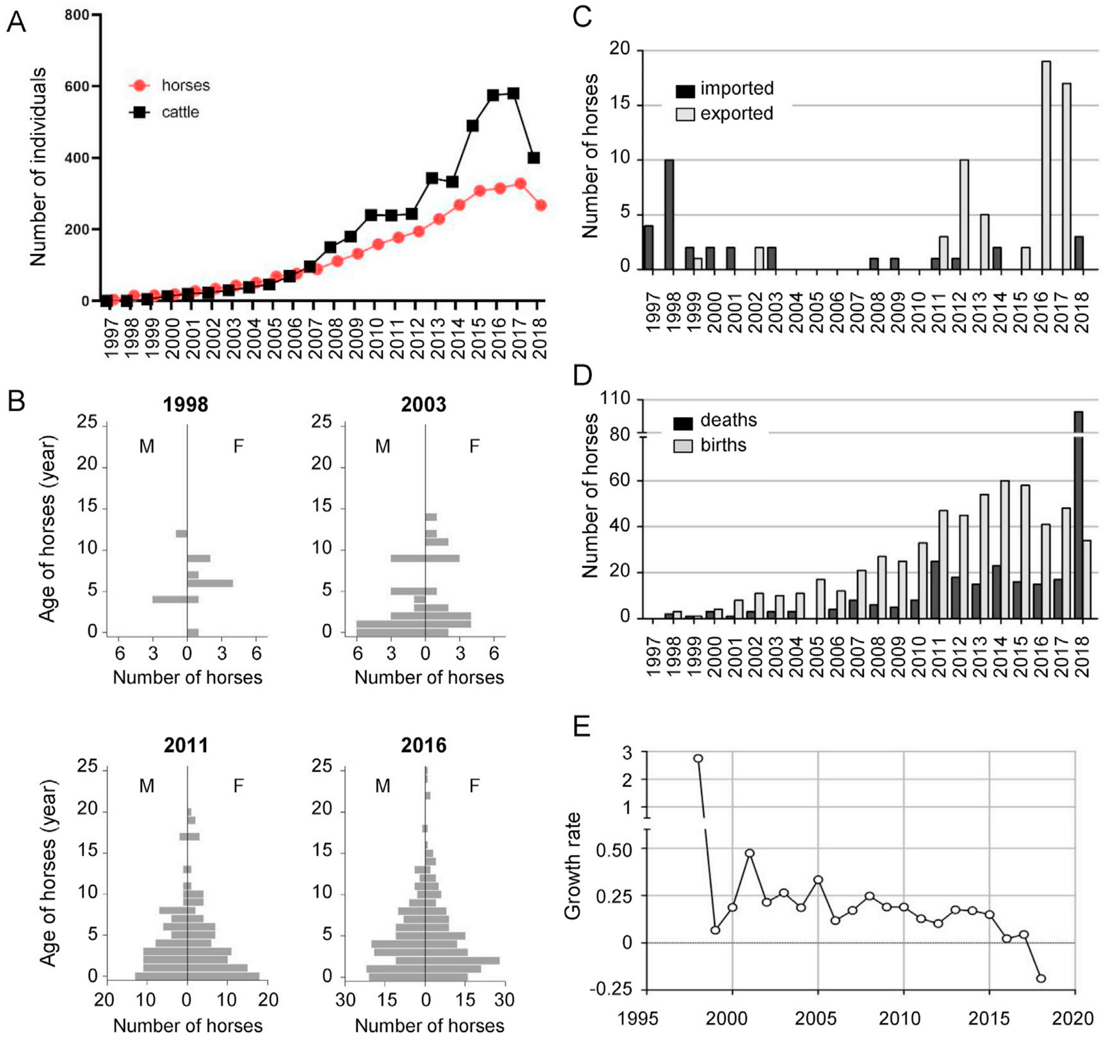

Fig. 1. Przewalski's horse population in Pentezug Reserve, Hortobagy National Park (HNP), Hungary between 1997 and 2018. (A) The number of Przewalski's horses and cattle in Pentezug Reserve from the initiation of the project. (B) Age-trees of horses in the reserve in the indicated years, separately for 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

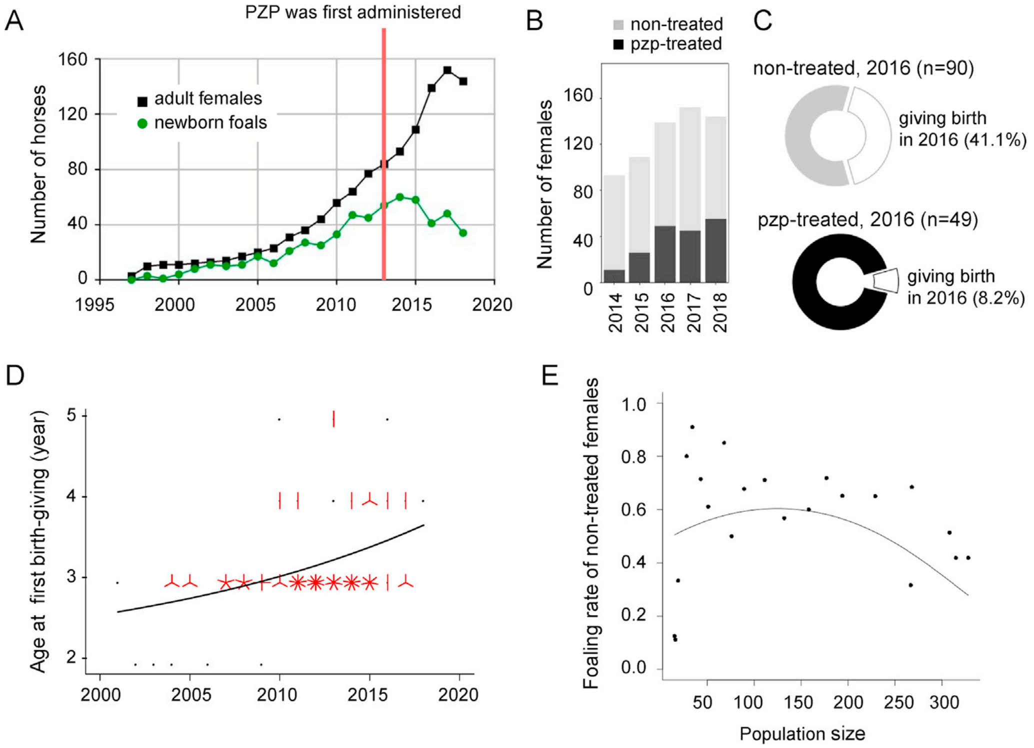

Fig. 2. Reproduction, birth control and foaling rates of Przewalski's horse population in Pentezug Reserve. (A) The number of adult females (2-year-old or older) and newborn foals in the reserve by years. (B) The numbers of PZP-treated (black) and non-treated females (grey) from 2014 (the first year 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.

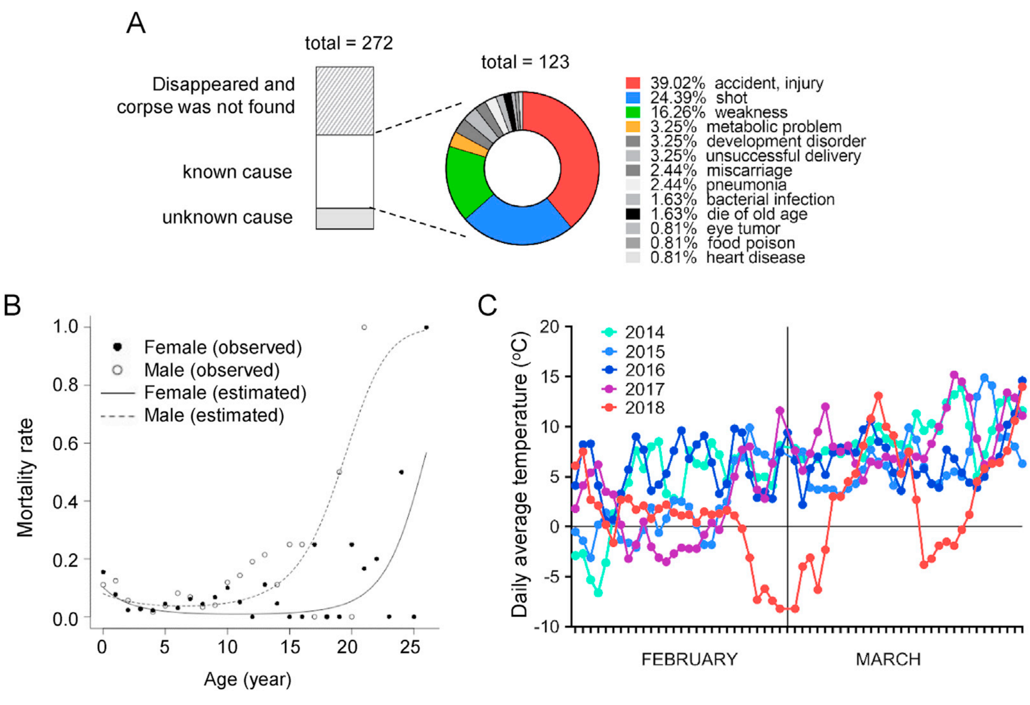

Fig. 3. Mortality in the Przewalski's horse population in Pentezug Reserve. (A) The percentages of various causes of death between 1997 and 2018 (n = 272). (B) Mortality rate versus age of horses between 1997 and 2017. White and black dots indicate the death rate of males and females, respectively. (C) 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).

what effects group size?
Male quality?
Age?
Sex ratio?
Something with genes?

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.

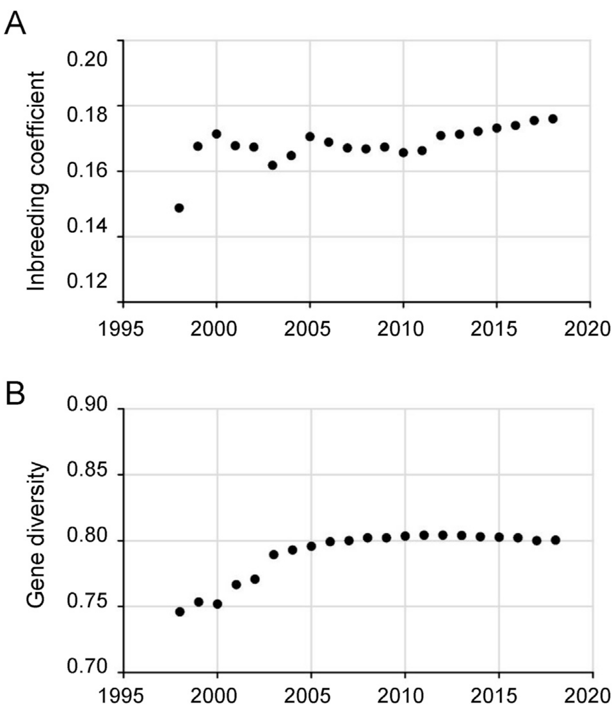

Fig. 4. Genetic characteristics of the Przewalski's horse population in Pentezug Reserve. (A) Pedigree mean inbreeding coefficients for the whole population by year. (B) Gene diversity based on kinship matrix by year.

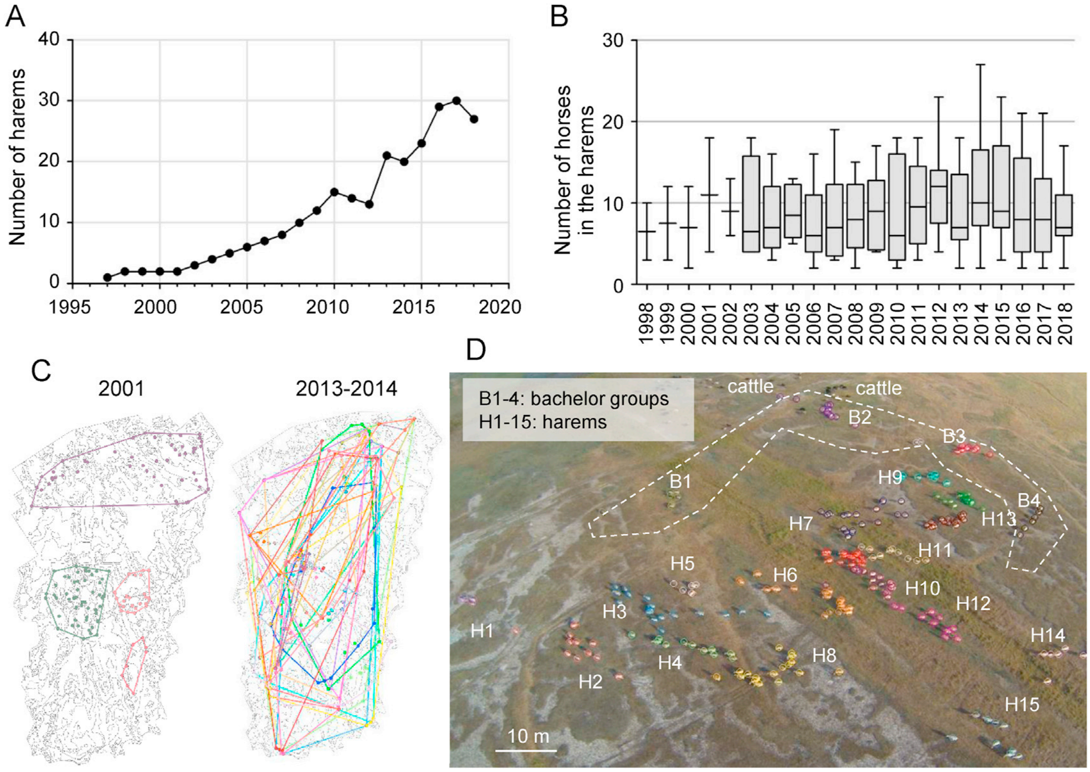

Fig. 5. Przewalski's horse harems in Pentezug Reserve. (A) The number of harems in the reserve by year. (B) The number of horses in harems as box-and-whisker plot. Median, first, and third quartile values are shown with boxes, while whiskers indicate the minimum and maximum values. (C) Home ranges (100% 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

Read these references!

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.

Older mares are treated now

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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