Human-controlled reproductive experience may contribute to incestuous behavior observed in reintroduced semi-feral stallions (*Equus caballus*).

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Short title: Incest avoidance in stallions.

Abstract

Equine reproductive behavior is affected by many factors, some remaining poorly understood. This study tested the hypothesis that a period of captivity during the juvenile period and human-controlled reproduction may potentially be involved in the disruption of the development of incestuous mating avoidance behavior in sanctuary-reintroduced male Konik polski horses. Between 1986 and 2000, cases of incestuous behavior in harem stallions born and reared until weaning in the sanctuary were studied. Eight males lived in the sanctuary's feral herd for the rest of their lives (the non-captive group; nC). They gained their own harem of mares without human intervention (no human-controlled reproductive activity, nHC). Another five stallions were removed as weanlings, reared in captivity and then reintroduced as adults (captive, C). Three of these C stallions were used as in-hand breeding stallions, one as a "teaser" (human-controlled reproductive activity, HC) and one was not used for reproduction in captivity (nHC). Reproductive records for 46 mares, daughters of all 13 harem stallions, were scrutinized and cases of incestuous breeding were recorded by interrogation of foal parentage records. C stallions failed to expel more daughters than nC stallions (33% vs. 18%, $P = 0.045$), and mated with significantly more of them (28% vs. 11%, $P = 0.025$). Interestingly, HC stallions expelled fewer (60%) and successfully mated with more (33%) daughters that nHC stallions (84% expelled, $P = 0.013$, and 10% successful mating with daughters, $P = 0.010$). All HC stallions bred incestuously at least once.

We propose that human intervention during a critical period of development of social and reproductive behavior in young stallions, by enforced separation from their natal herd and in-hand breeding, may contribute to their later aberrant behavior and disruption of inbreeding avoidance mechanisms in these stallions. The previous occurrence of human-controlled breeding may be one of the factors promoting incestuous behavior of stallions in natural

conditions. The uninterrupted presence of stallions in their harems and herd member recognition may also play important roles in inbreeding avoidance in horses.

KEYWORDS: Feral horse, Incest, Reproductive behavior, Inbreeding avoidance, Koniks

1. INTRODUCTION

Equine reproductive behavior is affected by many factors, some remaining poorly understood. Genetic [1, 2] and environmental [3, 4] effects have been confirmed to affect equine reproductive efficiency. Both domestic and feral equine reproduction could be compromised by environment-specific factors. Domestic horses experience different types of human-controlled reproduction; this includes seasonal "pasture breeding", when the stallion is only temporarily introduced to a group of mares [5], "in-hand" breeding, when both stallion and mare are restrained by humans during copulation [6], and artificial insemination as routine practice [4]. Genetic selection i.e. the breed [7] or a lack of socialization and familiarity with the mate may lead to silent estrus in mares, or decreased libido, sexual disinhibition or aggression in stallions [6, 8, 9]. Also, everyday conditions, such as confinement, short feeding duration and social restrictions, as well as caretakers' attitude, may influence the behavior and welfare of animals [6, 8, 10, 11].

In natural conditions, where horses are reared up to maturity in familial groups, and are free to choose their reproductive partner and to form stable social groups (harems), feral or semi-feral horses present high reproductive efficiency without human support [12]. However, this could be jeopardized by seasonal food scarcity [13], parasite infestation [14], or harassment by stallions [15]. In human-controlled semi-feral populations, the practices leading to

69 overpopulation [16] may influence the biological or behavioral balance and impact socio-sexual
70 behavior and related reproductive performance.
71 In both domestic and feral horses, a high level of inbreeding is one of issues that may have
72 negative effects on reproduction rates [17]. While in domestic horses, inbreeding levels could
73 be minimised by humans by selection of parental breeding stock, feral horses were observed to
74 avoid inbreeding by a specific behavior: dispersal. Juvenile dispersal is commonly considered
75 to be a behavioral strategy for the avoidance of inbreeding [18 - 22]. Maturing free-living horses
76 of both sexes disperse from their natal herds at aged one to five years old; males either join
77 bachelor bands or form their own harem, whilst females join new or existing harems [18, 23,
78 24].
79 Resident harem females may not play a role in offspring dispersal [19, but see 25 for a
80 counter-argument to this], and there is no consensus as to the role of the sire in inbreeding
81 avoidance in horses [26]. Generally, fillies leave either voluntarily [19, 23], or by forceful
82 expulsion by the sire [27, ZJ and MS personal observations]. Both avoidance of sexual attempts
83 by the father and increased sexual attraction to unfamiliar males have been proposed as
84 proximate causes of dispersal in fillies [19]. The indifference of the sire to attempted matings
85 by unfamiliar stallions has been also observed [23]. Although most fillies disperse, isolated
86 cases of stallions mating with non-dispersed daughters have been recorded [18, 21, 22]. Here
87 we sought to explore potential explanations for such incestuous matings in free-living horses.
88 For about 10 generations, free-living Konik horses in the Popielno sanctuary, Poland, have
89 been maintained under human surveillance. To prevent overpopulation, only some foals are left
90 in the herd to replace their parents [12, 28]; others are removed. Each season, most of the
91 weanlings are transported to nearby stables, when they are reared with their stable-born
92 conspecifics under human care. From this time onwards, they experience social contact only

93 with same-age and same-sex conspecifics. Upon sexual maturity, at 3-4 years old, occasionally,
94 selected young stallions are used for reproduction in domestic conditions.
95 In the sanctuary, remaining colts and fillies are actively expelled by the father upon reaching
96 sexual maturity [27, ZJ and MC personal observations]. Occasionally, some individuals from
97 the stable-reared group of captured yearlings are introduced to the sanctuary as adults. Although
98 this happens sporadically, it allows a unique opportunity to follow the breeding behavior of
99 reintroduced, captive-reared stallions. We were interested to know whether early experiences,
100 such as post weaning stabling, possibly affecting the social development of young stallions
101 (colts), and specifically human-controlled (HC) reproduction (where both male and female are
102 restrained, no familiarity or partner choice possible), can impact upon future reproductive
103 behavior. Since the development of an appropriate, species-specific courtship and coupling
104 repertoire during the individual's growth and maturation is essential for successful reproduction
105 [29], we hypothesize that human disturbance during a sensitive learning period, specifically
106 controlled reproduction, could significantly influence adult sexual behavior as proposed by Feh
107 and Munkhtuya [30]. In particular, we predict this could disrupt the mechanisms underlying
108 incest avoidance, leading to a failure to evict daughters and subsequently the occurrence of
109 incestuous matings.

110 ## 2. MATERIAL AND METHODS

111 ### *2.1 Ethical note*

112 The study involved the analysis of breeding records and regular observations of animals
113 from a distance. No experimentation was performed in view of European directive 2010/63/EU
114 and the Polish laws related to ethics in animal experimentation.

115 ### *2.2 Study site*

In the Popielno sanctuary, a 1600ha site mostly comprising forest habitat, there are on average 20 horses including 3-5 stallions (and their harems). The harems are almost evenly dispersed within the sanctuary, so the groups very rarely meet. Once expelled, when 1.9 ± 1.4 years old, the young females stay in close proximity to their natal herds; this could be for some time (few weeks to few months) since in reproductive seasons the stallions guard their mares rather than search for new ones. Finally, after mating with an unfamiliar stallion, the young mare enters a new harem and remains there for a number of years.

The horses are not dewormed, castrated, or their hooves trimmed. Supplementary food is only provided during harsh winters. Salt blocks are available for horses in chosen locations within the sanctuary. Once per year, all new foals are rounded up and the parentage of every foal is routinely tested from blood by a laboratory certified by ISAG (International Society of Animal Genetics) by microsatellite analysis. The genotypes of all stallions are known and so paternity can be reliably assigned. Horses in the sanctuary are monitored on alternate days for most of the year and on a daily basis during the breeding season; this means that information on the band to which each female belonged, including the identity of the harem stallion, was also available for all these individuals and so all dispersal events were known. Almost all offspring are transferred to the stables, where they are reared with stable-born counterparts and subsequently are sold. Only some foals are left to replace their parents; these are the core of reproductive semi-feral herds. Also, some horses from other sanctuaries are introduced sporadically to the sanctuary to prevent inbreeding.

All 13 harem stallions included in this study were born and reared until weaning (at around nine months old) in the sanctuary. Eight of them lived in the sanctuary's feral herd for the rest of their lives (the non-captive group; nC). They were expelled by their sires at around two years old and gained their own harem of mares later in life without human intervention (no human-controlled reproductive activity, nHC). Five stallions were, however, removed from the

sanctuary as weanlings or two-years-olds and kept in stable conditions (captive, C) with stable-born counterparts, where they were handled by humans, fed with hay and oats and pastured in single sex groups. Three of them were used as in-hand breeding stallions, one as a "teaser" (used to test for oestrus in females; these four stallions were all classed as "human-controlled reproductive activity", HC) and one was not used for any reproductive activity (nHC). These stallions were all managed in male-only groups and so lacked any interaction with females, apart from during in-hand mating or teasing eventsThe stallions were reintroduced to the feral herd at the age of 2.5, 4, 7 and 18 years old (Table 1).

Breeding records for 54 mares maintained for reproduction in the sanctuary between 1986 and 2020 were inspected. All mares were reared until dispersal in the harem of their biological father (43 females) or a non-related stallion (stepfather; three femalesthat were born in their harems after the stallion had taken control of mares that were already pregnant). All cases when the sire died before the filly became sexually mature or the harem was taken over by another stallion were excluded from analyses. Data for 46 mares and 13 harem stallions were retained and cases of incestuous breeding were recorded by interrogation of foal parentage records.

2.3 Statistical analyses

The effects of captivity and reproductive history on the numbers of subsequently expelled and mated daughters were tested by fitting generalized linear mixed models (GLMMIX, SAS System, 9.4), using a binary distribution. To consider the potential effect of premature separation from the natal herd, and the conditions experienced during maturation in domestic conditions, the fixed effect of captivity (or a free-living state) during post-weaning rearing (C, nC, binary outcome) was included. Reproductive history, i.e. whether or not stallions experienced "in-hand" breeding (HC, nHC, binary outcome), was included as another fixed effect. The individual effect of the stallion, i.e. all the variance that can be contributed to

166 an individual's possible psychological or genetic background, was included in the model as a
167 random effect (characteristic to given stallion). The results are presented, for better clarity, as
168 raw numbers and percentages in Fig 1.

169 **3. RESULTS**

170 Successful matings with on average 3.84 (SD = 1.67) fillies per stallion were scrutinized. The
171 stallions categorized as C and nC, as well as HC and nHC, differed in the probability to expel
172 and mate their daughter (Fig. 1). The probability to expel a daughter from the natal group was
173 significantly lower in C than in nC stallions ($F = 4.27$, $P = 0.045$; Fig. 1A). The C stallions bred
174 with significantly more of their daughters than did nC stallions ($F = 5.42$; $P = 0.025$; Fig. 1B),
175 which expelled most of them. Within non-expelled fillies, three cases of successful father-
176 daughter matings were recorded across two nC stallions. The one C stallion who never
177 experienced HC breeding expelled all his daughters.

178 HC stallions all bred incestuously at least once. For all possible father-daughter pairs, there
179 was therefore a higher probability for HC to fail to expel (and to mate with) their daughters
180 when compared with other males ($F = 6.63$, $P = 0.013$, Fig. 1C and $F = 7.16$, $P = 0.010$, Fig.
181 1D).

182 **4. DISCUSSION**

183 Since almost all reintroduced stallions were used for in-hand reproduction during the
184 stabling period, our results support the hypothesis that human-controlled conditions during
185 early reproductive activity may be, amongst other factors, those that disturb the development
186 of an appropriate mechanism for reduced sexual interest in daughters. Although we recorded
187 incestuous breeding by two free-living stallions with no captive history, experience of HC
188 breeding significantly increased the probability of inappropriate sexual behavior in Konik
189 stallions.

Whilst incestuous matings can occur in free-living populations at low rates [18, 23], a period of captivity, specifically where stallions experience HC mating, appears to significantly influence future reproductive behavior. Our results are in line with Duncan et al. [18] who observed incestuous matings in the first generation of feralized domestic horses. Human-controlled matings are frequently used to reduce the probability of injuries to stallions and mares in captive situations and can also be used to orchestrate incestuous matings [31]. It is already known that constrained reproduction in horses could provoke problems with the sexual behavioral repertoire [31, 32]. As stressed by McPhee and Carlsteed [29], maturing in captivity impacts the behavior of an animal since the captive environment includes a unique set of influential factors that do not exist in the wild environment and *vice versa*. Rearing in same-sex, same-age social groups, the physical restraint of both the stallion and mare during mating, or mating with a large number of receptive females in a short time, may all contribute to disturbed development of social and sexual behavior. This could potentially apply to both domestic and reintroduced stallions. Since nowadays, domestic mares can achieve high reproductive rates with assisted reproduction techniques [4], the role of social behavior and the welfare of the stallion are often neglected [10, 33]. Moreover, domestic males do not need to "work" for the possibility of mating and breeding with unfamiliar mares. As in the case of infanticide in equid stallions, which was suggested as being due to human "disturbance" by Feh and Munkhtuya [30], the indirect effects of human intervention earlier in life, particularly during mating, may have gone unnoticed when reintroduced stallions presented otherwise normal social male behaviour (e. g. herding, mate protection, mating).

Across wild living species that avoid mating with relatives here are various potential mechanisms for inbreeding avoidance [34]. Individual recognition, often involving kin, is one mechanism; this is essential for some social species as it underlies the construction of dominance hierarchies and also mate preferences [35]. Although some mechanisms for kin

recognition were proposed as being based on specific olfactory cues [36], kin discrimination is likely to be based on associative learning and familiarity [37]. Wild-born horses have been found to recognize and remember members of their natal group and reject them as future reproductive partners [19]. Thus all foals born in a harem, not only those who are direct kin, are thought to be considered relatives by the stallion. Our results support this cognitive hypothesis since nC stallions expelled all non-related stepdaughters from the natal group, despite a lack of close kinship. We also observed that despite one stallion failing to expel his daughter for five years, no successful mating resulted; similar outcomes were found by Keiper and Houpt [21], who found non-dispersing females showed lower reproductive rates In contrast to feral horses, year-round social recognition and memory are not possible for captive horses.

It should be acknowledged that the mechanism of incest avoidance in horses may be complex and our study focused mainly on one aspect of human-controlled reproduction. The stabled environment and routine differs significantly to the natural one and hence other, unnoticed factors could influence the development of socio-reproductive behavior of captive stallions. In natural conditions stallions are active reproductively for a relatively short period [21]. During social development, males undergo different roles, from a suckling foal, to a dispersed/expelled colt, followed by a period as a bachelor stallion, and, finally, some males become harem stallions [31, 38]. It can be suspected, that specific psychological and physical characteristics of the male contribute to successful harem formation and defense during, but also beyond the reproductive season [38]. Stallions carry out behaviors such as herding and harem protection [31], social interactions with foals [39], and, as observed in our study, offspring expulsion. It is suggested that experience gained in the natal band prior to dispersal is important for reproductive behavioral development in male horses [25, 38]. In domestic breeding, the stallion does not carry out any of these roles. It can be supposed that, for instance, the expulsion of the colt by the sire may be one of reasons for expelling his own daughters in

the future, since this paternal behavior provides a young male with a learning opportunity that could trigger unique neurobiological mechanisms [40, 41]. Being removed from the sanctuary before the onset of sexual maturity, then being reared in an all-male group without direct contact with cycling adult females, can have additional effects on species-specific sexual development in young males [42]. Here we suggest that social learning at the sub-adult stage could be important for later species-specific socio-reproductive behavior of stallions. Therefore, the uninterrupted presence of stallions in their harems and herd member recognition may play important roles in inbreeding avoidance in horses.

Our results have potential relevance for the reproductive management of domestic stallions. As shown by de Oliveira and Aurich [10] the domestic management of young stallions in social isolation may be detrimental. However, this could be mitigated by the company of adults during maturation [43] or by another male when adult [33]. Further research in this area is certainly needed so that the welfare and natural reproductive behavior of stallions can be maintained.

There are certain limitations to the conclusions we can make from our results. Although the number of mares per harem for every observed stallion does not differ from what is expected from available literature [44], we are aware that the annual removal of surplus offspring may be considered as disruption of the structure of the observed population of horses. This could potentially limit the breeding opportunities of harem stallions, the formation of bachelor bands, sneak copulations or affect the voluntary dispersal of fillies. Another limitation is the sample size of horses involved in the study. Nevertheless, to our knowledge, there is currently no domestic horse population which is left completely unmanaged and methods such as area restriction, contraception, or as in the case of our study, removal of some weanlings, are normally employed. Therefore, we provide here evidence available from the breeding records and personal observations for one study population that are likely to apply to other feral or semi-feral populations of horses.

CONCLUSIONS

We show here that experiences gained during the juvenile period in a captive environment, specifically human-controlled reproduction, can significantly impact the socio-reproductive behavior of adult harem stallions, specifically those related to inbreeding avoidance. We suggest that controlled breeding experience, which is practiced in the reproductive management of domestic horses all around the world, was one of the factors contributing to incestuous behavior of our harem stallions. Hence, this brief study may have important implications for equine management and welfare. We recommend further work be conducted in this area, with larger sample sizes, to verify these initial results.

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Conflict of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions

Conceptualization, A.G.-B., J.J., L.L.; resources, Z.J., J.J., M.S., I.W.-P.; writing—original draft preparation, AG-B.; writing—review and editing, A.G.-B., Z.J., J.J., M.S., CS, I.W.-P., L.L.

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Figure's caption

Fig 1. The number and percentage of fillies **A.** expelled from the natal group by C (captive) *versus* nC (non-captive) stallions, **B.** mated successfully by C *versus* nC stallions, **C.** expelled from the natal group by stallions that had experienced Human-Controlled breeding (HC) *versus* those that had not experienced this (nHC) and **D.** mated successfully by HC *versus* nHC stallions.

Table

Table 1. The reproductive history of captive (C) stallions.

Stallion Name	Age at removal from the sanctuary	Age at the reintroduction to the sanctuary	HC breeding activity	Incestuous
Nalewajko	1 year old	2.5 years old	Yes, in-hand breeding	Yes
Trzmiel	2 years old	18 years old	Yes, in-hand breeding	Yes
Tasznik	1 year old	5 years old	Yes, in-hand breeding	Yes

	Mor	2 years old	4 years old	Yes, teasing	Yes
	Nagaj	1 year old	2.5 years old	No	No

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