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JZAR Research article

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Asymmetric reproductive aging in cheetah (*Acinonyx jubatus*) females in European zoos

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Keywords: cheetah, captivity, reproduction, stress, suppression

Article history:
Received: 05 May 2018
Accepted: 19 Mar 2019

Abstract

For conservation breeding and management, such as re-introduction programmes, reproductively healthy animals are essential. Low reproductive performance in captive animals is therefore of great concern in species which are judged to be vulnerable or endangered in the wild. Hence, it is important to understand the mechanisms that lead to low reproductive performance in captivity. Here, a long-term study on 12 captive cheetah females is presented as an example to test predictions derived from three hypotheses, the captive 'stress', captive reproductive suppression and asymmetric reproductive aging hypotheses. Parous and nulliparous cheetah females did not differ in their faecal glucocorticoid concentrations, suggesting that allostatic load ('stress') might not affect reproductive performance. Consistent with the captive reproduction suppression hypothesis, females permanently kept together with other adult females (or males) did not reproduce until they were individually introduced to a single male when in oestrus. In addition, reproductive performance of these females was determined by their age and reproductive history, as predicted by the asymmetric reproductive aging hypothesis. Asymmetric reproductive aging arises when first breeding attempts and first pregnancies of females are substantially delayed, thereby ensuring frequent cycle fluctuations of oestrogen concentrations which subsequently result in reproductive tract pathologies. Our results suggest that conservation breeding facilities should breed cheetah females as early as possible, keep them separate from adult males or other females and introduce them to single males for breeding purposes when in oestrus only.

Introduction

Several mammalian species exhibit low reproductive performance in captivity (Carlstead 1996; Hermes et al. 2004, 2006; Hildebrandt et al. 2000; Steiner et al. 2015; Wielebnowski 1998). Successful reproduction in captivity is desirable because it can improve general and reproductive health and supplies the individuals required for conservation management activities, such as re-introduction programmes (Carlstead 1996; Ebenhard 1995; Hermes et al. 2004; Wielebnowski 1998). The cheetah (*Acinonyx jubatus*) is known to be difficult to breed in captivity (Lindburg et al. 1993; Marker and O'Brien 1989; Marker-Kraus and Grisham 1993; Versteege 2013; Wildt et al. 1993), as few breeding centres are consistently successful in their breeding attempts over longer periods of time (Bertschinger et al. 2008; Versteege 2013). Breeding in captivity will become more important in future as the global population of cheetah declines progressively (Durant et al. 2017; Weise et al. 2017). Cheetah females kept in zoological gardens have been observed to exhibit irregular cycling, anoestrous periods, reproductive suppression or lack of sexual arousal (Brown et al. 1996; Marker-Kraus and Grisham 1993; Wielebnowksi et al. 2002a). In contrast, free-ranging cheetah females have a higher reproductive performance in terms of conception rate than cheetahs in zoological gardens, resuming cycling shortly after the loss of a litter and readily becoming pregnant again (Caro 1994; Laurenson et al. 1992; Marker-Kraus et al. 1996; Wachter et al. 2011).

The possible reasons for this difference in performance between captive and free-ranging cheetahs have been previously discussed in the context of five hypotheses: (1) the "genetic monomorphism hypothesis", which suggests that the low genetic variability of cheetahs (Drake et al. 2004; Freeman et al. 2002; Menotti-Raymond and O'Brien 1993; O'Brien et al. 1983, 1985) is linked to low fertility and high cub mortality (Brown et al. 1996; Wildt et al. 1993); (2) the "captive 'stress' hypothesis", which suggests that unfavorable husbandry conditions increase allostatic load, for example, from crowded social living conditions (McEwen and Winfield 2010; Romero et al. 2009), which in turn suppresses ovarian activity (Jurke et al. 1997); (3) the "captive reproductive suppression hypothesis", which suggests that pheromones of jointly-housed females directly suppress ovarian activity of females living within the same enclosure (Kinoshita et al. 2011; Wielebnowski et al. 2002a); (4) the "innate rhythm hypothesis", which suggests that reproductive cycling is triggered by an endogenous circannual rhythm (Terio et al. 2003); and (5) the "asymmetric reproductive aging (ARA) hypothesis", which suggests that frequent fluctuations of oestrogen concentrations cause faster aging of and diminished functionality of reproductive organs and pathological lesions of the reproductive tract (Hermes et al. 2004; Wachter et al. 2011). This phenomenon has been described in African and Asian elephants (*Loxodonta africana* and *Elephans maximus*) and white rhinoceros (*Ceratotherium simum*) kept in zoological gardens (Hermes et al. 2004, 2006; Hildebrandt et al. 2000). It is observed in nulliparous females, particularly in older ones, because these individuals are exposed for the longest period to regular and frequent fluctuations of oestrogen concentrations. Such females enter into a non-reversible early reproductive quiescence in terms of hampered conception and lose a substantial part of their reproductive life compared to females which have successfully reproduced, are reproductively healthy and enter a natural senescence process (Hermes et al. 2004, 2006; Hildebrandt et al. 2000).

A link between nulliparity and endometrial hyperplasia, which can lead to reduced fertility, as well as between reproductive history and likelihood of future reproduction has also been described for several captive canine and feline species, including cheetahs (Asa et al. 2014; Crosier et al. 2011; Munson et al. 2002; Penfold et al. 2014; Saunders et al. 2014). These functional relationships are also encompassed by the ARA hypothesis, although the studies did not label them as such. Penfold et al. (2014) suggested for such relationships the term "use-it-or-lose-it hypothesis" as a more apt description. Contrary to the mechanism suggested by the ARA hypothesis, the captive reproductive suppression hypothesis assumes a mechanism that is a reversible process, because apparently reproductively suppressed captive cheetah females resumed cyclicity when they were separated from conspecifics (Wielebnowski et al. 2002a).

A study on free-ranging and captive cheetahs in Namibia, which simultaneously investigated all hypotheses except the captive reproductive suppression hypothesis found that the data were only consistent with the ARA hypothesis (Wachter et al. 2011) and rejected predictions from the genetic monomorphism, captive 'stress' and innate rhythm hypotheses. This study compared cheetahs held locally in captivity in very large enclosures of at least 10,000 m^2 per animal in natural habitat with free-ranging cheetahs in Namibia. Cheetahs living in such conditions of captivity therefore did not necessarily experience the same conditions as captive cheetahs kept in zoological gardens elsewhere. In particular, these conditions were unsuitable to test the captive reproductive suppression hypothesis which requires females to be jointly housed and in close proximity of each other. In the Namibian study, captive and free-ranging cheetahs did not differ in their size of adrenal glands, a proxy for chronic elevation of allostatic load ('stress') as measured by ultrasonography during immobilisation (Wachter et al. 2011). Cheetahs kept in zoological gardens, however, have larger adrenal glands and higher concentrations of faecal glucocorticoid metabolites (fGCM), the latter being a non-invasive hormonal measure and proxy for short-term increases in allostatic load, than free-ranging Namibian cheetahs (Terio et al. 2004). Increased allostatic load can suppress ovarian activity in many mammals (Breen et al. 2005; Dobson and Smith 1995; Ferris and McCue 2010; Rivier and Rivest 1991), although not necessarily in all of them (Hofer and East 1998). Thus, it is possible that in zoological gardens ARA and the effect of increased allostatic load both negatively affect reproductive health in female cheetahs.

In this study, parous and initially nulliparous female cheetahs kept in zoological gardens were investigated, with the latter being females that were brought into breeding situation but failed to breed. Predictions on reproductive age, reproductive history and allostatic load derived from the captive 'stress', captive reproductive suppression and ARA hypotheses were simultaneously tested. Here, the "genetic monomorphism hypothesis" and the "innate rhythm hypothesis" were not investigated because it was already demonstrated that the reproductive performance of cheetahs is not linked to their genetic makeup and that there is no endogenous circannual rhythm triggering reproductive activity (Wachter et al. 2011). The captive 'stress' hypothesis predicts that nulliparous females should show higher fGCM concentrations than parous ones. The captive reproductive suppression hypothesis predicts that cheetah females kept together with other females are unlikely to breed. Nulliparous captive Namibian cheetah females developed pathologies on their reproductive tract, such as hydrosalpinx, hydrometra and connective tissues on ovaries, all known to potentially hamper reproduction (Munson 1993; Munson et al. 2002; Wachter et al. 2011), at a mean age of 5.6±1.2 years. The ARA hypothesis therefore predicts that females kept in zoological gardens conceive their first litter when they are younger than 5.6 years of age. Females older than that and brought into a breeding situation for the first time would be likely to remain nulliparous. Pathologies on reproductive tracts may also occur in canine and feline females treated with contraceptive products (Asa et al. 2014; Munson et al. 2002; Penfold et al. 2014). It was therefore verified that none of the cheetah females in this study was ever treated with a contraceptive.

Methods

Study animals and facilities

In this long-term study, the reproductive history of eight adult cheetah females was monitored throughout their lifetimes (mean age at death: 9.9±2.0 years, range: 7.1–12.9), while four females were monitored most of their lifetime (their first 14.6±1.5 years of life) until the 31st of December 2018. According to the studbook (Versteege 2013), six females were nulliparous, five females had given birth previously to one (n=4) or two (n=1) litters and one female was pregnant (X012) during the period when faecal samples were collected for this study (see below). Thus, six females were classified as nulliparous and six females as parous and this data set was used to test the prediction derived from the captive 'stress' hypothesis (Table 1).

All animals were kept and monitored throughout their lives, in European zoological gardens. During the period of faecal sample collection, one animal was kept in Austria, three in Denmark, six in Germany, one in Portugal and one in Switzerland. Five females were kept alone in their enclosure, the others were kept together with another female (X011), a male (X008 and X013) or their offspring (n=4) aged 2.9, 3.4, 8.5 and 16.1 months at the start of faecal sample collection (Table 2). Prior to faecal sample collection, one animal was kept in Austria, two in the Czech Republic, one

Table 1. Identity of cheetah females (♀), social group in the enclosure before first potential sexual encounter (between a single female and a single male without other cheetahs in the enclosure), age of first potential sexual encounter, age at first conception, pathologies in reproductive tract detected by necropsy, and number of litters produced. Never refers to the date of death (X008: 10.2 years, X009: 12.9 years and X011: 12.2 years) or the end of the monitoring period on the 31st of December 2018 (X013: 15.7 years).

♀ ID	Conspecifics in same enclosure prior to first potential sexual encounter		Age at first potential sexual encounter (years)	Age when first litter was conceived (years)	Pathologies in reproductive tract	Reproduced	Number of litters
	♀	Male					
X002	-	-	2.9	2.9	Not investigated	Yes	1
X004	-	-	2.8	3.5	None found[a]	Yes	1
X005	-	-	1.9	4.4	Not investigated	Yes	2
X006	-	-	3.9	4.3	Not investigated	Yes	2
X007	-	-	3.8	5.0	Not investigated	Yes[b]	1
X008	1	-	8.9	Never	None found[a]	No	0
X009	Various	Various	8.6	Never	Not investigated	No	0
X010	Various	-	5.0	5.9	Paraovarian cysts	Yes[c]	1
X011	1	-	Unknown, most likely never	Never	Paraovarian and uterine cysts	No	0
X012	-	-	5.2	5.4	Not investigated	Yes[d]	1
X013	-	-	Never	Never	Not investigated	No	0
X014	-	-	4.1	4.3	Not investigated	Yes	1

a: X004 and X008 were diagnosed with gastritis; b: X007 was nulliparous during the faecal sampling period of this study, but gave birth 5.7 months after the sampling period terminated; c: X010 was nulliparous during the faecal sampling period of this study, but gave birth 7.3 months after the sampling period terminated; d: X012 was pregnant during the faecal sampling period of this study.

in Denmark, two in Germany, two in Ireland and four in the Netherlands. Back then, seven females were kept alone in their enclosure, X008 was kept together with her mother, X009 with other females and males, X010 with a variable number of other females, X011 with one other female and X013 with a male (Table 1). It is unknown whether—although it is suspected to be unlikely that—X011 was brought together with a male. Thus, to test the predictions derived from the captive reproductive suppression hypothesis, seven females were classified as being kept alone and three females (X008, X009, X010) as being kept with other females. X011 and X013 were excluded from this analysis.

After the collection of faecal samples from these females was completed, two of the nulliparous females (X010 and X007) and one female that had previously given birth to one litter (X006) gave birth to a litter 5.7, 7.3 and 20.3 months later, respectively (Table 1). Thus, to test the predictions derived from the ARA hypothesis, four females were classified as nulliparous and eight females as parous.

All cheetahs were zoo-born animals, fed with whole prey or beef meat and had water available ad libitum. Enclosure sizes and substrate in the facilities differed, but all animals had outdoor exhibits with natural fluctuations of daylight and huts or smaller facilities as indoor enclosures.

All procedures undertaken for this study were approved by the Ethics Committee on Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research (IZW).

Collection of faecal samples

Faecal samples were collected between February 2010 and May 2011 with sample periods ranging from 11 days to 23 days for each female. This sampling period is longer than commonly used

Table 2. Identity and origin of cheetah females (♀), their age, social group composition in the enclosure, including number and age of offspring, at the time when the collection of faecal samples commenced (between February 2010 and May 2011, depending on the zoo [zoological garden]).

♀ ID	Studbook number	Zoo	Age (years)	Conspecifics in same enclosure at start of faecal sample collection			
				♀	♂	Offspring	
						n	Age (months)
X002	2147	A	3.9	-	-	4	8.5
X004	2144	B	4.0	-	-	4	3.4
X005	1853	C	7.9	-	-	2	16.1
X006	2088	C	4.8	-	-	4	2.9
X007	2083	C	4.8	-	-	-	
X008	1769	D	9.4	-	1	-	
X009	1881	E	7.8	-	-	-	
X010	2060	F	5.5	-	-	-	
X011	1636	G	11.9	1	-	-	
X012	2137	H	5.6	-	-	-	
X013	1883	I	8.0	-	1	-	
X014	1979	F	5.8	-	-	-	

to determine levels of fGCM concentrations in feline species (Terio et al. 1999, 2004; Wells et al. 2004; Wielebnowski et al. 2002b). Faecal sampling was conducted at a random time period in relation to the age of the females and their potential breeding activities. The collection of faecal samples commenced when these females were between 3.9 and 11.9 years of age, with a mean of 6.6±2.4 years (Table 2).

Enclosures were checked for fresh faeces daily in the morning or in the morning and afternoon during husbandry routines. Eight to 24 faecal samples were collected per female for the measurement of fGCM concentrations. When more than one animal was kept in an enclosure (Table 2), food for the female in focus was prepared with blue food colorant (brilliant blue, FCF, Sensient Food Colors Europe, Geesthacht, Germany) to permit the allocation of faeces to the correct individual. All faecal samples were homogenised and frozen at -20 °C directly after collection and stored at -80 °C at the IZW until analyses were conducted.

Extraction of faeces and enzyme immunoassay (EIA)

First, 0.5 g of wet faeces were dried for 22 h in a freeze drier (EPSILON1-4, LSC plus, Martin Christ GmbH, Germany). After powdering the dried faeces, 0.1 g of well-mixed powder were extracted with 0.9 ml of 90% methanol with gentle shaking on a horizontal shaker for 30 min. After centrifugation at 3000 rpm for 15 min, the supernatant was transferred into a new tube and diluted 1:1 with water, and aliquots of 20 µl were subjected to the EIA to measure concentrations of fGCM.

The methanol extracts were analysed with a corticosterone-3-CMO immunoassay that was proven highly sensitive for measuring fGCM in cheetahs (Ludwig et al. 2013). The antibody of the EIA was polyclonal and raised in rabbit against corticosterone-3-CMO-steroid coupled with bovine serum albumin (BSA). The corresponding 3-CMO-peroxidase was used as label for the EIA (Ludwig et al. 2013).

Data analysis

Due to the small sample sizes non-parametric tests were conducted, that is, Mann-Whitney U and Fisher exact tests. Power analyses were performed for some of the non-significant results to ask what statistical power there was to discover a difference if there really was one. The calculations of the statistical power took into account how large the observed effect sizes were and considered the sample size or the degrees of freedom for each statistical test. For this purpose a general model was applied for power analysis developed and presented by Murphy et al. (2014), using their One-Stop-Calculator (Murphy et al. 2014, tab 'eResources' at the book's website, https://www.routledge.com/products/9781848725881). All other statistical analyses were performed with Systat 13 (Systat Software, Inc., San Jose, California, USA). The level of significance was set at 5% and all tests were two-tailed. All mean values are given with standard deviations (SD).

Results

Captive 'stress' hypothesis

The mean fGCM concentration of all 12 females was 1049.3±575.4 ng/g. There was no difference of fGCM concentrations between parous and nulliparous females (Mann-Whitney U test, n_{parous} 6, $n_{nulliparous}$=6, U=14, P=0.52, Table 1, Table 3). The result did not change when the two females that gave birth for the first time 5.6 and 7.3 months after collection of faecal samples were categorised as parous (n_{parous}=8, $n_{nulliparous}$=4, U=11, P=0.40). The power of finding a difference if there really was one was modest to moderate (0.121 for the equivalent two-sample t-test for the first comparison, 0.197 for the second), which was not surprising given the modest differences (effect size) observed between the two groups.

The pregnant female X012 had a mean fGCM concentration that was 4.0 times higher than the mean fGCM concentrations of the other 11 females (mean±SD: 840.3±435.0 ng/g). Exclusion of X012 from the analyses did not change the result (n_{parous}=5, $n_{nulliparous}$=6, U=14, P=0.86; n_{parous}=7, $n_{nulliparous}$=4, U=11, P=0.57). The power of finding a difference if there really was one was again modest to moderate (0.051 for the equivalent two-sample t-test for the first comparison, 0.103 for the second), which again was not surprising given the modest differences (effect size) observed between the two groups.

Captive reproductive suppression hypothesis

Four females were housed together with one or more other female(s) before collection of faecal samples commenced (X008, X009, X010, X011, Table 1). The managers of the facilities tried to breed X008, X009 and X010 with males when the females were thought to be in estrus, based on behavioral signs such as rolling, in these housing conditions. X011 is unlikely to have been brought together with a male. None of them produced litters while being housed together with other females. X008 was kept together with her mother until the latter died and from then on, at the age of 8.9 years, was permanently kept with a male. She never reproduced until she died at the age of 10.2 years. Female X009 was kept together with various numbers of females and males and was introduced alone to a single male for the first time at the age of 8.6 years, but did not reproduce until she died at the age of 12.9 years. Female X010 was kept together with several different females between her age of 2.6 years and 5.0 years, with several breeding attempts starting at the age of 2.6

Table 3. Number of faecal samples collected per cheetah female (♀), mean, SD and peak of faecal glucocorticoid metabolite (fGCM) concentrations in dry matter.

♀ ID	n	FGCM (ng/g)		
		Mean	SD	Peak
X002	8	924.3	297.0	1235.9
X004	11	1068.2	489.5	2108.2
X005	12	1034.5	406.9	1972.3
X006	18	711.2	330.5	1167.3
X007	16	1414.7	698.5	2813.0
X008	11	744.6	637.9	1971.5
X009	18	937.9	816.1	2777.1
X010	24	623.3	393.7	1661.7
X011	11	801.7	364.0	1289.9
X012	14	3348.3	2120.4	6491.7
X013	13	590.8	148.6	846.0
X014	20	392.5	201.9	778.1

years, but never reproduced. From the age of 5.0 years onwards, X010 was kept alone and at the age of 5.2 years was introduced alone to a single male for the first time, which was three months before the period of faecal sampling began. She was regularly introduced alone to the male after he advertised by vocalisations that she was in oestrus and she showed normal signs of oestrus in this constellation. She conceived her first litter at the age of 5.9 years. Another female (X013) was permanently kept together with a male between her own ages of 3.7 and 15.7 years (when the monitoring period ended) and never reproduced. Females housed on their own with no other adults present (n=7) had a much, and significantly, higher chance of successful reproduction (100%) than females jointly housed together with other females (n=3, 0%; Fisher exact test, P=0.0083).

Asymmetric reproductive aging hypothesis

Seven of eight parous females (87.5%) conceived their first litter when they were younger than 5.6 years of age, that is, the mean age when captive females in Namibia developed pathologies on their reproductive tracts (Wachter et al. 2011). The eighth female (individual X010) conceived her first litter at the age of 5.9 years (Table 1). Two females had also conceived at the age of 6.3 years (individuals X005 and X006) and were both, as predicted, pluriparous.

Two of the four nulliparous females were brought into a breeding situation, that is, an encounter between a single female and a single male without other cheetahs in the enclosure, for the first time at the age of 8.9 years (individual X008) and 8.6 years (individual X009), respectively (Table 1). Both females did not conceive until they died at 10.2 years and 12.9 years, respectively. Female X013 was 8.0 years old when faecal samples were collected for this study. She had been permanently kept together with a male since she was 3.7 years old and had never reproduced until the end of the monitoring period when she was 15.7 years old. From the studbook, it is not known whether female X011 was ever brought into a proper breeding situation, but she was 11.9 years old when faecal samples were collected for this study and had never reproduced until she died at the age of 12.2 years (Table 1). The association between presence (or absence) of successful reproductive activity if the first potential sexual encounter was initiated before (or after) the age of 5.6 years was highly unlikely to result from chance alone (Fisher exact test, P=0.01).

Necropsy reports were available from four females that died (X004, X008, X010, X011, Table 1), with information on pathologies of reproductive organs from three females (X008, X010, X011). The two nulliparous females X008 and X011 died at the age of 10.2 years and 12.2 years. X008 showed no pathologies on the reproductive tract, whereas X011 showed paraovarian and uterine cysts. The parous female X010 died at the age of 8.2 years and had paraovarian cysts. The parous female X004 died at the age of 7.1 years and was diagnosed with chronic gastritis and enteritis, whereas the nulliparous X008 was diagnosed with gastritis and nephritis.

Discussion

Low reproductive performance in zoological gardens can be problematic in species that are vulnerable or endangered in the wild (Arnold 1995; Brown et al. 2004; Marker and O'Brien, 1989; Marker-Kraus and Grisham 1993). Breeding and re-introduction programmes rely on reproductively healthy females (and males). It is therefore crucial to understand the mechanisms of low reproductive performance in captivity (Brown et al. 2004, Crosier et al. 2011; Saunders et al. 2014; Walker et al. 2004). Asymmetric reproductive aging (ARA) has been previously described in females of African and Asian elephants, as well as for white rhinoceros kept in zoological gardens (Hermes et al. 2004, 2006; Hildebrandt et al. 2000), in female domestic horses (*Equus caballus*) (Hinrichs 1997), female laboratory rats (*Rattus norvegicus*) (Sopelak and Butcher 1982), captive cheetah females in Namibia (Wachter et al. 2011) and free-ranging Sumatran rhinos (*Dicerorhinus sumatrensis harrissoni)* (Kretzschmar et al. 2016).

Our findings are consistent with the ARA hypothesis and our previous study in that the main factors for reproductive health in cheetah females are age and reproductive history. In one of the parous females, paraovarian cysts were detected during necropsy. Such cysts have been demonstrated not to hamper reproduction (Munson 1993; Wachter et al. 2011). In the nulliparous female, paraovarian and uterine cysts were found during necropsy, with the latter known to hinder reproduction in severe cases (Munson 1993), but this case was a mild one. This female was 12.2 years old when she died and it was not known whether she was ever put into a breeding encounter as a single female with a single male.

Most captive cheetahs in zoological gardens are managed by a breeding programme (Versteege 2013) providing recommendations to exchange animals between different institutions for breeding and conservation purposes to preserve genetic variability and prevent inbreeding. Due to logistical reasons, including official regulations on epizootic diseases, the transport of an animal from one facility to another can sometimes be delayed for months or even years. As a result, captive cheetah females often do not breed early in their life. This is unfortunate since early pregnancy and lactation are protective mechanisms against ARA, as demonstrated in several species such as African and Asian elephants, white rhinoceroses, cheetahs and tigers (*Panthera tigris*) (Hermes et al. 2004, 2006; Hildebrandt et al. 2000; Penfold et al. 2014; Sounders et al. 2014; Wachter et al. 2011).

The present findings are also consistent with the captive reproductive suppression hypothesis. Similar to other studies, females kept together with one or more other females did not reproduce (Kinoshita et al. 2011; Wielebnowski et al. 2002a). One of these females (X010) after being kept together with several other females until the age of 5.0 years, was introduced for the first time by herself to a single male during estrus and then produced a litter, demonstrating that the non-reproducing state can be reversible (Wielebnowski et al. 2002a).

The mechanism of reproductive suppression remains unclear. In mammalian species for which reproductive suppression is not a standard life history stage (Hofer and East 1998), suppression might be linked to agonistic behavior of behaviorally incompatible females (Wielebnowski et al. 2002a). In the wild, female cheetahs are solitary and only accompanied by their dependent offspring (Caro 1994; Marker 2002), thus they might not cope well when permanently housed with other females. Although two or more cheetah females kept in one enclosure do not show open aggression towards each other, such unnatural social grouping can sometimes result in agonistic interactions (Wielebnowski et al. 2002a).

One female (X013) was permanently kept together with a male and never produced a litter. With free-ranging cheetah males and females living separately except when mating (Caro 1994; Marker 2002), it might be likely that not enough sexual interest and arousal can be generated when both sexes are permanently kept together. The complex neuro-chemical system that controls the mechanisms necessary for mating behavior (Holstegge and Huynh 2011) is likely to be adversely affected by such housing situations.

Allostatic load in captivity may have many sources, although zoological gardens attempt to imitate the natural environment and many improvements in husbandry were implemented during the last decades (Hoage and Deiss 1996; Kuehn 2002; Morgan and Tromborg 2007; Shepherdson and Mellen 1998). Possible stressors might be nearby enclosures with natural predator species (Mellen 1991; Rawlins 1972; Wielebnowski 1998), artificial light, exposure to unnatural sounds or odors, uncomfortable temperatures or disturbance by visitors (Morgan and Tromborg 2007; Wielebnowski et al. 2002b). Yet the observed differences of dry fGCM concentrations between parous and nulliparous females in our population were not significant, although the power of finding a difference if there really was one was modest. One female had very high fGCM concentrations. Her pregnancy might have been responsible for inducing a high glucocorticoid level, as is well known for other species (Doerr et al. 1989; Raeside and Ronald 1981). A longer time period of faecal sample collection might have revealed a clearer result, and also additional measures to quantify allostatic load (Edes et al. 2018). Gastritis, a disease that develops in cheetahs under stressful conditions (Munson et al. 2005), was also found in parous and nulliparous females, suggesting no negative effect on reproduction, but also here, additional data would be valuable to further investigate this connection. Thus, with a caution note, the captive 'stress' hypothesis is unsuitable to explain the lack of reproduction in these cheetah females.

Conclusions

1. The present study suggests that females should be bred by the age of 5.6 years to maintain fertility for many years and to avoid the onset of asymmetric reproductive aging.
2. The study also suggests that females are more likely to come into oestrus when kept alone and only then should they be introduced to single males for breeding purposes.
3. This approach is likely to increase the reproductive performance and health of cheetah females in captivity and therefore increases the chances of success for breeding and re-introduction programmes.

Acknowledgements

We thank the participating zoos in Vienna (Austria), Ebeltoft (Denmark), Krefeld, Münster, Nuremberg, Stuttgart, Wuppertal (Germany), Lisbon (Portugal) and Basel (Switzerland) for their permission and vital support to conduct the study and Torsten Bohm and Stephanie Wandelmaier for their help in collecting the faecal samples. We also thank Marlies Rohleder and Katrin Paschmionka for their excellent technical assistance and Lars Versteege, the keeper of the European Endangered Species Programmes (EEP) studbook, for his continuous support. We thank the Messerli Foundation in Switzerland, the Westfälischer Zoologischer Garten in Münster, Germany, and the Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin, Germany for financial support of the study.

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