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Abstract: Re-introduction programs rely on reproductively sound individuals and high offspring survival once captive bred species are released into their natural habitat. Some species involved in captive breeding programs reproduce poorly: one prominent example is the cheetah (*Acinonyx jubatus*). Here we use the cheetah as a case study to test predictions from all current hypotheses explaining this phenomenon by monitoring postemergence cub survival and assessing the reproductive status of free-ranging and captive female cheetahs in Namibia with ultrasonography. We show that reproductive activity and health of cheetah females is determined by reproductive history and age rather than innate rhythms, captive stress, or lack of genetic diversity, and that postemergence cub survival under natural conditions in a predator-free habitat is high. Our results suggest that management practices of captive breeding and re-introduction programs should encourage early reproduction in females to induce long-lasting and healthy reproductive performance. With this practice, re-introduction projects might increase their chances of success.

# Reproductive history and absence of predators are important determinants of reproductive fitness: the cheetah controversy revisited

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## Keywords

Captivity; cheetah; conservation; free-ranging; re-introduction program; reproductive performance; survival.

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## Abstract

Re-introduction programs rely on reproductively sound individuals and high offspring survival once captively bred species are released into their natural habitat. Some species involved in captive breeding programs reproduce poorly: one prominent example is the cheetah (*Acinonyx jubatus*). Here we use the cheetah as a case study to test predictions from all current hypotheses explaining this phenomenon by monitoring postemergence cub survival and assessing the reproductive status of free-ranging and captive female cheetahs in Namibia with ultrasonography. We show that reproductive activity and health of cheetah females is determined by reproductive history and age rather than innate rhythms, captive stress, or lack of genetic diversity, and that post-emergence cub survival under natural conditions in a predator-free habitat is high. Our results suggest that management practices of captive breeding and re-introduction programs should encourage early reproduction in females to induce long-lasting and healthy reproductive performance. With this practice, re-introduction projects might increase their chances of success.

## Introduction

Captive breeding and re-introduction programs are an important aspect of the conservation of some threatened species (Ebenhard 1995; Seddon *et al.* 2005). With 172 re-introduction projects, mammals are a favorite taxon for these efforts (Seddon *et al.* 2005), and sustainable and healthy captive breeding is considered a prerequisite for re-introduction success (Ebenhard 1995; Williams & Hoffman 2009). Some captive populations, however, have worldwide poor reproductive performance—low conception rate, low fertility (offspring produced per female), and low juvenile survival. Prominent examples are cheetah (*Acinonyx jubatus*), white rhinoceros (*Ceratotherium simum*), African and Asian elephants (*Loxodonta africana* and *Elephas maximus*) and giant panda (*Ailuropoda melanoleuca*) (Wildt *et al.* 1993; Hildebrandt *et al.* 2000a;

Lu *et al.* 2000; Hermes *et al.* 2004). The impact of poor reproductive performance on captive populations is substantial as the projected decline of captive population size demonstrates (Olson & Wiese 2000). In lifetime models for African elephants, female populations were projected to decrease by 94% within 50 years under current management procedures, yet become nearly self-sustaining in a scenario with optimistic but feasible increases in reproductive success (Olson & Wiese 2000). Improving reproductive performance by obtaining a detailed understanding of factors promoting or reducing it, is therefore important to secure sustainable captive populations and thus increase the success of re-introduction programs (Caughley 1994; Lu *et al.* 2000; Williams & Hoffman 2009).

Here we use the cheetah as a case study to test four hypotheses explaining poor reproductive performance: the

(1) genetic monomorphism, (2) captive “stress,” (3) innate rhythm, and (4) asymmetric reproductive aging hypotheses. Although they are here formulated in terms of female cheetah biology, the arguments involved have been used to explain poor reproductive performance in other species. It is therefore timely to conduct a comprehensive assessment of all aspects of reproduction, since the cheetah has long been considered a challenge for conservation.

### Four hypotheses to explain poor reproductive performance in threatened mammals

Previous studies suggested that in captivity, irregular estrous cycles and anestrus periods of females (Wildt *et al.* 1993; Brown *et al.* 1996) and low cub survival (Marker-Kraus & Grisham 1993) were a consequence of a lack of genetic diversity in cheetahs (the genetic monomorphism hypothesis, O'Brien *et al.* 1983, 1985). A study on free-ranging cheetahs in the Serengeti, Tanzania, also documented low postemergence cub survival but concluded that this was mainly due to predation by lions (*Panthera leo*) and spotted hyenas (*Crocuta crocuta*) (Laurenson 1994). This still leaves the issue open to what extent genetic monomorphism contributes to cub mortality, since predation could have either pre-empted or worked in conjunction with its potential detrimental effects (O'Brien 1994; Laurenson *et al.* 1995). Other studies suggested that the low conception rate was a consequence of unfavorable or unnatural husbandry conditions, either alternatively or additionally to the lack of genetic diversity (Wildt *et al.* 1993; Caro & Laurenson 1994; Caughley 1994; Merola 1994; Brown *et al.* 1996; Wielebnowski 1996; Terio *et al.* 2003) but the mechanism remained elusive. Perhaps some husbandry conditions are a source of “stress,” leading to the suppression of ovarian activity in susceptible individuals (the captive “stress” hypothesis, Brown *et al.* 1996; Jurke *et al.* 1997). Captive female cheetahs held in a natural environment on Namibian farmland also showed anestrus periods (Terio *et al.* 2003) but only preceding a season of low rainfall, not a season of high rainfall. Thus, reproductive cycling might be triggered by an endogenous circannual rhythm not apparent in the conditions of zoos abroad, and anestrus periods preceding low rainfalls might prevent free-ranging females from raising offspring during periods with low prey availability (the innate rhythm hypothesis, Terio *et al.* 2003). Nulliparous females experience extensive periods of continuous estrous cycles. Older nulliparous females have irregular estrous cycles and eventually stop

cycling, as shown in captive female white rhinoceroses and elephants (Hildebrandt *et al.* 2000a; Hermes *et al.* 2004). The frequent fluctuation of estrogen concentrations in such females is likely to cause faster aging of reproductive organs than in breeding females and a premature postreproductive status (the asymmetric reproductive aging hypothesis, Hermes *et al.* 2004). Frequent maturation of follicles also leads to the development of hormone-dependent and age-related pathologic lesions of the reproductive tract, more frequent in nulliparous than in pluriparous females (Hermes *et al.* 2006).

No previous study has tested predictions from all four hypotheses and thus the mechanism(s) that cause anestrus periods and irregular estrous cycles of captive females and low cub survival in cheetahs or any other mammalian species are still not identified. We examined, in detail, the reproductive organs of free-ranging and captive female cheetahs on Namibian farmland and compared their chance of being in a reproductively active state and develop pathologies of reproductive organs. We also monitored postemergence cub survival in the same free-ranging population.

If reproductive inactivity or anestrus periods in Namibian captive female cheetahs are associated with genetic monomorphism, then the reproductive organs of captive and free-ranging cheetahs should not differ in their reproductive states, because both populations originate from the same genetic source. On Namibian farmland, lions and hyenas were driven to extinction in the last century, providing a “natural experiment” to measure postemergence cub survival in the absence of key predators. If postemergence cub survival is affected by genetic monomorphism, then cub survival should be low even in this environment. If reproductive inactivity or anestrus periods in Namibian captive female cheetahs are associated with stressful conditions, then captive females should exhibit signs of higher “stress” than free-ranging ones. If reproductive inactivity in cheetah females is triggered by environmental cues, inactive states of reproductive organs in captive females should be absent during months preceding high rainfall, and at any time of year in years with high rainfall. If asymmetric reproductive aging operates in cheetahs, reproductive organs of free-ranging females should be more likely to be in a reproductively active state than reproductive organs of captive females because most captive females were nulliparous (Table 1). Furthermore, captive females with inactive reproductive organs should be older than captive females with active reproductive organs and pathologies of reproductive organs should mainly occur in females with inactive reproductive organs and nulliparous females.

**Table 1** Reproductive state, reproductive history, and pathologies of reproductive organs of captive female cheetahs at the time of ultrasonographical examination.

Female	Age (years)	Examination date	Reproductive state	Reproductive history	Pathology of reproductive organs
A	2.5	April 2006	Juvenile	Nulliparous	None
B	2.0	June 2002	Active	Nulliparous	None
C	2.5	June 2002	Active	Nulliparous	None
D	3.5	April 2006	Active	Nulliparous	None
E	4.0	April 2006	Active	Nulliparous	None
F	4.0	June 2002	Active	Nulliparous	Hydrosalpinx
G	5.5	April 2006	Active	Nulliparous	None
H	4.5	June 2002	Inactive	Nulliparous	Hydrosalpinx
I	5.0	June 2002	Inactive	Unknown	Connective tissue on ovaries
J (1)*	5.0	June 2002	Inactive	Nulliparous	None
J (2)*	7.0	October 2004	Inactive	Nulliparous	Hydrometra
K (1)*	5.0	June 2002	Inactive	Nulliparous	None
K (2)*	7.0	October 2004	Inactive	Nulliparous	Connective tissue on ovaries
L (1)*	5.0	June 2002	Inactive	Nulliparous	Hydrosalpinx
L (2)*	6.0	October 2003	Inactive	Nulliparous	None
L (3)*	7.0	October 2004	Inactive	Nulliparous	None
M	6.5	June 2002	Inactive	One litter	Hydrosalpinx
N	6.5	April 2006	Inactive	Nulliparous	None
O	11.0	April 2006	Inactive	Nulliparous	None

\*Number in brackets represents the first (1), second (2), or third (3) examination of the female.

## Methods

### Study animals

Thirteen free-ranging adult and five juvenile females were captured and immobilized between June 2002 and December 2006 on farmland in central Namibia. Seventeen cubs were caught with the adults. Fourteen adults and one juvenile captive female, housed on seven farms and lodges in central, southern, and northern Namibia, were immobilized. All individuals were allocated to age classes as described by Caro (1994). Adult females were classified as "young" when they were full size, still had a circular face and retained some of their juvenile mane ( $\geq 2.0$  to  $< 3.5$  years, Caro 1994), and as "old" when they had no mane and calloused elbows with fur worn away ( $\geq 3.5$  years, Caro 1994). Captive females had been wild-caught as cubs, juveniles, or young adults and housed in social groups of different compositions in enclosures of various sizes and contact to people. Their age was either reported by the owners or estimated ( $n = 1$ ) by one author (ST).

### Immobilization

Most free-ranging (16/18) and captive (9/15) cheetahs were immobilized with Hellabrunn mixture (100 mg/ml ketamine, Kyron Laboratories, Benrose, RSA, and 125

mg/ml xylazine, Bayer, Isando, RSA) with a dosage of 0.04 ml/kg. For other free-ranging cheetahs, a mixture of ketamine (4.5 mg/kg) and medetomidine (0.08 mg/kg; Novartis, Spartan, RSA), and for captive animals a mixture of Zoletil (1.5 mg/kg; Virbac, RSA) and medetomidine (0.08 mg/kg) was used.

### Ultrasonography and measurements

All adult and juvenile females were assessed using ultrasonography, performed with a CS 9100 Picker using a 7.5 MHz fingertip transducer as probe (both Hitachi, Tokyo, Japan) and visualizing structures with a minimum size of 0.2 mm to an accuracy of 0.1 mm. The ultrasound probe was fixed to a rigid extension (Schnorrenberg, Woltersdorf, Germany) specifically designed for transrectal examination of medium-sized mammals (Hildebrandt *et al.* 2000b). Compared with conventional transabdominal ultrasound, transrectal ultrasound provides unmatched high-resolution images, since the acoustic coupling of the ultrasound probe on a wet, thin intestinal mucosa facilitates the accurate interpretation of fine tissue structures (Hermes *et al.* 2000). It can also distinguish between hydrosalpinx (oval shape, with fluid-filled lumen surrounded by thin, rough, and spiky wall) and paraovarian cysts, that is, remnants of Wolffian glands (round shape, with

fluid-filled lumen surrounded by thick, smooth, homogeneous wall). Females were classified as being reproductively active, reproductively inactive, or juvenile based on the presence, size, and echogenicity of functional structures on the ovaries and the echogenicity and structure of the vagina, cervix, and uterus (Thalwitzer 2007). To assess chronic "stress" in female cheetahs, we measured the size of the adrenal glands. These were determined as their cross-sectional area calculated as  $\pi/4 \times \text{maximum length} \times \text{maximum cross-sectional diameter}$ . To determine the allometric relationship between adrenal gland size and body length, the latter was measured dorsally with a flexible measuring tape from the tip of the nose to the sacro-coccygeal joint.

### Determination of litter size and postemergence cub survival

Eleven of 13 adult females were fitted with a VHF collar (Advanced Telemetry Systems, MN, USA) or a GPS collar (Vectronics Aerospace GmbH, Berlin, Germany) and tracked approximately twice a month by airplane between August 2002 and December 2007. Litter sizes were determined on capture day and/or by aerial tracking of the mothers. When unknown cubs were sighted with a female, special effort was made to estimate their age by flying low and reassessing the age during consecutive flights by different observers. Litter sizes were determined at 2, 4, and 14 months of age, indicating emergence from the lair, weaning, and earliest independence, respectively (Laurenson 1992) and compared with the results of the study in the Serengeti (Laurenson 1992). Sizes of all litters from the same mother were averaged. In this study, litter size at 2, 4, and 14 months of age were assessed in a more conservative way than in the Serengeti study (Laurenson 1992). When a cub was seen last at the age of, for example, 3 months and its death confirmed at the age of 5 months, it was scored as dead at the age of 4 months (age of weaning) for the Namibian study, whereas in the Serengeti study, it was scored as alive at the age of 4 months. Cub survival was determined from litters found in the lair or shortly after emergence and followed to 14 months of age, using one litter each per female in both this and the Serengeti study. In the Serengeti study, one female was observed while raising two litters, so one litter each was included in alternative analyses.

### Annual rainfall

Data were available from five representative farms in central Namibia where free-ranging cheetahs were captured. Mean rainfall was 233 mm in the rain year 2002/2003

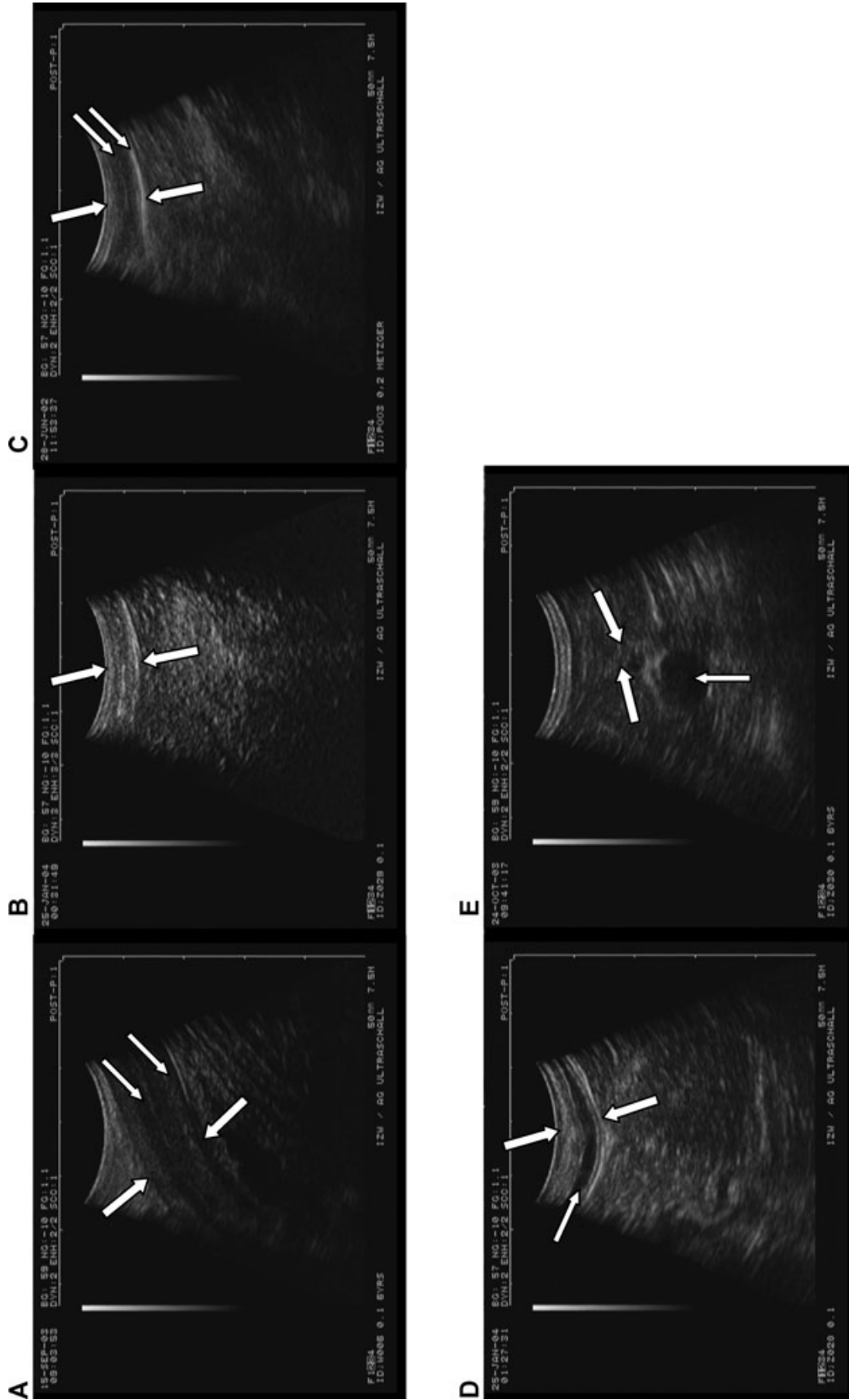
(June 2002 to May 2003), 425 mm in 2003/2004, 345 mm in 2004/2005, and 738 mm in 2005/2006. The same amount of annual rainfall can be expected in areas farther south and north (Mendelsohn *et al.* 2002) where some captive cheetahs were examined in this study and where the study of Terio *et al.* (2003) was conducted (northern Namibia). Assuming that annual rainfall is similar for both studies (Mendelsohn *et al.* 2002), rainfall in the years of Terio *et al.*'s study (2003) was 168 mm in 1994/1995 ("low" rainfall) and 181 mm in 1995/1996 ("high" rainfall). The rainy season usually lasts from January to April (Mendelsohn *et al.* 2002).

### Analysis

All tests were performed using Systat 13.0 (Systat Software Inc., Richmond, VA, USA). In the general linear model, we used the Lilliefors test to confirm that the residuals did not significantly deviate from normal distribution. Type I error probabilities for the Mann-Whitney *U* tests were based on exact *P*-values calculated with StatXact 8.0 (Cytel Inc., Cambridge, MA, USA) because sample sizes were below 30. Probabilities are for two-tailed tests and results are quoted as means  $\pm$  standard error of mean.

### Results

All 13 free-ranging adult females were in a reproductively active state; they were either in pro-estrous, estrous (Figure 1A), di-estrous, pregnant, or lactating. In contrast, 57% of 14 adult captive females (Table 1) were in a reproductively inactive state (Figure 1B), with reproductive organs resembling in size and appearance juvenile ones (Figure 1C). Free-ranging females were more likely to be in a reproductively active state than captive ones (Fisher's exact test,  $n = 27$ ,  $P = 0.0019$ ). Age structure among adult free-ranging and captive females was similar (young:  $n_{\text{free-ranging}} = 5$ ,  $n_{\text{captive}} = 2$ ; old:  $n_{\text{free-ranging}} = 8$ ,  $n_{\text{captive}} = 12$ ; Fisher's exact test,  $n = 27$ ,  $P = 0.21$ ). Similar results were obtained when pregnant and lactating females were excluded (free-ranging:  $n_{\text{cycling}} = 8$ ,  $n_{\text{inactive}} = 0$ ; captive:  $n_{\text{cycling}} = 5$ ,  $n_{\text{inactive}} = 8$ ; Fisher's exact test,  $n = 21$ ,  $P = 0.0068$ ; young:  $n_{\text{free-ranging}} = 4$ ,  $n_{\text{captive}} = 2$ ; old:  $n_{\text{free-ranging}} = 4$ ,  $n_{\text{captive}} = 11$ ; Fisher's exact test,  $n = 21$ ,  $P = 0.15$ ). All adult free-ranging females for which breeding information was obtained ( $n = 10$ ), were also fertile. Six females were associated with cubs at the time of examination and four females were observed with cubs after examination. The remaining three females were not observed after examination because two were not collared and thus not located again and one was examined during the last month of the study.



**Figure 1** Sonograms of healthy and pathologically altered reproductive organs of juveniles, reproductively active, and inactive cheetah females. (A) Uterus (band between bold arrows) in estrus with the characteristic dark bands reflecting edematization of the endometrium (thin upper arrow) and myometrium (thin lower arrow), respectively. (B) uterus (band between bold arrows) in inactive state with the characteristic light image reflecting high material density of the endometrium and myometrium, (C) uterus (band between bold arrows) in juvenile state with the endometrium (thin upper arrow) distinguishable from the myometrium (thin lower arrow), (D) hydrometra, characterized by a dark band between the endometrium layers reflecting fluid (thin arrow) in the uterus (band between bold arrows), and (E) hydrosalpinx, characterized by a dark structure reflecting fluid (thin arrow) in the oviduct (bold arrows).

Adrenal gland size did not differ between free-ranging and captive cheetahs ( $F_{1,11} = 0.260$ ,  $P = 0.62$ ) when accounting for body size in a general linear model ( $F_{1,11} = 1.583$ ,  $P = 0.23$ ,  $n = 14$ ). Rainfall during this study exceeded rainfall in 1995/1996 (the year of high rainfall during the study period of Terio *et al.* 2003) by 29%, 135%, 91%, and 308%. The 57% reproductively inactive captive females were in a reproductive inactive state in April or June, that is, just after the rainy season (Table 1).

The eight captive females with inactive reproductive organs were older ( $6.1 \pm 0.8$  years; youngest age used for females examined more than once) than the six females with active reproductive organs ( $3.6 \pm 0.5$  years,  $U = 5$ ,  $n_1 = 8$ ,  $n_2 = 6$ ,  $P = 0.0093$ ). Pathologies of reproductive organs such as dense connective tissue in the ovarian stroma, hydrometra (Figure 1D), and hydrosalpinx (Figure 1E) were only found in captive females 4 years of age or older ( $n = 7$ ); six of these seven females (86%) had inactive reproductive organs and four (57%) had inactive reproductive organs and were nulliparous (Table 1). The only genital lesion found in a free-ranging female was a paraovarian cyst in a pregnant female, confirming that this lesion does not hamper reproductive capacity (Munson 1993).

Fourteen cubs from three litters of three different females were seen in the lair or just after emergence, that is, at the age of 2 months or younger. At 14 months of age, eleven cubs (79%) from these three litters were still alive. In the Serengeti, 31 (or 32 if the larger litter of two litters of one female is considered) cubs from nine litters of nine females were seen at 2 months of age or younger; at 14 months of age seven cubs (23% or 22%) in three litters were still alive (Laurenson 1992). Thus, cubs on Namibian farmland were 3.4 (or 3.6) times more likely to survive from emergence to independence than Serengeti cubs (Fisher's exact test,  $n = 45$ ,  $P = 0.00072$ , or  $P = 0.00063$ ). Mean litter size of all litters were similar in both populations at 2 months of age [Namibia (this study):  $4.7 \pm 0.9$ , Serengeti (Laurenson 1992):  $3.5 \pm 0.2$ ,  $U = 6.5$ ,  $n_1 = 3$  females,  $n_2 = 9$  females,  $P = 0.19$ ] and larger in Namibia at 4 months of age [Namibia (this study):  $3.2 \pm 0.1$ , Serengeti (Laurenson 1992):  $2.0 \pm 0.0$ ,  $U = 0$ ,  $n_1 = 7$  females,  $n_2 = 6$  females,  $P = 0.00058$ ] and 14 months of age [Namibia (this study):  $3.2 \pm 0.5$ , Serengeti (Laurenson 1992):  $1.8 \pm 0.3$ ,  $U = 1.5$ ,  $n_1 = 5$  females,  $n_2 = 4$  females,  $P = 0.04$ ].

## Discussion

Four hypotheses have been proposed to explain poor reproductive performance, such as irregular ovarian cycles

and anestrus in females or low cub survival, in cheetahs and other mammalian species: the genetic monomorphism, captive "stress," innate rhythm, and asymmetric reproductive aging hypotheses. Our findings are consistent with the model of asymmetric reproductive aging and provide evidence that reproductive health in cheetah females is a function of reproductive history and age. Reproductively inactive states were only found in older captive females and genital pathologies mainly occurred in females with inactive reproductive organs and nulliparous females. Chronic "stress" leads to the production of glucocorticoids in the cortices of adrenal glands, eventually leading to enlarged cortices of adrenal glands (Suleman *et al.* 2000). There was no difference in adrenal gland size between captive and free-ranging females on Namibian farmland, suggesting that ovarian inactivity in captive females was unlikely to be caused by (chronic) "stress." These findings are consistent with studies on fecal corticoid concentrations, showing that on Namibian farmland, captive cheetah females have similar glucocorticoid concentrations as free-ranging ones (Terio *et al.* 2003, 2004). Reproductive cycling in cheetah females is also unlikely to be triggered by an endogenous circannual rhythm because our study was conducted during years with high rainfall, yet had a sizable proportion of captive females with inactive reproductive organs. Genetic monomorphism is unlikely to be a main factor reducing reproductive performance, be it conception rate, fertility, or postemergence cub survival. The high postemergence cub survival in free-ranging Namibian cheetahs confirms a previous study conducted in northern Namibia that found 75% of cubs surviving the first 12 months (Marker *et al.* 2003) and demonstrates that in a habitat free of natural predators, apparent low genetic diversity does not impair postemergence cub survival or recruitment. Reintroduction projects are therefore likely to be more successful in habitats with low predator density as long as female reproductive capacity at the time of release is not yet impaired by asymmetric reproductive aging. Thus, reproductive history and age as well as absence of predators are important for female reproductive fitness.

In free-ranging, long-lived mammal populations, juvenile survival is considered to be more important for population growth than conception rate and fertility (e.g., Heppell *et al.* 2000)—presumably because conception rate and fertility are usually high in natural populations of such species. However, if populations are small (and often threatened), conception rate and fertility can also become an important issue as the Allee effect clearly demonstrates (Courchamp *et al.* 2008). In the light of our findings, we suggest that captive breeding of species susceptible to asymmetric reproductive aging processes designed for reintroduction programs should ensure the availability of a

large number of animals in early adulthood because the irreversible processes of asymmetric reproductive aging and therefore reduced reproductive lifespan can be prevented by (1) breeding females as young adults (Hermes *et al.* 2004) and (2) encouraging lactation until the natural age of weaning, as it prevents frequent fluctuation of estrogen concentrations (Schmidt *et al.* 1983).

Since re-introduction programs rely on suitable field conditions before releasing captive bred individuals of a species, captive populations are often retained in captivity for several decades (Kleinman *et al.* 1994). To maintain the potential of a species to persist in the wild after re-introduction, genetic adaptations to captivity should be prevented (Kraaijeveld-Smit *et al.* 2006). One option, minimizing the number of generations in captivity (Frankham 2008; Williams & Hoffman 2009) either requires delayed reproduction or cryopreservation of gametes. Currently, cryopreservation of gametes is mostly practical for sperm (Crosier *et al.* 2006; Fickel *et al.* 2007), so that for females, only a delay in reproduction seems feasible. Our findings identified the mechanism why delaying reproduction is a dangerous road to follow, as previously noted (Frankham *et al.* 2002; Williams & Hoffman 2009), especially in species susceptible to asymmetric reproductive aging processes, as it decreases reproductive performance and reduces the number of females genetically represented in the next generation.

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