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Assessing puberty in ex situ male cheetahs (*Acinonyx jubatus*) via fecal hormone metabolites and body weights

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ABSTRACT

Cheetahs are one of the most heavily studied felid species, with numerous publications on health, disease, and reproductive physiology produced over the last 30 years. Despite this relatively long history of research, there is a paucity of crucial biological data, such as pubertal onset, which has direct and significant applications to improved management of *ex situ* cheetah populations. This study aimed to determine age of pubertal onset in *ex situ* male cheetahs using non-invasive fecal steroid hormone monitoring and body weights. Fecal samples from 12 male cheetahs from four institutions were collected 2–3 times weekly from 1 to 42 months of age. Fecal androgen and glucocorticoid metabolites were analyzed using enzyme immunoassays previously validated for use with cheetah feces. Animal body weights were recorded monthly. Fecal hormone and body weight data were analyzed using generalized linear mixed models. Androgen concentrations exhibited an increase to levels similar to those observed in adult males by 18–24 months of age, and males attained adult body weights by 21 months of age. Based on these weight data and the initial increase in androgens toward adult concentrations, males were considered pubertal from 18 to 24 months of age. Glucocorticoid concentrations and amplitude of concentration over baseline were also increased during this period. Knowledge about the physiological changes associated with puberty is useful for management and improving reproductive success of cheetah populations under human care, particularly for determining timing of litter separation from dam, littermate dispersal and when to introduce potential breeding pairs.

1. Introduction

The cheetah (*Acinonyx jubatus*) is one of the most well-studied felids, including extensive investigations on reproduction (Brown et al., 2001; Brown and Wildt, 1997; Crosier et al., 2009; Howard et al., 1992, 1997; Roth et al., 1995; Wildt et al., 1988) health and disease (Bolton and Munson, 1999; Franklin et al., 2015; Munson et al., 2005, 2002), and behavior (Wielebnowski, 1999; Wielebnowski and Brown, 1998). Influential studies on cheetah biology include the discoveries of low genetic diversity (O'Brien et al., 1985, 1983), the documentation of the species' poor sperm quality and routine production of ~75% malformed spermatozoa per ejaculate (Crosier et al., 2007; Donoghue et al., 1992; Roth et al., 1995; Wildt et al., 1983, 1993, 1987), and the recent discovery that heterozygosity was not correlated with sperm quality (Terrell et al., 2016). While the ultimate goal of intensely studying the species is to support *in situ* populations, it can be difficult to ascertain biological data from free-ranging animals. Therefore, it is advantageous to have a self-sustaining *ex situ* population to increase our knowledge of this species, as well as provide a genetic and demographic reservoir for the future. Despite the similar high incidence of structurally abnormal sperm in captive and free-ranging males (Crosier et al., 2007), cheetahs still manage to reproduce successfully in the wild (Caro, 1994). However, their *ex situ* counterparts do not exhibit similar success, with ~70% of the Association of Zoos and Aquariums (AZA) Species Survival Plan (SSP) population failing to reproduce (Crosier et al., 2017). The discrepancy between the two populations suggests that factors associated with management, husbandry and/or the captive environment may be contributing to reduced fecundity of the *ex situ* population

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rather than only genetic or sperm morphology concerns (Crosier et al., 2007; Wildt et al., 1993).

To determine how management and environmental factors influence male reproductive function, there is a need to understand basic male biology. Previously, the majority of studies focused primarily on spermatozoa structure and function (Crosier et al., 2007; Terrell et al., 2012; Wildt et al., 1983, 1993, 1987). Only recently have longitudinal androgen and glucocorticoid profiles been elucidated in male cheetahs 2–12 years of age (Koester et al., 2015a). Biological assay validation by Koester et al. (2015a) reported increased mean androgen concentrations in 29 study males > 2 years of age when compared to seven males < 2 years of age. Androgens in males > 2 years of age were highly variable both within and between males, but concentrations did not vary based on season nor did the data directly correlate with ejaculate quality, potentially indicating that once androgens reach adult concentrations, variations above such a threshold level are not predictive of ejaculate quality (Koester et al., 2015a). These results, along with studies on free-ranging Namibian cheetah sperm production (Crosier et al., 2007) and year-round cub births recorded internationally (Marker, 2015), provide additional evidence for the absence of seasonality in male cheetahs (Koester et al., 2015a). These discoveries also highlight the impact social (Koester et al., 2015b) and environmental factors (Crosier et al., 2007; Koester et al., 2015a) have on physiological traits of adult *ex situ* males. For example, group management of males revealed improved ejaculate quality compared with that for males housed singly (Koester et al., 2015b). However, the numbers of other conspecifics housed at the same institution did not influence either fecal androgen concentrations or ejaculate quality (Koester et al., 2015a). Given the large amount of physiological data compiled on male cheetahs, there remains a paucity of data on the biology of immature males, or indeed the onset of puberty.

Puberty is an important biological process culminating in the achievement of the physiological capability of fertilization, which leads to the ability to successfully produce offspring. This process includes the activation of the hypothalamic-pituitary–gonadal (HPG) axis and rise of androgen concentrations to mature adult levels and release patterns (Ebling, 2005; Plant and Witchel, 2006). This in turn facilitates the acquisition of breeding behaviors (Hull et al., 2006), and the initiation of spermatogenesis (O'Donnell et al., 2006). In mammals, to be considered sexually mature, a male must produce competent sperm capable of fertilization, as well as exhibit proper breeding behaviors required for successful mating and insemination of the female (Ebling, 2005). Puberty has been assessed in other species using breeding behaviors (Romeo et al., 2002), presence of sperm in seminiferous and epididymal tubules (Stewardson et al., 1998), the first presence of sperm in ejaculation (Asa, 2010), and spermaturia (Nysom et al., 1994). Unfortunately, due to the challenges of performing repeated procedures requiring anesthesia on an individual of a nondomestic species, seminal parameters could not be used here. Breeding behaviors are also difficult to measure in cheetahs, as it is difficult to ascertain whether a lack of appropriate breeding behavior in juvenile cheetahs is due to pubertal timing or rather a response to a myriad of environmental factors. Reports of sexual behavior in male juvenile cheetahs are mostly anecdotal. Based on observations from the wild, mixed sex sibling groups leave their mother around 18 months of age. Within the following six or so months, males split from their sisters to form lifelong coalitions with their brothers (Caro, 1994). The timing of this sibling separation may be an indicator of pubertal onset. Additionally, the measurement of androgens is used to assess pubertal development, including in felids. In domestic cats, an increase in mean serum testosterone concentrations was observed between 9 and 12 months of age, with the peak levels occurring at 12 months of age (Tarttelin et al., 1998); leading the authors to determine that males in that study were pubertal between 10 and 12 months of age.

Currently, little is known of hypothalamic–pituitaryadrenal (HPA) axis activity during the pubertal process of non-human mammals. In adults of many species, fecal glucocorticoids have been shown to increase around the time of other major physiological events, such as pregnancy (Cavigelli, 1999; Dantzer et al., 2010; Fanson et al., 2012; Weingrill et al., 2004) and at the beginning of breeding season (Eggermann et al., 2013; Fanson et al., 2012; Kersey et al., 2010; Pavlova et al., 2014), as part of the response to an intensification of metabolic demand (Romero, 2002). Due to substantial physiological changes that occur, pre- and peri-pubertal intervals are highly sensitive periods of development in mammals. Disturbance of the hypothalamic-pituitary–gonadal (HPG) axis during neonatal development may lead to delayed onset of puberty (Carranza et al., 2014; Risso et al., 2012), or decreased reproductive function through stunted sexual development (Mann et al., 1998) that may carry into adulthood (Kolho and Huhtaniemi, 1989). One way in which the HPG axis can be disrupted is via increased hypothalamic–pituitaryadrenal activity (Hardy et al., 2005; Orr et al., 1994). In recent studies of adult male cheetahs, no correlation was found between glucocorticoids and either androgen concentrations or ejaculate quality (Koester et al., 2015a). However, following significant management/husbandry changes, such as moving to a new institution, some animals exhibit major glucocorticoid fluctuations (Wells et al., 2004). Increased adrenal activity can also be associated with increased metabolic demands (Uchoa et al. 2014). As animals begin a major physiological transition, such as during puberty, it may be likely that glucocorticoid production patterns vary as well.

Body weight, condition score, and nutrition have been shown to influence hypothalamic pubertal onset, where animals must reach a threshold weight or fat percentage as seen in dairy cows (Macdonald et al., 2005), lambs (Boulanouar et al., 1995), rats (Ojeda and Skinner, 2006), nonhuman primates (T.M. Plant and Witchel, 2006), and humans (Baker, 1985), making body weight a good indicator of pubertal development. This is best documented in livestock, such as cattle, where onset of puberty begins after attainment of 60% of the adult body weight (Freetly et al., 2011). Body weights of free-ranging adult male cheetahs vary widely, and ranges of 38.6–62.0 kg have been reported (Du Preez, 1976; Labuschagne, 1979; Marker and Dickman, 2003; McLaughlin, 1970). Four to eight year old healthy, *ex situ* male cheetahs average 45.8 kg ± 2.6 with a range of 38.4–51.0 kg (Crosier, unpublished data). However, similar to previous studies on hormone data, little to no information exists on growth patterns in cheetahs < 24 months of age. Tracking body weights in young cheetahs over time would help with our understanding of when the pubertal process occurs, and is a husbandry practice that is done on a routine basis, making it a useful management strategy to track early development.

No data are available for pubertal processes in free-ranging male cheetahs as mating events are rarely witnessed (Caro, 1994), and the ability to assess individual hormonal production over time is not feasible due to the limitations associated with regular collection of biological materials from free-ranging animals. Managed populations of cheetahs provide a unique opportunity to biologically monitor these individuals as they develop. Understanding the physiological changes that occur during puberty in male cheetahs is critical to ensure appropriate environmental conditions are provided to support successful reproductive capabilities into adulthood. In this study, we set out to investigate physiological changes, through gonadal and adrenal hormone monitoring, occurring at the same time as significant life events such as timing of offspring separation from dam, sibling separation by sex, transfer to new facilities and breeding introductions, all of which routinely take place in sub-adult cheetahs in managed populations. Specifically, we aimed to determine age of pubertal onset in male cheetahs ranging from 1 month to 42 months of age. Due to the limited access to seminal and behavioral characteristics, and because a pubertal rise in androgens is necessary for culmination of spermatogenesis, for the purposes of this study we define pubertal onset as the age in which androgen concentrations significantly rise to that expected of adult male cheetahs. In this study, we investigated pubertal onset using two mechanisms: 1) analysis of longitudinal fecal gonadal hormone