# 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 to 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.



**Keywords: glucocorticoids, androgens, sexual maturity, population management**


**Running title: Male cheetah puberty**

**Highlights:**

- Androgen concentrations increased to adult levels by 18 to 24 months of age
- Males attained adult body weights by 21 months of age
- Male cheetahs are considered pubertal from 18 to 24 months of age
- Glucocorticoid concentration and amplitude also increased during this time period

## 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 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, 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, 2015b) and environmental factors (Crosier et al., 2007; Koester, 2015a) have on physiological traits of adult *ex situ* males. For example, *g*roup 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-pituitary-adrenal (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-pituitary-adrenal 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 to 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 metabolite profiles, and 2) documentation of changes in body weight through monthly measurements. We also identified non-reproductive

physiological changes during this time period through analysis of longitudinal fecal adrenal hormone metabolite profiles. To our knowledge, this is the first study to characterize longitudinal gonadal and adrenal hormone profiles and body weights in male cheetahs under 24 months of age.

## 2. Materials and Methods

### **2.1** *Experimental animals*

Twelve male cheetahs from four institutions participating in the AZA Species Survival Plan were included in this study. All study animals were captive born with an age range of 1-42 months. Cheetahs were located at the Smithsonian Conservation Biology Institute (SCBI; n=9; Front Royal, VA; 38° 53' 9.35" N 78° 09' 55.1" W), National Zoological Park (NZP; n=1; Washington D.C.; 38° 55' 31.13" N 77° 02' 35.42" W), White Oak Conservation (WOC; n=3; Yulee, FL; 30° 45' 29.6" N 81° 45' 52.3" W), and Dallas Zoo (DZ; n=2; Dallas, TX; 32° 47' 0.9060 N 96° 48' 18.2772" W). Three males began the study at WOC at 13 months of age and were relocated to SCBI at 18 months of age. The 12 cheetahs included in this study were born across most of the months of the year, with births occurring from April through December. Ten males in the study were mother-reared with siblings. To mimic behaviors in the wild, mother-reared cubs remained with their mother and siblings until 15 to 18 months of age. After this, a "soft" separation occurred, by which males were removed from their mothers/sisters but allowed visual access to them. Because males in the wild typically stay together for life and little evidence of aggression has been shown in captivity (Chadwick et al., 2013), sibling males from the same litter were kept together as a coalition when possible, or non-related coalitions established (occurred in one instance when two males were introduced at 14 and 19 months of age). All but two males (NZP n=1 and SCBI n=1) were part of a male coalition during at least one stage of the study. Two male siblings (DZ n=2) in this study were removed from their mother within 4 days of birth and hand-raised together by zoo staff to become ambassador program animals. These two males remained together during the hand-raising period and lived in a coalition similar to those males being mother-reared.

Across all institutions, unless stated otherwise, cheetahs were fed either a commercial carnivore beef-based diet (Natural Balance Pet Foods Inc., Burbank, CA) or a horse-based diet (Milliken Meat Products, Ltd, Ontario, Canada) or a combination of the two, and water was available *ad libitum*. Cheetahs located at SCBI lived in 2000 $m^2$ enclosures with free access to both indoor and outdoor areas. Animals located at NZP were in outdoor yards ranging from 28.1 $m^2$ to 6000 $m^2$ and in overnight holding stalls ranging from 4.5 $m^2$ – 6 $m^2$. WOC cheetahs lived in outdoor enclosures ranging from 1000 $m^2$ to 6000 $m^2$ with wooden dens for shelter. DZ animals were hand-raised and fed Kitten Milk Replacer (Pet-Ag, Hampshire, IL) until they were old enough for the commercial beef-based diet. Indoor and outdoor enclosures at DZ range from 46.5 $m^2$ 650.3 $m^2$.

### **2.2** *Fecal samples*

Fecal samples were collected 2 to 3 times weekly for each individual for varied age intervals (Table 1). Cheetahs housed with conspecifics received a non-digestible, and non-toxic fecal marker (i.e. glitter) in their food to differentiate individual fecal samples (Koester et al., 2015). Sample collection for cheetahs less than 6 months of age began when the opportunity arose to separate cubs from their mother and siblings for feeding of individual fecal markers (approximately 2 to 4 months of age). Individual fecal samples were placed into clean, labeled plastic bags, shipped (when necessary) and stored frozen (-20°C) until lyophilized (Labconco, Kansas City, MO) and processed at the SCBI.

### **2.3** *Sample processing*

Fecal steroid hormone extractions followed previously described procedures for cheetah (Crosier et al., 2016; Koester et al., 2015a). Steroid extraction efficiencies were determined with the addition of radiolabeled hormone ($^3$H-testosterone or $^3$H-cortisol; 4,000-8,000 dpm) to each sample prior to extraction. Mean (± standard error of the mean [SEM]) radiolabeled hormone recovery after extraction was 78.9% ± 16.8% for all samples. Fecal extracts were diluted in dilution buffer (0.039 M $NaH_2PO_4$, 0.061 M $Na_2HPO_4$, 0.15 M NaCl, pH 7.0) as necessary for androgen and glucocorticoid assays (1:20, 1:100, 1:200, and 1:500). Sample extracts were stored at -20°C until utilized for hormone assays.

**2.4** *Enzyme immunoassays*

Androgen metabolite concentrations were quantified from diluted fecal extracts using a single polyclonal antibody (No. R156/7; C. Munro, University of California, Davis, CA) enzyme immunoassay (EIA) previously validated in the cheetah (Koester, 2015a). In brief, 96-well microtiter plates (Nunc-Immuno, Maxisorp; Thermo Fisher Scientific; Waltham, MA) were coated with antibody (0.05 ml; 1:8000) and incubated for 12 to 48 h (4ºC). Excess unbound antibody was removed with wash solution (1.5 M NaCl, 0.5% Tween 20). Diluted samples (0.05ml; 1:20) and controls (0.05ml) in duplicate, and standards in triplicate (0.05 ml; 46 – 12,000 pg/ml; 17β-hydroxy-4-androstein-3-one; Steraloids, Newport, RI) were loaded into plate wells followed by a horseradish peroxidase enzyme-conjugated testosterone (0.05 ml; 1:20,000; C. Munro). Plates were incubated at room temperature (RT; approximately 23ºC) for 2 h before being washed three times to remove unbound components. A chromogen solution (0.10 ml) was added to each well. Following a 30 min incubation period at 23ºC, the reaction was stopped with 1N HCL and optical densities were determined using a microplate reader (Dynex MRX, reading filter 405nm, reference filter 540 nm).

The cross-reactivities of the R156/7 antibody have been previously published (Koester, 2015a). Sensitivity of the testosterone assay at 100% binding was 2.3 pg/well. The inter-assay coefficients of variation (CVs) were 7.90% and 9.55% for high and low synthetic controls (n = 82 assays), and the intra-assay CVs were 8.9%, 9.2%, and 9.8% for high and low synthetic and biological controls, respectively. CVs for all samples run in duplicate were below 10%. This immunoassay was biochemically validated for measuring androgen metabolites in male cheetah fecal extracts through parallelism and matrix interference assessment. Serially diluted pooled fecal extracts demonstrated displacement curves parallel to those of standard hormone preparations ($y = 0.910x + 2.207$, $R^2 = 0.986$, $F_{1,7} = 487.604$, $P<0.001$). Addition of diluted fecal extract to synthetic standards demonstrated no evidence of matrix interference ($y = 1.079x – 3.797$, $R^2 = 0.994$; $F_{1,6} =$, $P < 0.001$). The androgen assay has been previously validated for measuring androgen metabolites in male cheetah feces in the same laboratory using the described methodology (Koester, 2015a).

Glucocorticoid metabolite concentrations were quantified using a polyclonal primary antibody (R4866; 1:8500; C. Munro, University of California, Davis, CA) enzyme immunoassay previously validated in the cheetah (Koester, 2015a). A double antibody system was utilized with a secondary goat-anti rabbit IgG antibody (A009, Arbor Assays, Ann Arbor, MI). In brief, secondary antibody (0.15ml; 10 µg/ml) was added to 96-well microtiter plates (Costar, Fisher Scientific) followed by incubation at RT for 15-24 h. After incubation, coating buffer (X108, 20X, Arbor Assays, Ann Arbor, MI) and unbound antibodies were washed from wells. Blocking solution (X109, 10X, Arbor Assays, Ann Arbor, MI) was added to each well (0.25ml) and left to incubate for 4 to 24 h at RT. Following incubation, blocking solution was removed and plates were dried at RT in a desiccator cabinet. After drying, plates were packaged in vacuum-sealed bags and stored at 4°C until use. Diluted fecal extracts (0.05ml; 1:20) and controls (0.05ml) were added to plate wells in duplicate, followed by cortisol standards (0.05ml; 78-20,000 pg/ml; Sigma Diagnostics, St. Louis, MO) in triplicate. A horseradish peroxidase enzyme-conjugated cortisol (0.025 ml; 1:20,000; C. Munro, University of California, Davis, CA) was added to all wells. The primary cortisol antibody (0.025 ml; 1:8500) was added to all wells except for the non-specific binding (NSB) wells followed by incubation for 1 h at RT. Unbound components were removed with wash solution followed immediately by the addition of a chromagen solution (0.1 ml, X019, Arbor Assays, Ann Arbor, MI) to each well. Another incubation period of 15 min at RT occurred before the reaction was ended with the addition of stop solution (0.05ml, X020, Arbor Assays, Ann Arbor, MI) and optical densities were determined using a microplate reader (Dynex MRX, reading filter 405nm, reference filter 540 nm).

The cross-reactivities of the R4866 antibody have been previously published (Young et al., 2004). Sensitivity of the glucocorticoid assay at 100% binding was 3.9 pg/well. The inter-assay coefficients of variation (CVs) were 7.51% and 9.28% for high and low synthetic controls (n = 82 assays), and the intra-assay CVs were 2.6%, 9.0%, and 2.9% for high and low synthetic and biological controls, respectively. CVs for all samples run in duplicate were below 10%. This immunoassay was biochemically validated for measuring glucocorticoid metabolites in male cheetah fecal extracts through parallelism and matrix interference assessment. Serially diluted pooled fecal extracts demonstrated displacement curves parallel to those of standard hormone preparations ($y = 0.980x - 3.755$, $R^2 = 0.998$, $F_{1,6} = 2773.101$,

P<0.001). Addition of diluted fecal extract to synthetic standards demonstrated no evidence of matrix interference ($y = 1.308x – 25.870$, $R^2 = 0.995$; $F_{1,6} =$, $P < 0.001$).

**2.5** *Body Weights*

Cheetah body weights were measured in kilograms (kg) every three months using a platform scale (either Tru-Test, Auckland, New Zealand or H & C Weighing Systems, Columbia, MD). Animals were conditioned to stand on the scale for voluntary weight measurements.

**2.6** *Data analyses*

The birth dates of all study animals were known from the international studbook (Marker, 2015), and ages were calculated in months. Animals were assigned to age groups in 6 month increments from ≤6 months to >36 to 42 months (see Table 1). When an animal was one day older than the upper limit of an age group, that data point was included in the next oldest age category.

Raw data for fecal androgen and glucocorticoid metabolite concentrations were used to calculate overall mean, standard deviation (SD), minimum and maximum values for each individual in each age group (Table 1). Fecal androgen and glucocorticoid metabolite data were then used to create two variables for each individual and each of the six-month age categories from 0 to 6 months through >36 to 42 months of age. These included: 1) a baseline concentration calculated following an iterative process where all values greater than the mean plus 1.5 SD were excluded (Brown et al., 1994) (baseline); and 2) amplitude over baseline which includes the mean of the distance between the baseline value and all concentrations over baseline value (amplitude).

Dependent variables of body weight, daily androgen/glucocorticoid metabolite concentrations, and the two calculated variables of baseline and amplitude for each hormone metabolite were analyzed using separate generalized linear mixed models (GLMM) in MLwiN version 2.02 (Rasbash et al., 2005). This approach allowed the incorporation of random effects (individual and sample date [daily androgen/glucocorticoid metabolite

concentrations and amplitude over baseline] or individual [body weight, baseline]) to control for repeated fecal samples and measurements across individuals. GLMMs were built for each dependent variable, incorporating the same random (individual and sample, or individual) and categorical fixed effects (age) across all models. The oldest age categories (42 months for body weight and >36 to 42 month age category for androgen and glucocorticoid metabolite variables) were chosen as reference categories because males over 3 years of age are likely to be most comparable to previously published adult male data (Koester, 2015a, 2015b). Because individuals were housed at four different facilities, we included facility as a covariate in each GLMM to account for variation in the data that might be associated with location. However, due to the uneven distribution of both individuals and age-classes across facilities, and the difficulty in determining other confounding factors associated with location, comparisons were not made directly between facilities. Coalition status (yes or no) was also added as a covariate in the model but was later removed from the analysis as it was non-significant.

Fixed effects of facility, age and coalition status were entered into the GLMM together before non-significant terms were dropped sequentially until only those that explained significant variation in the dependent variable remained. All statistics reported are taken from this, the minimal model. Each dropped term was subsequently re-entered to the minimal model individually to obtain their level of non-significance. A normal error structure was used for all models and the significance of each fixed effect (main effects and post-hoc comparisons) was determined using the Wald statistic and chi-squared ($\chi^2$) distribution, where significance was defined as $P < 0.05$. Raw fecal androgen and glucocorticoid metabolite concentrations (mean, SD, minimum, and maximum) for each individual at each age group are presented in Table 1. Data are presented as the mean prediction ± standard error (SE) from the minimal model (µg/g dry feces or kg body weight), which takes into account the non-independence of data as defined by the random effects.

## 3. **Results**

### **3.1** *Fecal androgen metabolites*

Differences in fecal androgen metabolite concentration were observed among institutions, and were taken into account by inclusion of facility as a covariate in the minimal model for each dependent variable. After taking facility into account, age was a significant predictor of daily androgen metabolite concentrations, baseline, and amplitude above baseline (Fig. 1).

Fecal androgen metabolite concentrations (Fig. 1A) were not significantly different between the 0 to 6 month (0.405 ± 0.035 µg/g dry feces) and >6 to 12 month (0.395 ± 0.027) groups ($\chi^2 = 0.126$, df = 1, P = 0.723). However, concentrations were lowest ($P \leq 0.022$ for all pairwise comparisons) in the >12 to18 month group (0.337 ± 0.024) and highest ($P \leq 0.027$ for all pairwise comparisons) for both the >30 to 36 (0.497 ± 0.024) and >36 to 42 (0.479 ± 0.027) month old males. Compared to the >6 to 12 month age group, androgen concentrations declined at >12 to 18 months ($\chi^2 = 8.668$, df = 1, P = 0.003). Thereafter concentrations then increased with age at >18 to 24 months (0.405 ± 0.023; $\chi^2 = 21.748$, df = 1, $P < 0.001$) and again from >24 to 30 (0.410 ± 0.023) to >30 to 36 months ($\chi^2 = 54.622$, df = 1, $P < 0.001$).

Baseline fecal androgen concentrations (Fig. 1B) followed similar patterns to mean concentrations, with initially high values (0.394 ± 0.046) observed in the youngest males. These concentrations were followed by a decline between the > 6 to 12 month (0.369 ± 0.032) and >12 to 18 month (0.261 ± 0.032) groups ($\chi^2 = 5.778$, df = 1, P <0.016). Similar to mean androgen values, baseline concentrations for the >12 to 18 month group were lower ($P \leq 0.018$ for all pairwise comparisons) than all other age groups, with the exception of the >24 to 30 month (0.340 ± 0.032) group ($\chi^2 = 3.339$, df = 1, P = 0.068). Baseline androgen concentration then increased with age, from > 18 to 24 month old males (0.357 ± 0.025) through to the oldest males (>36 to 42 months; 0.464 ± 0.043). This oldest age group exhibited the highest androgen baseline concentrations compared with all other groups ($P \leq 0.032$), except the youngest males (0 to 6 months; $\chi^2 = 1.227$, df = 1, P = 0.268 and >6 to 12; $\chi^2 = 3.132$, df = 1, P = 0.077) and the next closest in age, >30 to 36 months old (0.407 ± 0.031; $\chi^2 = 1.172$, df = 1, P = 0.279).

Overall, androgen amplitude over baseline (Fig. 1C) increased with age. All males from the 0 to 6 month (0.067 ± 0.031), >6 to 12 month (0.088 ± 0.021), and >12 to 18 (0.103 ± 0.016) month groups had lower ($P \leq 0.047$ for all pairwise comparisons) amplitude values

than males over 24 months of age. The amplitude for the >18 to 24 month (0.121 ± 0.013) group exhibited similar amplitude to both the younger (≤ 18 months; $P \geq 0.084$) and older males (> 24 to 30 months; 0.137 ± 0.013; $\chi^2 = 1.353$, df = 1, P = 0.245). The >30 to 36 month age group (0.172 ± 0.014) exhibited the highest amplitude compared to all other males ($P < 0.009$) except for >36 to 42 month old males (0.151 ± 0.021; $\chi^2 = 1.025$, df = 1, P = 0.311).

**3.2** *Fecal glucocorticoid metabolites*

Similar to fecal androgen data, the minimal model for each glucocorticoid variable included both facility and age. After taking this into account as a covariate, age was a significant predictor of daily glucocorticoid metabolite concentrations, baseline, and amplitude above baseline (Fig. 2).

Fecal glucocorticoid metabolite concentrations (Fig. 2A) fluctuated across ages. Interestingly, the youngest males (0 to 6 months; 0.675 ± 0.043 μg/g dry feces) had higher mean concentrations than all other age groups ($P < 0.001$ for all pairwise comparisons). Mean glucocorticoid concentrations then declined with age, where >12 to 18 month olds (0.301 ± 0.022) exhibited the lowest ($P \leq 0.010$) mean concentrations compared to all other age groups, except the >30 to 36 month males (0.333 ± 0.019; $\chi^2 = 1.662$, df = 1, P = 0.197). Compared to the preceding age group, concentrations then increased in the >18 to 24 age group (0.407 ± 0.018; $\chi^2 = 20.737$, df = 1, $P < 0.001$), decreased again by > 24 to 30 months of age (0.365 ± 0.018; $\chi^2 = 4.768$, df = 1, P = 0.029) and remained at this level through 36 months ($\chi^2 = 2.618$, df = 1, P = 0.106). The >36 to 42 age group increased again (0.395 ± 0.029; $\chi^2 = 4.365$, df = 1, P = 0.037), reaching similar to concentrations observed in >6 to 12 (0.380 ± 0.027; $\chi^2 = 0.145$, df = 1, P = 0.703) and >18 to 24 month old males ($\chi^2 = 0.175$, df = 1, P = 0.676).

Baseline glucocorticoid concentrations (Fig. 2B) were highest ($P \leq 0.024$) in the youngest males (0 to 6 months; 0.407 ± 0.056), compared to all other age groups. A significant decrease ($\chi^2 = 5.116$, df = 1, $P < 0.024$) occurred between the 0 to 6 and >6 to 12 (0.267 ± 0.038) month age groups; followed by a second decrease ($\chi^2 = 4.349$, df = 1, $P < 0.037$) between >6 to 12 and >12 to 18 (0.155 ± 0.038) months of age. After which, baseline concentrations remained relatively constant through 42 months of age (> 18 to 24: 0.189 ±

405 0.030; >24 to 30: 0.196 ± 0.038; >30 to 36: 0.205 ± 0.037; >36 to 42: 0.196 ± 0.052; P ≥
406 0.337 for all comparisons).

407 Average amplitude over baseline glucocorticoids (Fig. 2C) depicted a "wave-like"
408 pattern across ages. Similar to both average and baseline glucocorticoids, 0 to 6 month old
409 males exhibited the highest (0.467 ± 0.047 µg/g) amplitude values compared to all other age
410 groups ($P < 0.001$ for all comparisons). Amplitude then declined at each age group until >12
411 to 18 months (0.189 ± 0.022 µg/g; $P < 0.078$)). At >18 to 24 months (0.292 ± 0.017),
412 amplitude increased ($\chi^2 = 15.561$, df = 1, $P < 0.001$) from the previous age group. Values then
413 declined ($\chi^2 = 11.778$, df = 1, $P < 0.001$) between the >18 to 24 and >24 to 30 (0.217 ± 0.016)
414 month age groups, remaining low in the >30 to 36 month age group (0.190 ± 0.018; $\chi^2 =$
415 1.418, df = 1, P = 0.234), before rising ($\chi^2 = 6.233$, df = 1, $P < 0.013$) again at >36- to 42
416 (0.274 ± 0.030) months of age. However, it should be noted the "wave peaks" at >18 to 24
417 and >36 to 42 months were not significantly different, and were similar ($P \geq 0.188$) to the
418 waning value of the >6 to 12 (0.250 ± 0.028) month group.

419

420 **3.3** *Body Weights*

421 Facility was not a significant predictor of body weight, and was therefore removed
422 from the minimal model. Male cheetah body weight increased significantly ($P \leq 0.015$**)** during
423 each three-month interval evaluated from 3 months of age (7.1 kg ± 0.9) until 21 months of
424 age (44.5 kg ± 0.9) (Fig. 3). After 21 months, weights dropped slightly, but did not differ ($P \geq$
425 0.111) through 42 months of age (42.8 kg ± 1.4). Body weights at months 39 ($\chi^2 = 1.366$, df =
426 1, P = 0.243) and 42 ($\chi^2 = 0.898$, df = 1, P = 0.343) did not differ from those at 18 months of
427 age (41.2 kg ± 0.8).

428

429 4. **Discussion**

430 Identifying when males become pubertal is important for *ex situ* management as it
431 allows animal care staff, population managers and researchers to determine when it is
432 necessary to separate males from their dam and female siblings, and when they can begin to
433 participate in an SSP breeding program either through natural mating or sperm donation. In

young cheetahs, this time period is especially sensitive as significant management events occur, such as separation from the natal unit and formation of coalitions, generating a complex physiological and behavioral time period. For the first time, we have utilized our unique access to a large number of juvenile cheetahs to explore the physiology associated with onset of puberty in this species, monitoring cheetahs from as early as just under 2 months of age, up to 42 months. Our data suggest that pubertal onset in *ex situ* male cheetahs occurs at 18 to 24 months of age, supported by an increase in mean and baseline fecal androgen production and a transition towards adult fecal androgen amplitude production in the >18 to 24 month old group. Additionally, all males in the current study achieved adult body weights at 21 months of age and demonstrated altered glucocorticoid production across this pubertal time interval.

The rise in androgens observed in our study in males from >18 to 24 months old to concentrations within the range of fecal androgen levels measured in adult cheetahs (>24 months of age) in a previous study (Koester et al., 2015a) may be reflective of the initiation of testosterone production prior to reaching threshold values necessary for spermatogenesis. While the duration of spermatogenesis is not known in the cheetah, one full cycle takes roughly 60 days in both jaguars (*Panthera onca*) (Costa et al., 2008) and ocelots (*Leopardus pardalis*) (Silva et al., 2010). Before spermatogenesis can successfully occur, there must be adequate concentrations of circulating testosterone. Therefore, peripheral testosterone concentrations will be noticeably increased before production of fertile sperm is observed in the ejaculate. Based on previous data, the youngest *ex situ* male cheetah to sire a litter was just over 22 months old (Marker, 2015). This is a rare case, as the average age at first siring a litter in captivity is 5.8 years (Marker, 2015), though it should be noted that this is confounded by a plethora of management factors and likely does not accurately capture the physiology of these animals. Even in what appears to be a comparatively young sire (22 months), the androgen concentrations would presumably have to increase, at the very latest, by approximately 20 months of age to allow spermatogenesis to occur, fitting well into our predicted pubertal timeframe of 18-24 months. It is also important to note that early spermatogenesis may not result in the same quality of sperm as older males with longer exposure to elevated androgen concentrations. Indeed, both free-ranging and captive juvenile (< 2 years) male cheetahs in Namibia exhibited decreased sperm motility, forward progressive status, seminal volume and

motile spermatozoa compared to males > 2 years (Crosier et al., 2007). In the same study, three males approximately 14 months of age were sampled. Only one of these 14 month old males produced mature sperm, and the concentration and quality of the sperm and ejaculate were much lower than the older males sampled in the study (Crosier et al. 2007).

By >30 to 42 months of age, males in the present study had androgen baseline concentrations that fit into the range previously published for adult males (Koester et al., 2015a), with no significant differences observed between the oldest two age groups. In adult cheetahs, androgen concentrations varied by individual; however, provided a threshold level of production was maintained, this variation did not have a significant influence on sperm production as all adult males produced viable sperm (Koester et al., 2015a,b). All of these factors are evidence for increasing androgen production from developing testes as necessary to support successful production of viable mature spermatozoa. While overall it appeared androgen concentrations increased at >18-24 months of age, it should be noted that among the raw data individual distinctions were observed. Just as in humans and many other species, puberty is not a "one size fits all" event and some individual variation in age of onset should be expected.

Due to the great variation within an individual's daily androgen concentration values over time, it was of interest to measure this disparity across age groups. Amplitude, or concentration above baseline, was utilized to quantify this variation in androgen profiles. Although in adult male cheetahs, amplitude variation had no correlation with ejaculate quality (Koester et al., 2015a), increased androgen production is associated with testicular activation and increased sperm production in hyenas (van Jaarsveld and Skinner, 1991) and many seasonal wild canids including the grey wolf (Kreeger, 2003), red wolf (Walker et al., 2002), and coyote (Minter and DeLiberto, 2008). Overall, androgen amplitude increased with age in young male cheetahs indicating intensification of testosterone production may be required to initiate and maintain sperm production. This pattern suggests an intensification of testicular activity, with an increase in androgen output as easily measurable evidence. The >18-24 month group was similar to both young (0 to 12 month) and older (>24 to 30, >36 to 42 month) males indicating a possible transitional period between juvenile and adult testosterone production patterns. While previously published androgen amplitude data do not exist for adult male cheetahs, it is clear that the increase in amplitude does not vary significantly, but

496 remains high, between >30 to 36 and >36 to 42 month olds in this study. Relatively high
497 androgen amplitude suggests the older males have reached threshold androgen production for
498 maintaining spermatogenesis and may continue at this magnitude for the duration of a male's
499 reproductive lifespan.

500 One unexpected, yet interesting finding from this study was the baseline
501 concentrations of androgens in the youngest age group were similar to those of the older
502 males, as we expected them to exhibit the lowest androgen concentrations. This is presumably
503 unrelated to the onset of puberty, but may be a 'post-natal surge', or resulting from lactational
504 transfer as seen for cortisol in humans (Neelon et al., 2015) and other primates (Hinde et al.,
505 2015). The post-natal surge, or "mini-puberty", of gonadal steroids have been documented in
506 a number of species such as the rat (Ojeda and Skinner, 2006), chimpanzee (Winter et al.,
507 1975), and human (Forest et al., 1973; Gendrel et al., 1980). In both humans and
508 chimpanzees, the post-natal surge is described as a response of the infant's hypothalamic-
509 pituitary-gonadal (HPG) axis to the immediate removal from the maternal endocrine
510 environment (Winter et al., 1975). In humans, androgen concentrations of "mini-puberty"
511 peak between 1-3 months of age and decline around 6 months of age (Winter et al., 1976).
512 Recently, the post-natal surge has also been documented in the domestic cat (Faya et al.,
513 2013), whereby fecal gonadal metabolite concentrations were greater in the first four weeks
514 following birth compared with values obtained during weeks 5-14 of life (Faya et al., 2013).
515 These domestic cat patterns were similar to those described in humans, but occurred at a
516 much faster rate, however, the differences in age at which the pattern change occurred are
517 expected given the size and development difference between the species. Therefore, it seems
518 plausible that the unexpected high baseline concentrations of fecal androgens seen in 0-6
519 month old cheetahs in the present study may be evidence of the post-natal surge in gonadal
520 steroids. It is worth noting that the elevated concentrations of fecal androgens did not last the
521 full duration of the 6 months. In fact, when fecal androgens were first observed in the two
522 youngest males included in this study (SCBI n = 2; not from the same litter) at 1.9 months and
523 3.3 months of age, the values, which were well above any other recorded data point for this
524 study, had already peaked and continually declined through 6 months of age (data not shown).
525 This may also explain why there is no significant difference between the daily and baseline
526 androgen concentrations of the 0-6 and <6-12 month old groups. Interestingly, the only two

hand-raised male cheetah cubs (DZ n=2) in this study did not exhibit similarly elevated androgen production during the 0 to 6 month age group, suggesting there may be some maternal influence in the observed increase. The 0-6 month old group may have exhibited even greater androgen concentrations in the absence of the hand-raised males. Thus, the lower androgen concentrations from the hand-raised males and the consequence of our 6-month categorical grouping, may mask the true post-natal surge in the youngest age group. Identifying the post-natal surge in future studies would be of interest, particularly if there are aftereffects from management decisions early on that may affect future reproductive capabilities.

Cheetahs in this study also exhibited increased concentration and amplitude of glucocorticoids at approximately the same time as pubertal onset, between 18 and 24 months of age, when compared with both the preceding and following age groups. This increase concentration and variability may be a consequence of the increased metabolic demand of puberty, or may reflect associated physiological changes occurring within the hypothalamic-pituitary-adrenal axis. Previous studies have shown that in rodents, pre-pubertal males exhibited prolonged corticosterone production in response to the same stressor stimulus when compared to adults (Goldman et al., 1973; Romeo et al., 2004a, 2004b; Vázquez and Akil, 1993). The physiological consequences of the extended hormone response and subsequent exposure remain to be elucidated, but due to the important role of corticosterone in energy metabolism, pre-pubertal animals may exhibit different metabolic demands during a stress response compared to adults (Klein and Romeo, 2013). Throughout the course of puberty in rodents, changes in an individual's stress response were abrupt, shifting from a pre-pubertal to a more adult-like pattern of ACTH and corticosterone release (Foilb et al., 2011). Therefore, it is possible the glucocorticoid concentrations in <12--18 month old cheetah males are indicative of pre-pubertal hormone release patterns. Glucocorticoid patterns after 24 months of age, particularly the baseline concentrations, do not express dramatic variation across age, suggesting a matured HPA axis and adult glucocorticoid release pattern.

Alternatively, the increased glucocorticoid production at 18-24 months compared to both >12-18 and >24-30 months of age may be explained by the many management housing changes occurring for these males during this time. Data on fecal steroid hormone metabolites in the cheetah provide pooled information from an entire day (Pribbenow et al. 2016). If an

558 animal has a prolonged stress response, hormone is produced, metabolized, and excreted in
559 higher quantities over a longer period. For example, institutions typically will begin
560 separating cubs from their mother around 18 months of age to mimic independence based on
561 wild observations (Caro, 1994). Shortly after separation from their mother, the males are
562 slowly removed from female siblings. These dramatic management changes could prompt a
563 stress response and account for the increased glucocorticoid concentrations in the >18-24
564 month group. Interestingly, just as observed with androgen concentrations, the youngest
565 males (0-6 months) exhibited unexpectedly high glucocorticoid concentrations. Whether the
566 increase in glucocorticoid concentrations is also in relation to the early activation of
567 hypothalamic-pituitary axes, lactational transfer, or simply responses to stressful stimuli
568 remains to be studied. Similar to that observed for the androgen concentrations, the two hand
569 raised males in this study did not exhibit the high concentrations of glucocorticoids that was
570 observed in mother reared males of the same age. Again, this suggests that lactational transfer
571 may be responsible for this increase in hormone concentrations.

572 Prior to this study, information regarding body weight in cheetahs under 24 months of
573 age was scarce. As expected, body weight increased with age in young male cheetahs and
574 continued to increase until 21 months of age. This measurement has been shown to be an
575 indicator for pubertal onset, for example in ewes (Levasseur and Thibault, 1980), rats (Ojeda
576 and Skinner, 2006), humans (Baker, 1985), and non-human primates (T. M. Plant and
577 Witchel, 2006). Many mammals, particularly females, become pubertal after achieving a
578 particular body weight (Garcia et al., 2002). Rhesus macaques (*Macaca mulatta*) with higher
579 body weights, achieved first ovulation earlier than those with lower body weights (Zehr et al.,
580 2005). Mechanisms for this include linear rising concentrations of serum leptin from an
581 increasing number of adipocytes acting as a proposed signal for pubertal onset in rodents
582 (Ahima et al., 1997; Chehab et al., 1996; Cheung et al., 1997) humans (Frisch, 1984; Issad et
583 al., 1998; Quinton et al., 1999), and cattle (Wiltbank *et al.*, 1966; Garcia *et al.*, 2002). While
584 the majority of these "body weight thresholds" for pubertal onset research have been geared
585 towards females, it should be considered that males would reproductively benefit from
586 achieving a particular body size. Larger males are better suited for maintaining territory and
587 competing for access to females. If 21 months is the age at which captive male cheetahs attain

adult size, it can be assumed pubertal onset would have occurred before or around this age, supporting the endocrine findings from this study.

After 21 months of age, small, non-significant fluctuations in weight were documented, most likely due to adjustments in husbandry and feeding management. As young cheetahs grow, diets are increased to accommodate developmental demands. However, once the animal reaches maximum body size, excess calories may be stored as fat. There is likely an adjustment period for animal care staff to provide adequate calories without allowing excessive weight gain. Until this plateau is reached, it is expected that the animals' weights may fluctuate. This may explain the slight dip in weight after 36 months of age as adult diets perhaps were adjusted accordingly. From a management perspective, understanding the weight gain of captive cheetahs is important to ensure nutritional needs are being met while avoiding overfeeding and obesity, as carrying excessive weight has been linked in other species to decreased fertility (Fan et al., 2015; Jungheim et al., 2012; Michalakis et al., 2013). By providing a first look into the weight gain patterns of young cheetahs, this information will aid in creating a guide to help influence management decisions regarding nutritional and developmental requirements.

Due to the non-invasive approach of this study, the metrics by which we defined pubertal onset, fecal androgen concentrations and body weights, are not without limitations. As mentioned earlier, puberty in traditional model and domestic species is often defined by behavioral observations (Romeo et al., 2002), first presence of sperm in seminiferous and epididymal tubules (Stewardson et al., 1998), ejaculate (Asa, 2010), or urine (Nysom et al., 1994) indicating successful spermatogenesis and fertility. While informative, these are difficult measurements to obtain from cheetahs. Currently, the method by which semen is routinely collected from cheetahs is via electroejaculation, which requires anesthesia. It is not feasible to attempt repeat electroejaculation procedures on an individual during the timeframe of suspected pubertal onset. Likewise, it is also difficult to obtain opportunistic urine samples from individual males to observe any evidence of spermituria, especially in group living situations as is often the case with male cheetah coalitions. While androgen concentrations elucidate part of this physiological story, they are not informative of either the spermatogenic state or behavior of these males. It would be interesting to collect semen and perform routine

behavioral observations from this proposed 18 – 24 month old age group to get a more rounded view of the pubertal process in captive male cheetahs.

In conclusion, it was determined that captive male cheetahs begin puberty around 18-24 months of age based on increasing androgen concentrations, and reach adult body size at 21 months of age. This timeline supports previous observations from the wild where sibling groups become independent from their mother at approximately 18 months of age (Caro, 1994). As males become pubertal it would make sense for them to disperse from their mothers to avoid inbreeding as well as avoid confrontation with fully developed adult males. Glucocorticoid patterns indicated high concentrations in young males, followed by a decline and subsequent rise in concentration around puberty. The effects of external stressors and HPA activity during this developmental period in the cheetah is outside the scope of this study and is suggested for future research as this is important for the successful development, management, and propagation of *ex situ* cheetahs. Additional research on the effects of mother-rearing compared to hand-raising cubs in both physiological and behavioral development, as well as what components are in cheetah milk that may be transferred to offspring, are warranted. Increasing our understanding of captive cheetah behavior, health, and reproductive milestones, such as onset of puberty, is essential to improving management techniques and reaching a self-sustaining captive population.

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**Figure 1:** Mean (± SEM) prediction from the generalized linear mixed model (GLMM) for daily concentration (A), baseline (B) and amplitude over baseline (C) androgen concentration across male cheetah age groups, taking into account non-independence of data. Letters denote significant differences ($P<0.05$) within predicted hormone concentrations across age-categories. N denotes the number of cats in each category.

**Figure 2:** Mean (± SEM) prediction from the generalized linear mixed model (GLMM) for daily concentration (A), baseline (B) and amplitude over baseline (C) glucocorticoid concentration across male cheetah age groups, taking into account non-independence of data. Letters denote significant differences ($P<0.05$) within predicted hormone concentrations across age-categories. N denotes the number of cats in each category.

**Figure 3:** Mean (± SEM) prediction from the generalized linear mixed model (GLMM) for body weight across male cheetah age groups, taking into account non-independence of data. Letters denote significant differences ($P<0.05$) in body weight across age-categories.

**Table 1**: Fecal androgen and glucocorticoid concentrations (µg/g dry feces) per cheetah by age group. n denotes sample number analyzed per age group for each male. Coalition mates are denoted by matching symbols. The hand-raised coalition is denoted by an asterisk (*).

| | | | Raw Androgen Data (µg/g dry feces) | | | | Raw Glucocorticoid Data ( µg/g dry feces) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Animal ID** | **Age group (months)** | **Location** | **Mean** | **SD** | **Min** | **Max** | **Mean** | **SD** | **Min** | **Max** | **n** |
| 1❖ | >6-12 | A | 0.49 | 0.07 | 0.41 | 0.63 | 0.52 | 0.26 | 0.25 | 0.96 | 8 |
| | >12-18 | A | 0.34 | 0.12 | 0.12 | 0.87 | 0.35 | 0.23 | 0.13 | 1.25 | 52 |
| | >18-24 | A | 0.41 | 0.12 | 0.16 | 0.69 | 0.37 | 0.20 | 0.16 | 1.17 | 65 |
| | >24-30 | A | 0.50 | 0.25 | 0.14 | 1.38 | 0.31 | 0.19 | 0.11 | 1.12 | 55 |
| | >30-36 | A | 0.57 | 0.21 | 0.24 | 1.35 | 0.34 | 0.24 | 0.09 | 1.57 | 44 |
| 2 | 0-6 | A | 0.47 | 0.34 | 0.31 | 1.97 | 0.61 | 0.54 | 0.08 | 2.22 | 22 |
| | >6-12 | A | 0.41 | 0.13 | 0.21 | 1.05 | 0.26 | 0.26 | 0.06 | 1.53 | 50 |
| | >12-18 | A | 0.49 | 0.18 | 0.31 | 0.89 | 0.10 | 0.08 | 0.04 | 0.31 | 13 |
| 3◆ | >12-18 | B | 0.31 | 0.10 | 0.11 | 0.50 | 0.17 | 0.08 | 0.08 | 0.33 | 17 |
| | >18-24 | A | 0.46 | 0.13 | 0.23 | 0.84 | 0.34 | 0.26 | 0.05 | 1.59 | 56 |
| | >24-30 | A | 0.45 | 0.11 | 0.25 | 0.83 | 0.37 | 0.20 | 0.12 | 1.12 | 76 |
| | >30-36 | A | 0.57 | 0.15 | 0.23 | 1.23 | 0.34 | 0.20 | 0.10 | 1.15 | 74 |
| | >36-42 | A | 0.50 | 0.13 | 0.05 | 0.86 | 0.32 | 0.19 | 0.04 | 0.93 | 42 |
| 4◆ | >12-18 | B | 0.41 | 0.11 | 0.25 | 0.70 | 0.28 | 0.31 | 0.08 | 1.38 | 16 |
| | >18-24 | A | 0.54 | 0.21 | 0.26 | 1.63 | 0.51 | 0.42 | 0.14 | 2.35 | 57 |
| | >24-30 | A | 0.40 | 0.10 | 0.07 | 0.68 | 0.38 | 0.19 | 0.17 | 1.15 | 76 |
| | >30-36 | A | 0.53 | 0.17 | 0.25 | 1.28 | 0.31 | 0.20 | 0.10 | 0.96 | 76 |
| | >36-42 | A | 0.49 | 0.14 | 0.25 | 0.92 | 0.48 | 0.33 | 0.14 | 1.52 | 43 |
| 5 | >12-18 | C | 0.41 | 0.18 | 0.19 | 0.89 | 0.20 | 0.09 | 0.04 | 0.50 | 34 |
| | >18-24 | C | 0.60 | 0.24 | 0.25 | 1.43 | 0.16 | 0.09 | 0.04 | 0.55 | 50 |
| | >24-30 | C | 0.68 | 0.27 | 0.24 | 1.36 | 0.20 | 0.18 | 0.06 | 0.76 | 29 |
| 6* | 0-6 | D | 0.34 | 0.07 | 0.22 | 0.51 | 0.18 | 0.11 | 0.04 | 0.46 | 23 |
| 7❖ | 0-6 | A | 0.52 | 0.17 | 0.35 | 1.03 | 1.08 | 0.98 | 0.43 | 4.45 | 14 |
| | >6-12 | A | 0.32 | 0.09 | 0.19 | 0.55 | 0.30 | 0.17 | 0.12 | 1.06 | 38 |
| | >12-18 | A | 0.32 | 0.10 | 0.11 | 0.57 | 0.25 | 0.12 | 0.10 | 0.56 | 53 |
| | >18-24 | A | 0.38 | 0.11 | 0.14 | 0.67 | 0.38 | 0.30 | 0.05 | 1.63 | 74 |
| | >24-30 | A | 0.35 | 0.13 | 0.14 | 0.89 | 0.39 | 0.28 | 0.13 | 2.09 | 75 |
| | >30-36 | A | 0.57 | 0.30 | 0.19 | 1.85 | 0.43 | 0.29 | 0.14 | 1.57 | 62 |
| 8∞ | >6-12 | A | 0.41 | 0.06 | 0.30 | 0.50 | 0.54 | 0.41 | 0.17 | 1.79 | 19 |
| | >12-18 | A | 0.28 | 0.08 | 0.13 | 0.58 | 0.38 | 0.34 | 0.09 | 1.71 | 51 |
| | >18-24 | A | 0.21 | 0.07 | 0.08 | 0.37 | 0.39 | 0.25 | 0.09 | 1.03 | 31 |
| 9 | >18-24 | A | 0.51 | 0.13 | 0.39 | 0.79 | 0.52 | 0.36 | 0.14 | 1.02 | 6 |
| | >24-30 | A | 0.33 | 0.13 | 0.05 | 0.83 | 0.30 | 0.18 | 0.10 | 1.02 | 78 |
| | >30-36 | A | 0.26 | 0.11 | 0.15 | 0.87 | 0.36 | 0.17 | 0.15 | 1.27 | 78 |
| 10◆ | >12-18 | B | 0.37 | 0.10 | 0.20 | 0.66 | 0.30 | 0.23 | 0.09 | 0.95 | 18 |
| | >18-24 | A | 0.52 | 0.27 | 0.11 | 2.29 | 0.45 | 0.46 | 0.11 | 2.82 | 58 |
| | >24-30 | A | 0.46 | 0.10 | 0.27 | 0.82 | 0.44 | 0.34 | 0.13 | 2.21 | 73 |
| | >30-36 | A | 0.58 | 0.16 | 0.29 | 1.30 | 0.25 | 0.14 | 0.09 | 0.83 | 77 |
| | >36-42 | A | 0.62 | 0.17 | 0.28 | 0.99 | 0.42 | 0.36 | 0.04 | 1.62 | 41 |
| 11* | 0-6 | D | 0.33 | 0.10 | 0.18 | 0.52 | 0.13 | 0.06 | 0.05 | 0.29 | 21 |
| | >6-12 | D | 0.49 | 0.23 | 0.15 | 1.08 | 0.19 | 0.13 | 0.05 | 0.61 | 32 |
| 12∞ | >6-12 | A | 0.35 | 0.04 | 0.29 | 0.43 | 0.26 | 0.15 | 0.11 | 0.71 | 20 |
| | >12-18 | A | 0.38 | 0.22 | 0.15 | 1.71 | 0.30 | 0.24 | 0.07 | 1.19 | 54 |
| | >18-24 | A | 0.30 | 0.09 | 0.12 | 0.44 | 0.53 | 0.58 | 0.11 | 2.66 | 30 |

922