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Effect of age and body condition score on reproductive organ size and sperm parameters in captive male African lion (*Panthera leo*): Suggesting a prime breeding age

Imke Lueders [a,b,c,*], Johanna Reuken [c,d], Ilse Luther [e], Gerhard van der Horst [b,e], Antoinette Kotze [b], Adrian Tordiffe [b,f], Harald Sieme [d], Ulrike Jakop [g], Karin Mueller [g]

[a] GEOlifes - Animal Fertility and Reproductive Research, Berger 12c, Nordkirchen 59394, Germany
[b] National Zoological Gardens of South Africa, 323 Boom Street, Pretoria 0110, South Africa
[c] Westfälischer Zoologischer Garten Münster GmbH, Sentruper Str. 315, Münster 48161, Germany
[d] Unit for Reproductive Medicine of the clinics, University of Veterinary Medicine Hannover, Foundation, Bünteweg 15, Hannover 30559, Germany
[e] Department of Medical Bioscience, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa
[f] Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa
[g] Department of Reproduction Biology, Leibniz Institute for Zoo and Wildlife Research, Reproduction Biology, Alfred-Kowalke-Straße 17, Berlin 10315, Germany

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ABSTRACT

Sexual maturity and body condition are interconnected in many mammals. For non-domestic felids held in human care, the effect of body condition on male fertility has not been studied, although obesity is a recognized problem for many species such as the African lion *(Panthera leo*) under captive conditions.

Here, we assessed body weight, body condition via body condition score (BCS), reproductive organ appearance and size by ultrasonography and semen parameters as a function of age in a large population of captive male lions housed in different facilities in South Africa. Of 59 individuals (age range 2.5–10 years), we rated 21 (36 %) of the males ($\geq$ 4 years) as highly obese (BCS 8–9). Semen collection via urethral catheter was successful in 49 males (83.0 %). Sperm were found in 44 males (74.6 %) across all age groups. Sperm motility and detailed sperm morphology was assessed in 42 and 18 lions, respectively.

As expected, body weight and reproductive organ size increased with age, but only the testis size increased in proportion to BCS. Although our data are unbalanced, as older animals (already after five years of age) were more often obese, they suggest an optimum age and body condition for potential fertility with adequate physical maturity in male lions. Physical maturity appeared to be reached between the ages of three and five years, which is later than sexual maturity in male lions.

Lions that far exceeded the normal body weight range and had a BCS of 8 or 9 had lower semen quantity and quality. Therefore, male lions in captivity are in prime breeding condition when physical maturity is combined with a BCS $<$ 8.

Our data contribute towards interpretation of fertility assessments in managed breeding programs for lions. Ultrasonographic appearance of the reproductive organs, BCS and semen parameters can help to identify and select breeding males.

1. Introduction

The African lion (*Panthera leo*) represents one of the largest felids and, along with the Asiatic lion (*Panthera leo persica*), is the only big cat that lives permanently in social groups [1]. Latest estimates suggest that Africa's wild lion numbers have declined by ~75 % during the last five decades [2]. As wild lion populations continue to decline, the species is currently classified as "vulnerable" by the International Union for Conservation of Nature and Natural Resources (IUCN) [3] and the value of captive individuals as a potential pool of genetic resources increases. Thus, a priority is to uphold genetic diversity and high fertility to maintain healthy captive lion populations. Compared to smaller, solitary

* Corresponding author at: GEOlifes - Animal Fertility and Reproductive Research, Berger 12c, Nordkirchen 59394, Germany.
E-mail address: imke.lueders@gmail.com (I. Lueders).

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roaming felid species, lions appear to reproduce with relative ease in captivity. However, assisted reproduction techniques (ARTs) may offer additional tools to support breeding of endangered felids as well as to connect *in situ* and *ex situ* populations [4,5]. In this context, comprehensive research and fundamental understanding of lion reproductive anatomy and physiology is particularly important to identify breeding males and, at the same time, risk factors for male sub- and infertility.

Ultrasonography is a basic, non-invasive approach for the *intra vitam* assessment of reproductive organs in many domestic and non-domestic species [6]. Several studies exist, looking at the relation between ejaculate quality and ultrasonographic male reproductive organ characteristics for different mammal species. In domestic and non-domestic species such as the dromedary camel (*Camelus dromedarius*) [7], domestic cattle (*Bos taurus*) [8], or elephants (*Loxodonta africana and Elephas maximus*) [9], it was found that ultrasonographic examinations of the male genital tract facilitates predictions of fertility and semen quality. This information would also be helpful in breeding soundness evaluation of lions. However, to date, no information on male reproductive sonomorphology and the relations between organ size, age, body condition and sperm quality is available for lions or other feline species.

Recently, a study assessed the on onset of puberty in captive male lions [10]. Puberty was defined as the process of acquiring reproductive competence [10]. The sexual maturity is then the functional capability of an organism to reproduce (sperm production), while physical maturity is reached, when full body size and weight are reached. Prime breeding age is therefore defined as the age at which both, sexual and physical maturity are reached and therefore the physical capability to reproduce is gained. This is reflected in full body weight, fully grown reproductive organs and high-quality ejaculates.

For many male mammalians it is suggested that reproductive success increases shortly after reaching sexual maturity, and forms a short plateau phase during a certain age, therefore giving males a certain window for peak fertility [11]. Male African lions reach sexual maturity (sperm production) at the age of two years in the wild and as early as 1.2 years in captivity [10], but have not gained their maximum body mass at this age [12]. Therefore, they may not have the opportunity to reproduce (lack of physical maturity). Male lions are usually only able to defend females against other mature males once they have gained the physical ability, which is not reached before the age of four to six years in the wild [1,13,14]. The breeding age (age of first sired young) as the beginning of the reproductive lifespan occurs therefore two to four years after reaching sexual maturity. Several authors assume an average reproductive period of three years for male lions, which reflects the time span between the onset of breeding opportunities with females after the establishment of a harem until the death or displacement of the male from the pride [1,13,14]. Although males can lead a pride as early as 2.8 years, this only appears to occur in reserves free of older males [15].

Lions in captivity have a tendency to become overweight, due to a lack of opportunities to burn calories while being supplied energetic nutrition [16]. It has been shown already that captive lions gain faster in body weight and appear to reach puberty much earlier compared to wild counterparts [10]. As the breeding age depends not only on age, but also on body condition, we hypothesize that sexual maturity and physical maturity may occur closer together in captive male lions compared to wild males.

To find characteristics for the postulated prime breeding age in lions (sexual and physical maturity), we measured reproductive organ sizes, determined sperm parameter, and examined their associations with age and body condition in 59 African lions in different captive populations within South Africa.

2. Material and methods

2.1. Study animals

This study was performed on 59 captive born, male lions housed in six different breeding facilities in South Africa, with an age range of 2.5–10 years. Data were collected during routine health checks and always in accordance with the Directive 2010/63/EU EEC for animal experiments and all procedures had ethical committee clearance in South Africa (National Zoological Gardens Research Ethics and Scientific Committee Project number NZG/ P12/17). All lions were kept in outdoor enclosures permanently, containing natural substrate and wooden or brick shelters and further structures such as natural trees, logs and/or rocks. The male lions were kept with one or more conspecifics (male or female) or temporary solitary with visual and olfactory contact to other lions. The lion diets consisted of beef (entire carcasses or meat with bones) and entire chicken carcasses.

2.2. Body weight and condition scoring

Once anaesthetized, body weight was recorded prior to reproductive examination. Two different portable scale systems were used: a High Precision Heavy Duty hanging crane scale a weight beam floor scale with adjustable platform. The animals were either hung to the crane scale in a stretcher or moved onto the platform of the beam scale. The weight of the stretcher/carry canvas was subtracted.

A 9-point body condition scoring system for lions, with body condition scores (BCS) ranging from 1 (emaciated) to 9 (extremely obese) according to the Association of Zoos and Aquaria (AZA) Animal Care Manual [17] and originally adapted from domestic cats [18,19], was applied. The detailed scoring scale for lions used in this study has been applied to assess lions in U.S. zoos [20]. The BCS < 4 are equaling too low body weight, BCS 4–5 are regarded as ideal, BCS 6–7 as slightly to moderately overweight and BCS 8–9 as obese to grossly obese.

Two exemplary photographs of lions with BCS 9 are shown in Fig. 1. In extremely obese animals (BCS 9), per AZA definition, the neck region was bulging and convex, and appeared continuous with head and shoulder. Excessive pendulous fat accumulated beneath neck, chest and abdomen. Back, hips and waist were rounded, filled and continuous and their tail base was noticeably thickened [17].

2.3. Definition of terms

For this study and the discussion of results, we have defined certain recurring terms in connection with male lion reproductive activities as follows: Sexual maturity is defined here as the status when gonads are active and semen is produced. Physical maturity is defined here as the status when a sexually mature lion male is fully grown in body weight and reproductive organs are fully developed in size (maximum values reached). Fertility is defined here as the ability to produce viable sperm and the absence of reproductive tract pathologies, irrespective of the actual ability/opportunity to mate.

Prime breeding age is defined here as achievement of both, sexual and physical maturity. Reproductive lifespan is defined as the time between first successful reproduction (actual mating and impregnating a female) and last successful reproduction of an individual. Species reproductive lifespan is defined as the age range in which naturally successful reproduction may be observed in lions. We anticipate that successful breeding males have reached sexual maturity, fertility and physical maturity at prime breeding age and that this represents the beginning of the reproductive lifespan.

2.4. General Anesthesia

Lions were immobilized with two different drug combinations: Either with (1) medetomidine (0.015–0.025 mg/kg IM; 20 mg/ml,

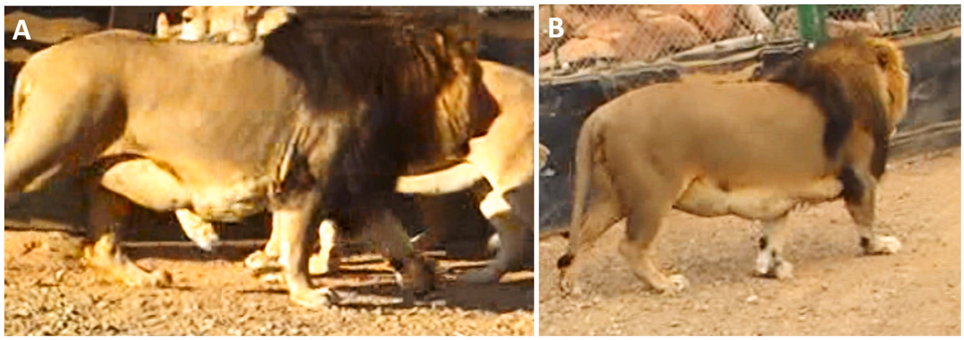


Fig. 1. Two examples for severely obese captive male African lions with a body condition score (BCS) of 9.

Kyron laboratories Pty (Ltd), Johannesburg, South Africa) and zolazepam-tiletamine (0.6–1.0 mg/kg IM; Zoletil 100®, Virbac RSA Pty Ltd., Centurion, South Africa); or with (2) medetomidine (0.03–0.04 mg/kg IM) and ketamine (2.0–4.0 mg/kg IM, Ketamine 10 %, Kyron laboratories Pty (Ltd), Johannesburg, South Africa). The drugs were delivered intramuscularly via remote dart gun. Total procedure time ranged from 40 to 115 minutes.

The alpha-2-agonist was reversed with either yohimbine (0.175–0.25 mg/kg IM, Yohimbine 6.25 mg/ml, Kyron Laboratories Pty (Ltd), Johannesburg, South Africa) or with atipamezole (0.2 mg/kg IM, Antisedan®, 5 mg/ml, Orion Corporation, Espoo, Finland) administered by hand injection.

2.5. Ultrasound examination

The ultrasonographical examination of lions was carried out as described in a previous report [21]. A portable, battery driven ultrasound machine (Logic e, General Electric Healthcare GmbH, Solingen, Germany) equipped with a 7–10 MHz endolinear probe (i739L-RS) was used. The ultrasound probe head was fixed by taping it into a PVC extension made of a 50 cm long hollow PVC pipe with a diameter of 1.5 cm. A schematic overview of the lion urogenital anatomy is given in Fig. 2 (left).

Transscrotal ultrasound was performed to evaluate the testes (Fig. 2 A, B) and the epididymides (Fig. 2 C). Testes were measured without scrotal skin. Instead of shaving the testes, alcohol-based disinfectants were used to wet the fur and ensure good coupling. After emptying the rectum with a tap water enema, transrectal ultrasound was applied to visualize the accessory sex glands (Fig. 2 D-F) and anal scent glands (Fig. 2 G). The prostate was divided into the right and left prostate lobe (Fig. 2 D) connected by the prostate midpiece (Fig. 2 E) above the urethra (Fig. 2 E, H).

Video sequences of 15 seconds or 2D still images (Fig. 2 A-H) of each organ were recorded. Measurements were obtained retrospectively from these records. To facilitate statistical evaluation of ultrasonographically measured organ dimensions in relation to age, BCS or semen quality, an approximation for the area (cm^2) was calculated using length (cm) × height (cm) at the largest expansion of the respective organ. For paired organs (testes, prostate lobes, bulbourethral gland), a mean of the area for left and right is given. For the caput epididymis, which appeared round in the cross-section ultrasound, the largest diameter was determined, and the mean diameter of both sides is given.

2.6. Semen collection

Semen collection was performed via urethral catheter (UC) as previously described for lions [21]. Prior to the UC semen collection, an enema was given to remove feces from the rectum, an external and internal ultrasound examination was carried out, and each testicle was gently pulled to stretch the spermatic cord and facilitate sperm release into the urethra. For the semen collection, the penis was extended beyond the prepuce, cleaned, and a lubricated (non-spermicidal lube (ReproJelly®, Minitube, Tiefenbach, Germany), commercial dog urinary catheter (Buster, sterile dog catheter, WDT, Garbsen, Germany), either 2.6 mm×500 mm or 3.3 mm×500 mm was inserted into the external urethral opening. The catheter was advanced within the urethra

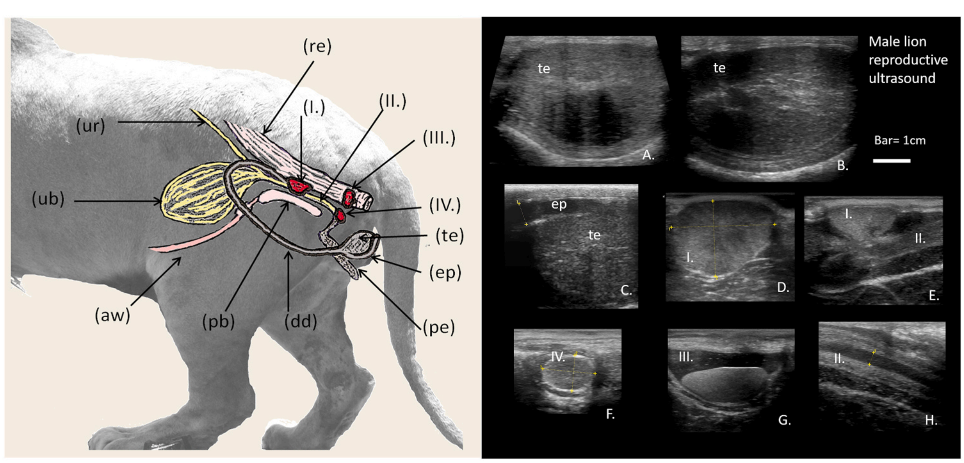


Fig. 2. Schematic image of the urogenital tract (left) and sonoanatomy of the respective organs in male lion (right). The drawing serves and an overview of location of organs observed by transrectal ultrasonography: I. prostate, II. pelvic urethra, III. anal sent gland, IV. bulbourethral gland. Other structures for orientation are: aw = abdominal wall, dd = ductus deferens, ep = epididymis, pb = pelvic bone, pe = penis, re = rectum, te = testis, ub = urinary bladder, ur = ureter. Reference ultrasound images show by transcutaneous scanning: A./B. testicle; C. corpus epydymidis; by transrectal scanning: D. one prostate lobe; E. prostate midpiece bridging atop the urethra; F. bulbourethral gland; G. anal scent gland; H. urethra with fluid filling. All ultrasound images in relative size, normalized to 1 cm (bar).

under ultrasonographic control until the prostate was reached. Once at the prostate, the catheter was slowly retracted. The catheter was inserted as deep as 30 cm. To avoid reaching the bladder, 20–25 cm may be deep enough to catch a sperm rich semen fraction in male lion. When semen was present within the catheter lumen by capillary forces, it was subsequently ejected into a prewarmed 1.5 ml Eppendorf tube for further processing by previously published methods [22]. To prevent small amounts of semen from drying out, these samples were immediately diluted with a certain volume (1:2) of pre-warmed tissue culture medium M199 (Hepes modification M7528, Sigma-Aldrich, Germany) and immediately given to the field lab for analysis.

2.7. Semen analysis

Collected semen samples were processed as previously described [22]. In brief, each sample was immediately evaluated for volume and sperm motility. After dilution of a semen aliquot with prewarmed tissue culture medium M199 and five minutes of incubation at 38°C, total motility (the percentage of motile sperm) was subjectively estimated by two independent and skilled observers under a Nikon E50i microscope (Nikon, Japan) with a heating stage (38°C) and phase contrast optics (100× magnification). A further semen aliquot of 10 µl was diluted with distilled water at a ratio 1:10 up to 1:200 (v/v) to determine the sperm concentration in a counting chamber (Neubauer improved, Paul Marienfeld GmbH & Co. KG, Germany) as described previously [23]. The total sperm count was determined by multiplying the concentration by the original volume collected.

For morphological evaluation of the sperm, an aliquot of semen was fixed with 1 % formaldehyde in Dulbecco's phosphate buffered salt solution (DPPS, D8537, Sigma-Aldrich, Germany) at a ratio of 1:10 (v/v) within 15 min after collection and transferred to the lab facility where the microscope (Axioskop, Zeiss, Germany) was available and further diluted by DPBS as required for assessment. At least 200 sperm per sample were evaluated under phase contrast optics and 1000× magnification for defects of their acrosome, head, midpiece, tail or both, head and tail. The number of sperm in each category was related to the total number of 200 cells. Since cytoplasmatic droplets and the particular anomaly of "knobbed" acrosomes were observed in several morphological categories, their total percentages across all categories were given separately. Since the majority of cytoplasmic droplets were distal droplets, no further distinction was made between proximal and distal positions.

2.8. Dataset and statistical evaluation

Table 1 shows the numbers of the 59 examined animals classified by age and BCS. The assignment of the animals of different age and BCS classes to the facilities are shown in Suppl. Table 1. Lions of this study ranged from BCS 4–9. Table 2 shows the number of animals in which organ parameters were measured, semen collection was attempted, semen/sperm collection was successful and semen parameters were determined. Due to time constraints on site, cauda diameter visualization was only carried out in 52 males, bulbourethral gland area measurement in 58 males, and sperm motility data were only determined in 42 out of the 44 individuals with successful sperm retrieval. A detailed morphological evaluation of the sperm was only carried out on a subset of only 18 (9 animals each from Facility A and B) in a limited study period. The number of individuals tested (N) is always indicated in tables and figures.

Data analysis was performed using IBM SPSS Statistics 24 (SPSS Inc., IBM, NY, USA). It is evident that there is a bias in the data as older males ($\geq$ 5 years) are more likely to be obese (BCS 8 or 9) compared to the younger individuals (Table 1). This makes it difficult to clearly distinguish between the influence of age and BCS. To test, whether age or BCS have a main or an interactive effect on body weight, reproductive organ sizes as well as on semen/sperm parameters, a generalized linear model (GzLM) was used. Parameters were considered as dependent variables, age and BCS were considered as main factors, and an interaction term (age × BCS) was included in the model. In some cases, selected parameters were directly correlated with each other using Spearman's rho. Effects and correlations were considered statistically significant if $P<0.05$.

Visualization of data in scatter plots or box plots was performed by Sigma Plot 10.0 (Systat Software, Inc., CA, USA). To visualize the expected growth of the animals and their reproductive organs, the age dependence of body weight and organ sizes is shown in scatter plots. If the GzLM confirmed an effect of age, the data were exponentially fitted (solid line) to the maximum (plateau) and the 95 % confidency band is shown (Figs. 3–4). For this purpose, only animals with a BCS < 8 were included in the growth fit. Dark-filled data points are added for the obese males (Figs. 3–4). Box plots were used to visualize the effect of BCS on semen parameters (Fig. 5). Data points were also represented by different symbols to indicate the age of the males.

3. Results

3.1. Age, body weight and body condition score (BCS)

The body weight was dependent on age, BCS and a two-way interaction of both factors (Table 3). Our BCS ranged from 5 to 9 with only one animal classified at BCS 4, thus covered only normal to grossly overweight body conditions. Of the 39 lions $\geq$ 5 years of age, 19 (49 %) developed obesity (BCS 8) or severe obesity (BCS 9; Table 1). Only two of the 20 males < 5 years of age (10 %) were already categorized as obese at an age of 4 years (Table 1). In total, 21 males (35 % of all 59 males) were characterized by a BCS 8 or 9. Eleven males of BCS 9 had a body weight > 260 kg, and ten males of BCS 8 had a body weight > 230 kg (Fig. 3 A). One five -years- old male of more than 230 kg was only classified as BCS 7. The heaviest male weighed 314 kg at an age of six years.

If the body weight of the non-obese animals (BCS < 8) was exponentially fitted to maximum as a function of age, the growth curve reached its maximum at a plateau of 203.6 kg (Table 4, Fig. 3A). The average body weight of three- and four-year-old lions reached already > 90 % of the plateau body weight, and most (7/10) non-obese five-year-old males had reached the plateau (Suppl. Table 2). The mean body

Table 1
Numbers of examined captive African lions in different age classes. Individuals were classified by different body condition scores (BCS).

N	Age [years]											
	2.5	3	3.5	4	4.5	5	5.5	6	7	8	9	10
BCS 4	0	1	0	0	0	0	0	0	0	0	0	0
BCS 5	2	0	0	4	1	0	0	0	0	1	0	0
BCS 6	1	1	2	1	1	4	1	0	0	3	0	1
BCS 7	0	2	1	1	0	6	1	0	0	2	0	1
BCS < 8	3	4	3	6	2	10	2	0	0	6	0	2
BCS 8+9	0	0	0	2	0	5	1	4	3	3	3	0
%	*0*	*0*	*0*	*25*	*0*	*33*	*33*	*100*	*100*	*33*	*100*	*0*
total	3	4	3	8	2	15	3	4	3	9	3	2

Table 2
Numbers of captive African lions examined for organ parameters (ultrasound), semen collection via urethral catheter (collection attempt), presence (semen successful) and quality of sperm (sperm successful). Individuals were classified by different body condition scores (BCS).

N	Individuals	Organ parameters* determined	Collection attempt	Semen successful	Sperm successful	Motility* tested	Morphology* evaluated
BCS < 8	38	38	38	34 (89 %)	30 (79 %)	29	11
BCS 8+9	21	21	21	15 (71 %)	14 67 %)	13	7
total	59	59	59	49 (83 %)	44 (75 %)	42	18

* Due to technical problems, the total number of cauda diameter measurements was only 52 with 33 and 19 individuals for BCS < 8 and BCS 8+9, respectively. The bulbourethral gland was only measured in 58 individuals, data of one obese male is missing. Motility was not recorded in two males (8 years, BCS 8 and 4 years, BCS 5). Morphology was only determined for 18 samples in a limited study period.

weight of obese lions exceeded the plateau level and reached 130 % of the plateau body weight.

3.2. Ultrasonographic appearance of reproductive organs

The lion reproductive tract anatomy appeared similar to that of other wild felid species and the domestic cat (Fig. 2). Lion testes were oval shaped and symmetrical (Fig. 2 A, B). The testicular echotexture was homogeneous with a comparable echogenicity typically seen for the spleen. The mediastinum or rete testis appeared as a hyperechogenic, central line or lines (Fig. 2 A, B). The mean (across all ages, all BCSs and both sides) length of testes ± SD measured 4.1 ± 0.7 cm (range 3.2–5.2 cm). The corresponding heights were 2.5 ± 0.4 cm (range 1.7–3.7 cm). No major pathologies were recorded, besides two cases of testis abnormalities: A size asymmetry was observed in one animal (9 years, 258 kg, semen collection not successful), the dimensions of the smaller, rather normal-range, testis was used for statistical calculations. An inhomogeneous parenchymal echotexture was observed in one testis of another male (3 years, 220 kg, semen collection successful). The epididymes were much less echogenic compared to the testes (Fig. 2 C). The corpus epididymis was detectable along the testis in longitudinal view (Fig. 2 C) and the caput was measured at the cranial pole of the testis and appeared as the most frequently identifiable part measuring 0.5 ± 0.1 cm (range 0.3–1.0 cm) in diameter. When inserting the endolinear probe rectally, following on the urethra, caudal of the bladder neck, the prostatic gland appeared as most prominent structure (Fig. 2 D, E). The prostate consisted of two lobes, one each left and right to the urethra (Fig. 2 D) which were connected by the prostatic midpiece (Fig. 2 E), which bridged on top of the urethra. Left and right lobes of the prostate appeared roundish to plum shaped in the ultrasound picture measuring 2.8 ± 0.4 cm (range 1.8–3.9 cm) in length and 2. ± 0.4 cm (range 1.2–3.4 cm) in height. The mid piece had rather a flat to triangular shaped appearance in the longitudinal view. Its length was 2.4 ± 0.3 cm (range 1.8–3.3 cm), and its height was 1.0 ± 0.2 cm (range 0.5–1.4 cm). The texture of this gland was homogeneous and spleen-like, varying slightly in echogenicity between males. The other accessory sex gland was the paired bulbourethral gland (Fig. 2 F). The rather round bulbourethral glands became visible, located left and right from the caudal pelvic urethra, measuring 1.6 ± 0.2 cm (range 1.3–2.2 cm) in length and 1.3 ± 0.2 cm (range 0.9–1.8 cm) in height. They formed an irregular circle with a hyperechogenic capsule and an inhomogeneous texture (Fig. 2 F). The most caudal structures visible were the anal scent glands on either side of the anus. The anal glands usually had hypoechogenic to slightly echogenic content and their size and shape varied greatly depending on the filling (Fig. 2 G) with a mean length of 3.2 ± 0.7 cm (range 1.6–4.7 cm) and a mean height of 1.9 ± 0.6 cm (range 0.5–4.1 cm). Occasionally, hyperechogenic sediments appeared accumulated at the bottom of the gland (Fig. 2 G). When scanning ventrally in the midline of the rectum, the urethra was visualized in longitudinal view and was distinct by a hyperechogenic muscular wall (Fig. 2 H). Fluid accumulation, seen as anechoic content within the urethral lumen, was usually an indicator for successful semen collection by urethral catheter (Fig. 2 H).

In the same individual, the measurements of left and right testes, epididymes and accessory glands were not significantly different between the sides (with the one exception of testicular asymmetry). The mean values of both sides for the size of the reproductive organs in non-obese animals are shown in Suppl. Table 2 for different age classes. For comparison, the data for obese males are shown separately in Suppl. Table 2.

3.3. Age, BCS and reproductive organ size

An age dependency was evident for the size of all reproductive organs, with the exception of the caput diameter (Table 3). Figs. 3 and 4 provide an overview of the measurements of the reproductive organs as a function of age, using the mean values of the areas (length × width) or of the single diameters (width only in case of caput epididymis) of both sides. If the GzLM confirmed an effect of age, an exponential growth function was used to describe the development of organ size as a function of age (Table 4). To do this, we only included the non-obese individuals in the fit and added the data points for the obese animals to these graphs (Figs. 3 and 4). In Suppl. Table 2, the mean values of the organ sizes in different age classes (3, 4, 5, 8 years) were summarized and related to the plateau value (maximum) of the respective fitted growth curve. In most cases (except for the prostate lobe area), the non-obese males exceeded 90 % of the maximum value already at the age of three years. The plateau values were reached for all organ sizes at around the age of five years.

A dependency on both age and BCS was only confirmed for the testis area, and an interactive effect of age and BCS was found for the size of the bulbourethral gland (Table 3). This is also illustrated by the fact that the testis areas of the obese animals were mostly above the fitted line and the 95 % confidence band in Fig. 3 B. The interactive effect of age and BCS was evident, as the bulbourethral gland areas of young obese males were below the "normal" growth curve, while the data points for old, obese males are above it (Fig. 4 A). The mean values for the testis area of obese lions reach 120 % of the plateau value of non-obese lions (Suppl. Table 2). The size of the bulbourethral gland and prostate lobe of obese lions did not differ from the values of non-obese animals (104 % and 100 % of the plateau values, respectively). Growth of accessory sex glands was therefore not associated with the BCS.

As with BCS, the testis area naturally increased with body weight. This applied to all lions (*Rho* 0.494, $P < 0.001$) and to the non-obese lions only (*Rho* 0.447, $P = 0.005$), when looked at separately. However, there was a tendency for a decreased testis area to body weight ratio in males with the highest BCS of 8 and 9 (not shown).

3.4. Age, BCS and sperm parameters

Ejaculates containing spermatozoa were obtained from 44 out of the 59 animals (74.6 %). Urethral semen collection was successful in 89 % of males with a BCS < 8, compared to 71 % successful collections in males with a BCS 8 or 9. Sperm were present in 67 % of the ejaculates of the obese males compared to 79 % in the other group (Table 2).

The mean (across all individuals, all ages, all BCSs) ± SD for semen

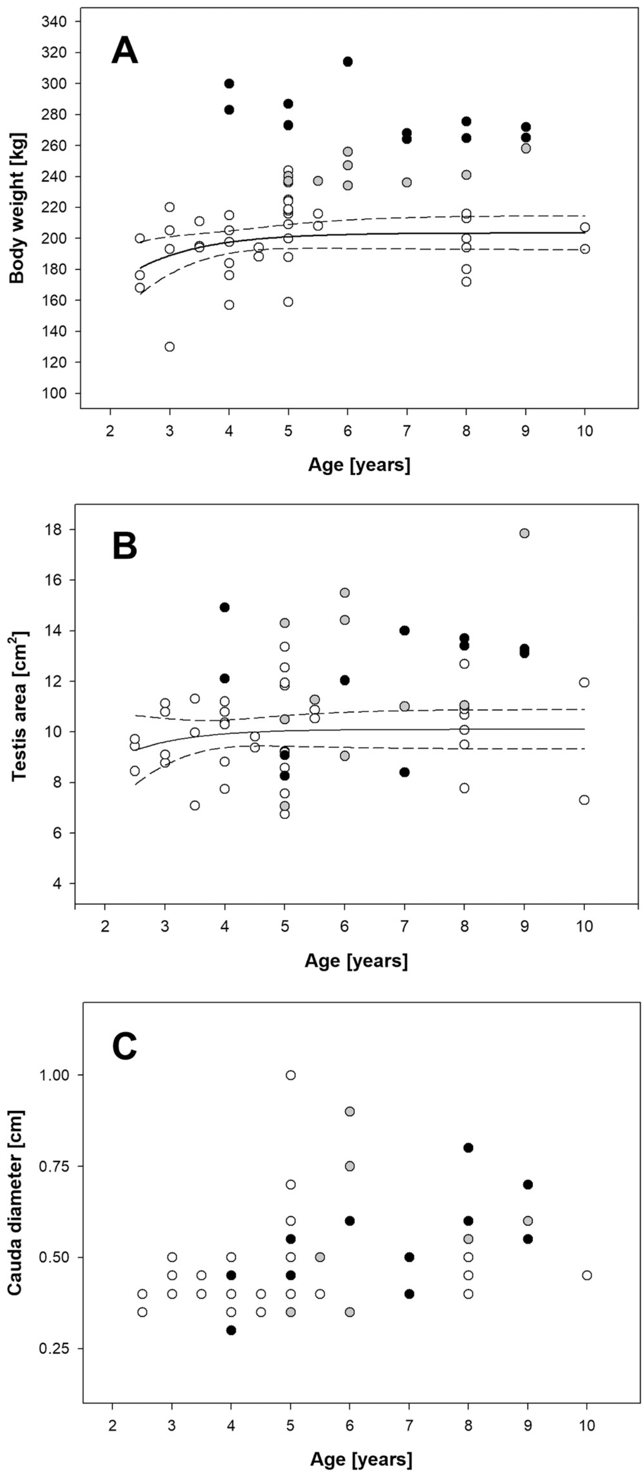


Fig. 3. Body weight (A), testis area (B), and cauda epididymis diameter (C) as a function of age in captive male African lions. Exponential fits as rise to maximum (plateau) and 95 % confidency bands are shown for the body weight and testis area of individuals with a body condition score (BCS) <8 (BCS 4–7, white symbols). Grey and black symbols represent individuals with a BCS of 8 and 9, respectively.

volume, sperm concentration, total sperm count, total motility and normal sperm were 268 ± 219 µl (range 5–900 µl), 1385 ± 1944 $\times 10^6$/ml (range 0–6500 $\times 10^6$/ml), 485 ± 517 $\times 10^6$ (range 0–2250 $\times 10^6$), 59 ± 28 % (range 5–96 %), 19 ± 15 % (range 0–47 %), respectively. The mean values of semen/sperm parameters in non-obese animals are shown in Suppl. Table 3 for different age classes. For comparison, the data for obese males are shown separately.

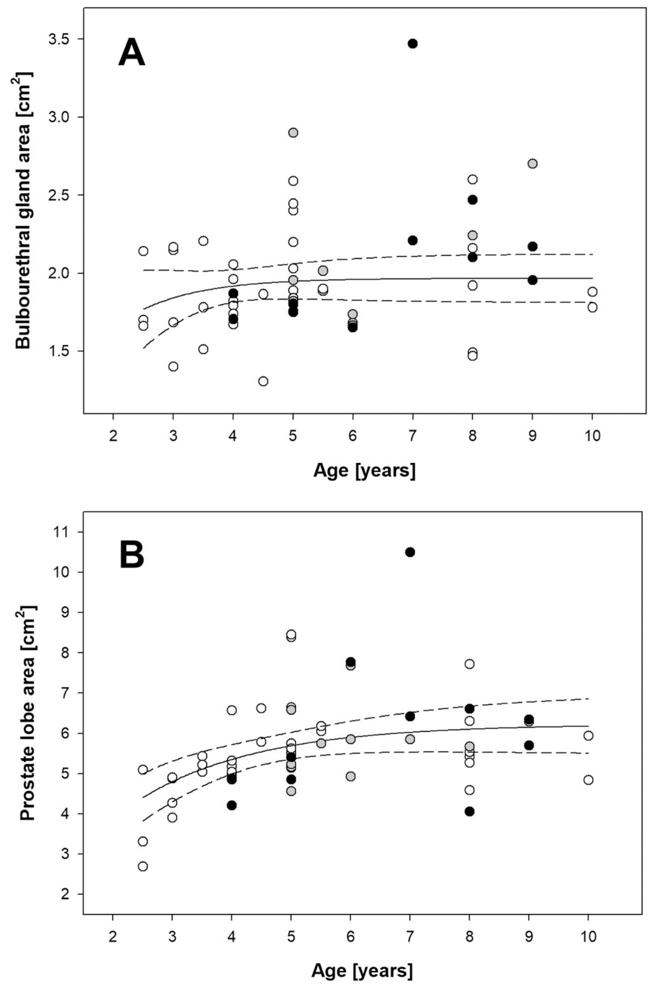


Fig. 4. Areas of accessory sex glands, namely Bulbourethral gland (A) and Prostate lobes (B) as a function of age in captive male African lions. Exponential fits as rise to maximum (plateau) and 95 % confidency bands are shown (see Table 4). Grey and black symbols represent individuals with a body condition score (BCS) of 8 and 9, respectively. White symbols belong to individuals with a BCS < 8 (BCS 4–7).

The interactive effect of age and BCS in the GzLM is particularly pronounced for the semen and sperm parameters (Table 3). This interaction made it impossible to separate the effects of age and BCS. Visualization of the data in dependence of BCS showed similar semen volumes in all groups with BCS > 5, including quite high volumes in severely obese lions (Fig. 5 A). In contrast, the total sperm count was low in severely obese males (Fig. 5 B). The highest absolute values for the sperm count were achieved at BCS 6 and the highest medians and means occurred at BCS 5–7 (Fig. 5 B). Within the cohort of males studied, highest absolute, median and mean motility values were achieved at BCS 6 and 7 (Fig. 5 C). Note that 18 (47 %) of the 38 non-obese lions with BCS < 8 were younger than five years of age. Ten (26 %) of these lions were only five years old.

The morphological analysis presented in Table 5 revealed that the most frequently detected sperm abnormalities were defects of the tail and the midpiece as well as "knobbed" acrosomes. The percentage of defect midpieces increased (*Rho* 0.567, P = 0.014) significantly with higher BCS. In contrast, sperm with "knobbed" acrosomes such as sperm with cytoplasmic droplets appeared to be less common in older animals and animals with high BCS (Table 5). Knobbed acrosomes occurred in nine males < 5 years, in four males at 5 years, and only in three males >

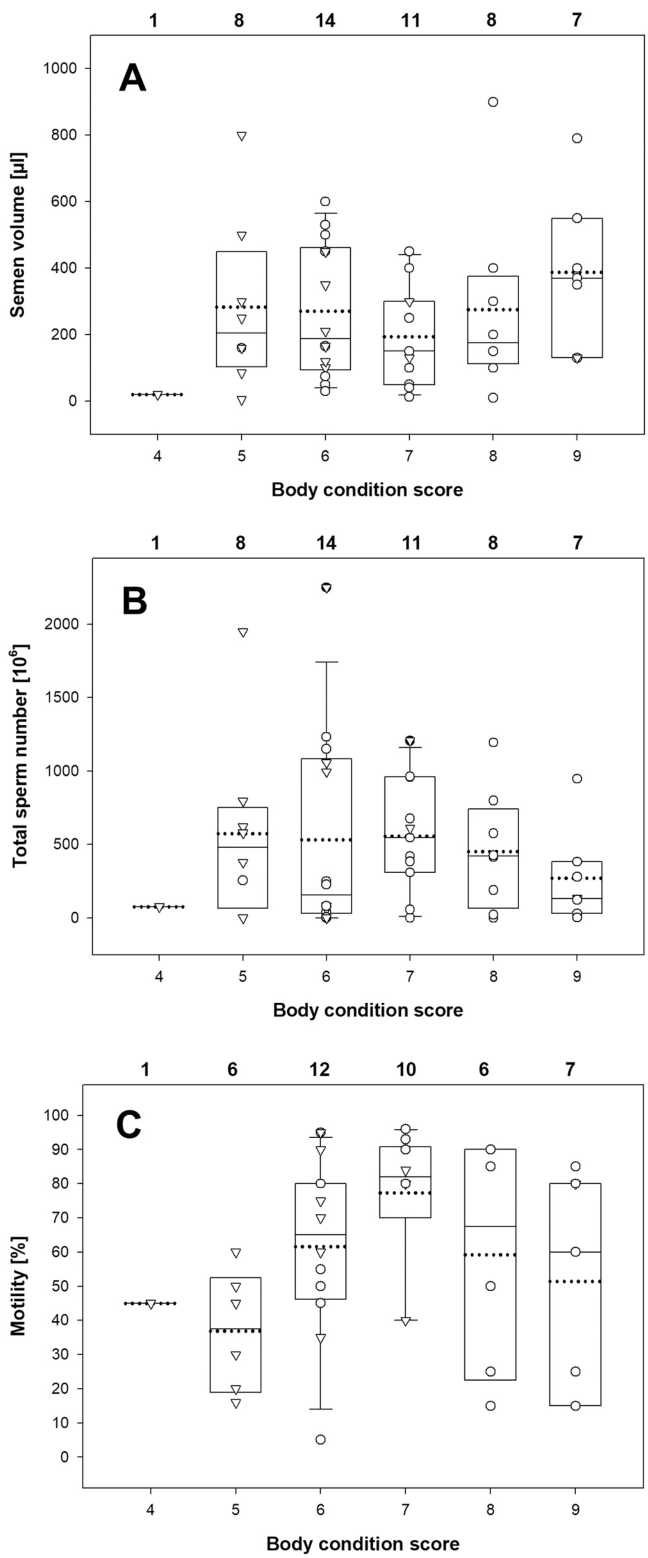


Fig. 5. Box plots for the semen volume (A), the total sperm count in ejaculate (B) and the sperm motility (C) in dependence of body condition score (BCS) in captive male African lions. The number of individuals per BCS is given above the respective box on top of the graph. The means are shown as dotted lines. Triangles represent young individuals (2.5–4.5 years), circles represent older individuals (5–10 years).

Table 3

Effects of age and body condition score (BCS) of captive male African lions on body weight, organ sizes and semen parameters in a generalized linear model. P-values for the main effects and interaction are given and considered significant if $P < 0.05$ (light grey) or $P < 0.01$ (grey).

		Wald-Chi-Quadrat	df	Sig.
Body weight	BCS	671.114	6	0.000
	Age	31.651	11	0.001
	Age * BCS	50.018	16	0.000
Testis area	BCS	19.883	6	0.003
	Age	34.514	11	0.000
	Age * BCS	24.597	16	0.077
Cauda diameter	BCS	7.287	6	0.295
	Age	16.817	11	0.113
	Age * BCS	20.935	15	0.139
Bulbourethral gland area	BCS	11.171	6	0.083
	Age	49.594	11	0.000
	Age * BCS	27.104	15	0.028
Prostate lobe area	BCS	7.861	6	0.248
	Age	35.583	11	0.000
	Age * BCS	22.178	16	0.138
Semen volume	BCS	22.606	6	0.001
	Age	14.025	11	0.232
	Age * BCS	67.324	13	0.000
Sperm concentration	BCS	10.83	6	0.094
	Age	20.376	11	0.040
	Age * BCS	22.014	13	0.055
Total sperm number	BCS	17.703	6	0.007
	Age	56.407	11	0.000
	Age * BCS	69.86	13	0.000
Motility	BCS	19.259	6	0.004
	Age	20.541	11	0.038
	Age * BCS	33.691	11	0.000

Table 4

Exponential fit of body weight, testis area, bulbourethral gland area and prostate lobe area as a function of age in captive male African lions. Only individuals with body condition scores < 8 were included. Plateau and b-values are given according to the formula for rise to maximum (plateau).

y = max(1 - e$^{-b\ *\ \mathrm{Age}}$)		
y	**max (plateau)**	**b** [years^{-1}]
Body weight	203.6 kg	0.8775
Testis area	10.10 cm^2	1.0055
Bulbourethral gland area	1.97 cm^2	0.9194
Prostate lobe area	6.22 cm^2	0.4932

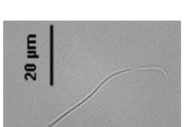
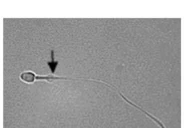
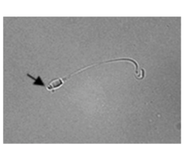
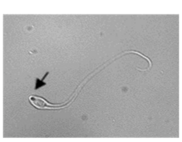
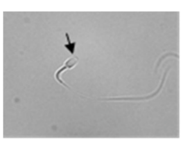
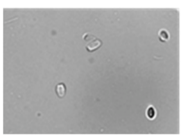
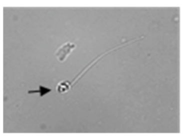
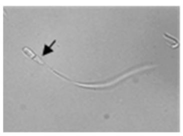
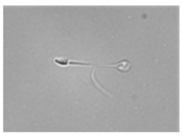
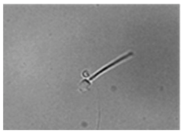
5 years of age. Cytoplasmic droplets occurred in eight males < 5 years, in four males at 5 years, and in five males > 5 years of age. In two individuals (3 and 4 years old) even more than 50 % of the sperm were counted in both categories. Both sperm with cytoplasmic droplet and sperm with "knobbed" acrosome were able to move if no other defect was evident.

3.5. Reproductive organs and sperm parameters

To answer whether ultrasonography may be useful to predict sperm quantity and quality, testis and accessory sex gland sizes were correlated with the main sperm parameters (volume, concentration, total sperm count, total motility, percentage normal sperm). No significant correlation was found between the size of the accessory sex glands and the sperm parameters, neither when all males nor only the non-obese males were considered (not shown).

However, the testis area was positively correlated to sperm concentration (*Rho* 0.729, $P < 0.001$), total sperm count (*Rho* 0.514, $P = 0.002$) and motility (*Rho* 0.556, $P = 0.002$) in non-obese lions. The strength of the correlation (*Rho*) decreases when the obese males are included. This corresponds to the fact that testis size did not develop proportional to the body weight in males with an extremely high BCS, where the ratio of testis area to body weight tended to become low (not shown). As already

Table 5
Morphological sperm characteristics in ejaculates obtained from captive African lions with a body condition score (BCS) of 4–5 (n = 5), of 6–7 (n = 6), and of 8–9 (n = 7). Mean ± SD (range) is shown for the percentages in each category. Cytoplasmic droplets and "knobbed" acrosomes are also summarized across all categories (*).

	Normal sperm	*Cytoplasmic droplet total across all categories**	*„Knobbed" acrosome*	„Knobbed" acrosome only	Detaching or lost acrosome	Loose head	Defect head	Defect midpiece	Defect tail	Defect head and tail
										
BCS 4–5	23.2 ± 11 (20–40)	*39.0 ± 23 (8–64)*	*34.6 ± 28 (1–68)*	15.4 ± 16 (0–34)	2.8 ± 2 (1–5)	3.0 ± 2 (1–5)	4.6 ± 4 (0–10)	8.2 ± 5 (2–14)	33.8 ± 18 (13–58)	9.0 ± 4 (4–15)
BCS 6–7	22.3 ± 18 (4–46)	*15.2 ± 13 (0–32)*	*11.2 ± 9 (3–28)*	2.5 ± 3 (0–8)	3.3 ± 3 (0–7)	7.2 ± 4 (2–13)	6.0 ± 6 (1–17)	12.8 ± 4 (6–19)	27.5 ± 19 (4–51)	18.3 ± 17 (4–44)
BCS 8–9	13.1 ± 16 (0–47)	*11.1 ± 9 (1–25)*	*6.4 ± 8 (0–24)*	0.4 ± 1 (0–2)	3.1 ± 4 (0–10)	7.4 ± 5 (2–15)	1.6 ± 1 (0–3)	23.1 ± 19 (0–57)	30.9 ± 19 (6–54)	20.3 ± 12 (4–40)

shown in Fig. 5, the total count and motility of sperm is low in many animals with BCS 8 or 9 from which semen was obtained. In addition, semen collection success was lower in obese males (Table 2) compared to BCS <8.

4. Discussion

This study reports on reproductive physiology of male African lions as a function of age and body condition. In a large number of male lions, born and bred in different breeding centers across South Africa, we analyzed age, body weight, BCS, and measured reproductive organ size ultrasonographically as well as semen parameters from samples collected via urethral catheter.

With regard to the growth of the 2.5–10-year-old captive animals, it was expected that the animals – similar to the captive lion males in zoos – would grow faster than their wild counterparts and may reach their adult weight already at the age of 3–4 years [24]. The constant supply of optimum nutrition under human care and missing natural challenges such as conflict with conspecifics or diseases may account for this. The growth curve, which reflected the mean body weights as a function of age, had its plateau at a body weight of ~204 kg, if the obese animals (BCS 8+9) were excluded. The average weight gain ceased only at the age of five years when the maximum body weight of the cohort of non-obese animals was reached. This growth period was closer to the situation reported for the wild lions with 2250 days until physical maturity [24]. However, there was a great variability between individuals of the present study. Many males were considerably heavier than the mean body weight of 187 ± 5 kg recorded for adult free roaming lions [25], a weight that was determined in the current study at an average age of three years. Recent data on captive lions in western zoos [17] indicate for males at the age of seven years a mean body weight of 190 kg (range: 140 – 240 kg). Thus, on average, adult non-obese lions in our study were more than 10 kg heavier compared to wild and zoo lions. In our total cohort, 17 males weighed more than 240 kg, seven of them even had a body weight of more than 270 kg. In contrast, the heaviest wild lion male on record weighed 272 kg (Mount Kenya) [24].

Body weight alone may not always give a good indication for the body condition as it also correlates with body size. Therefore, we performed a body condition scoring and 49 % (29/59) of all animals in this study were in moderate overweight body condition (BCS 6–7). Additional 21 males (36 %) were even classified with extremely high BCS according to the AZA scoring system (BCS 8–9) indicating severe obesity. Two of the latter males were only four years old. Previous assessments have shown that the BCS has a strong linear relationship with the body weight of lions [24]. Extrapolating BCS from body weights, it was suggested that wild lion BCS range from 2.5 to 5.25 with an average BCS of 4.9 [26]. However, the body size and frame also play a role, and very large males with higher body weights are not necessarily overweight. In our study, BCS was assessed independently from body weight and provides additional information. We may conclude that the captive lions examined were on average fully grown at five years of age, but 36 % had developed obesity between the ages of four to ten.

Ultrasonographic results are not only the basis for the investigation of physiological and pathological processes, but potentially also for the differentiation between fertile, sub- and infertile conditions. Here we applied ultrasonography to monitor the lion's reproductive organs size and appearance. Analyzing the size development of reproductive organs as a function of age, their growth (like the body weight gain) is completed on average at five years. An association to the BCS was only found for the testis area, which is larger in males with higher BCS. However, in a number of obese animals (BCS 8–9), testis size did not follow weight gain and the ratio of testis size to body weight decreased (not shown). This is an indication of a natural limit to a species' testicular size. Overall, lions in captivity can be expected to reach physical maturity by the age of five, as measured by both body weight and reproductive organ size, with exceptional weight gain having no effect on the size of the bulbourethral glands or prostate lobes and no further increase in testicular size.

Only two pathologies (3.4 %) of the urogenital tract were observed. This may be a first indication that common problems found in domestic cats such as testicular neoplasia, infection of the urogenital tract, testicular hypoplasia or degeneration [27], which are associated with a negative impact on spermatic parameters, are not common in captive lions. However, the individuals studied here were relatively young (2.5–10 years). The average life expectancy in captive lions is reported to be around 20 years [28], and it is well known that the prevalence of pathologies, such as tumors rises with animal age in carnivores. The influence of human breeding selection is also lower in captive lion populations than in domestic species. Captive lion populations may still benefit from natural selection in previous non-captive generations, which leads to elimination of genetic predisposition of reproduction related pathologies.

There are only few studies on semen quantity and quality in lions. Semen collected by UC in the present study had a higher sperm concentration and a lower volume than in other studies with lions in which the ejaculates were obtained by electro-ejaculation (EE) [29,30]. The high sperm concentration and low semen volume of ejaculates collected by UC are consistent with previous studies of this method in lions [21,22] and tomcats [31]. As already described by Zambelli et al. in 2010, one of the big differences between semen collection by UC and EE is the lower volume of accessory sex gland fluids for the UC method [32].

Within the age range of the lions available for the study, sperm was successfully collected at any age. With the successful sperm collection from males at the age of 2.5 years, we confirmed basic sexual maturity at that age. Although male lions can live up to 16 years in the wild, they usually only reach a maximum age of 10–12 years [33]. In the present study, we thus covered the age range after reaching sexual maturity and the reported species reproductive lifespan of wild lion males (4–9 years). High total sperm counts ($\geq 1\times10^9$) were collected from males between 2.5 and 8 years of age, but most of these males had a BCS 6. Almost all obese males with BCS 9 had a lower sperm counts ($<0.5\times10^9$).

In the present study, the average sperm motility of UC semen was within the range of other studies: total motility was slightly higher in samples obtained from adult wild lions by EE (mean of 83–91 %), but comparable to results from EE of captive zoo lions (61.0 %) [29] and young wild lions between 3.3 and 4.5 years (59–72 %) [30]. In a previous study of ours, UC total motility was also higher than our current results, averaging 84 % [20]. In contrast to all other studies, which examined a maximum of 7–17 lion male lions, here we have motility results for 44 animals from different age classes and BCS. A larger variability in sperm motility is therefore not surprising. Motility values higher than 70 % were found in our cohort of males across all ages, but most of these males had a BCS 6 or 7. A BCS > 5 proved to be a precondition, but not a predictor of high motility values, as sperm motility was on average tending to be worse in obese males.

Males with BCS 5, which were scored for sperm motility, were all younger than five years and their motility was below average. This contrasted with the relatively good morphology of these samples in comparison to semen samples from the obese males. However, the samples from males with BCS 4 or 5 had also the highest average percentages of sperm with cytoplasmic droplets and "knobbed" acrosomes. Sperm with cytoplasmic droplets are in principle motile but can be considered as immature, because the cytoplasmic droplets were not properly removed during epididymal transit and ejaculation. With increasing BCS (essentially associated with higher age), maturation obviously improved, but midpiece defects and double defects of head and tail increased at the same time. These defects occurred most frequently in obese lions and are fatal for sperm functionality because energy supply, motility and proper nucleus and acrosome formation are impaired.

The proper formation of the acrosome normally occurs during the final steps of spermiogenesis in the testis. A particular anomaly of “knobbed” acrosome was observed in a number of the lions studied and was counted separately due to the increased occurrence in young animals, in this case immature lions. However, three of the older males (> 5 years) were also affected, albeit to a lesser extent. In some domestic species, this defect is a heritable defect that is associated with impaired fertility. It has a variable ultrastructural appearance and has also been observed in rhinoceroses and cheetahs (for review see [34]). It is very likely that a genetic background favours this anomaly in captive lions in South Africa, as they probably share a common genetic origin [35]. Although sperm with a “knobbed” acrosome are in principle able to move, their plasma membrane function is probably impaired, as has been observed in bull sperm [36].

Sexual maturity and fertility are not simply related to the age at which sperm are produced, but also to physical body condition, as mammals reach sexual maturity precociously when a certain body weight/body fat mass is attained [37–39]. This has also been shown to apply to male lions in captivity [10]. In our study, BCS 6 proved to be optimal for sperm count and motility but is one score higher than the “normal” BCS of adult wild lions and is already considered the onset of overweight. The faster weight gain of male lions under captive conditions to higher maximum weights is the reason why among the males in our study group which were ≥5 years, only one individual had a BCS of <6. These data on the non-obese lions (BCS<8) suggest that physical maturity can be reached in captivity as early as at three years of age, but certainly by five years of age. About three years after sexual maturity, by the age of five, fully grown gonads and accessory sex glands had developed and a higher BCS (>6) had been reached in almost all studied males. At BCS 6 or 7, sperm quantity and quality were highest. We are aware that the evaluation of only a single ejaculate limits the significance of the data. However, in all other studies on lion semen mentioned in this paragraph and in other studies on felid species [28] only one sample was utilized per individual. In addition, we have unpublished data from another study in which the same lions were used for two semen collection methods seven days apart, demonstrating similar individual quality in both runs. We also assume that fertile lions, as non-seasonal breeders [1], produce semen continuously. Breeding males usually copulate several times within a relatively long receptive period of the female.

Despite a higher weight gain which resulted in generally heavier animals, our findings for the postulated prime breeding age fit the reported onset of breeding activities with 4–5 years and the natural age in which a pride may be conquered earliest by a male or several males in the wild (physical maturity) [33]. A study on mane length and darkness found that darker, longer manes appear to reflect maturation and testosterone level and reach a maximum after the age of 5 years in wild lions in the Serengeti [40]. Since sperm production may occur as early as 1.2 years in captive males [10], our findings underline the gap between sexual and physical maturity in lions. Male lions need a minimum body size (physical maturity) to take a pride over and typically succeed to remain in their position for up to three years, which reflects their reproductive lifespan [1,13,14]. A study on captive lion breeding success also found an average reproductive lifespan of 3.3 years for male lion [41]. These findings are interesting because, despite the human influence on social composition and feeding regime, the natural timing of sexual and physical maturity development in breeding males appears to remain relatively unaffected.

Extremely high body fat accumulation in obese animals is known to be negatively correlated to health and longevity in domestic felids and other mammalian species including human [42]. The effect of excessive body weight on male fertility is primarily monitored in men for decades, but controversially discussed, because most studies are population-based and molecular mechanisms behind have only recently become a field of research [43,44]. However, decreases in sperm count, concentration, motility, morphology, and an increase in DNA damage have been reported, leading to reduced sperm performance in assisted reproduction *in vitro* [43].

Our study covers a cohort of over-conditioned male lions, reared in captivity on high caloric diet based on whole chicken carcasses with a high content of subcutaneous fat. As with the studies on humans and mice, the effects of obesity on sperm parameter were not drastic, but they do have consequences if the semen is to be used for sophisticated assisted reproduction techniques. In a previous study, we observed that lion sperm survived cryopreservation better, if the seminal fluid from their ejaculates had a high capacity to remove chemical radicals [19]. This high radical reduction capacity was associated with a low incidence of lipid oxidation products in erythrocytes of the lion [22]. In men, data suggest that the function of pro-oxidant and antioxidant systems in semen may directly influence basic semen parameters [45] and that this function may be reduced in obese males which show higher oxidative distress and lipid peroxidation [46,47]. Further studies are warranted to test if altered antioxidant activity in seminal plasma and sperm may play a role in sperm parameters of obese lions. Certainly, a BCS of 6 has revealed superior in young adult lion males in terms of sperm output and motility compared to BCS 4 or 5 in the youngest males of the study.

Finally, the present work provides the first large scale description of male lion reproductive sonoanatomy in connection to spermatology. Similar to other studies in domestic and wildlife species, we found correlations between testicle size and sperm parameters. The testis size is associated with a higher sperm motility and a high testis size in relation to body weight indicates a good sperm quality. A low testis size/body weight ratio in adult lions suggests a potential obesity with the risk of loss of sperm quantity and quality.

5. Conclusion

Captive male lions become overweight at relatively young ages. The incidence of moderate overweight (BCS 6–7) and even severe obesity (36 % with BCS 8–9) was already high from the age of 4 years in our study population. But only severe obesity may have an impact on semen quality, whereas light over condition does not seem to affect fertility in male lions. The BCS 6–7 revealed as optimal BCS in terms of ejaculate quality in the population studied here, with the highest sperm motility together with highest total sperm counts, while the average percentage of sperm defects, especially midpiece defects and double defects of head and tail, increased from BCS 4–9 in the subset of 18 samples analysed. We found only severely obese males are at higher risk of producing low sperm quantity and quality.

Our data suggest that prime breeding age is achieved at around 5 years, when plateau values for body weight and reproductive organ size are reached on average. Although the absolute body weights were somewhat higher compared to other published data, the timeframe to reach a plateau equals that for wild lions. Although due to higher nutrition and less mobility, puberty may be reached earlier in captive compared to wild lions [10], the timespan to reach prime breeding age may remain conserved.

This study also provides the first description of male reproductive sonoanatomy, and ultrasonographic examination of reproductive organs provides an indication of possible fertility, as testis size was associated with sperm motility and morphology. The growth of testis was partly associated to the BCS, but testis size in relation to the body weight decreased in obese males. Therefore, low relative testis size in older males is indicative for obesity and low sperm quantity and quality.

6. Limitations

This study concerned a large number of captive male lions in different facilities across South Africa. Semen collection in lions is only possible under full anaesthesia and therefore was only performed at a single point in time. The catheter semen collection appears however as a very practical and compelling method for lions and is established in other non-domestic feline species. Nevertheless, multiple additional factors may influence semen quality.

We had only a small number of “normal weight” lions. Ideally, a larger number of lions in BCS 4–5 would have been included in this study for better comparison. These animals were however not available and may reflect the problem of overfeeding in certain facilities in South Africa. While semen volume and motility were recorded in all successful collections, detailed analysis of the sperm morphology was only performed in 18 males.

Finally, the effect of human selection and thus genetic influences on fertility may remain a factor in isolated captive breeding situations. A recent genetic assessment of captive South African lions found however that the genetic composition is comparable to existing wild South African lion populations [35].

CRediT authorship contribution statement

Antoinette Kotze: Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Gerhard Prof. van der Horst:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Data curation. **Ilse Luther:** Writing – review & editing, Resources, Investigation, Data curation. **Johanna Reuken:** Writing – review & editing, Investigation, Funding acquisition, Formal analysis, Data curation. **Imke Lueders:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Karin Müller:** Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ulrike Jakop:** Writing – review & editing, Formal analysis, Data curation. **Harald Sieme:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Adrian Tordiffe:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that no generative AI and AI-assisted technologies were used in the writing process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2024.100093.

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