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Computer Assisted Sperm Analysis (CASA) in the Critically Endangered Captive Arabian Leopard (*Panthera pardus nimr*): A Multivariate Clustering Analysis

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Abstract

Rationale: Big felids including the *Panthera* genus are under tremendous stressful conditions that threaten the very existence of wild populations around the world. Survivability is commonly linked to numerous factors such as poaching, habitat fragmentation, inbreeding depression and lack of prey. A crucial element that is used to mitigate endangerment risk is the enhancement of reproductive performance with the use of assisted reproductive technologies. Amongst them is computer assisted sperm analysis (CASA) that digitally evaluates the kinematics of individual spermatozoa. Regrettably, this powerful tool is overlooked in all big felids due to the lack of a universal setting.

Objective: To conduct a comparative CASA with several species modules and to deploy it for the first time in the critically endangered Arabian leopard.

Results: The progressive motility was variable amongst all settings, whereby the highest in the bovine standard (82.9%), lowest in the stallion setting (12%), subjective (85%) and average at 50.1%. The combination of all motility parametrics, indicate a progressive joining of two minor and two major clusters with a very high distance of 93% and a linkage space of approximately 42%. This in turn demonstrate notable divergence of two important kinematic settings.

Conclusion: The current study illustrates the inconsistent and incompatible readings amongst various CASA species modules. This affirms the urgent need to establish CASA exclusively customized for the *Panthera* genus to maximize the reproductive potential.

Keywords: Semen evaluation; CASA; Arabian leopard; *Panthera pardus nimr*; Endangered species

Abbreviation

CASA: Computer assisted sperm analysis; ITIS: Integrated Taxonomic Information System; CR: Critically endangered; ART: Assisted Reproductive Technologies; EEJ: Electro-ejaculator; PBS: Phosphate buffer saline; PCA: Principal components analysis.

Introduction

The taxonomical hierarchy list of the *Panthera* genus shows 9 genetically proven *Panthera pardus* subspecies according to the integrated taxonomic information system (ITIS) [1] including the Arabian leopard (*Panthera pardus nimr*) [2]. The Arabian leopard is the largest living felid in the mountainous region of the Arabian Peninsula [3,4], but the smallest leopard subspecies [5]. Currently, it is indexed on the IUCN red list as critically endangered (CR) [6]. Notably, the world population of Arabian leopards has substantially declined over the past 30 years [3,7]. Retrospectively, not more than 200 animals have been known to inhabit the entire Arabian Peninsula [8], approximately 50 in Oman [9] and declared instinct in parts of the Middle East such as Jordan and Iraq [10,11]. The emergence of maladies in carnivores has been described for some time [12], but only recently, a plethora of diseases has been reported in big cats [13-20], including the Arabian leopard [21]. Moreover, poisoning [22], infanticide [23], climate change, and rising sea levels [24-26] are expected to increase the vulnerability and decrease the distribution and/or abundance of big cats around the world. The current habitat of wild Arabian leopards in Oman is under environmental stress [9] and disturbance in neighboring Yemen by the ongoing conflict prohibits cross-border movement of animals leading to genetic isolationism. Additional factors, including solitary animals inhabiting large ranges, seasonality, habitat fragmentation, and short breeding seasons, provide additional constraints [27].

While the challenges facing the survivability of large felids are numerous, it is prudent to deploy highly affective assisted reproductive technologies (ART) to mitigate endangerment risk, inbreeding

constraints and a growing aging population of the Arabian leopard. Amongst those technologies is computer assisted sperm analysis (CASA) that was designed almost four decades ago to digitally capture and objectively evaluate the kinematics of individual spermatozoa [28,29]. CASA has been recognized throughout the years as a practical, accurate and revolutionary method for analyzing sperm parameters [30-35]. While CASA is carried out routinely in livestock animals such as, bulls [36], goats [37], rams [38], buffaloes [39], and stallions [40], it is rarely used or scantly reported in the *Panthera* sp. This is largely due to the lack of affordability and computerized accessibility in several ex situ conservation sites and zoological institutes. Additionally, modern semen analysis is hindered by the fact that big cat ejaculates are small in volume, highly diluted, and inconsistent in color. Furthermore, the presence of urine, lack of a standard diluent, limited rounds of collection, absence of detailed sperm morphometry, and most importantly, high percentage of defective spermatozoa increases the difficulties in working with these samples [41-44]. The present study was conducted to establish CASA threshold values from various species settings comparable to the routinely used conventional subjective method in the big cat species. Additionally, the data herein would facilitate the shift from the routinely used subjective sperm analysis method in big cats that provide imprecise and variable readings to the more accurate and informative objective method.

Materials and Methods

Animals and study area

A wild born Arabian leopard male living in captivity at the Omani Wildlife Animal Breeding Center (N23.70 E58.09 A5.80 m) was admitted to the veterinary clinic for a routine physical examination and semen collection. The male leopard had no known disease nor received any type of medication that influenced the reproductive performance for one year prior to admission. Body condition score was adequate and no symptoms of dehydration were observed. Animal health was monitored throughout the year and was recorded as outstanding. The daily diet consisted of frozen/thawed chicken and extra lean goat or sheep meat with weekly supplements of vitamins and minerals. The leopard was reared in captivity from a very early age and housed individually in a large indoor/outdoor enclosure with exposure to natural light and temperature. The enclosure had sand flooring and a limited number of shrubs and trees. Natural photoperiod and ambient temperature were maintained in the enclosure. The animal was occasionally paired with a female for mating. Feed and water were withdrawn at least 12 hours prior to semen collection. Animal care and veterinary services were provided by experienced practitioners and volunteers following big cat husbandry protocols.

Restraint and Immobilization

The male leopard was captured using standard protocols, including a blow dart tranquilizer followed by an anesthetic protocol developed in house. The entire process of darting, anesthetizing, semen collection and recovery was less than 100 minutes during which time the leopard was blindfolded. The experiment was conducted in the winter (February) with a maximum and minimum day temperatures at the time of investigation of 25°C and 19°C respectively. The relative humidity on the day of the procedure was 48% and no rain precipitations were witnessed in the 7 days prior and post experimentation. The UV index was at 7 degrees (scale 0-11), length of the day 13 h 34 m and the length of visible light was 14 h 25 m. Semen collection took place indoors in a semi-ventilated area.

Semen collection

Semen collection was carried out as previously described in the Arabian leopard [41]. The penis was wiped with a cotton gauze soaked in saline. Urine was drained from the bladder via a sterile 5 Fr long catheter to eliminate the possibility of urine contamination of the ejaculate. No feces were observed around the anal area. The electro-ejaculator (EEJ) probe used was adapted from a model routinely applied in Canid species consisting of two ½" electrodes. The probe diameter, length, circumference and the total length were 1.27 cm, 6.35 cm, 4.5 cm and 22 cm respectively. The probe was connected to an EEJ apparatus (Minitube, Germany) that is manually regulated. Extensive non-spermicidal lubricant (BOVI-VET Gel, Kruuse) was used on the probe prior to insertion in the rectum. The probe was positioned with the electrodes directed ventrally on top of the male reproductive glands. The EEJ sequence consisted of a total of 80 stimuli given in three series (I, II, III) with increasing voltages ranging from 2-6 volts. Semen was collected into a sterile 50 ml polypropylene conical centrifuge tube (Eppendorf, USA). New sterile tube was used after every burst/ejaculate of semen (total 3). Semen collection procedure was carried out within 25 min.

Semen preparation

The ejaculate was liquefied at 21°C in a sterile water bath for 10 minutes. The sample was washed twice by adding filtered phosphate buffer saline (PBS) (Sigma, USA) as a washing buffer and centrifuged at 2000 rpm for 3 minutes. After centrifugation, the pellet was suspended in 1 ml of filtered PBS and mixed gently. To eliminate aggregates, clumps, debris and other gel particles the specimen was passed through a 40 µm nylon mesh filter (BD, USA). The ejaculate was transported within 1 hour after collection to the lab in a portable temperature adjusted incubator set at 22°C Minitube, Germany). Semen was extended in a pre-warmed egg yolk free media containing antibiotics (AndroMed®, Minitube, Germany) at a 1:1 ratio to obtain a dilution of 2-25 × 10^6 sperm/ml.

Conventional subjective sperm evaluation

Spermatozoa count and concentration were visually measured with a hemocytometer (Bright-Line, USA) under a light microscope (YS100, Nikon, China) at 100 x magnification [45] fitted with a heated stage (Minitube, Germany) set at 38°C. Motility was assessed in an aliquot diluted with filtered DPBS (Sigma, USA) at 1:100 and mounted (10 µl) onto a pre-warmed slide (38°C ± 2°C). Motility (MOT) and progressive motility (P. MOT) and linearity (0%=no forward movement, 100%=rapid linear movement) were subjectively evaluated as a percentage of total cells and evaluated from 6 fields by 3 different practitioners. Technical staff were unaware of their own or each other's subjective threshold values. The mean value of all the six fields was used as the final parametric score.

Objective sperm evaluation

Sperm motility parameters were carried out by loading 2 µl of the diluted semen aliquot onto a pre-warmed disposable 4 chamber counting glass slide (Leja, Nieuw-Vennep, The Netherlands) and visualized under a CASA microscope (Zeiss, Axiostar Plus, Germany) equipped with a motorized heated stage and a digital video camera of

black and white phase contrast (AccuPiXEL, Germany). Ten seconds after mounting the slide onto the microscope heated stage, sperm motility parameters were analyzed by Sperm Vision® software (Version 3.7.2, Minitube). The magnifying lens used was 20 x and a total of eight fields were recorded from at least 300 cells. The fields were selected randomly by the software setting that scanned the chamber from top to bottom at different points, bypassing aggregates and unidentified sections. Objects beyond the automated range specified are considered debris. CASA analytical parameters were adapted from preinstalled manufacturer settings and listed in Table 1. The following sperm variables were measured by the CASA operating system: motility (MOT, %), progressive motility (P. MOT, %), linearity (LIN, %)[VSL/VCL], straightness (STR, %)[VSL/VAP] and wobble coefficient (WOB, %)[VAP/VSL].

General parameters	
Depth of chamber	20 µm
Volume per chamber	2 µl
Temperature	37°C
Sperm concentration	Fixed
Time from extension	20 min
Lens magnification	20 x
Motility parameters	
Pixel to µm ratio	Up to 100
Area of sperm heads	22 to 99 μm^2
Maximum area	2-4 times sperm heads area
Local motility	DSL<5 micron
Display curved line (DSL)	On
Fields	8
Field analysis	2 s/field
Field view depth	20 micron
Number of cells/field	Min 300
Frame rate	60 frame/sec
Immotility parameters	
Immotile	AOC<7, DSL<3
AOC (degrees)	less than 5
BCF (hertz)	less than 0.01

Table 1: CASA configuration used to analyze the Arabian leopard sperm.

Statement of bioethics

Restraint, anesthesia and semen collection procedures were done according to well defined international protocols and approved by the bioethics committee at Sultan Qaboos University and the animal care unit of the Oman wildlife animal breeding center at the royal court affairs. Additionally, the project adhered to the association for the study of animal behavior guidelines for the use of animals in research (http://asab.nottingham.ac.uk/ethics/guidelines.php).

Statistical and data analysis

Data analysis was performed using the paleontological statistics software package (PAST3; 2016) version 3.10 (Oslo, Norway). Absolute values were analyzed with a multivariate hierarchical clustering scheme by using the Ward's method (non-constrained with Euclidean distances) [46] and set at an agglomerative bottom up direction to show the most closeness value in addition to the second and third values. The same software was used for principal components analysis (PCA) by producing new variables that are linear combinations of the original variables [47].

Results

CASA was carried out under various species settings and the data were evaluated by multivariate hierarchical clustering and principal component analysis (PCA) approach.

Average (AVG) motility reading and an exogenous bull standard (Bovine S.) was used to broaden the comparative spectrum. Objective motility analysis with CASA with several species settings (Table 2) in addition to the subjective (SUB) assessment revealed that the maximum motility was achieved under the porcine setting (88.3%) compared with the lowest in the stallion setting (37.90%).

Motility parameters (CASA)				
Species	**Local-MOT (%)**	**Non-MOT (%)**	**MOT (%)**	**P. MOT (%)**
Bovine S	2.26	14.84	85.2	82.9
Porcine	10.31	11.71	88.3	77.96
Caprine	23.94	15.65	84.3	60.39
Ovine	33.17	23.6	76.4	43.22
Equine	29.68	35.39	64.6	34.92
Bovine	8.54	52.14	47.9	39.32
Stallion	25.84	62.14	37.9	12.01
AVG	19.1	30.78	69.22	50.1
SUB	-	24	70	85

Table 2: Motility parameters displayed in percentages as local motility (local-MOT), non-motile sperm (non-MOT), motility (MOT), progressive motility (P. MOT) and average motility (AVG).

Progressive motility (P. MOT) a known factor used for grading sperm ejaculates recorded the highest activity in the Bovine S. at 82.9%. Interestingly, SUB and AVG motility readings were very close at 70% and 69.22% respectively. Inversely, SUB and AVG non-motile sperm analysis were close at 24% and 30.78% respectively. This indicated that both measurements are somehow interrelated. Moreover, progressive motility showed the highest percentage in the bovine standard (82.9%), and lowest in the stallion setting (12%), SUB (85%) with AVG at 50.1% amongst all settings. This suggests the existence of considerable variability in progressive motility amongst all species setting.

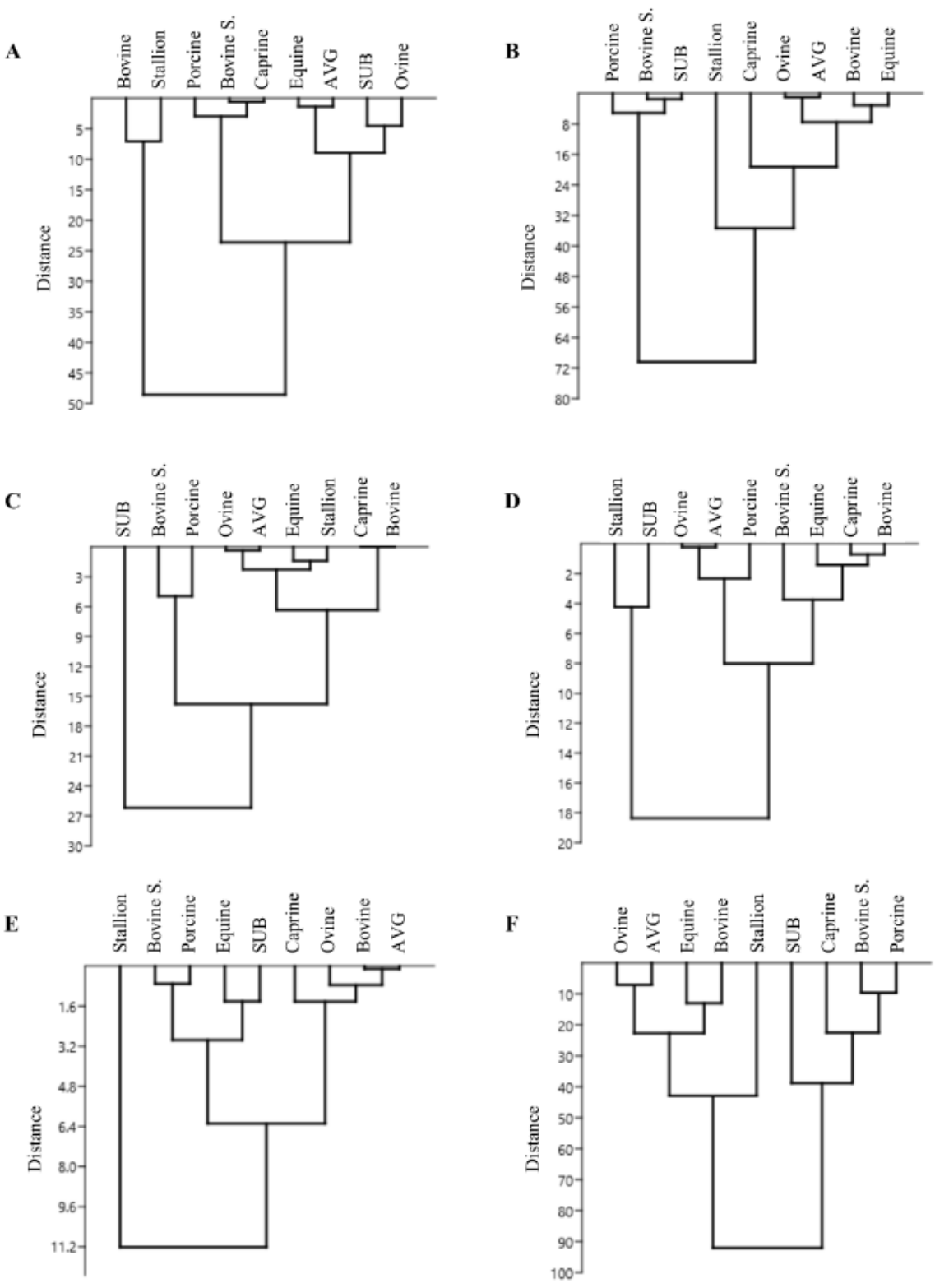

Figure 1: Bottom up dendrogram showing a multivariate hierarchical clustering scheme (Ward's method) in all species kinematic settings. MOT (A) P.MOT (B) WOB (C) LIN (D) STR (E) combined clustering plot (F) SUB; subjective, AVG; Average.

To minimize and maximise the variability between species settings in algorithmic groupings, multivariate hierarchical clustering was used (Figure 1).

The distribution of intra-species settings of motility generated 4 major clusters with the SUB and ovine settings clustered together in a distance less than 5%. A sub cluster was formed that brought ovine, SUB, AVG and equine settings together (distance less than 8%). The tree diagram of P. MOT shows two large clusters at the linkage distance of approximately 68% of its maximum length. Another scheme was depicted in WOB dendrogram whereby a crossing of two major clusters with the SUB data and a relatively low distance.

Principal components	Parameter	Eigen value	Individual variance (%)	Cumulative variance (%)
PC 1	MOT	841.076	79.474	79.474
PC 2	P. MOT	147.49	13.937	93.411
PC 3	WOB	45.872	4.3345	18.2715
PC 4	LIN	19.6479	1.8565	6.191
PC 5	STR	4.2152	0.3983	2.2548

Table 3: Principal components result: sperm parameters, eigenvalues, individual variance (%) and cumulative variance (%).

Indicating that WOB measurement in intra-species settings might produce reserve algorithms. Interestingly, LIN produced low distance and STR generated the lowest amongst all the parameters. Indicating that LIN and STR parametrises are tangled in all species settings. A combination of all motility parametrics (Figure 1F), indicate a progressive joining of two minor and two major clusters with a very high distance (93%) and a linkage space of approximately 42%, thus the presence of intra species settings variability between groupings. Five motility parameters were linearly combined to generate principal components plots and analysed according to eigenvalues, individual variance and cumulative variance (Table 3).

PCA plots were based on component I (motility) scores as displayed in Figure 2. Our data show that two separate aggregates were evident in motility PCA plot whereby SUB, bovine S, porcine and caprine readings were placed in the upper right quadrant and the remaining parameters in the lower left quadrant. Scattering pattern were more visible in the other four parametric data namely, P. MOT, WOB, LIN and STR evident by the distribution of species settings into four squadrons. In general, two species settings, SUB and porcine displayed close relatedness by forming a clustering pattern around one squadron under MOT, LIN and STR and close proximity of WOB. Another interesting relatedness event emerged by forming a singular cluster around AVG and ovine species settings in the P. Mot, LIN, STR and close proximity in the WOB metrics. Collectively, PCA reveals a wide divergence amongst species analytical settings compared to the commonly used SUB analysis. Moreover, scree plot with a downward curve shows percentages of eigenvalues with MOT at 79%, P. MOT 14% and all the remaining 3 kinematic parameters (WOB, LIN and STR) leveling off the curve and cumulatively under 4% (Figure 2f). Taken together, the present study demonstrates variation amongst a number of CASA species settings in sperm kinematics.

Discussion

The Arabian leopard is the last big cat species in the Middle East with very low connectivity between patches [48]. It is assumed that the only remaining wild population is confined to a tiny area in Dhofar Governorate of Oman, specifically, at the Jabal Samhan (Samhan Mountain; elevation 1800–2200 ft.) natural reserve [48-51], evident through a recent spotting of twins by camera traps [50]. Of note, in neighboring south eastern Yemen, the transboundary protected area of Hawf is thought to have a wild population that is nearly extinct with last sightings occurring half a decade ago [52-55].

It remains to be elucidated whether a cross border corridor that links Dhofar and Hawf exists currently. Alternatively, a range breakdown of the wild Arabian leopard population might become palpable and remanence to other leopard range collapse in south east Asia [56] and restricted corridors movement in Russia [57] and [58]. The geographically isolated and relatively small wild populace of the Arabian leopard would inevitably undergo loss of diversity, spatial homogeneity and genetic drift due to a systematic inbreeding structure. Inbreeding depression is notoriously known to contribute to reduce genetic variation [59] and species extinction [48,60,61]. On the other hand, captive population numbers of the Arabian leopard constitute only 47 males and 32 females at 9 institutions [62], thereby representing a non-viable breeding program that fails to contribute to heterozygosity and analogous to the extremely low wild populace that is only existing in Oman and ranges between 44 to 58 leopards [63]. Alarmingly, the current wild population trends of the Arabian leopard are largely ambiguous with scant and non-authenticated data with a reported loss of 98% of the historic range [48]. Taken together, reproductive management including intensive deployment of ART in the Arabian leopard is a crucial pillar that ensures longevity, permanence and revival of this critically endangered species. Amongst the numerous ART applications, CASA is overlooked in the *Panthera* sp. While CASA in the felidae family was first described in the domestic cat [64] then used on a routine basis [65], to date only three studies have reported the use of CASA in the *Panthera* sp. [66-68] and not a single CASA has been documented in the Arabian leopard to the best of our knowledge.

Hence the current study was carried out to investigate the Arabian leopard sperm kinematics with the use of multivariate clustering, a method that has been used for a while in other species [69,70] and combining it with different species settings to broaden the analytical spectrum. Our data show the low distance spreads in multivariate hierarchical clustering of WOB, LIN and STR. Similarly, the very three kinematics elbowed at the bottom of the scree slope with only 3% variance indicating the possibility of applying alternative species settings as reference values in the *Panthera* sp. To the contrary, divergence was visible in P. MOT and MOT evaluations in all aspects. However, compromising on such key kinematic features will hinder any progress in the ART outcome of the *Panthera* sp. given their usage as a predictive parameter in semen fertilization capacity [71]. The divergence of inter species CASA modules compared to the leopard, renders this vital tool ineffective. This observation is in support of a historical [72] and recent study [73] that highlight CASA inter species compatibility concerns.

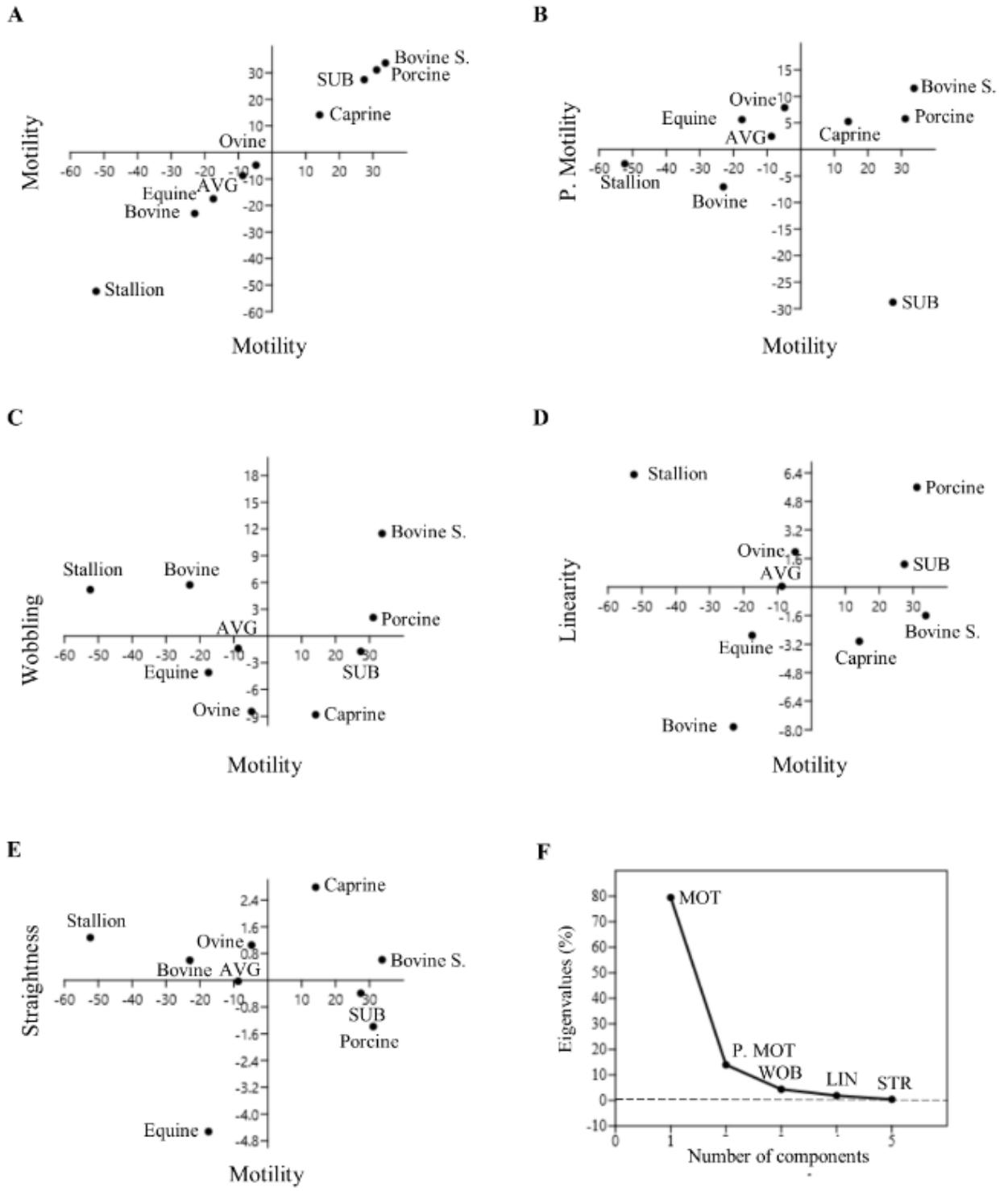

Figure 2: Principal Component Analysis (PCA). Scattered plot of the first principal component motility verses motility (A) motility verses progressive motility (B) motility verses wobbling (C) motility verses linearity (D) motility verses straightness (E) in all species settings. Scree plot with all components combined and respective eigenvalues in percentages (F).

Compounding the precariousness of sperm analysis is the tendency to disregard semen evaluation by CASA prior to pairing animals in captive breeding programs or the exclusive dependence on subjective assessment of sperm motility in the *Panthera* sp. This latter approach is a method that is based on light microscopy limitations [74-77], relies solely on the skills and interpretation of the practitioner with much variability amongst inter [78,79] and intra laboratory technicians [80]. Thus, the development of a revamped CASA system that is customized exclusively to leopards would serve as a pivotal tool in identifying reproductive potential in this exotic wild animal and potentially enhancing species revival. This notion is supported by a number of studies that recommend proper CASA programming of species-specific settings [81,82]. It is anticipated that a number of aspects would radically improve semen photometry screening and ultimately CASA. Firstly, microscopic enrichment of birefringence, brightness by dynamic LED illumination, lenses designed for high resolution imagery and capable of ultra-wide apertures. Secondly, substantial revamp in computer hardware speed, processing proficiency and enhanced motion plugins. Thirdly, improvement of visual and video capture by dynamic image stability, superior single pixel resolution, deblurring capabilities, time lapse monitoring, extra contour and contrast filters. Notably, imaging software have been advanced further to screen additional features not included in the standard CASA metrics such as 3D capture. Namely, Image J (NIH, Chicago, IL, USA) use of 3D imaging to screen the acrosome and the nucleus. Likewise, Imaris (Bitplane, CT, USA) utilizes a 3D and 4D image stack of non-deconvoluted segments that enables visualization of the interior of single sperm cells. Thereby, facilitating sperm evaluation by numbering motile sperm that are morphometrically intact and free of vacuoles. For instance, while vacuolization pattern has been shown to be associated with chromatin condensation failure [83,84], it is a parameter that is overlooked in the big cat species. Hence, a next generation CASA system is expected to be highly accurate, exceedingly reproducible, multifactorial, have asymmetry recognition with peculiar morphometric depiction thorough revamped semen algorithmic trackability. Currently, the majority of CASA operating systems permit manual entry of sperm dimensions for species that are not listed in settings. However, sperm metrics are routinely derived from light microscopy measurements that are neither accurate nor compatible with the next generation CASA module. To overcome this hurdle, morphometric analysis obtained by scanning electron microscopy (SEM) with additional parameters such as roughness, roundness, regularity and elongation would provide superior tools for uncovering sperm anomalies and tracing sperm trajectory. The combination of a detailed sperm morphometric analysis generated by SEM with CASA would presumably eliminate the inconsistent motility readings generated by various CASA operating systems [85] and annulling biased assumptions based on CASA artifacts [86]. In fact, it has been shown that merging both measures can provide complementary data that contribute to fertility prediction [87]. This in turn is a crucial step needed to enhance reproductive assessment in the *Panthera* sp. that is known to harbor poor sperm morphometrics, kinematics and breeding potential [7,88,89].

Conclusion

Taken together, our results represent the first detailed information about alternating CASA species settings in correlation to the commonly used subjective analysis in big cats. The disproportionate levels of divergence and variability shown in this study necessitate the development of specific CASA settings purposely customized for the *Panthera* sp. by empowering and refinement of CASA modules. Unless revolutionary CASA systems are deployed and fully utilized in the *Panthera* sp., the fate of the Arabian leopard is reminiscent to a relatively close subfamily namely; the cheetah (Acinonyx jubatus) whereby a continued decline in genetic diversity [90] and loss of habitat and displacement is evident, an environmental recipe for imminent wild animal extinction [91].

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