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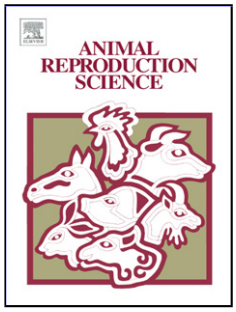

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Kinematic and head morphometric characterisation of spermatozoa from the Brown Caiman (*Caiman crocodilus fuscus*)

Running Title: Brown caiman sperm analyses

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ABSTRACT

The development of analytical methods for the evaluation of crocodilian semen is an important component for the assessment of male breeding soundness and the development of assisted breeding technology in this taxon. Computer-Assisted Semen Analysis (CASA) technology is becoming an increasingly common technique in seminal evaluations for animals but there has been no application of this technique for reptilian spermatozoa. The aim of this study was to analyse sperm kinematic and morphometric variables in *Caiman crocodilus fuscus* semen samples and to determine whether there were sperm subpopulations. Four ejaculates from four sexually mature captive caimans were used for this study. A CASA-Mot and CASA-Morph system was used with an image acquisition rate of 50 Hz for 2 seconds of capture. The ISAS®D4C20 counting chambers were used and spermatozoa incubated at 25 °C. Total and progressive motilities did not differ among animals (P >0.05). There was a significant animal effect in the model with respect to sperm morphometry, and kinematic indices including linearity (LIN) and straightness (STR) (P< 0.05). Results for principal component (PC) analysis indicated variables were grouped into four components: PC_1 related to velocity, PC_2 to progressivity, PC_3 to oscillation and PC_4 to sperm path cross-linking. Subpopulation (SP) structure analysis indicated there were four groups, namely, rapid non-progressive (SP_1), slow non-progressive (SP_2), rapid progressive (SP_3) and medium progressive (SP_4), representing 14.5%, 45.4%, 18.7%, and 21.4% respectively. Findings in the present study indicate the importance of continuing development of reliable protocols regarding the standardisation of computer-based semen analyses in reptilian species.

Keywords: Andrology; Caiman; Crocodylia; Sperm analysis; Reptile reproduction

1. Introduction

The *Caiman crocodilus fuscus* (Cope 1868), originally known as *Caiman crocodilus chiapasius* (Linnaeus, 1758) or *Alligator* (*Jacare*) *chiapasius* (Bocourt 1876) and colloquially known as the "Guajipal" or Brown Spectacled Caiman, belongs to the order Crocodylia, Family Alligatoridae and Class Reptilia (Huchzermeyer, 2003; Martin, 2008). Species distribution has been cited as occurring mainly in the Pacific from Oaxaca, Mexico, to Guayaquil, Ecuador (Carvajal et al., 2005) and in the Atlantic from the Yucatan peninsula (Charruau et al., 2015) to the northwest of Venezuela (Meden, 1981, 1983; Goombridge, 1982). *Caiman crocodilus* is found in Appendix II of (CITES, 2017) (Convention on International Trade in Endangered Species of Wild Fauna and Flora), where it is listed as lower risk (LR) or least concern (LC).

Knowledge on the reproduction of the caiman is a key element for understanding its conservation and in the development of captive breeding expertise for other closely related endangered species of Crocodylia. There, therefore, has been recent interest in the development of assisted breeding technology of captive crocodiles for both production and endangered species propagation (Johnston et al., 2014a), including refinement of semen collection protocols and characterisation of seminal variables (Romero-Solórzano et al., 2010; Johnston et al., 2014b; Fitri et al., 2018;). There have also been studies determining the physio-chemical requirements of sperm diluents (Johnston et al., 2014c), assessment of sperm DNA quality (Johnston et al., 2015; Gosálvez et al., 2016) and preliminary attempts at sperm cryopreservation (Johnston et al., 2017); all in which there was examination of the spermatozoa of the salt water crocodile (*Crocodylus porosus*).

Assessment of sperm motility and morphometry is now commonly performed by means of computer assisted semen analysis (CASA) technologies that allow objective and accurate assessment of sperm variables such as kinematics (Gallagher et al., 2018; van der Horst et al., 2018; Yániz et al., 2018), morphometry (Maroto-Morales et al., 2016; Soler et al., 2016; Valverde et al., 2016; Yániz et al., 2016) and DNA fragmentation (Sadeghi et al., 2016). The CASA systems can be used to provide information based on values of thousands of individual sperm tracks of the sample (Amann and Waberski, 2014). The CASA analysis also allows identification of motile sperm subpopulations that have characteristic kinematic and morphometric patterns, but the biological meaning of these different sperm subpopulations and interactions is not always clear. Studies of sperm subpopulations have been conducted for cattle (Valverde et al., 2016; Yániz et al., 2018), sheep (Yániz et al., 2015), pigs (Gil et al., 2009; Valverde et al., 2018), cat (Gutiérrez-Reinoso and García-Herreros, 2016), poultry (García-Herreros, 2016), salmon (Caldeira et al., 2018), fox (Soler et al., 2017, 2014) and primate species (Valle et al., 2013), but there are currently no descriptions documenting reptile spermatozoa.

Knowledge of the sperm kinematic and morphometric subpopulation structure will not only increase understanding of male crocodilian breeding soundness and assisted breeding technology, but also help to quantify and further characterise reptile sperm metabolism and physiology, such as capacitation (Nixon et al., 2016). Consequently, the aim of this study was to analyse sperm kinematic and morphometric variables using CASA and to determine whether there are sperm subpopulations in *Caiman crocodilus fuscus*.

2. Materials and methods

2.1. Study site

This study was conducted as part of the crocodile management and exhibition facilities associated with the Scientific Ecotourism Project (EcoTEC) based at the School of Agronomy, Costa Rica Institute of Technology, San Carlos Campus, Alajuela, Costa Rica (10°21'52'' N, 84°30'31' W). The facility is located at an altitude of 170 metres above sea level, in a tropical wet forest with a basal altitudinal floor, according to the Holdridge life zones system (Holdridge, 1967). According to the data recorded at the closest weather season (069567, St Clara, University Campus), the crocodile facility has annual minimum and maximum temperatures of 21.7 and 30.7 °C, respectively, and a relative humidity of 88.5%, with the rainfall rate of 3321.1 mm per year.

2.2. Animal husbandry

The experiment was conducted conditions consistent with the laws and regulations controlling experiments on live animals in Costa Rica and without any requirement of approval from the animal research committee of the Costa Rica Institute of Technology. Nevertheless, this study was conducted with the approval of the National System of Conservation Areas (SINAC-Costa Rica) and Arenal Huetar Norte Conservation Area (ACAHN) Scientific Purposes Permit (SINAC-ACAHN-SCH-818-18). Four sexually mature healthy male caimans were used as semen donors in this study. The animals were housed together with 11 females in the same pond. All caimans were estimated to be between 14 and 16 years of age. The EcoTEC crocodile facility was designed to allow the animals to be housed in conditions that mimic their natural habitat, in which they have access to natural and artificial fresh-water ponds, native vegetation, shaded areas and sunbathing and shelter.

The caimans were fed pieces of lean meat (pork, chicken, beef - which can be fed on the bone in larger adults) which was supplemented with additional calcium at 1.9% to 2.4% dry-matter.

2.3. Timing of semen collection

From observations of caiman behaviour at the EcoTEC crocodile facility since 2008, it was concluded that *C. c. fuscus* express courtship and mating behaviours from February to June, the females deposit eggs and incubate the eggs from June to September and subsequently lay eggs from September to December (Castro-Morales, Personal Observations). In the present experiment, sexually mature specimens were used, with weights between 12.0 and 14.2 kg and lengths between 72 and 77 cm (Castro-Morales, Personal Observations).

2.4. Animal restraint

Semen was collected from restrained caiman without the need for sedation or drugs for immobilisation. Briefly, this included the removal of the animal from the fresh-water pond with a 13 mm diameter rope that was secured around the upper jaw of the animal (Fig. 1A). A moistened cotton cloth was then placed directly over the animal's eyes (Fig. 1B) before two expert handlers physically restrained the animal (Fig. 1C), while another secured the top and bottom jaw with vinyl tape (Fig. 1D). The front and hind legs were then carefully tied caudal to the shoulders and pelvis of the animal to prevent possible injury to semen collector or caiman (Fig. 1E). All animal restraint procedures were conducted without incident.

2.5. Semen collection and processing

Semen collection was conducted by digital manipulation (Fig 1F) as previously described by (Johnston et al., 2014b). A gloved hand was introduced into cloaca to gently exteriorise the phallus; once it was exteriorized the fore and index fingers were used to gently massage – stroke the terminal portions of the vas deferens immediately cranial to the *urodeum*. In mating season (February – June), an erection response to manual stimulation typically occurred in less than 5 minutes after initiation of manual stimulation. Following massage semen flowed down the sulcus of the phallus and was carefully lavaged into a collection vessel. Small volumes of ejaculate (e.g., 1.0 mL) were recovered in to a 1.5 mL Eppendorf® microtube (Sigma-Aldrich, St. Louis, MO, USA) aided by use of a micropipette fitted with a 10 to 100 µL pipette tip. Semen was recovered from the sulcus with approximately 100 µL of buffered Dulbecco's phosphate-buffered saline (DPBS, pH 6.8, Sigma-Aldrich). The total time handling the animal for semen collection typically did not exceed 30 minutes. All semen collection procedures were conducted without incident.

2.6. Assessment of sperm variables

The pH of undiluted semen was determined using narrow range pH paper strips (± 0.3-0.4 pH unit; Sigma-Aldrich). For the analysis of motility and kinetic variables, ISAS®D4C20 disposable counting chambers (Proiser R+D, S.L., Paterna, Spain) were used after being pre-warmed to 25 °C. After a thorough mixing of the diluted semen samples, 3 µL diluted semen was dispensed along the counting chamber tracks by capillarity. A further dilution 1:10 in DPBS diluent was used for motility and kinematic assessment before loading the sperm suspension in the counting chamber. Analyses were conducted using the CASA-Mot system ISAS®v1 (Integrated Semen Analysis System, Proiser R+D, Paterna, Spain). The video-camera was a Proiser 782M (Proiser R+D), with a frame rate of 50 fps and a final

resolution of 768 x 576 pixels. The camera was attached to a microscope UB203 (UOP/Proiser R+D) with a 1X eyepiece and a 10X negative-phase contrast objective (AN 0.25) and an integrated heated stage maintained at 25 ± 0.5 °C. Sperm concentration ($x10^9$/mL) was estimated in the CASA-Mot system after accounting for the initial dilution of the semen sample.

The morphology of live spermatozoa was observed by using the Trumorph® device (Proiser R+D) that briefly, increases the temperature of the sample to 45 °C for stopping sperm motility and applies a light pressure of 6 kP for extending the volume of the sample (2 µL) in a conventional slide and 22 mm x 22 mm cover slide. The chamber depth between slide and coverslide is ~6 µm and the spermatozoa are restricted in movement so as to expose their flat upper surface parallel with the coverslip (Soler et al., 2015). Morphological observations were conducted with the UB203 microscope using 40x negative phase contrast.

The CASA analyses were performed in seven microscope fields on a total of at least 600 cells per sample (S1. Video file, see Supplementary Material). Seven fields were used for statistical analyses. The CASA-Mot variables assessed in this study included straight line velocity (VSL, µm/s) - corresponding to the straight line from the beginning to the end of the track; curvilinear velocity (VCL, µm/s) - measured for the actual point-to-point track followed by the cell; average path velocity (VAP, µm/s) - the average velocity over the smoothed cell path; amplitude of lateral head displacement (ALH, µm) - defined as the maximum of the measured width of the head oscillation as the spermatozoa swim, beat-cross frequency (BCF, Hz) - defined as the frequency with which the actual track crosses the smoothed track in either direction, motility (%) - the percentage of the total motile cells and progressive motility (%) - corresponding to spermatozoa swimming rapidly forward in a

straight line (assessed as straightness index ≥45%; VAP ≥25 μm/s). Three progression ratios, expressed as percentages, were calculated from the velocity measurements described above: linearity of forward progression (LIN = VSL/VCL·100), straightness (STR = VSL/VAP·100), and wobble (WOB = VAP/VCL·100).

2.7. Computerised morphometric analysis

For morphometric analysis, semen smears were prepared, and slides stained with the Diff‑Quik kit (Medion Diagnostics, Dudingen, Switzerland), following the instructions of the manufacturer. All the slides were identified and then permanently sealed with Eukitt mounting medium (Kindler & Co, Freiburg, Germany) under a cover slip and analysed in a double‑blind scheme. Microscope slides were analysed for sperm head morphometry using the CASA-Morph system ISAS®v1. The equipment comprised a microscope (UOP200i/Proiser Valencia, Spain) equipped with a 40X bright-field objective (AN 0.7). A video camera (Proiser 782M, Valencia, Spain) was mounted on the microscope to capture the images and transmit them to the computer. The array size of the video frame grabber was 768 × 576 × 8 bit, providing digitized images of 442,368 pixels and 256 grey levels. Resolution of images was 0.21 μm/pixel in both the horizontal and vertical axes. Sperm head images were obtained randomly in different fields, rejecting only those that overlapped with background particles or other cells that interfered with subsequent image processing. Initial erroneous definition of the sperm head boundary was corrected by varying the analysis factor of the setup of the CASA-Morph system. Following the criteria of (Boersma et al., 1999) at least 100 sperm heads were measured on each slide for two primary variables of head size (area [A, μm^2] and perimeter [P, μm]).

2.8. Statistical analysis

The data obtained from the analysis of all sperm variables were first assessed for normality and homoscedasticity by using the Shapiro-Wilks and Levene tests. A normal probability plot was used to assess for a normal distribution. The kinematics sperm variables did not satisfy the normality requirement for a parametric analysis of variance. Non-parametric analyses, therefore, were performed using a Kruskal–Wallis test. When statistically significant differences were detected using this test, the non-parametric Mann–Whitney U-test was used to compare pairs of values directly.

Clustering procedures were performed to identify sperm subpopulations from the set of sperm motility data. All the values for kinematic (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) and morphometric (area-head, perimeter-head) variables were standardised to avoid any scale effect. The first process was to perform a principal component analysis (PCA) of these data (each variable was weighted with their variances extracted for that principal component, known as eigenvectors) to derive a small number of linear combinations (PCs) that still retained information from the original variables as much as possible. The number of principal components (PC) used in the next process of the analysis was determined from the Kaiser criterion, namely selecting only those with an eigenvalue (variance extracted of each PC) >1. Furthermore, Bartlett's sphericity test and the KMO index (Kaiser-Meyer-Olkin) were performed (Spencer, 2013). As a rotation method, the varimax method with Kaiser normalisation was used (Kaiser, 1958); the rotation is a procedure to help in the interpretation of the importance of each principal factor in the factorial weight matrix (Everitt and Hothorn, 2011). The third process was conducted to perform a non-hierarchical analysis with the k-means model that uses Euclidean distances from the quantitative variables after standardisation of these data, so the cluster centres were the means of the observations assigned to each cluster (Kaufman and Rousseeuw, 1990). The

multivariate k-means cluster analysis was made to classify the spermatozoa into a reduced number of subpopulations (clusters) according to kinematic and morphometric variables. In the final process, to determine the optimal number of clusters, the final centroids were clustered hierarchically using the Ward method (Murtagh and Legendre, 2014). Thus, the clustering procedure (*k*-means model and Euclidean distance) enables for the identification of sperm subpopulations because each cluster contributed to a final cluster formed by the spermatozoa linked to the centroids. The ANOVA and $\chi 2$-test procedures were applied to evaluate statistical differences in the distributions of observations (individual spermatozoa) within the steps and subpopulations (percentages of spermatozoa assigned to each cluster), and then a generalized linear model (GLM) procedure was used to determine the effects of the steps, as well as variation, on the relative distribution frequency of spermatozoa within subpopulations. The GLM procedure was also used to evaluate the influence on the mean kinematic and morphometric parameter values defining the different sperm subpopulations (i.e., the cluster centres). Differences between means were then analysed by the Bonferroni test and Mann-Whitney U-test. Pearson correlation was calculated for sperm head morphometric and kinematic values. Results are presented as mean ± standard error of the mean (SEM). Statistical significance was considered at $P< 0.05$. All data were analysed by using IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Sperm motility and kinematics

There was no animal effect ($P >0.05$) on total sperm motility or progressive motility. The range of least squares mean (LSM ± SEM) for total and progressive motility was 45.86 ± 4.17 - 53.0 ± 4.50 and 21.71 ± 3.25 - 25.71 ± 3.26, respectively (Fig. 2). The mean (± SE)

sperm concentration ($x10^9$/mL) of the samples was 3.80 ± 0.4 with a range of 1.3 to 6.6. Mean (± SE) pH of the samples was 6.4 ± 0.1. The values for kinematic variables corresponding to the whole analysed sperm population are included in Table 1.

The majority of the kinematic variables indicated there were differences in values among animals, with VCL, VAP, LIN, STR and ALH being the most variable. Only the values for WOB were not significantly different among animals (Table 2).

Values for principal component analysis indicated there were four PCs, named velocity (PC1), progressivity (PC2), oscillation (PC3) and sperm path crossing (PC4), with a total variance of 95.45% explained. These results indicated that sperm velocity and progressivity have a relatively greater effect on the total variance than the other variables (Table 3).

The data from the subsequent cluster analysis indicated there were four subpopulations. The kinematic values corresponding to each subpopulation were characterised as: rapid non-progressive (SP1), slow non-progressive (SP2), rapid progressive (SP3), and medium progressive (SP4) (Table 4, Fig. 4). The distribution of subpopulations was similar among animals, with the slow non-progressive spermatozoa being the most frequent (Table 5).

3.2. Sperm morphology

Normal sperm morphologies in live cells, using the Trumorph® technique, indicated there was the typical filiform head and a tail that was approximate three times as long as the head (Fig. 3). The area of the sperm head was different among animals but there was no difference in the perimeter of the sperm heads among animals (Table 2).

Correlations between the values for sperm kinematics and head morphometric variables were performed and with a greater velocity there was a lesser morphometric area of the spermatozoa (Table 6).

4. Discussion

Using the same principals in the present study for breeding soundness in mammalian species (Valverde et al., 2016, 2018; Soler et al., 2017), there was an enhanced understanding of caiman male reproductive physiology that will consequently lead to improvements in reproductive management of these species both in wild and captivity. While results from previous studies of sub-therian vertebrate species [fowl (Froman et al., 1999), turtle (Gist et al., 2000) and snake (Tourmente et al., 2011)] have all indicated sperm movement speed is directly correlated with fertilization success, the findings in the current study represent the first computer assisted sperm assessment in a crocodilian species.

The mean total sperm motility reported in the present study (49.6 ± 10.7%) is similar to that reported previously after post-mortem epididymal recovery of sperm (Larsen et al., 1982, 1992). Depending on the study and recovery technique, the motility described for *Crocodylus porosus* is also in the same range or slightly less (Fitri et al., 2018: 45.0 ± 17.6%, Johnston et al., 2014c: 63.4 ± 3.2%, both after sedation; Johnston et al., 2014b: 50.7 ± 4.2%, after digital massage). Sperm motility can be important in competition for fertilisation (Birkhead et al., 1999). A greater sperm motility may be favoured when conditions induce sperm competition, and there may be lesser motility in some species when there is sperm storage (Gist et al., 2000). In ectotherms, female body temperature modulates sperm motility after mating (Uller et al., 2010). In turtles there is an increase in sperm motility at lesser body temperatures, suggesting local adaptation of sperm motility in relation to environmental

temperature during the timing of copulation (Gist et al., 2000). In the case of the Leopard tortoise, values for total sperm motility indicate there is a large amount of variability ranging from 10% to 80%, which may indicate adaptation of sperm motility when there is sperm competition occurring (Gist et al., 2000; Zimmerman and Mitchell, 2017) that could also be related to semen collection success or technique. Sperm motility and kinematic variables have been reported in other reptiles, including corn snakes (Fahrig et al., 2007), lizards (Aranha et al., 2008; Blengini et al., 2014; López Juri et al., 2018), green iguanas (Zimmerman et al., 2013), turtles (Gist et al., 2000), black and white tegu lizards (*Tupinambis merianae*) (Young et al., 2017) but the number of frames per second used in some of these previous studies were few (30 fps); (Gist et al., 2000).

The use of the CASA systems typically provide an objective and repeatable assessment of the proportion of motile sperm cells in a sample, as well as quantification of kinematic and morphometric variables (Amann and Waberski, 2014; Bompart et al., 2018). Such differentiation not only allows for identification of semen samples with poor sperm motility but may also be useful in selecting the most desirable males for artificial insemination programs or assessing sperm preservation and storage protocols (e.g., cryopreservation, capacitation or activation). Data resulting from use of the CASA technology, particularly when based on kinematic and morphometric variables, have been important in the last 30 years in many species in the fields of research, seminal dose production and conservation programmes (Waberski et al., 2008; Soler et al., 2005; Cucho et al., 2016).

The curvilinear velocity (VCL; 54.16 ± 0.48 μm/s) in the present study is similar to that reported for the *Boa constrictor* (58.97 μm/s) (Tourmente et al., 2007), whereas straight-

line velocity (VSL) in the *Boa constrictor* (50.22 μm/s) is much greater than that for the caiman (14.15 ± 0.21 μm/s). The relatively greater percentage for sperm motility of snake spermatozoa could be an adaptation to sperm competition pressures (Snook, 2005; Tourmente et al., 2007) or be a consequence of collection techniques or sperm dilution. For *B. constrictor* spermatozoa, Tourmente et al. (2007) also reported the linearity of forward progression (LIN) as being 80% which is substantially greater than that for caiman in the present study (28%) even though the analyses were conducted at the same temperature (25 °C).

While results related to sperm motility in most studies are typically indicative of the whole sperm population in the ejaculate (normal distribution model) the increasing use of the CASA analytical systems has allowed for reporting and identifying sperm subpopulations, (Amann and Hammerstedt, 1993; Hirai et al., 2001; Thurston et al., 2001; Soler et al., 2014; Gallego et al., 2015; Valverde et al., 2016; Vásquez et al., 2016; Yániz et al., 2016; Soler et al., 2017; Caldeira et al., 2018; Yániz et al., 2018). In the present study, the use of the cluster analysis of caiman spermatozoa allowed for classification of spermatozoa into four clusters with the following characteristics: spermatozoa with relatively greater velocities (VCL and VAP) but less linearity, which was defined as SP_1 or "rapid non-progressive"; a second cluster of sperm with a relatively lesser VSL, VAP and LIN that were considered the SP_2 or "slow non-progressive"; a third cluster with relatively greater velocities (VCL, VSL, and VAP) and greater linearity (LIN, STR) designated SP_3 or "rapid progressive"; forth cluster (SP4) of sperm with moderate velocity and relatively greater linear movement of cells (great LIN and STR) considered as a "medium progressive" subpopulation. Some plots representing different sperm head digitalized models were quite similar, however, the trajectory plots were observed to be different. This can be explained because with the use of correlation

assessments there was elucidation of possible associations between values for sperm kinematics and head morphometric variables (the correlation coefficient between head area and VCL was -0.38). These observations prompted the question whether reptilian sperm size, more specifically head area, was associated with progressivity. Furthermore, sperm head size should be attributable to differences in chromatin (Gosálvez et al., 2016). The inter-animal effects on the proportions of each sperm subpopulation were very apparent and provide the basis of future studies to explore the relevance of these sub-populations to fertilisation capacity of sperm.

Morphologically normal sperm cells of *C. c fuscus* possess a filiform nucleus and intact acrosome with a small midpiece (Fig. 2) and are similar to those observed in the spectacled caiman (*Caiman crocodilus* (Assumpção et al., 2017) and crocodile (Jamieson et al., 1997; Gribbins et al., 2011; Johnston et al., 2014b). Assumpção et al., (2017) reported that spectacled caiman spermatozoa had a head length of 20.09 ± 0.85 μm, a midpiece length of 2.40 ± 0.16 μm and tail (principal + end piece) of 58.49 ± 0.29 μm for a total mean length of 80.98 ± 1.29 μm. In the present study, the use of the CASA-Morph system resulted in morphometric values of head area and head perimeter with range of 4.95 to 6.50 ± 0.11 μm^2 and 14.82 to 15.15 ±0.19 μm, respectively. Furthermore, it is necessary to evaluate whether the use of Diff-Quik staining procedures leads to the introduction of some artifacts. These artifacts are common for all the species (Soler et al., 2016), but particularly important in crocodilian sperm which do not contain cysteine in the protamine, thus, there is a special tendency for de-condensation when the smears are dried (Cummins, 1980). The use of Trumorph™ technique allowed for assessment to the actual morphology of the caiman sperm, but unfortunately, there is no CASA-Morph system available for the measurement of

this image source. Future studies will be conducted to explore the use of CASA based systems to identify sperm abnormalities in crocodilian ejaculates.

5. Conclusion

The evaluation of seminal quality is important in species conservation, so that results of research contribute to the development of reliable protocols regarding the standardisation of computer-based semen analyses in reptilian species. This would allow for collection of values for kinematic and morphometric variables which could be used to evaluate the reproductive potential of the crocodilian populations and allow for more objective measurements of sperm variables. The use of CASA-based systems for crocodilian spermatozoa assessment will also facilitate studies of sperm physiology and preservation. There are currently extremely limited studies documenting crocodilian reproduction so that the techniques described in the present study provide a basis for the standardisation of procedures for reptilian sperm assessments.

Author contributions

MM was involved with all laboratory experiments and experimental design together with OC, AG and AV. MM was involved in the semen collection procedures with OC. SJ contributed ideas for this article and manuscript editing together with AV and CS. AV contributed to interpretation of the data and was responsible for the data analysis. AV wrote the initial draft of the paper. All were involved in revision and approval of the final version of the manuscript.

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Competing interest

The authors declare that no competing interests exist.

CONFLICT OF INTEREST FORM

Manuscript tittle:

Kinematic and head morphometric characterization of spermatozoa from the Brown Caiman (*Caiman crocodilus fuscus*)

Author statement:

I not have a potential personal conflict of interest.

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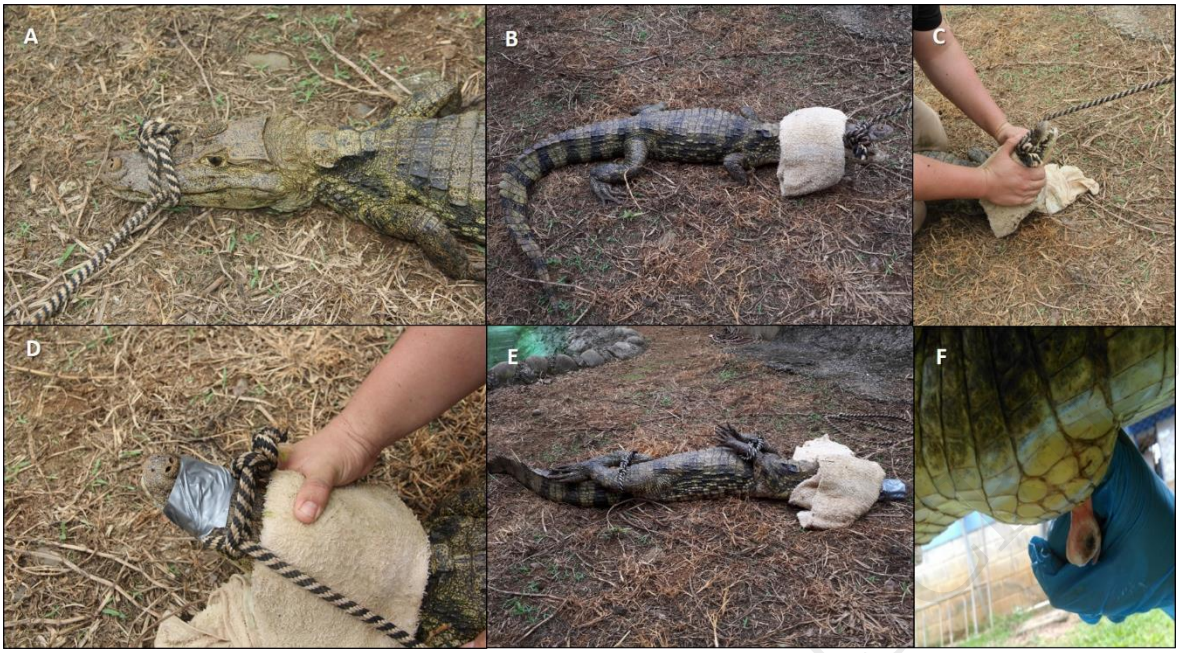

Fig. 1. Caiman restraint by removal of the animal from the fresh-water pond (A); A moistened cotton cloth was then placed directly over the animal's eyes (B); Two expert handlers physically restrained the animal (C); Secure the top and bottom jaw with vinyl tape (D); Front and hind legs were then tied caudal to the shoulders and pelvis of the animal (E); Sperm collection by manipulation and digital massage of the penis and ductus deferens, introducing a gloved hand in the opening cloaca (F); (*C. c. fuscus*)

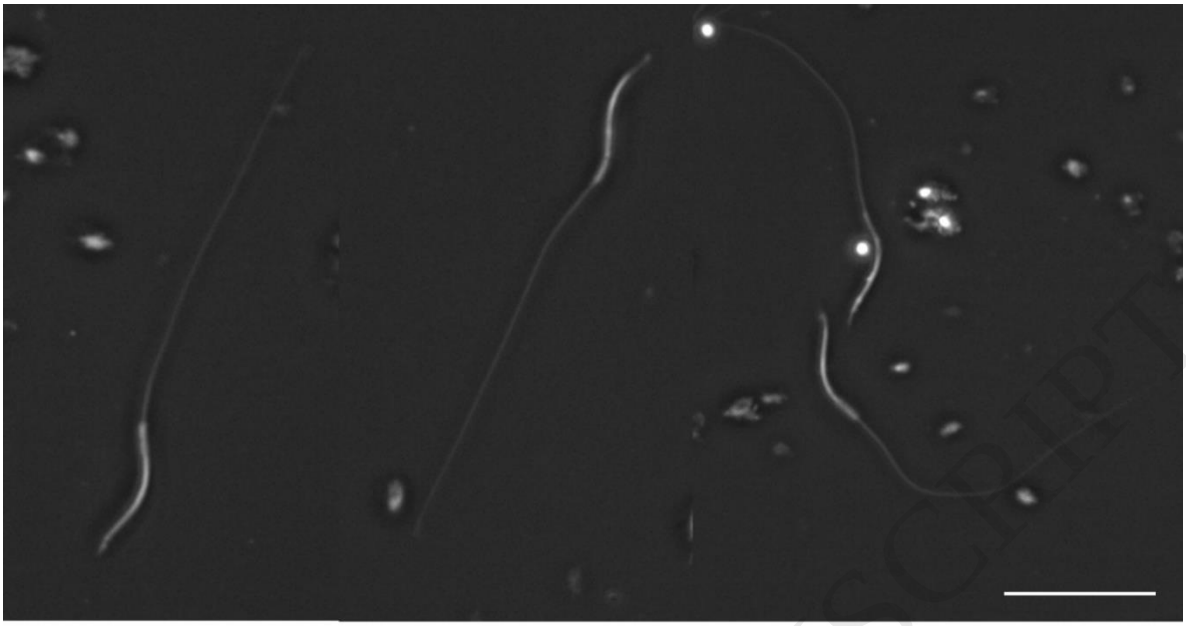

Fig. 2. Normal caiman (*C. c. fuscus*) spermatozoa as assessed using the Trumorph® system; Scale bar = 10 μm

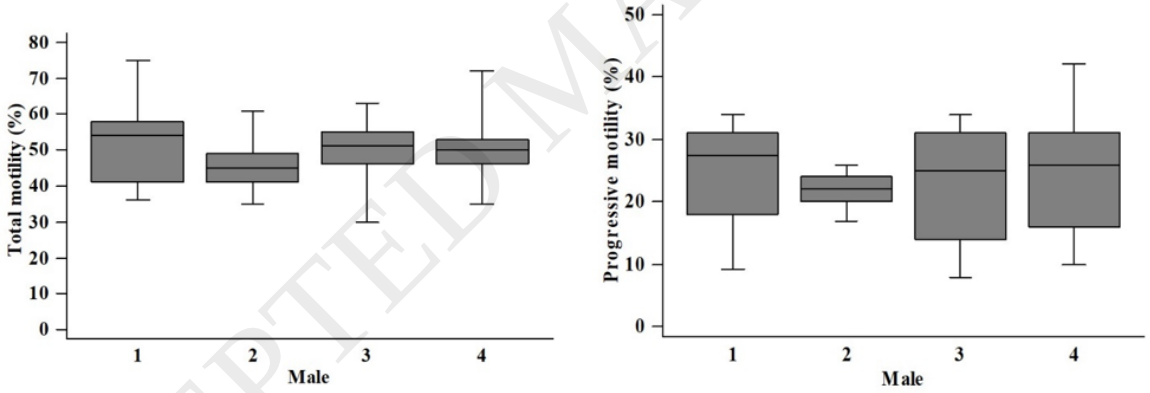

Fig. 3. Boxplot (25th/75th percentiles, -: median; ⊥ ┬: Values considering three standard deviation) graphics of four *Caiman crocodilus fuscus*: total and progressive (STR >45%) motility

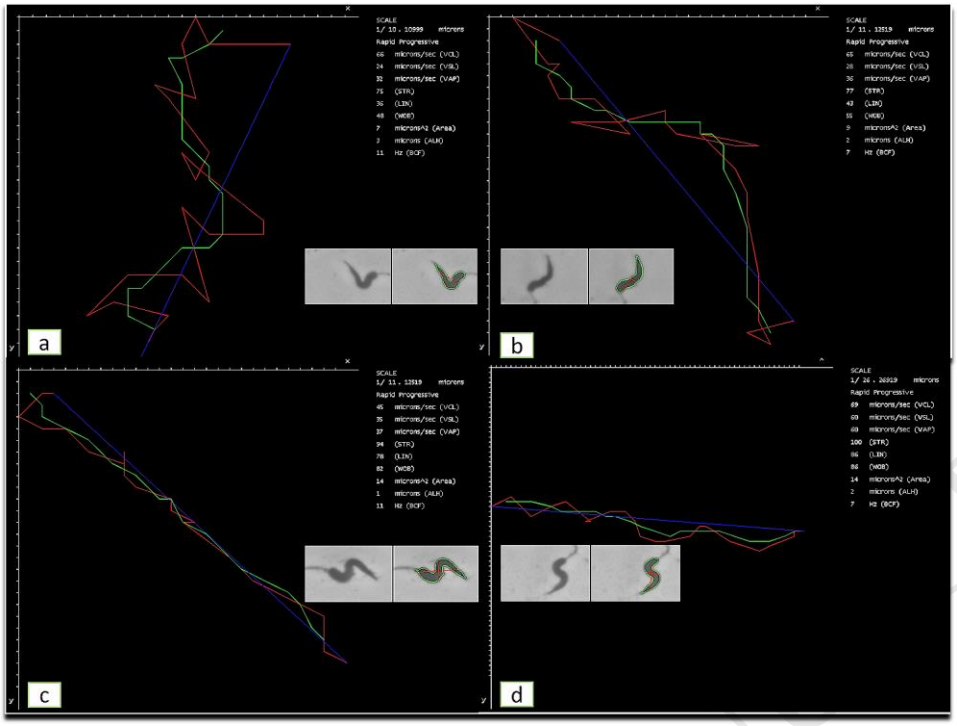

Fig. 4. Representative trajectories of a subpopulation of caiman spermatozoa (*C. c. fuscus*) analysed with the ISAS®v1 CASA-Mot system; a: rapid non-progressive (SP1); b: slow non-progressive (SP2); c: rapid progressive (SP3); d: medium progressive (SP4); Lines: Blue, VSL; Red, VCL; Green, VAP. VCL, curvilinear velocity (µm/s); VSL, straight line velocity (µm/s); VAP, average path velocity (µm/s); SP: subpopulation

Table 1

Sperm kinematic variables (mean and dispersion) in four ejaculates of four Brown Caiman (*Caiman crocodilus fuscus*) (*n* = 8; two ejaculates/male)

Male	Mean ± SEM	SD$^{\alpha}$	Min$^{\beta}$	Max$^{\gamma}$	Q1$^{\delta}$	Q3$^{\varepsilon}$	Skewness	Kurtosis
VCL[1]	54.16 ± 0.48	25.43	10.50	226.40	37.20	66.80	1.38	3.70
VSL[2]	14.15 ± 0.21	11.31	0.90	79.50	6.20	18.90	1.53	2.47
VAP[3]	23.64 ± 0.22	11.44	4.60	79.60	15.30	29.50	1.11	1.25
LIN[4]	27.90 ± 0.35	18.61	0.70	98.10	13.60	38.40	0.90	0.40
STR[5]	57.29 ± 0.52	27.48	2.10	100.0	35.60	80.50	-0.08	-1.06
WOB[6]	46.11 ± 0.30	15.79	9.10	100.0	34.70	55.70	0.60	0.14
ALH[7]	2.58±0.02	1.16	0.40	9.00	1.80	3.10	1.11	2.21
BCF[8]	4.40±0.05	2.53	0.00	15.30	2.50	6.00	0.56	0.26

Number of cells = 2 840; [1]VCL, curvilinear velocity (μm/s); [2]VSL, straight line velocity (μm/s); [3]VAP, average path velocity (μm/s); [4]LIN, linearity of forward progression (%); [5]STR, straightness (%); [6]WOB, wobble (%); [7]ALH, amplitude of lateral head displacement (μm); [8]BCF, beat-cross frequency (Hz); SEM, standard error of the mean; $^{\alpha}$SD, standard deviation; $^{\beta\,\gamma}$Min-Max, minimum and maximum values; $^{\delta}$Q1: lower quartile; $^{\varepsilon}$Q3: upper quartile

Table 2

Sperm kinematics and morphometric (mean ± SEM) variables in caiman (*Caiman crocodilus fuscus*; *n* = 8; two ejaculates/male)

Male	1	2	3	4
Kinematics/*n*	721	615	835	670
VCL[1]	50.02 ± 0.95^{a}	54.99 ± 1.03^{bc}	57.25 ± 0.88^{c}	52.95 ± 0.98^{b}
VSL[2]	12.48 ± 0.42^{a}	14.07 ± 0.46^{b}	15.24 ± 0.39^{b}	14.02 ± 0.44^{b}
VAP[3]	22.23 ± 0.43^{a}	23.80 ± 0.46^{b}	25.11 ± 0.40^{c}	22.54 ± 0.44^{a}
LIN[4]	26.01 ± 0.70^{a}	26.69 ± 0.76^{ab}	28.23 ± 0.65^{bc}	29.41 ± 0.72^{c}
STR[5]	52.99 ±1.04^{a}	55.38 ± 1.12^{ab}	57.07 ± 0.96^{b}	61.03 ± 1.08^{c}
WOB[6]	46.28 ± 0.60^{a}	44.60 ± 0.65^{a}	46.09 ± 0.55^{a}	46.20 ± 0.62^{a}
ALH[7]	2.15 ± 0.04^{a}	2.69 ± 0.05^{bc}	2.81 ± 0.04^{c}	2.62 ± 0.04^{b}
BCF[8]	4.49 ± 0.09^{a}	4.43 ± 0.10^{a}	4.41 ± 0.09^{a}	4.13 ± 0.10^{b}
Morphometric/*n*	95	98	99	100
Head area (μm^2)	6.03 ± 0.12^{a}	6.50 ± 0.11^{b}	4.95 ± 0.11^{c}	4.99 ± 0.11^{c}
Head perimeter (μm)	15.15 ± 0.19^{a}	14.87 ± 0.19^{a}	15.07 ± 0.19^{a}	14.82 ± 0.19^{a}

[1]VCL, curvilinear velocity (μm/s); [2]VSL, straight line velocity (μm/s); [3]VAP, average path velocity (μm/s); [4]LIN, linearity of forward progression (%); [5]STR, straightness (%); [6]WOB, wobble (%); [7]ALH, amplitude of lateral head displacement (μm); [8]BCF, beat-cross frequency (Hz); SEM, standard error of the mean; [a-d]Different superscripts within row indicate differences among subpopulations $P < 0.05$

Table 3

Eigenvectors of principal components (PCs) for caiman (*C. c. fuscus*) sperm kinematic variables

Principal component[*/a]	PC1	PC2	PC3	PC4
VCL[1]	0.945			
VSL[2]	0.411	0.803		
VAP[3]	0.749		0.543	
LIN[4]		0.864	0.446	
STR[5]		0.977		
WOB[6]			0.948	
ALH[7]	0.937			
BCF[8]				0.982
Variance explained (%)	32.16	30.53	19.09	13.67

Total variance explained = 95.45%; [*]Expresses the more important variables in each PC; Only eigenvectors > 0.4 are presented; [a]Rotated component matrix; [1]VCL, curvilinear velocity (μm/s); [2]VSL, straight line velocity (μm/s); [3]VAP, average path velocity (μm/s[1]); [4]LIN, linearity of forward progression (%); [5]STR, straightness (%); [6]WOB, wobble (%); [7]ALH, amplitude of lateral head displacement (μm); [8]BCF, beat-cross frequency (Hz)

Table 4

Sperm subpopulations for kinematic variables (means ± SEM) in caiman (*C. c. fuscus*; *n* = 8; two ejaculates/male)

	SP1	SP2	SP3	SP4
n/%	412/14.5	1 288/45.4	531/18.7	609/21.4
VCL[1]	93.95 ± 0.88[a]	43.46 ± 0.50[b]	63.13 ± 0.78[c]	40.94 ± 0.73[d]
VSL[2]	15.87 ± 0.47[a]	7.90 ± 0.26[b]	24.02 ± 0.41[c]	16.91 ± 0.39[a]
VAP[3]	32.81 ± 0.44[a]	15.95 ± 0.25[b]	31.13 ± 0.39[c]	26.50 ± 0.36[d]
LIN[4]	17.26 ± 0.75[a]	19.31 ± 0.43[a]	38.50 ± 0.66[b]	42.55 ± 0.62[c]
STR[5]	46.45 ± 1.29[a]	49.92 ±0.73[a]	72.97 ± 1.14[b]	63.43 ± 1.06[c]
WOB[6]	36.26 ± 0.56[a]	37.79 ± 0.31[a]	50.37 ± 0.49[b]	65.47 ± 0.46[c]
ALH[7]	4.51 ± 0.04[a]	2.11 ± 0.02[b]	2.64 ± 0.04[c]	2.19 ± 0.03[b]
BCF[8]	3.97 ± 0.09[a]	3.67 ± 0.05[b]	8.00 ± 0.08[c]	2.96 ± 0.07[d]

[1]VCL, curvilinear velocity (µm/s); [2]VSL, straight line velocity (µm/s); [3]VAP, average path velocity (µm/s); [4]LIN, linearity of forward progression (%); [5]STR, straightness (%); [6]WOB, wobble (%); [7]ALH, amplitude of lateral head displacement (µm); [8]BCF, beat-cross frequency (Hz); SEM, standard error of the mean/ [a-d]Different superscripts within row indicate /differences among subpopulations $P < 0.05$

Table 5

Percentage subpopulation distribution of caiman spermatozoa (*C. c. fuscus*) depending on animal

Caiman/Subpopulation	Rapid non-progressive	Slow non-progressive	Rapid progressive	Medium progressive
1	73 (10.1)	380 (52.7*)	122 (16.9)	146 (20.2)
2	87 (14.2)	278 (45.3*)	133 (21.7)	116 (18.9)
3	148 (17.7)	337 (40.4*)	164 (19.6)	186 (22.3)
4	104 (15.5)	293 (43.7*)	112 (16.7)	161 (24.0)

Each row indicates the percentage of spermatozoa belonging to the different cluster (sum of percentage for each animal = 100); *indicates difference within column regarding to animal, chi squared (χ^2) test, $P < 0.05$

Table 6

Pearson correlations between morphometric and kinematic sperm variables in caiman (*C. c. fuscus*)

	HA	HP	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
HA[1]	1	0.693*	-0.381*	-0.069	-0.220*	0.118*	0.099*	0.082	-0.196*	-0.078
HP[2]		1	-0.007	-0032	0.033	-0.027	-0.073	0.057	0.013	-0.007
VCL[3]			1	0.372*	0.652*	-0.187*	-0.058*	-0.298*	0.866*	0.288*
VSL[4]				1	0.688*	0.749*	0.699*	0.366*	0.233*	0.290*
VAP[5]					1	0.277*	0.092*	0.425*	0.578*	0.389*
LIN[6]						1	0.821*	0.636*	-0.225*	0.070*
STR[7]							1	0.206*	-0.134*	0.043*
WOB[8]								1	-0.232*	0.103*
ALH[9]									1	0.143*
BCF[10]										1

[1]HA, head area; [2]HP, head perimeter; [3]VCL, curvilinear velocity (µm/s); [4]VSL, straight line velocity (µm/s); [5]VAP, average path velocity (µm/s); [6]LIN, linearity of forward progression (%); [7]STR, straightness (%); [8]WOB, wobble (%); [9]ALH, amplitude of lateral head displacement (µm); [10]BCF, beat-cross frequency (Hz); *P <0.05