APPLICATION OF CASA TECHNOLOGY AND MULTIVARIATE ANALYSIS TO OPTIMIZE THE SEMEN EVALUATION IN DOMESTIC AND WILD ANIMAL SPECIES

VNIVERSITAT DE VALÈNCIA

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PROGRAMA DE DOCTORADO EN BIODIVERSIDAD Y BIOLOGÍA EVOLUTIVA 3001

Application of CASA technology and multivariate analysis to optimize the semen evaluation in domestic and wild species

by

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Tesis presentada por ANTHONY VALVERDE ABARCA para optar al grado de Doctor en Ciencias Biológicas por la Universidad de Valencia, con el título

“Application of CASA technology and multivariate analysis to optimize the semen evaluation in domestic and wild species”

Firmado: Anthony Valverde Abarca

VNIVERSITAT DE VALÈNCIA Faculty of Biological Sciences

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En Carles Soler Vázquez, Professor Titular del Departament de Biologia Cel·lular, Biologia Funcional i Antropologia Física de la Facultat de Ciències Biològiques de la Universitat de València i **Na Raquel Ortells Bañeres**, Professora Contractada Doctora del Departament de Microbiologia i Ecologia de la Facultat de Ciències Biològiques de la Universitat de València,

CERTIFIQUEN que **En Anthony Valverde Abarca**, *Ingeniero Agrónomo Zootecnista* per la *Universidad de Costa Rica*, ha realitzat, sota la nostra direcció i tutela respectivament, i amb el major dels aprofitaments, el treball d'investigació titulat: "**APPLICATION OF CASA TECHNOLOGY AND MULTIVARIATE ANALYSIS TO OPTIMIZE THE SEMEN EVALUATION IN DOMESTIC AND WILD SPECIES**", i que havent estat conclòs, autoritzem la seua presentació amb la finalitat que puga ser jutjat pel tribunal corresponent i optar així al grau de Doctor en Ciències Biològiques per la Universitat de València, dins del Programa de Doctorat en Biodiversitat i Biologia Evolutiva.

I per a que així conste, en compliment de la legislació vigent, signe el present certificat en València, a 8 d'octubre de 2018.

Signat: Carles Soler Vázquez

Director de la Tesi

Signat: Raquel Ortells Bañeres

Tutora de la Tesi

En Carles Soler Vázquez, Professor Titular del Departament de Biologia Cel·lular, Biologia Funcional i Antropologia Física de la Facultat de Ciències Biològiques de la Universitat de València.

CERTIFICA que les revistes on s'han publicat o sotmès per a la seua publicació els articles que s'integren en la de la Tesi Doctoral d'En **Anthony Valverde Abarca**, titulada: "**APPLICATION OF CASA TECHNOLOGY AND MULTIVARIATE ANALYSIS TO OPTIMIZE THE SEMEN EVALUATION IN DOMESTIC AND WILD SPECIES**", es troeben indexades en els principals índex com ara MEDLINE/Index Medicus, PubMed Central, Scimago Journal Ranking, SCOPUS, Science Citation Index Expanded y Web of Science. Així mateix a continuació s'especifica per a cada revista el factor d'impacte així com l'ISI Journal Citation Reports@ Ranking, tots dos actualitzats al 2017

	Impact Factor 2017
Asian Journal of Andrology	3.259
Reproduction in Domestic Animals	1.422
Livestock Science	1.204
Andrologia	1.588
Andrology	2.734

	ISI Journal Citation Reports @ Ranking: 2017
Asian Journal of Andrology	1/6 (Andrology)
Reproduction in Domestic Animals	19/60 (Agriculture, Dairy & Animal Science
Livestock Science	22/60 (Agriculture, Dairy & Animal Science
Andrologia	4/6 (Andrology)
Andrology	2/6 (Andrology)

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INFORME per tal de donar compliment a la legislació vigent en la Universitat de València, pel que fa a la presentació de Tesis Doctorals per compilació d'articles, que la Tesi Doctoral d'En **Anthony Valverde Abarca**, titulada: "**APPLICATION OF CASA TECHNOLOGY AND MULTIVARIATE ANALYSIS TO OPTIMIZE THE SEMEN EVALUATION IN DOMESTIC AND WILD SPECIES**", inclou tres articles publicats i altres tres que es troben en procés d'avaluació en les revistes indicades en el document que correspon.

Cal assenyalar que en un dels tres articles publicats jo soc el primer firmant, mentre que el present candidat a Doctor signa en tercer lloc. Hem decidit incorporar el susdit paper per la seua rellevància, car és la conclusió d'un número especial de la revista número ú en el rànking corresponent a l'apartat d'Andrologia (*Asian Journal of Andrology*) en el qual del doctorand va actuar com a editor invitat. La seua tasca va centrar-se en l'edició de tres dels papers incluïts en el mateix. Així mateix, cal indicar que la resta de signants del susdit paper són Doctors, per la qual cosa, el treball no ha estat, ni estarà, formant part d'altra Tesis doctoral.

I per a que així conste, en compliment de la legislació vigent, signe el present informe en València, a 8 d'octubre de 2018.

Signat: Carles Soler Vázquez

Director de la Tesi

A mis padres, Patricia y Rafael

A Manri

Cuando emprendas el viaje hacia Itaca

ruega que sea largo el camino,

lleno de aventuras, lleno de experiencias.

Konstantino Kavafis (Alejandría, 1863-1933)

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tecnología al sector productivo, sobre todo pequeños y medianos productores, que tanto necesitan del apoyo de la academia en Costa Rica.

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Tu gente cercana, si… en alma y espíritu, porque por diversos menesteres, la vida los lleva lejos y la cercanía ya no es presencial, pero no importa, te han dado momentos valiosos, recordarán los pormenores de tu vida, compartirán y se alegrarán de tus logros porque los mueve el amor y son esencia del ser que eres. Esto es para tía Nelsy, tía Olga, tía Betty (y Ricardo), tía Daisy, Maribel (y Greivin), tío Álvaro, Ninito, Roxsette, Nane, Kattia y Kristel, Claudia e Isabelita, Kattia y Juliancito, Mónica y mis sobrinos Gabriel y Marcelo, Nía, Kharol, Daniel y Kenito.

A toda mi familia, sin excepción.

Anthony Valverde Abarca

Valencia, octubre 2018

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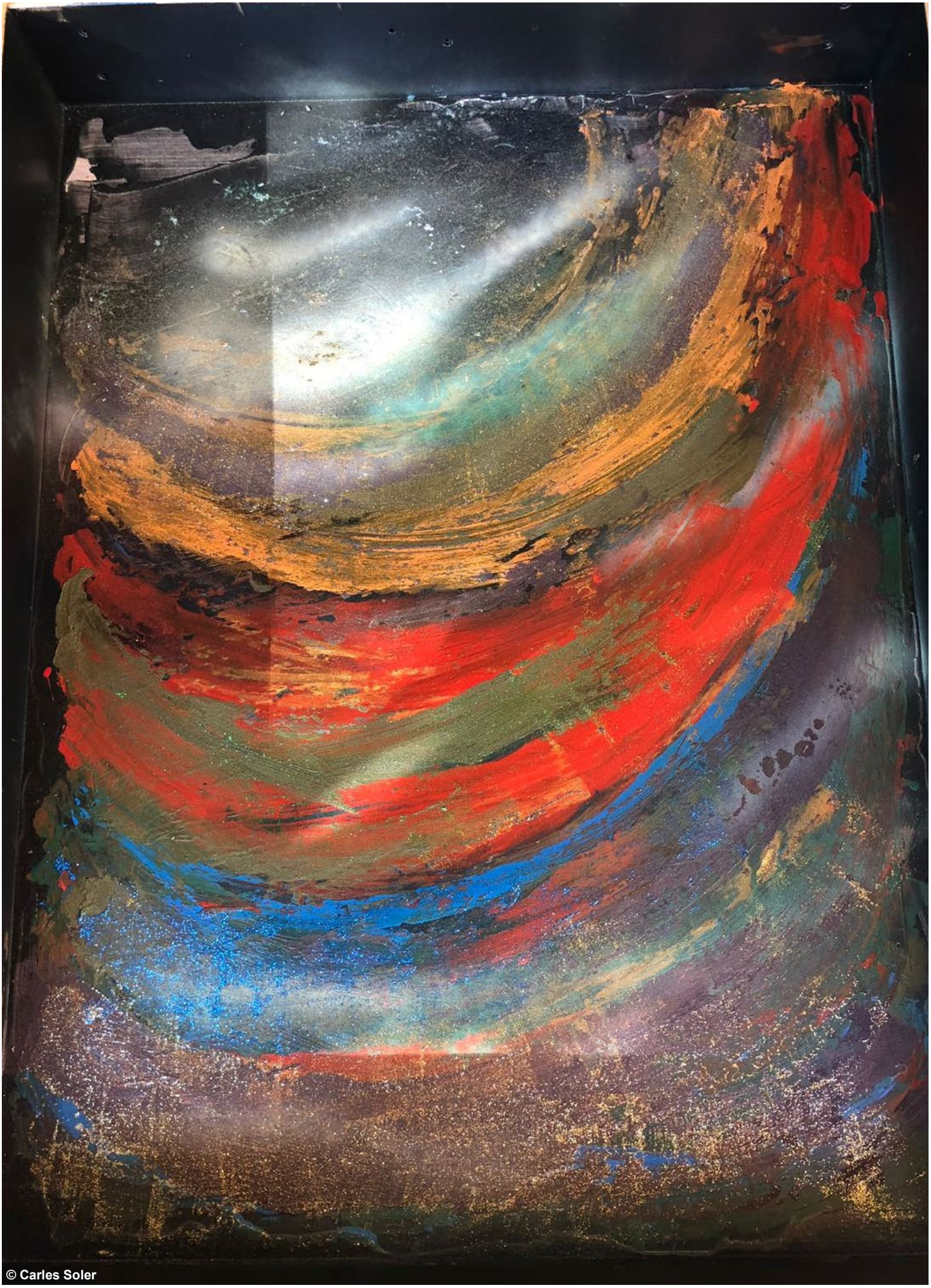
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RESUM

La reproducció assistida en l'àmbit veterinari

En l'actualitat, la majoria de les espècies d'interès ramader es reprodueixen utilitzant alguna tècnica de reproducció assistida, sent la inseminació artificial (IA) la més utilitzada. La pràctica de la IA implica les següents etapes: recol·lecció del semen, avaluació del a qualitat seminal, dilució, transport (amb refrigeració o congelació) i inseminació.

La recol·lecció del semen es pot realitzar per masturbació (rabosa), vagina artificial (bou), electro-ejaculació (quan altres mètodes no són factibles), manipulació digital (caiman), derivació del vas deferens (alpaca) o recuperació epididimària (particularment d'animals d'alt valor que presenten problemes de ejaculació o al poc temps post-mortem).

L'anàlisi tradicional de la qualitat seminal inclou l'avaluació de la concentració i la mobilitat (total i progressiva), paràmetres en els que es basa el càlcul del nombre de dosis que es pot produir a partir d'un ejaculat concret. Aquesta valoració es realitza, habitualment, utilitzant l'aproximació del 5% més pròxim, el que significa que per tal d'assegurar la utilitat de les dosis es sol acudir a un excés de cèl·lules, el que redunda en una deficient productivitat. Val a dir que amb l'ús de la tecnològica CASA (computer assisted semen analysis, veure més endavant), s'ha afegit el càlcul de la cinètica, i encara, de la morfometria com a nous paràmetres més sensibles i reproduïbles.

La dilució del semen és un element clau i ha comportat a la definició de medis de dilució específics tant respecte de la espècie com del sistema de preservació escollit. A la fi l'objectiu és el manteniment de la qualitat espermàtica durant el major temps possible, el que es tradueix en una major distància potencial de transport. Òbviament, la millor de les solucions és la criopreservació (temps il·limitat, si més no teòricament), encara que la refrigeració resulta més pràctica quan la vàlua genètica dels individus no justifica la inversió requerida per a la criopreservació.

Finalment, la inseminació implica el desenvolupament de cànules de inseminació adequades a cada espècie.

La IA ha facilitat la disseminació ràpida i universal del material genètic a partir d'un nombre relativament menor (encara que assegurant la necessària i desitjable biodiversitat) de sementals. Potser, l'espècie on més evident s'ha fet aquest fenomen ha estat la porcina, on de cada ejaculat s'obtenen entre 10 i 20 dosis de inseminació.

Com s'ha esmentat anteriorment, la introducció (quan no la substitució) de sistemes automatitzats d'avaluació espermàtica basats en sistemes CASA va suposar una revolució en el conjunt del procés productiu. Ara bé, val a dir que aquesta tecnologia ha estat generalment implantada sense una anàlisi crítica de les seus limitacions i dependències en diversos factors metodològics, el que, com es comentarà més endavant, constitueix un dels eixos vertebradors de la present Tesi Doctoral.

En qualsevol cas, els sistemes CASA permeten l'anàlisi d'una gran quantitat de cèl·lules en un temps molt curt, aportant també una considerable bateria de dades quantitatives sobre la cinètica o la morfometria espermatozoide a espermatozoide, amb la qual cosa és possible optimitzar la quantitat i la fiabilitat de les dosis seminals produïdes.

Finalment s'ha de fer menció de la importància dels sistemes CASA en la definició de programes de Control de Qualitat en el procés de producció de dosis d'inseminació.

L'analítica Seminal

Recentment, el nostre grup de recerca ha participat en l'edició y publicació de dos números especials de les revistes *Asian Journal of Andrology* (Vol. 16, número 6 de 2016) i *Reproduction Fertility and Development* (Vol. 30, número 6 de 2018) en els que se ha posat al dia el coneixement general sobre el significat de l'anàlisi seminal en allò referent a la morfologia/morfometria i a la mobilitat/cinemàtica dels espermatozoides en una gran varietat d'espècies. Alguns dels treballs constitutius de la present Tesi Doctoral han format part dels esmentats números especials.

Com a resum, val a dir que, una vegada esgotat el significat de la valoració subjectiva de la qualitat seminal, el present i el futur passa per l'ús de les tècniques CASA, i no ja aquelles que han estat sent utilitzades en les darreres èpoques sinó en altres que marquen el camí futur, com és el cas de la refent a la valoració de la mobilitat amb l'ús de microscòpia làser, en un treball també presentat pel nostre grup.

Un element que ha quedat perfectament establert en el conjunt de treballs publicats suposa un canvi substantiu de paradigma conceptual respecte de què és un ejaculat. Val a dir que fins temps recents es va considerar que la considerable població (milions i milions) d'espermatozoides estava format per cèl·lules "equivalents" amb un objectiu comú: ser el que finalment fecundés l'oòcit. La comprovació del fet que el conjunt d'espermatozoides s'agrupa en subpoblacions ben definides, en quant a les seus característiques cinètiques i/o morfomètriques obre el camí cap a una visió

més cooperativa. A un nivell col·loquial e podria dir que s'ha passat de pensar en una marató a pensar en un joc d'equip. Endemés, s'ha vist que la distribució sub poblacional és diferent entre diferents individus, el que sembla indicar diferents estratègies que es poden entendre dins d'un altre paradigma, qual és el de la competència espermàtica entre diferents ejaculats. És cert que encara no coneguem el paper de les diferents subpoblacions o, dit d'altra forma, que no coneguem l'esport que es juga. Però el futur haurà d'anar en eixa direcció. Els treballs que composen aquesta Tesi Doctoral intenten aportar un nou esglaó en aquest sentit.

Objectius generals

L'eix vertebrador de la present Tesi Doctoral omplir el buit detectat en la literatura respecte de l'optimització dels protocols d'avaluació espermàtica. Tan sols en el cas de l'espècie humana, s'ha fet un esforç en aquest sentit el que s'ha traduït en l'elaboració de successius manuals per part de la OMS (Organització Mundial de la Salut). De tota manera, fins i tot, en el cas humà coexisteixen protocols alternatius reconeguts per altres organitzacions internacionals.

Centrant-nos-en la tecnologia CASA, per tal d'obtindre dades quantitatives fiables cal definir protocols òptims per a l'avaluació de cada paràmetre seminal que asseguren la consistència i universalitat de l'aplicació dels resultats. Ara bé, hem constatat que aquesta mena de treball d'estandardització dels protocols encara no s'ha realitzat d'una forma integrativa, la qual cosa configurarà el **primer objectiu general** de la present Tesi Doctoral. Tres són els aspectes fonamentals a considerar en el procés d'optimització que es proposa per a l'ús dels sistemes CASA: el tipus i la profunditat de les càmeres de recompte, el medi de dilució i la freqüència de captura de les imatges. En aquest darrer aspecte, la nova disposició de tecnologia de vídeo a preus assumibles ha mostrat que el increment en la susdita freqüència implica una variació molt significativa en algun dels paràmetres cinètics. Els aspectes tècnics relatius a l'estudi de la morfometria espermàtica no s'inclouen en la present Tesi Doctoral, per quant constitueixen l'objectiu d'altra Tesi Doctoral en marxa en el nostre grup de recerca.

El **segon objectiu general** es centra en l'estudi de l'estructura subpoblacional en diferent espècies i condicions, així de com influencien en la mateixa les diferents condicions tècniques i operatives avaluades.

Objectius específics

1. Comparar diferents aproximacions estadístiques per a l'avaluació de la morfometria espermàtica del bou amb la finalitat de definir el mètode més útil i precís i d'aplicar-lo a

l'avaluació de diferents ejaculats i mostres rere congelació i descongelació del mateix individu.

2. Determinar l'efecte de la freqüència de captura de imatges sobre l'avaluació dels paràmetres cinemàtics en els espermatozoides de porc i de la seua importància en la correcta determinació de la estructura de subpoblacions d'acord a la cinètica.
3. Determinar l'efecte del temps de captura en l'avaluació de la mobilitat i la cinètica en semen de porc per tal d'optimitzar el protocol d'anàlisi de les mostres.
4. Col·laborar en la definició i l'establiment del coneixement del significat de la morfometria espermàtica amb l'ús de la tecnologia CASA-Morph. Aquest objectiu es va completar sent un dels Editors Invitats del número especial corresponent de la revista *Asian Journal of Andrology* de 2016, abans esmentat.
5. Analitzar, en semen de bou, la distribució espermàtica diferencial i les característiques de mobilitat i cinètica a llarg de tres càmeres comercials de recompte (CellVu®, Leja® i Makler®), així com l'efecte de dos medis comercials de dilució (Biladyl® i Andromed®), amb la finalitat d'optimitzar l'ús dels sistemes CASA-Morph.
6. Avaluar la mobilitat i la cinètica, mitjançant un sistema CASA-Mot, en diferents races de gos (Canis familiaris), per tal de definir si la perllongada selecció artificial en aquesta espècie ha conduït a un significant procés de diferenciació gamètica entre races.
7. Caracteritzar la cinemàtica i la morfometria espermàtica del caiman (*Caiman crocodilus fuscus*) i determinar l'existència de subpoblacions al si del ejaculat.

Metodologia

Mostres

La procedència de les mostres va ser:

Bou (*Bos taurus*), de la raça Holstein: Xenética fontao S.A. (42º58' N, 7º34' O), Lugo, Espanya (Objectius 1 i 5).

Porc (*Sus scrofa*), de la raça Pietrain i de les seus línies genètiques derivades: Semen Cardona, S.L. (41°54' N, 1°43', E), Cardona, Barcelona, Espanya (Objectiu 2) i Mejoramiento Porcino S.L. (10°05' N, 84°06', W), San José de la Montaña, Costa Rica (Objectiu 3).

Gos (*Canis familiaris*), de diverses races: Reprovalcan and Clínica Veterinaria Sangüeso (39°29' N, 0°22', W), València, Espanya (Objectiu 6).

Caiman (*Caiman crocodilus fuscus*): Scientific Ecotourism Project (EcoTEC), at the School of Agronomy, at Costa Rica Institute of Technology (10°21' N, 84°30', W), San Carlos Campus, Alajuela, Costa Rica (Objectiu 7).

Recollida i processament de les mostres

En el cas dels experiments amb semen de bou, les mostres es van obtindré amb l'ús d'una vagina artificial en les condicions habituals per a la recollida amb finalitat d'elaboració de dosis seminals comercials. El volum inicial, entre 5 i 10 mL, es va mesurar amb un tub cònic graduat (0.1 mL) i la mobilitat global es determinà ficant 20 µL de semen fresc en un portaobjectes pre-escalfat a 37ºC. Les mostres es diluïren a una concentració final de $100x10^6$, preparant-se les corresponents palletes per a la seua congelació. Per tal d'assegurar, en la mesura del possible, la formació de sub-mostres representatives i evitar imprecisions, les mostres s'homogeneïtzaren sensiblement i es distribuïren en palletes de 0,25 mL, utilitzant una màquina automàtica d'emplenament i segellat i foren immediatament congelades emprant un congelador programable amb una corba de refredament: 4°C a -10°C a -5°C/min; -10°C a -100°C a -40°C/min; -110°C a -140°C a -20°C/min i, llavors, immersió en nitrogen líquid. De cada mostra es descongelaren en un bany d'aigua a 37ºC durant 30s, buidant-se el contingut de les palletes en un tub eppendorf a la mateixa temperatura.

En els experiments realitzats en porc en Costa Rica les mostres s'obtingueren a primera hora del matí, una vegada a la setmana, utilitzant el mètode mà enguantada "gloved-hand" i s'introduïren immediatament en un bany a 38ºC. Es consideraren les fraccions riques en espermatozoides, diluïdes en el medi Androstar Plus®. En aquells fets a València, s'utilitzaren dosis seminals comercials de l'empresa Semen Cardona que es transportaren fins el laboratori a 17ºC, tal i com son distribuïdes a les granges de mares. Una vegada en el laboratori, les mostres s'homogeneïtzaren intensament, agafant-se 1mL que es dipositá en un tub eppendorf i es mantingueren a 37ºC durant 30 min abans del seu ús.

Pel que fa al treballs amb diverses races de gos les mostres s'obtingueren per estimulació manual, dipositant-se l'ejaculat en vasos de recollida estèrils. Les mostres es diluïren a una concentració final $<50 \times 10^6$ espermatozoides/mL amb el medi CaniPlus Chill abans del seu anàlisi. Les races considerades foren: Staffordshire Bull Terrier, Labrador Retriever, Mastí Español, Ratoner Valencià Dog, Buldog Anglés i Chihuahua.

Finalment, en el cas del caiman la col·lecció seminal es realitzà sense anestèsia, rere immobilització de l'animal i per estimulació manual durant l'època reproductiva natural (febrer-

Juny). L'ejaculat (de poc volum) es recollí en un tub eppendorf i es diluí per a la seua estabilització amb medi DPBS.

Anàlisi de les variables espermàtiques

En tots els casos s'utilitzaren sistemes CASA de la marca ISASv1®, encara que en diferents condicions de hardware i software, com s'especifica més endavant.

Els paràmetres obtinguts amb el sistema CASA-Mot foren: velocitat lineal (VSL), velocitat curvilínia (VCL), velocitat mitja ponderada (VAP), amplitud del desplaçament lateral del cap (ALH) i freqüència de batuda de la cua (BCF), així com els índex de mobilitat (LIN, STR i WOB). Endemés, quan es considerà rellevant, es valorà la mobilitat total i la mobilitat progressiva.

Pel que fa a les mesures morfomètriques, les cèl·lules es seleccionaren aleatòriament, evitant aquelles sobreposades a altres cèl·lules o a detritus del fons. S'analitzaren els següents paràmetres del cap de l'espermatozoide: àrea, perímetre, longitud, amplària, àrea corresponent a l'acrosoma (en aquelles espècies on es fa visible), elongació, el·lipticitat, rugositat i regularitat. Endemés, en algunes espècies es varen incloure les següents mesures de la peça intermitja: àrea, amplària, distància i angle de la inserció.

En el primer treball en bou, que versà en morfometria, s'utilitzà un microscopi Olympus BH-2 amb un objectiu de camp clar de 100x i un foto ocular de 3,3x, acoblat a una càmera digital Basler A312 de 768x576x8 bits amb 256 nivells de gris i una resolució, tant horitzontal com vertical de 0,08 µm/píxel. En el segon treball en aquesta espècie, ara sobre mobilitat, el microscopi utilitzat va ser un Nikon Eclipse E600 amb una platina termostatizdada a 37ºC i un objectiu de contrast de fase negatiu de 10x i una càmera digital de vídeo Proiser 782M amb una resolució en els dos eixos de 0,84 µm/píxel. El "setting" utilitzat amb el sistema CASA-Mot ISAS®v1 va capturar a una freqüència de 30 imatges per segon, amb una grandària de partícula de 14-80 μm^2 i una connectivitat de 14 µm. S'avaluà l'efecte de l'ús de tres càmeres de recompte (Leja®, CellVu®, Makler®) i dos medis de dilució, Andromed® i Biladyl®.

Als dos estudis amb mostres de porc, s'utilitzaren càmeres de recompte d'un sol ús ISAS®D4C20, prèviament escalfades a 37ºC, que es carregaren per capil·laritat amb 3 µL de mostra. El microscopi utilitzat fou el UOP/Proiser UB203, calefactat a 37ºC i amb una vídeo-càmera Proiser 782M amb una resolució de 0,84 µm/píxel.

Aquesta mateixa configuració es va utilitzar en el cas del treball realitzat en caiman.

A l'últim, en el cas del treball amb races de gos, les mostres es van mantindre al llarg dels experiments a una temperatura de 24ºC i, utilitzant en mateix hardware abans esmentat, es van realitzar les captures a una freqüència de 25 imatges per segon.

Anàlisi estadística

Les dades obtingudes dels diferents experiments es testaren, en primer lloc, per a la seua normalitat i homoscedasticitat utilitzant els test de Shapiro-Wilks i Levene, respectivament. Així mateix, es va realitzar els corresponents gràfics de distribució per a la seua valoració visual. Quan no es va observar la normalitat, es va realitzar la transformació de l'arrel quadrat de l'arcsinus abans de la realització dels corresponents estudis d'ANOVA de mesures repetides. Quan es mostraren diferències significatives, el test a posterior per tal de definir les possibles diferències entre grups va ser el de Bonferroni.

Quan les variables, fins i tot després de la seua transformació, no seguiren una distribució normal, es va procedir a la realització de tests no paramètrics, el Kruskal-Wallis en una primera etapa i, en cas d'observar diferències, el Test-U de Mann-Whitnay per a comprar entre els diferents grups experimentals.

Per a tots els tests les diferències amb una $P<0,05$ es consideraren com a estadísticament significatives.

L'anàlisi discriminant s'utilitzà en el treball de gos per tal d'avaluar el poder predictiu de classificació de les diferents races en funció dels paràmetres cinemàtics corresponents. L'execució de l'anàlisi discriminant canònic generà una sèrie de funcions ortogonals de forma que la implicació de cada raça en la variable canònica fora el més diferent possible per la maximització de la variància inter-classe al mateix temps que minimitzava la variància intra-classe.

En referència als procediments de clústers, es realitzaren per tal de determinar la presència de subpoblacions espermàtiques a partir dels conjunts de dades tant de cinemàtica com de morfometria. Totes les dades s'estandarditzaren de forma prèvia per tal d'evitar la possible influència d'escala. L'anàlisi de clústers es va efectuar en dues etapes, sent la primera la corresponent a la realització de l'anàlisi de component principals (CP), de forma que cada CP es va contrapesar amb les seues variàncies, conegudes com a "eigenvectors". La valoració final va considerar el nombre menor de CPs que retingueren el màxim possible de la variància original. Aquestes CPs foren utilitzades en la segona etapa de l'anàlisi, que es definiren tot seguint el criteri de Kaiser i emprant per a la definició final la rotació del mètode varimax amb la normalització de

Kaiser. La segona etapa va seguir l'anàlisi no jeràrquica pel mètode de k-means i utilitzant les distàncies d'Euclides a partir de les variables quantitatives rere l'estandardització de les mateixes dades, de forma que els centres dels clústers foren els valors mitjans de les observacions assignades a cada clúster. En aquesta darrera etapa, per tal de determinar el nombre òptim de clústers, els centroides finals se clusteritzaren jeràrquicament emprant el mètode de Ward. A continuació s'aplicaren els test D'ANOVA i de χ^2 per avaluar les diferències en les distribucions de les observacions (espermatozoides individuals) en les diferents etapes i subpoblacions (percentatges d'espermatozoides assignats a cada subpoblació). A continuació s'aplicà el model lineal generalitzat (GLM) per determinar els efectes de les etapes, així com la seua variació, sobre la distribució relativa de freqüències dels espermatozoides en l'interior de les subpoblacions. Aquest darrer procediment se utilitzà també per tal d'avaluar la influència dels paràmetres cinemàtics o morfomètrics en la definició de les diferents subpoblacions. Les diferències entre valors mitjans entre subpoblacions s'analitzaren pel tes de Bonferroni.

Els resultats s'expressaren com a la mitja $\pm$ l'error estàndard de la mitja (SEM) i la significació es considerà a partir de $P<0.05$. totes les dades s'analitzaren utilitzant el software InfoStat (v. 2008) per a Windows, paquet IBM SPSS, versió 23.0.

Resultats i valoració

En el primer treball (capítol IV), l'aproximació "clàssica", basada en l'ANOVA, va mostrar-se insuficient per a la correcta definició de la realitat present en la població total d'espermatozoides, no convenientment representada per tendències centrals, encara que va posar de manifest diferències entre animals, ejaculats i palletes. L'anàlisi de CP mostrà que el conjunt de les variables morfomètriques analitzades s'agruparen en una CP relativa a la grandària una segona CP relacionada amb la forma del cap de l'espermatozoide. L'anàlisi subpoblacional evidencià la presència de quatre subpoblacions referides a caps grans, xicotets, curts i estrets que representaren el 31, 27, 24 i 18% del total de la població, respectivament. La distribució de subpoblacions va ser diferent entre animals i ejaculats, encara que no sempre entre palletes. Aquesta nova aproximació que considera al conjunt dels espermatozoides de una mostra dividit en diferents subpoblacions funcionals en funció de paràmetres quantificables generats per sistemes CASA-Morph obre una nova visió de la funció espermàtica.

En el segon treball, en porc (capítol V), es va posar de manifest que la freqüència de captura d'imatges va afectar sensiblement els paràmetres cinemàtics, sent la VCL la variable més

sensible. Tots els paràmetres mostraren diferències entre animals. L'estudi de correlació no lineal va mostrar una corba asimptòtica amb un punt de saturació per a la VCL de 212 imatges per segon, mostrant-se com el paràmetre més sensible. En referència a la valoració de la mobilitat total i la progressiva 50 i 100 imatges per segon foren suficients, respectivament. L'anàlisi de components principals va oferir tres CPs (velocitat, progressivitat i oscil·lació), que es mostraren independents de la freqüència de captura utilitzada. Finalment, les cèl·lules s'agruparen en quatre subpoblacions que si variaren, tant en la seua cinètica com en la seua distribució, segons la freqüència de captura. Fins al present les càmeres de vídeo presentaven limitacions en la capacitat de incrementar la freqüència de captura el que ha tingut un efecte negatiu en la validesa dels resultats.

En el tercer treball (capítol VI), es va observar, en mostres de porc, que tant la mobilitat total com la progressiva no es veien afectades per la duració del vídeo analitzat, mentre que tenia un efecte significatiu tant respecte de les velocitats com dels índex, però no en l'ALH o la BCF. Tots els paràmetres mostraren diferències entre animals. Els resultats demostraren doncs que la valoració de la cinètica espermàtica es veu afectada pel temps d'anàlisi el que es va posar de manifest amb l'anàlisi de clústers que va mostrar el fet que una durada curta de la gravació pot sobreestimar el percentatge de cèl·lules ràpides no-progressives. Així doncs, es recomana analitzar al menys mig segon per a l'avaluació de la mobilitat i dos segons per a la cinètica.

En el quart treball (capítol VII) posarem de manifest que l'acrònim CASA (Computer Assisted Semen Analysis) per sí mateix resulta insuficient, donat que l'anàlisi pot referir-se a diferents aspectes de la funcionalitat espermàtica, com ara la concentració, mobilitat/cinètica, morfologia/mofometría, Fragmentació del DNA... o combinacions dels mateixos. Com a conseqüència proposarem una nova terminologia, basada en l'ús del terme CASA per a referir-nos-en a la tecnologia, seguit de l'abreviació corresponent que indique l'aspecte estudiat, com ara CASA-Conc (per a concentració), CASA-Mot (per a la mobilitat, tot incloent-hi la cinètica) o CASA-Morph (per a la morfologia i morfometria).

En el cinquè treball (capítol VIII) s'avaluà l'efecte del tipus de càmera i del medi de dilució en la valoració cinemàtica de mostres descongelades de bou. En les càmeres emplenades per capil·laritat (Leja® i CellVu®) s'analitzaren nou posicions, considerant les àrees central i lateral (sumant els dos laterals), així com l'eix longitudinal. En el cas de la càmera Makler® (de forma circular) s'analitzaren també nou camps però no es va poder establir una variació direccional. Independentment del medi de dilució (Biladyl® o Andromed®), hi hagué variacions en les característiques de mobilitat entre les diferents càmeres, indicant que l'eix longitudinal introdueix

variacions en els valors obtinguts. Així mateix va haver un efecte del medi de cultiu. Aquests resultats indiquen la necessitat de realitzar un mostreig representatiu (cosa infreqüent fins al present) i definir protocols adients a cada espècie i condició per al correcte ús de la tecnologia CASA-Mot.

El sisè treball (capítol IX) va evidenciar que els paràmetres cinemàtics estudiats mostrares diferències entre les diverses races de gos. Els espermatozoides amb major velocitat foren els corresponents als Stafforshire Bull Terriers i els de menor al Chihuahua. El coeficient de variació intra-mascle fou major que el inter-mascle per a totes les races, sent el Stafforshire Bull Terrier el que mostrà els valors menors. L'anàlisi discriminant mostrà una elevada capacitat de predicció de la raça a la que pertanyien els espermatozoides. L'anàlisi de clústers mostrà una classificació jeràrquica prou coincident a la referida a filogènies basades en marcadors genètics. Futurs treballs sobre espermatozoides de gos hauran de tindre present les diferències entre races, de forma que els resultat no poden ser transferits d'una a l'altra. Els resultats obtinguts indiquen que el gos potser considerat un bon model en biologia evolutiva per a examinar canvis en diversos paràmetres reproductius associats a processos de selecció.

Finalment, en el setè treball (capítol X), relatiu a mostres de semen de *Caiman crocodilus fuscus*, tant la mobilitat total i progressiva es mostraren constant entre diferents individus, però si hagué diferències significatives respecte dels paràmetres de morfometria i cinètica (tant velocitats com índex). L'anàlisi de CPs va agrupar les variables en quatre components, relatives a la progressivitat, la velocitat, l'oscil·lació i la grandària del cap de l'espermatozoide. Es van obtindre quatre subpoblacions corresponents a les cèl·lules xicotetes de velocitat mitjana i no progressiva, grandària mitja lents no progressius, grans mitjos progressius i xicotets ràpids progressius. La distribució de subpoblacions varià entre animals el que pot presentar relacions ambla fertilitat.

Conclusions

La present Tesi Doctoral ha analitzat l'aplicació de la tecnologia CASA i de l'anàlisi estadística multivariant per tal d'optimitzar l'avaluació seminal en espècies domèstiques i salvatges. Les conclusions a que s'ha arribat són:

1. Si més no, en el cas del boví, les aproximacions tradicionals per a l'estudi de la morfometria espermàtica, basades en l'ANOVA, no son suficientment satisfactòries per definir les subpoblacions espermàtiques, el que fa necessari l'ús de l'estadística multivariant basada en l'anàlisi de components principals. Les diferències observades en l'estructura

subpoblacional de diferents palletes del mateix animal posen en dubte l'anterior idea que la susdita estructura era característica de cada animal, el que indica que també pot estar relacionada amb respostes fisiològiques en resposta al medi ambient.

2. Assumint la BCF i la VCL com els paràmetres més sensitius a la modificació de la freqüència de captura, es recomana capturar el més proper a 225 imatges per segon per a l'avaluació cinemàtica del semen de porc. Per contra, si l'objectiu és mesurar la mobilitat total 50 imatges per segon són suficients i en el cas de la mobilitat progressiva caldrien 150. Qualsevol treball futur ha de tindre present aquest resultats tant en els camps de la recerca com de la producció.
3. Es recomana capturar seqüències de dos segons per a l'estudi cinètic de mostres de porc, sent suficient l'anàlisi de mig segon per avaluar les mobilitats total i progressiva.
4. Suggerim la següent terminologia per tal d'evitar confusions respecte de la tecnologia CASA: l'ús genèric del terme CASA per referir-se a la tecnologia en sí mateixa, que es seguirà de l'abreviatura que indique l'anàlisi específic a que cada sistema es refereix. Per exemple CASA-Mot (per a la mobilitat, incloent-hi la cinètica) i CASA-Morph (per a la morfologia, incloent-hi la morfometria), que són els dos sistemes utilitzats en la present Tesi Doctoral.
5. Si més no, en el cas del boví, l'ús de diferents càmeres de recompte i de medis de dilució causen canvis significatius en l'estimació de la cinètica espermàtica. Així mateix és necessari tindre en compte l'àrea de recompte dins la càmera perquè hi ha diferències, particularment respecte de l'eix de desplaçament vertical.
6. En base a les diferències significatives observades en les característiques cinemàtiques entre diferents races de gos posa en evidència l'error que ha suposat la repetida generalització del concepte "gos", barrejant races incontroladament. Les races de gos poden constituir un model molt útil per examinar la diversitat de respostes en estudis reproductius així com per a la millor comprensió de diversos aspectes de la biologia reproductiva de la femella que podrien estar influenciats per l'evolució de les característiques espermàtiques.
7. L'avaluació de la qualitat seminal resulta molt important en els programes de conservació d'espècies, com hem posat de manifest en el cas del caiman. Els resultats obtinguts en la present Tesi Doctoral poden contribuir al desenvolupament de protocols fiables respecte de l'anàlisi seminal d'espècies de rèptils que poden ser usats per a la determinació del seu potencial reproductiu. L'avaluació espermàtica basada en tecnologia CASA pot facilitar estudis de fisiologia espermàtica i de preservació d'aquestes espècies.

SCIENTIFIC PUBLICATIONS FROM THE PRESENT Ph.D. THESIS

The results of this thesis have been published, accepted or sending for its publication in the following scientific journals:

- **Anthony Valverde**, Héctor Arenán, María Sancho, Jesús Contell, Jesús Yániz, Alejandro Fernández, Carles Soler. (2016) Morphometry and subpopulation structure of Holstein bull spermatozoa: variations in ejaculates and cryopreservation straws. ***Asian Journal of Andrology*** **18, 851–857; doi: 10.4103/1008-682X.187579**

- **Anthony Valverde**, Monica Madrigal, Carina Caldeira, Daznia Bompart, Javier Nunez de Murga, Sandra Arnau, Carles Soler. (2018) Effect of frame rate capture frequency on sperm kinematic parameters and subpopulation structure definition in boars, analyzed with a CASA-Mot system. ***Reproduction in Domestic Animals.*** **(*In press*). doi: 10.1111/rda.13320**

- **Anthony Valverde**, Mónica Madrigal-Valverde, Johan Lotz, Daznia Bompart, Carles Soler. (2018) Effect of video capture time on sperm kinematic parameters in breeding boars. ***Livestock Science*** **(Submitted)*.***

- Carles Soler, Trevor G Cooper, **Anthony Valverde**, Jesús L Yániz. (2016) Afterword to Sperm morphometrics today and tomorrow special issue in Asian Journal of Andrology. ***Asian Journal of Andrology*** **18, 895–897; doi: 10.4103/1008-682X.188451**

- **Anthony Valverde**, Hector Areán, Alejandro Fernández, Daznia Bompart, Almudena García-Molina, Javier López-Viana, Carles Soler. Combined effect of type and capture area of counting chamber and diluent on Holstein bull sperm kinematics. ***Andrologia*****, (Submitted)**.

- **Anthony Valverde**, Sandra Arnau, Almudena García-Molina, Daznia Bompart, Marcos Campos, Eduardo Raúl S Roldán, Carles Soler. Dog sperm swimming parameters analysed by the CASA-Mot system reveal major breed differences. ***Reproduction in Domestic Animals*** **(Submitted)**.

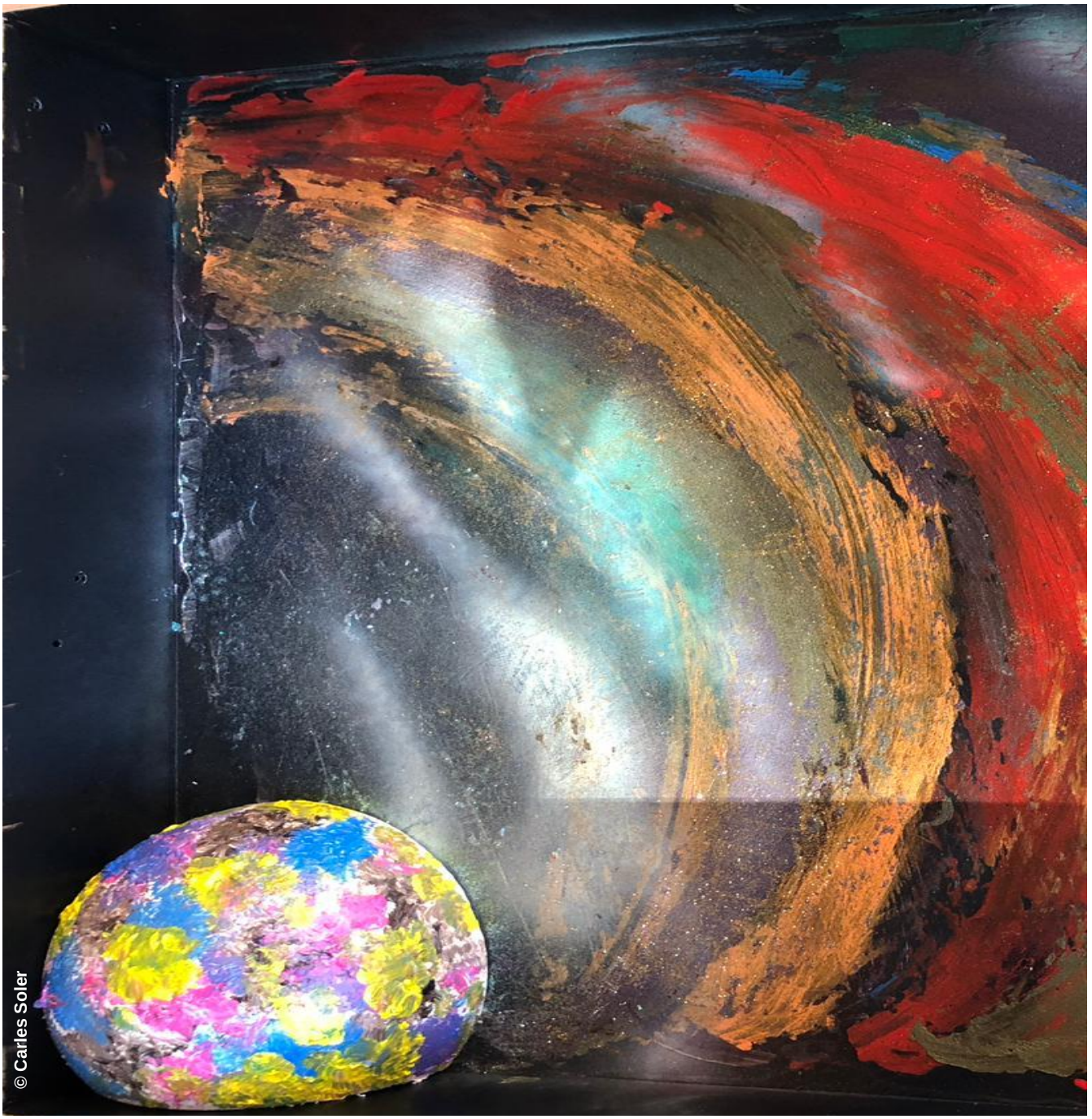
- **Anthony Valverde**, Mónica Madrigal-Valverde, Olivier Castro-Morales, Arnoldo Gadea-Rivas, Stephen Johnston, Carles Soler. Kinematic and head morphometric characterization of spermatozoa from the Brown Caiman (*Caiman crocodilus fuscus*). ***Andrology*** **(Submitted)**.

OTHER PUBLICATIONS RELATED TO THE PRESENT Ph.D. THESIS

During these studies I have also participated in the following publications within the frame of the semen analysis with CASA technology research line:

- Aydee Meza, Carina Caldeira, **Anthony Valverde**, César Ordóñez, Enrique Ampuero, Hernán Cucho, Carles Soler. (2018) Sperm kinematic characterization of alpaca (Vicugna pacos L.) during the reproductive season. **Reproduction in Domestic Animals doi:10.1111/rda.13284 (*In press*)**
- Carles Soler, José Á. Picazo-Bueno, Vicente Micó, **Anthony Valverde**, Daznia Bompart, Francisco J. Blasco, Juan G. Álvarez, Almudena García-Molina. (2018) Effect of counting chamber depth on the accuracy of lensless microscopy for the assessment of boar sperm motility. **Reproduction Fertility and Development 30 (6), 924-934 doi:10.1071/RD17467**
- Carina Caldeira, Almudena García-Molina, **Anthony Valverde**, Daznia Bompart, Megan Hassane, Patrick Martin, Carles Soler. (2018) Comparison of sperm motility subpopulation structure among wild anadromous and farmed male Atlantic salmon (Salmo salar) parr using a CASA system. **Reproduction Fertility and Development 30 (6), 897-906 doi:10.1071/RD17466**
- Daznia Bompart, Almudena García-Molina, **Anthony Valverde**, Carina Caldeira, Jesús Yániz, Manuel Núñez de Murga, Carles Soler. (2018) CASA-Mot technology: How results are affected by the frame rate and counting chamber. **Reproduction Fertility and Development 30 (6), 810-819 doi:10.1071/RD17551**
- Carles Soler, Jesús Contell, Lorena Bori, María Sancho, Almudena García-Molina, **Anthony Valverde**, Jan Segarvall. (2017) Sperm kinematic, head morphometric and kinetic-morphometric subpopulations in the blue fox (Alopex lagopus**). Asian Journal of Andrology 19 (2), 154-159 doi:10.4103/1008-682X.188445**
- Carles Soler, Ana Alambiaga, Maria A Martí, Almudena García-Molina, **Anthony Valverde**, Jesús Contell, Marcos Campos. (2017) Dog sperm head morphometry: Its diversity and evolution. **Asian Journal of Andrology 19 (2), 149-153 doi:10.4103/1008-682X.189207**
- Fernando Vásquez, Carles Soler, Patricia Camps, **Anthony Valverde**, Eduardo Bustos-Obregón, Almudena García-Molina. (2016) Spermiogram and sperm head morphometry assessed by the ISAS®v1 CASA-Morph system: multivariate cluster analysis results during adolescence (12-18 years) and the effect of varicocele. **Asian Journal of Andrology 18 (6), 824-830 doi:10.4103/1008-682X.186873**
- Sara Sadeghi, Almudena García-Molina, Ferran Celma, **Anthony Valverde**, Sogol Fereidounfar, Carles Soler. (2016) Morphometric comparison by the ISAS® CASA-DNA system of two techniques for the evaluation of DNA fragmentation in human spermatozoa. **Asian Journal of Andrology 18 (6), 835-839 doi:10.4103/1008-682X.186875**

CHAPTER I: GENERAL INTRODUCTION



1.1 Male fertility

1.1.1 Sperm production

The testis is an ovoid gland that produces spermatozoa that leave the testes from the bottom and are stored near the top of the testicle and secretes steroid hormones (Amann, 1986). Sperm cells are produced only within the seminiferous tubules of the testes. Sperm production occurs along the entire length of these densely packed tubes within the testes. All of the separate sperm producing tubes, however, eventually converge into a single collection tube in the center (rete testes) of the testicle (Knox, 2003).

The process of reproduction is initiated and regulated by hormones at the level of the brain (Fig. 1). The hypothalamus serves as both a neural and endocrine (hormonal) organ and is located near the base of the brain. The release of anterior pituitary hormones is regulated by hypothalamic hormones (releasing [GnRH Gonadotropin Releasing Hormone] or inhibitory), which are synthesized in the cell bodies of neurons. In response to neural activity, the hypothalamic hormones are released from the nerve endings into the hypophyseal portal blood and are then carried down to the anterior pituitary or Adenohypophysis (Hong, Payne, & Jane, 2016).

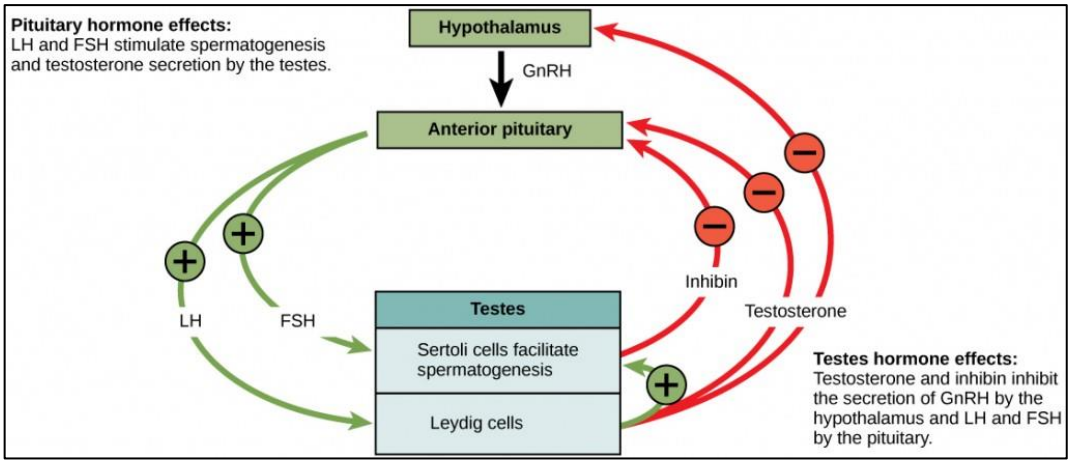


Fig. 1. Hormones control sperm production in a negative feedback system. Image adapted from https://opentextbc.ca/biology/chapter/24-4-hormonal-control-of-human-reproduction/

The GnRH is essential because it is responsible for inducing the release of FSH (Follicle Stimulating Hormone) and LH (Luteinizing Hormone) from the Adenohypophysis, which is located just below the hypothalamus. In males, FSH is required for spermatogenesis, and LH stimulates testosterone secretion by Leydig cells (ElSayed & Bhimji, 2018).

The process of meiosis (Fig. 2) reduces by half the somatic or diploid number of chromosomes (2*n*, where *n* equals the number of pairs of chromosomes), including the two sex chromosomes. In de male case, the male has an XY complement of chromosomes, and his spermatozoa can carry either the X or the Y chromosome. In spermatogenesis, the meiotic prophase is the same as in oogenesis, but after that, it differs in several respects. It does not begin until around the time of puberty, but once it has started, it then continues in an uninterrupted manner throughout adult life (Lawrence, Fowler, & Novakofski, 2012).

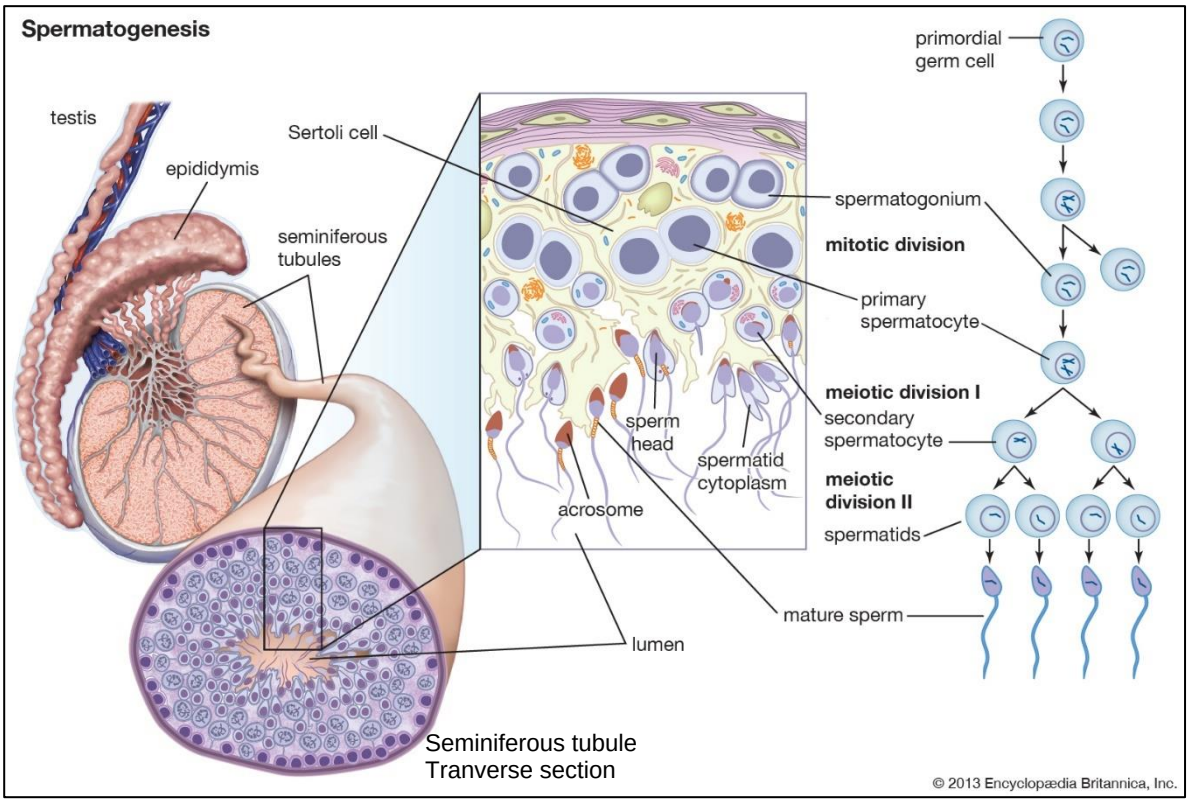


Fig. 2. During spermatogenesis, four sperm cells are produced from each primary spermatocyte. Spermatogenesis begins when the 2*n* (diploid) spermatogonium undergoes mitosis. The spermatogonia undergo meiosis I, producing haploid (1*n*) secondary spermatocytes, and meiosis II, producing spermatids. Differentiation of the spermatids results in mature sperm. By courtesy of Encyclopædia Britannica, Inc., copyright 2013; used with permission.

1.1.2 Epididymal factors to sperm maturation and storage

The epididymal function is vital for the fertility of male mammals because their sperm is infertile when they leave the testes and only acquire the ability to fertilize an ovum during passage through the epididymides. It is relevant that the epididymides accumulate and store sperm as it, depending upon species, it takes 0.5–2 days for the testes to produce the number of sperm in a normal ejaculate (Jones, 1999; Jones, Dacheux, Nixon, & Ecroyd, 2007). The success of sperm storage in the epididymis is so high which makes that fertile sperm can survive in an isolated epididymis for several days at 4 °C (Dacheux et al., 2009).

Although all mammals have an initial segment of the epididymis with distinctive characteristics, there is variation between species in the structure and length of the different segments, suggesting some variation in post-testicular sperm maturation and storage (Jones, 2002). Due to the variations in epididymides between species, it is considered that there must be variations between species in the changes in protein composition throughout the epididymis (Dacheux, Gatti, & Dacheux, 2003) and probably indicating the relative significance of sperm maturation and storage between species.

1.1.3 Fertility evaluation

It was well established that seminal characteristics are related to the fertility of the samples (Flowers, 2009). However, fertility is multifactorial, and several factors including season could influence the outcome, the number of sperm, the timing of copula before ovulation, and the individual sire's seminal plasma profile (Flowers, 2009; Vesseur, Kemp, & Den Hartog, 1996), and if that was not enough it must be considered the female effect.

Furthermore, functional and structural sperm parameters such as motility, kinematic, viability, acrosome and DNA integrity, mitochondrial function, morphology, and morphometrics (Gillan, Evans, & Maxwell, 2005) may be associated with fertility process.

Concerning seasonal effect, boar or bull is not usually considered a seasonal breeder, can also occur seasonal variations of semen quality (Ibănescu et al., 2018). Variations of sperm parameters between summer and winter months have been partially attributed to related changes of scrotal thermoregulation and heat dissipation mechanisms (Menegassi et al., 2015).

1.2 Assisted reproduction in animal species

Reproduction within animal farms is usually by artificial insemination (AI), reflecting the importance of this procedure that has increased and developed globally after its introduction in the first half of the 20th century (Amann & Waberski, 2014; Hansen, 2014). Regarding males, the AI implies the following steps: semen collecting, assessment, dilution and frozen-thawed, and insemination.

The collection can be performed by using an artificial vagina (Barszcz, Wiesetek, Wasowicz, & Kupczynska, 2012), masturbation (Knox, 2016) and after electro-ejaculation (Palmer, Amundson, Brito, Waldner, & Barth, 2004). The sperm motility (total, progressive), kinetics, and concentration are the principal variables assessed at the AI centers or farms to calculate AI doses. For dilution of the samples species, specific extenders must be defined both for refrigeration or cryopreservation of the samples (Foote, 2002). In the case of cryopreservation specific protocols for frozen-thawed, including vitrification, must be defined for each species (Sieme & Oldenhof, 2015; Yeste, 2016). Finally, the insemination requires the development of specific catheters (Hernández-Caravaca et al., 2017).

The AI facilitates the rapid dissemination of genetic material from a small number of genetically superior sires to a large number of females of the population (Vishwanath & Shannon, 1997), is the most important technique to facilitate the genetic improvement of animals (Howley, Donoghue, & Heanue, 2012).

Besides, the AI reduces the need for studs in the farms and improve the accuracy of reproductive records (Funk, 2006). For instance, in swine, the rate of genetic progress can be dramatically increased with AI. Semen could be collected from top sires and use by mating 10 to 20 females with a single boar ejaculate (Johnson, Weitze, Fiser, & Maxwell, 2000). With advancements in the development of longer-term extenders, the ability to spread the doses transport distances increased a lot allowing the rapid adoption of AI around the globe (Knox, 2016).

1.2.1 Seminal doses production

The primary objective is to obtain the higher number of doses from each ejaculate (or pool, depending on species) having enough good number of spermatozoa to assure the pregnancy of the inseminate female (Tsakmakidis, Lymberopoulos, & Khalifa, 2010). Traditionally, the semen evaluation (see below for details) was performed using the 5 % approximation by a subjective approach, what suppose a much loss of precision and reliability. The typical way to overpass these limitations as to put more cells than that needed (Soler et al., 2017).

The introduction of computer-assisted semen analysis (CASA) technology has revolutionized the semen evaluation process for seminal doses production and the quality control planned for marketing or research (Amann & Waberski, 2014; Didion, 2008; Feitsma, Broekhuijse, & Gadella, 2011), however this evaluation could be affected by several factors (Kathiravan, Kalatharan, Karthikeya, Rengarajan, & Kadirvel, 2011). The computers have the capabilities for rapid counts of hundreds of sperm in seconds being able to analyze motility, kinematics, morphometrics, and concentration and subsequently for optimized the number and reliability of the final produced seminal doses.

The success of AI in animals is also related to the ability of farm staff to detect estrus, their skills during actual insemination, as well as semen quality (Holt, Holt, Moore, Reed, & Curnock, 1997). When semen parameters are sub-optimal as volume, sperm number, motility or sperm morphology, conception rates can be affected (Flowers, 1997). In this context, several authors have demonstrated the correlations among some semen parameters, including those evaluated using CASA systems and fertility indices (Budworth, Amann, & Chapman, 1988; Hirai et al., 2001; Hirano et al., 2001; McPherson, Nielsen, & Chenoweth, 2014; Sutkeviciene, Andersson, Zilinskas, & Andersson, 2005).

Finally, the introduction of Quality Control Programs during all the process for the production of AI doses is needed, and the use of CASA technology makes it easy, reproducible, and reliable (Gadea, 2005). These programs have been successful for many years and have been important for assuring successful results in AI around the world (Maes, López Rodríguez, Alfonso, Rijsselaere, Vyt, & Van Soom, 2011).

1.3 Semen analysis

Microscopic sperm motility observation started a soon as the 1600s (Sztein, Takeo, & Nakagata, 2018), and motility is remaining as the most used parameter for semen quality evaluation in AI centers (Lenz, Kjelland, VonderHaar, Swannack, & Moreno, 2011). The evaluation of sperm motility provides relevant information on the energy status of mammalian sperm (Quintero-Moreno, Rigau, & Rodríguez-Gil, 2004; Roldan, 1998). Furthermore, the motility function has played an essential role once spermatozoa reach the utero-tubal junction, which contains mucus (Jansen, 1978; Jansen & Bajpai, 1982) and has served as a barrier to sperm with poor motility (Mortimer, 1997).

Motility evaluation has presented a degree of subjectivity, due to a visual estimation of the percentage of total motile spermatozoa, which have reduced its potential as a fertility marker (Walker, Winet, & Freund, 1982). Subjective estimation of motility is affected by different factors as technician effect (Gallego, Herranz-Jusdado, Rozenfeld, Pérez, & Asturiano, 2018; Rijsselaere et al., 2005; Rijsselaere, Van Soom, Maes, & Kruif, 2003; Verstegen, Iguer-Ouada, & Onclin, 2002).

As it was pointed out before, the typical approaches are using a 5% approximation of motility, and a classification of 1 to 5 (slowest-to-fastest) speed of progression (SOP) score to characterize overall motility of a semen sample and to assess sperm quality. This approach is lacking the precision that is required for accurate motility estimation (Zhao et al., 2004).

On the other hand, sperm morphology is also considered an important part of the semen analysis reflecting the genetics of the spermatozoa (Murphy, Fahey, Shafat, & Fair, 2013; Thurston, Watson, Mileham, & Holt, 2001). Sperm cells with abnormal morphology have been associated with low fertility rates (Barth, Bowman, Bo, & Mapletoft, 1992; Chandler, Painter, Adkison, Memon, & Hoyt, 1988; Jasko, Lein, & Foote, 1990b). However, subjective estimates of sperm morphology imply lack of precision, repeatability, and accuracy (Hidalgo, Rodríguez, & Dorado, 2006). Besides, in species as ram the number of obvious morphological abnormal cells is meager (Sancho, Pérez-Sánchez, Tablado, de Monserrat, & Soler, 1998). Moreover, most of the morphological observation techniques imply high time consuming (Soler et al., 2005). All these limitations have conducted to the fact that sperm morphology is no commonly evaluated during seminal doses production and, if it is, limited to the evaluation of cytoplasmic droplets or too obvious teratozoospermic samples (Thundathil, Palasz, Barth, & Mapletoft, 2001; Zou & Yang, 2000).

All these limitations were the basis for the advent of the CASA technology at the beginning of the 80's of the last century (Bompart et al., 2018; Yániz, Silvestre, Santolaria, & Soler, 2018).

1.4 Computer Assisted Semen Analysis (CASA) systems

As it was indicated before, CASA technology has been commercially available since mid-1980s and provide a more objective sperm characteristics evaluation (Holt, Cummins, & Soler, 2018; Soler, Cooper, Valverde, & Yániz, 2016). The main components of a CASA system include a microscope equipped with a heated stage and negative phase contrast optical device with an attached video camera. The signal of the camera is arriving at a computer which has specific software for different kind of analysis (Fig. 3)

The measurement of sperm motility and kinematics has been a target of semen research for over 25 years (look the special number of Reproduction Fertility and Development (RFD) 30(6), 2018 for a thorough review of state of the art). CASA-Mot systems offer a big battery of motility parameters in addition to the general motility evaluation. During this time it was showed that these parameters are sensitive to several hardware and software parameters, as well as the variability of semen samples (Bompart et al., 2018; Castellini, Dal Bosco, Ruggeri, & Collodel, 2011; Yeste, Bonet, Rodríguez-Gil, & Rivera Del Álamo, 2018).

Fig. 3. Views of a 2018 CASA system. CASA, computer-assisted semen analysis. Image provided by C. Soler.

In parallel to the development of CASA-Mot systems, CASA-Morph commercial systems were designed to reduce the subjectivity of sperm morphology assessment (Yániz, Soler, & Santolaria, 2015). Even no in the same volume that kinematics studies, morphometry has also been widely considered in the last few years (Yániz et al., 2015), (look also the special number of the Asian Journal of Andrology (AJA) 18(6), 2016 for complete review of the state of the art). When morphometric instead morphological criteria were used the predictive ability of morphological aspects of sperm quality was highly increased (Soler et al., 2005).

In general, the CASA parameters enable the comparison of results, both inside the laboratory and between laboratories, and makes it possible to detect quantitative differences in seminal parameters. These facilities apply to studies analyzing different experimental or productive situations (Palacín, Vicente-Fiel, Santolaria, & Yániz, 2013) and to find differences between males or treatments and interactions inside one experiment (Verstegen et al., 2002).

Moreover, the capacity of CASA for generating large datasets comprising motility data from thousands of spermatozoa has been overlooked in use favor of the summary statistics provided by the software, which do not show the intrinsic variability of the semen sample (Martínez-Pastor, Tizado, Garde, Anel, & de Paz, 2011). The first step in the evaluation of CASA datasets is a data outlier evaluation (Martínez-Pastor et al., 2011) as it will be developed in the following sections.

1.5 Technical conditions and limitations of CASA technology

Although CASA systems have demonstrated to be more accurate than traditional methods (Broekhuijse, Šoštarić, Feitsma, & Gadella, 2011; Didion, 2008; Krause, 1995), errors in the automatic detection of sperm tracks and silhouettes can occur (Amann & Waberski, 2014; Broekhuijse et al., 2011; Simonik et al., 2015). The final results can be influenced by many unrelated to the semen samples factors (Ehlers, Behr, Bollwein, Beyerbach, & Waberski, 2011), such as dilution of semen sample, time elapsed since ejaculation until examination, sample temperature, counting chamber type used (Del Gallego et al., 2017; Bompart et al., 2018) and location inside the camera (Nöthling & dos Santos, 2012), number of analyzed fields (Broekhuijse et al., 2011), recording frame rate (Castellini et al., 2011; Wilson-Leedy & Ingermann, 2007), staining technique for morphological evaluation (Soler et al., 2005), and type of CASA system used (Jasko et al., 1990a; Boryshpolets et al., 2013).

Attention must be devoted to the counting chamber used. Different types of counting chambers can be used on the CASA-Mot systems that differ widely of volume inside the chamber, depth, shape and loading modality (for revision look (Bompart et al., 2018)). Some studies have shown that counting chamber used for the semen analysis had a significant effect on sperm kinetics in cattle (Gloria et al., 2013), goat (Del Gallego et al., 2017), human (Peng, Zou, & Li, 2015), ram (Palacín et al., 2013), and stallion (Hoogewijs et al., 2012).

Another significant factor is related to the CASA system design. Although most of them are based on similar principles, they differ regarding optics, hardware and software characteristics, particularly in that referred to the algorithms used for sperm segmentation, identification and trajectory reconstruction and silhouette identification (Kraemer et al., 1998; Yániz et al., 2017). The CASA systems differ in their grayscale bit-depth, frame rate (Wilson-Leedy & Ingermann, 2007), number of consecutive frames analyzed, grayscale thresholding method, image segmentation method to determine the pixel coordinates of the sperm, head versus midpiece tracking, and strategies for handling collisions between spermatozoa (Shi, Nascimento, Berns, & Botvinick, 2006), as well as to define the different morphological components of the cell (Yániz, Capistrós, Vicente-Fiel, Hidalgo, & Santolaria, 2016).

Furthermore, CASA systems can provide high accuracy and repeatability (Davis, Rothmann, & Overstreet, 1992; Farrell, Trouern-Trend, Foote, & Douglas-Hamilton, 1995). Standardization of equipment used in the process can further influence results (Verstegen et al., 2002), along with the training level or expertise of the technician (Comhaire, Huysse, Hinting, Vermeulen, & Schoonjans,

1992; Ehlers et al., 2011; Holt, Watson, Curry, & Holt, 1994), the technical settings (Mortimer, Aitken, Mortimer, & Pacey, 1995; Rijsselaere et al., 2003), the type of software (Tejerina, Buranaamnuay, Saravia, Wallgren, & Rodriguez-Martinez, 2008) and the type of chamber used for analysis (Gloria et al., 2013; Hoogewijs et al., 2012; Kraemer et al., 1998; Lenz et al., 2011; Tomlinson, Turner, Powell, & Sakkas, 2001).

In general, the effect of the different technical settings was found as showing high relevance for the kinematics (Contri, Valorz, Faustini, Wegher, & Carluccio, 2010) and morphometrics (Yániz et al., 2015) evaluation. All these factors claim for well-defined inclusive protocols for the standardization of results output, being one of the central objectives of the present work.

1.6 CASA parameters

1.6.1 Motility and kinematic parameters

Most of the CASA-Mot systems plot the movement of a sperm head centroid creating a trajectory and analyzing the kinematic parameters of the resulting track. Sperm kinetics include measuring the distance between each head point for a given sperm during the acquisition period. Sperm motion parameters are mainly composed of three values on sperm motion velocity, three on velocity ratio and two reflecting sperm wobble characteristics. The main parameters are: curvilinear velocity (VCL, $\mu m \cdot s^{-1}$) is measured by summing the distance between the sperm head centroid positions frame by frame, divided by the elapsed time; the straight line velocity, (VSL, $\mu m \cdot s^{-1}$) is the distance between the first and last points of the sperm track, divided by the elapsed time; the average path velocity (VAP, $\mu m \cdot s^{-1}$) is the average path length, determined by smoothing the sperm head position in a running average (what algorithm change among CASA-Mot brands), divided by the elapsed time; linearity (LIN, %) measures the level of linear progression and is calculated as the ratio VSL/VCL in percent; the straightness (STR, %) is the ratio VSL/VAP in percent and is a measure of track compactness; the wobble (WOB, %) refers to the oscillation of the actual path about the average path and is expressed as ratio VAP/VCL in percentage; the amplitude of lateral head displacement, (ALH, µm) is the amplitude of the approximately sinusoidal oscillation of the sperm head about the track (can be considered as the maximum or the mean value along the track); the beat-cross frequency (BCF, Hz) is the frequency with which the sperm head crosses the average path line during acquisition (Kay & Robertson, 1998) (Fig. 4).

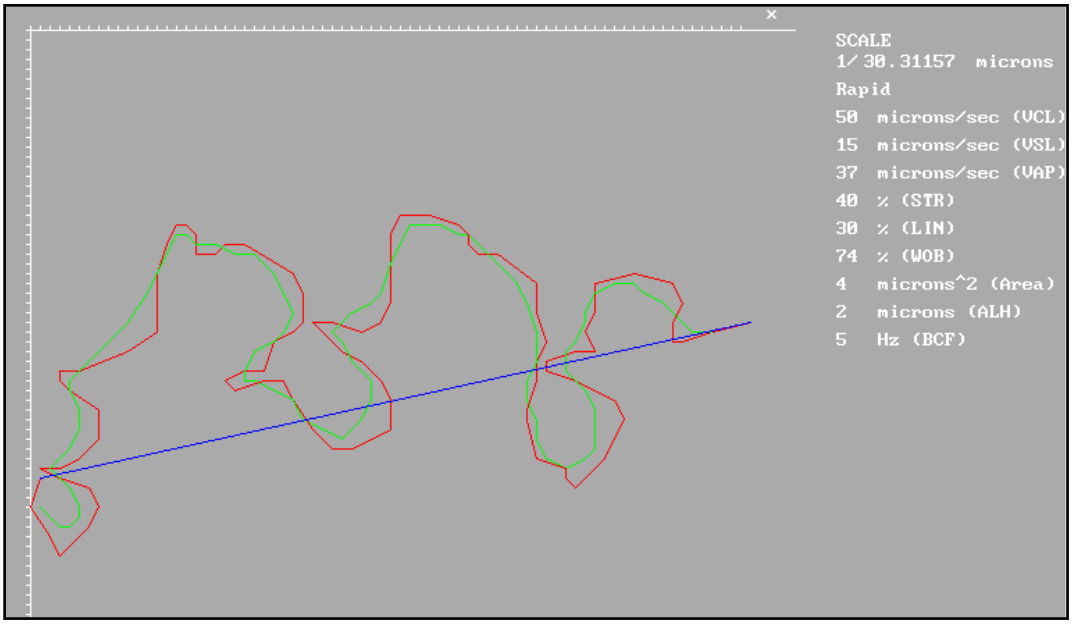


Fig. 4. Kinematic parameters as presented by the ISAS®v1CASA system. The red line shows curvilinear velocity (VCL), the blue line shows straight line velocity (VSL) and the green line shows average path velocity (VAP). STR, straightness (= VSL/VAP*100); LIN, linearity (= VSL/VCL*100); WOB, wobble (= VAP/VCL*100); ALH, amplitude of lateral head displacement; BCF, beat-cross frequency (Soler et al., 2018).

As it was mentioned previously, some variables as sample concentration and dilution, the frame rate (Castellini et al., 2011; Morris, Coutts, & Robertson, 1996), the frequency of image acquisition (Acosta & Kruger, 1996), algorithm for the reconstruction of the trajectories (Morris et al., 1996), the number of fields analyzed, and counting chamber used can affect motility results in semen evaluation even with the same CASA device (Rijsselaere et al., 2003, Bompart et al., 2018).

Maybe the FR is the key factor, and it was conditioned for a long time by the available video-cameras. At the begging of the technology as low as 16 or 25 frames per second (fps) were used, but in the recent times it is more frequent, particularly for human samples an acquisition frequency rate of 50–60 Hz is having been recommended (Morris et al., 1996). However, when cells display high speed and low linearity, such as the hyperactivated sperm, an increased frame rate is recommended (Mortimer & Swan, 1999). Recent works of our group are indicating that for most of the species this remains wholly inadequate being necessary higher FR for assuring the veracity of the measurements (unpublished results), being one of the objectives of the present Thesis in the studied species.

1.6.2 Morphometry

Most published studies on sperm morphometry have been focused mainly on the sperm heads, although some of them have also measured other parts of the sperm cell structure, such as the nucleus, acrosome, midpiece or the whole flagellum (tail, including the midpiece) (Yániz et al., 2015). Different parameters have been used to describe the morphometry of sperm heads, but the most commonly accepted are: primary parameters that provide information on sperm head dimensions, and usually include length (L, μm), width (W, μm), area (A, μm^2), and perimeter (P, μm), and derived parameters as approximations to head shape using a series of mathematical formulae, including, among others, Ellipticity = L/W, Rugosity (also known as roughness) = $4\pi A/P^2$, Elongation (lack of roundness) = (L − W)/(L + W), and Regularity = πLW/4A (Fig. 5). To some authors, ellipticity and elongation provide redundant information as they describe the same phenomenon: the ratio between sperm head lengthening and widening (Sánchez, Bastir, & Roldan, 2013), but, in general, the multivariate mathematical analysis includes both as significant (Vásquez, Soler, Camps, Valverde, & García-Molina, 2016).

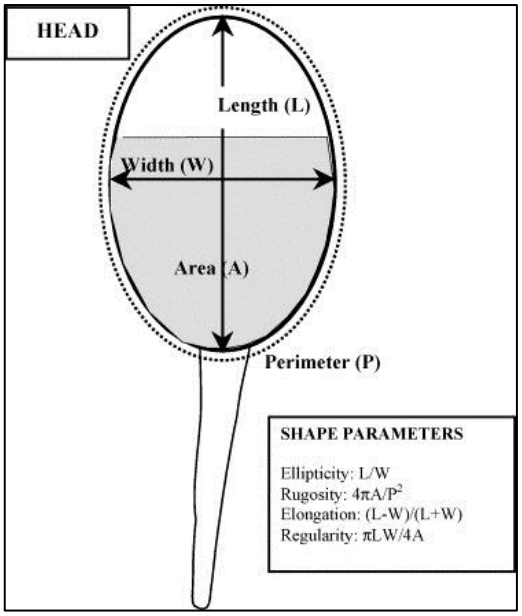


Fig. 5. Morphometric sperm-head parameters. The length (**L**, along with the major axis), width (**W**, along with the shortest axis), area (**A**) and perimeter (**P**) of the head are self-evident. Shape parameters are mathematical combinations following the correspondent expressions (Soler et al., 2005).

Morphometric results may be influenced by factors intrinsic and extrinsic to the male. Intrinsic factors may include genetic or environmental factors, age and sexual maturity, and sampling frequency. Nevertheless, the most important factor is the introduction of artifacts as a consequence of the

staining technique. In one or another way this process implies the dehydration of the cells what is introducing a sensitive change in the final observed morphology (Soler & Cooper, 2016; Yániz et al., 2015). However, also, the fixation of the samples using chemical fixatives introduces dramatic changes in the final cell morphometry (Sancho et al., 1998; Soler et al., 2000). To solve this handicap a new technique was proposed (Soler, García-Molina, Contell, Silvestre, & Sancho, 2015; Soler et al., 2016) to observe and analyze the sperm morphometry on cells in suspension without staining, named Trumorph®, that was used in part in the present Thesis.

In all the studied species individual differences in sperm head morphometry were observed (see Yániz et al., 2015b and the special number of AJA 18(6), 2016). Differences in sperm morphometry have also been described between different related species (Soler et al., 2014), subspecies (Beletti, Costa, & Viana, 2005) and breeds (Martí, Aparicio, & García-Herreros, 2011; Saravia et al., 2007). Extrinsic factors may include those related to the influence of environment on the donor (Immler, Pryke, Birkhead, & Griffith, 2010; Yániz et al., 2015).

1.7 Sperm subpopulation concept

The spermatozoon is a dynamic cell, and their biochemicals process modify the sperm physiology throughout maturation, ejaculation, transport in the female genital tract and fertilization (Chamberland et al., 2001). These physiological changes relate flagellar beating, thus spermatozoa show different swimming patterns in the epididymis, seminal plasma, cervical mucus and oviduct (Hamamah & Gatti, 1998; Tash & Bracho, 1998). Sperm samples are heterogeneous, implying that spermatozoa with different motility values coexist in the same ejaculate (Chantler, Abraham-Peskir, & Roberts, 2004; Katz & Davis, 1987; Katz, Erickson, & Nathanson, 1979; Neill & Olds-Clarke, 1987). Also, the morphology is heterogeneous in different levels depending on the species, being the human one of the most heterogenous inside mammals (Yániz et al., 2015).

The historical view conceived the ejaculate as a conjunct of “equivalent” cells competing for arrival to the oocyte (like in a marathon). However, this conceptual approach is in contradiction with the observed heterogeneity. To solve this, with the arrival of the XXI century, and using the quantitative sperm parameters obtained with CASA technology and multivariate analysis, the paradigm is changing to a subpopulation approach (Quintero-Moreno, Miró, Rigau, & Rodríguez-Gil, 2003; Valle et al., 2013). As indicated previously, the specials numbers of AJA 18(6), 2016 and RFD 30(6), 2018, define the stat of the art from morphometry and kinematic parameters.

Nowadays, we can consider well defined the sperm subpopulation structure in all the studied species, but the real biological significance remains to be defined. The present Thesis had the increase our knowledge about this as the second general aim.

1.7.1 Principal components analysis

Principal component analysis (PCA) is a multivariate technique that is being used for the dimension-reducing CASA data (Caldeira et al., 2018; Dorado, Molina, Muñoz-Serrano, & Hidalgo, 2010; Maroto-Morales et al., 2016; Martínez-Pastor et al., 2011; Ramón & Martínez-Pastor, 2018; Soler et al., 2017). In brief, PCA replaces the variables in a multivariate data set by an uncorrelated set of derived variables (linear combinations of the initial variables) called principal components (Fig. 6). This enables the selection and use of only the principal components conveying most of the total variance, thus reducing the number of variables or variable selection. Also, the conceptual weight of the new variables, that integrate some coherent individual ones increases the significance of the derived results (Ramió et al., 2008).

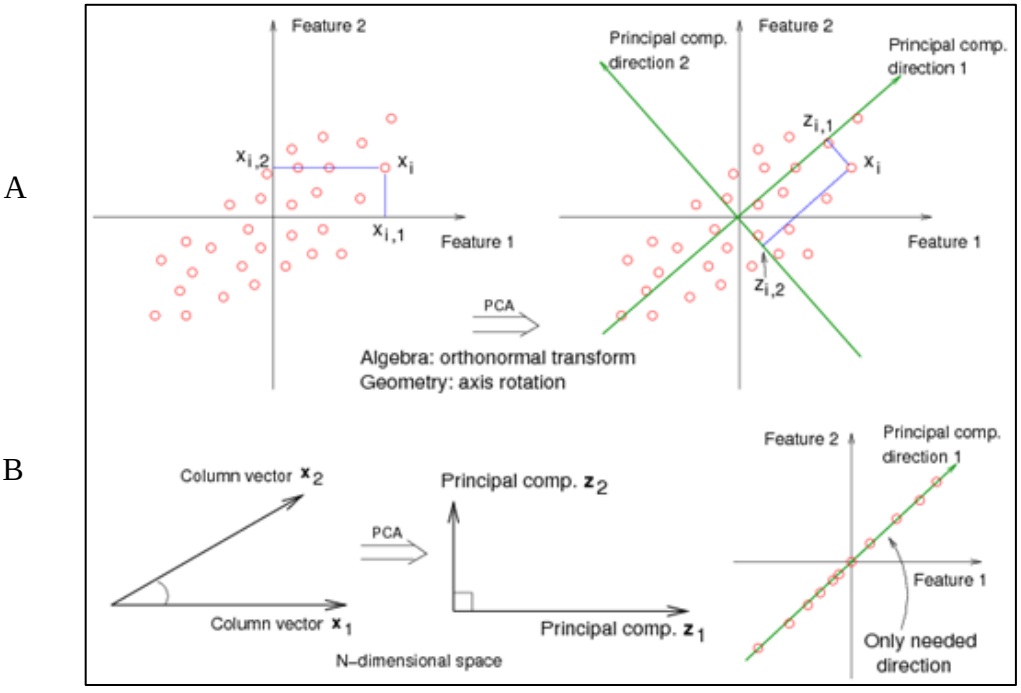


Fig. 6. **A:** Principal Components Analysis chooses the first PCA axis as that line that goes through the centroid, but also minimizes the square of the distance of each point to that line. Equivalently, the line goes through the maximum variation in the data. The second PCA axis also must go through the centroid and goes through the maximum variation in the data, but with a certain constraint: It must be completely uncorrelated (i.e., at right angles, or "orthogonal") to PCA axis 1. **B:** Consider an extreme case, (lower right), where your data all lie in one direction. Although two features represent the data, we can reduce the dimension of the dataset to one using a single linear combination of the features (as given by the first principal component). Image adapted from https://onlinecourses.science.psu.edu/stat857/node/154/

1.7.2 Clustering methods

There are two different approaches to multivariate analysis for classification: the discriminant and cluster analysis. The first one is based on an *a priori* classification based on canonical subjects of well-predefined classes (male/female) while the second one is just looking for the intrinsic mathematical distances among the considered variables to define a conjunct of classes, being needed to *a posteriori* definition of their meaning (Kaufman & Rousseeuw, 2005; Spencer, 2013).

Cluster analysis is a technique for multivariate statistical data analysis that allows unsupervised grouping of observations into subsets (called clusters) so that observations in the same cluster are similar depending on a given criterion (Everitt, Landau, Leese, & Stahl, 2011; Kaufman & Rousseeuw, 1990; Xu, Wunsch, Xu, Wunsch, & Wunsch, 2005). “Unsupervised” implies that there is not an *a priori* grouped dataset to guide the grouping. Therefore, cluster analysis is suited to resolve the heterogeneity of sperm motility data in discrete subpopulations, helping to take advantage of the information contained in CASA datasets (Martínez-Pastor et al., 2011).

The cluster analysis aims to divide the observations into homogeneous and distinct groups. The objective of cluster analysis is to assign observations to groups ("clusters") so that observations within each group are similar to one another concerning variables or attributes of interest, and the groups themselves stand apart from one another (Everitt et al., 2011). Cluster analysis involves several formulating problems as the selection of distance measure, clustering procedure, number of clusters, and the interpretation of the profile clusters and the assessment of the clustering validation.

The datasets are must first explore before the clustering process. Testing that should be made as to lack of fit for normal distribution, skewness, outliers, extreme values, data “noise,” a weak clustering structure, and multicollinearity among different variables (Spencer, 2013). Nevertheless, we must take into account that datasets are expected to bear non-normal distribution and skewed variables. Thus, the presence of such features should not be automatically taken as a sign of incorrect data (Martínez-Pastor et al., 2011).

Datasets should be examined for extreme or unreliable data, which could profoundly affect clustering results (Martínez-Pastor et al., 2011). Nevertheless, it is often difficult to determine if an event is a real outlier or a real value belonging to an underrepresented cluster. Typical clustering methods to outliers as the k-means method, that tend to group outliers in a few clusters can be used to remove them. Moreover, some clustering methods can deal with noise or outliers as the model-based clustering (Fraley & Raftery, 2002). The CASA systems provide a high number of kinematic variables that can be redundant. It is because many variables convey similar information as the velocities VCL,

VAP, and VSL, whereas other are derived as linearity (LIN) is the VSL/VCL ratio. Therefore, it is desirable to reduce the number of variables before running the clustering algorithm, for reducing both dimensionality and redundancy. Moreover, not all variables contribute equally in the cluster structure, and an incorrect variable selection could result in inaccurate clustering (Steinley & Brusco, 2008). A Pearson correlation analysis enables to determine subsets of highly correlated variables, suggesting redundant ones.

Clustering procedures in cluster analysis may be a hierarchical, or non-hierarchical procedure (Kaufman & Rousseeuw, 2005; Spencer, 2013). In the partitional or non-hierarchical methods, the final number of clusters (k) is decided by the user before carrying out the cluster running. Then, the algorithm begins assigning the observations to the k clusters, iteratively recalculating cluster membership, and seeking for the optimal partitioning of the data. The k-means algorithm was the most used in this kind of method, but it has some drawbacks, as sensitiveness to outliers and data-noise (Kaufman & Rousseeuw, 1990).

On the other hand, hierarchical clustering methods are based on multiple-step procedure, that can mainly be categorized into agglomerative (bottom-up) and divisive (top-down) procedures (Castro, Coates, & Nowak, 2004; Leonard & Droege, 2008; Wang et al., 2015) (Fig. 7). In agglomerative procedures, each sample is initially assumed to be a cluster. The two nearest clusters (based on a distance measure) are then merged at a time. This merger continues until all the samples are clustered into one group. Consequently, a tree-like structure, known as a dendrogram, is yielded. As an alternative, if the number of clusters is provided, the process of amalgamation of clusters can be terminated when the desired number of clusters is obtained. The first step of an agglomerative procedure considers all the possible mergers of two samples, which requires $n(n-1)/2$ combinations (where n depicts the number of samples) (Sharma, López, & Tsunoda, 2017). Among the agglomerative algorithms, Average linkage (UPGMA) or Ward's averaging method may be more appropriate for clustering CASA systems data (Martínez-Pastor et al., 2011).

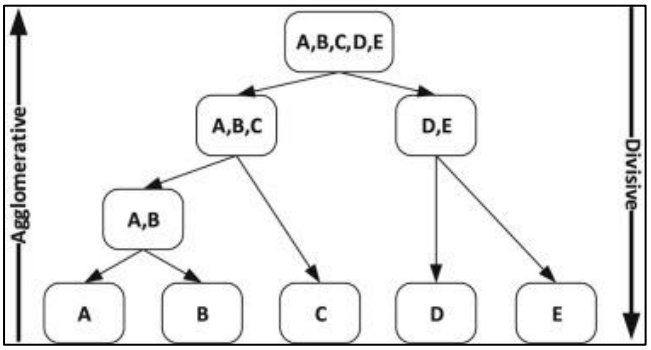


Fig. 7. Example of agglomerative and divisive hierarchical clustering. Adapted from (Everitt et al., 2011).

Following this, divisive procedures perform clustering in an opposite way than agglomerative methods. They begin by considering a group (having all samples) and divide it into two groups at each stage until all the groups comprise of only a single sample (Duda, Hart, & Stork, 2001). In the first step of a divisive procedure, all the partitions of a sample set are considered, which amounts to $2^{n-1}-1$ combinations. This number of combinations grows exponentially and practically makes divisive clustering a difficult procedure to implement. In hierarchical classifications, each sub cluster can be formed from one larger cluster split into two, or the union of two smaller clusters. Thus, divisive procedures, which start with the entire dataset, are in general considered safer than agglomerative methods (Kaufman & Rousseeuw, 2005). Therefore, the accuracy of a divisive procedure is usually higher than that of an agglomerative procedure (Roux, 2015). However, the high computational demand ($O(2^n) \sim O(n^5)$) of divisive procedures has severely restricted their usage (Roux, 1991). The number of bipartitions is $O(n^2)$; therefore, the complexity of one divisive step is $O(n^4)$. As the construction of the full binary hierarchy needs n – 1 steps, the overall complexity of the proposed divisive algorithms is $O(n^5)$ (Roux, 2015). This involves a heavy computer task. This has made the divisive procedure has not been generally used for hierarchical clustering, remaining largely ignored in the literature (Sharma et al., 2017).

As a result, clustering analysis allows to distribute the observations (spermatozoa) in clusters (subpopulations). The samples can be characterized by calculating the respective average values of the CASA parameters as the median or confidence intervals. The frequencies of each subpopulation within males or treatments can be estimated, obtaining in this way different subpopulation patterns (Martínez-Pastor et al., 2011). These population frequencies can be used for carrying out further statistical analyses, for example, regression, that can be used to relate subpopulations to other sperm features (Quintero-Moreno, Rigau, & Rodríguez-Gil, 2007).

1.7.3 Sperm subpopulation structure

It is needed to remember that the primary requirement for subpopulation analysis is that the CASA systems provide accurate data. The optimization of new methodological approaches, we were commented previously, makes necessary reconsideration of the former work developed on this topic. The combination of clearly acquired image-sequences and sophisticated image processing allows obtaining reliable kinematic and morphometric sperm parameters, resulting in improved datasets, making possible to better define real and significant subpopulation structures species by species (Martínez-Pastor et al., 2011).

Even with the previous technological analysis limitations, many studies have explored the use of cluster analysis to identify subpopulation patterns in sperm samples. Several works have considered kinematics data (Ortega-Ferrusola et al., 2009; Soler, García, Contell, Segervall, & Sancho, 2014; Yániz et al., 2018; Yániz, Palacín, Vicente-Fiel, Sánchez-Nadal, & Santolaria, 2015), morphometric (Aggarwal et al., 2007; Álvarez et al., 2008; Esteso et al., 2009) or a combination of both (Soler et al., 2017; Vásquez et al., 2016).

In reference to motility, each subpopulation may be characterized accordingly to its average kinematic variables. For example, a subpopulation with high-velocity values and high linearity could be defined as “fast, linear,” whereas another could be defined as “slow, non-linear” (Martínez-Pastor et al., 2011). Then, the frequencies of these subpopulations can be calculated, and variations in these frequencies can be associated to individual variations among ejaculates and males (Núñez-Martínez, Moran, & Peña, 2006), to sperm freezability (Martinez-Pastor et al., 2005), or to sperm fertility (Quintero-Moreno et al., 2003).

Independently on the species, different works have indicated that kinematic subpopulation structure was composed of three or four subpopulations. The presence of a “fast and linear” subpopulation has been proposed as a good indicator of sample quality, whereas a predominant “slow and non-linear” subpopulation would be a marker of poor quality (Martínez-Pastor et al., 2011). In any case, the final structure is animal dependent, and it means different animals present different subpopulation structure (Soler et al., 2017).

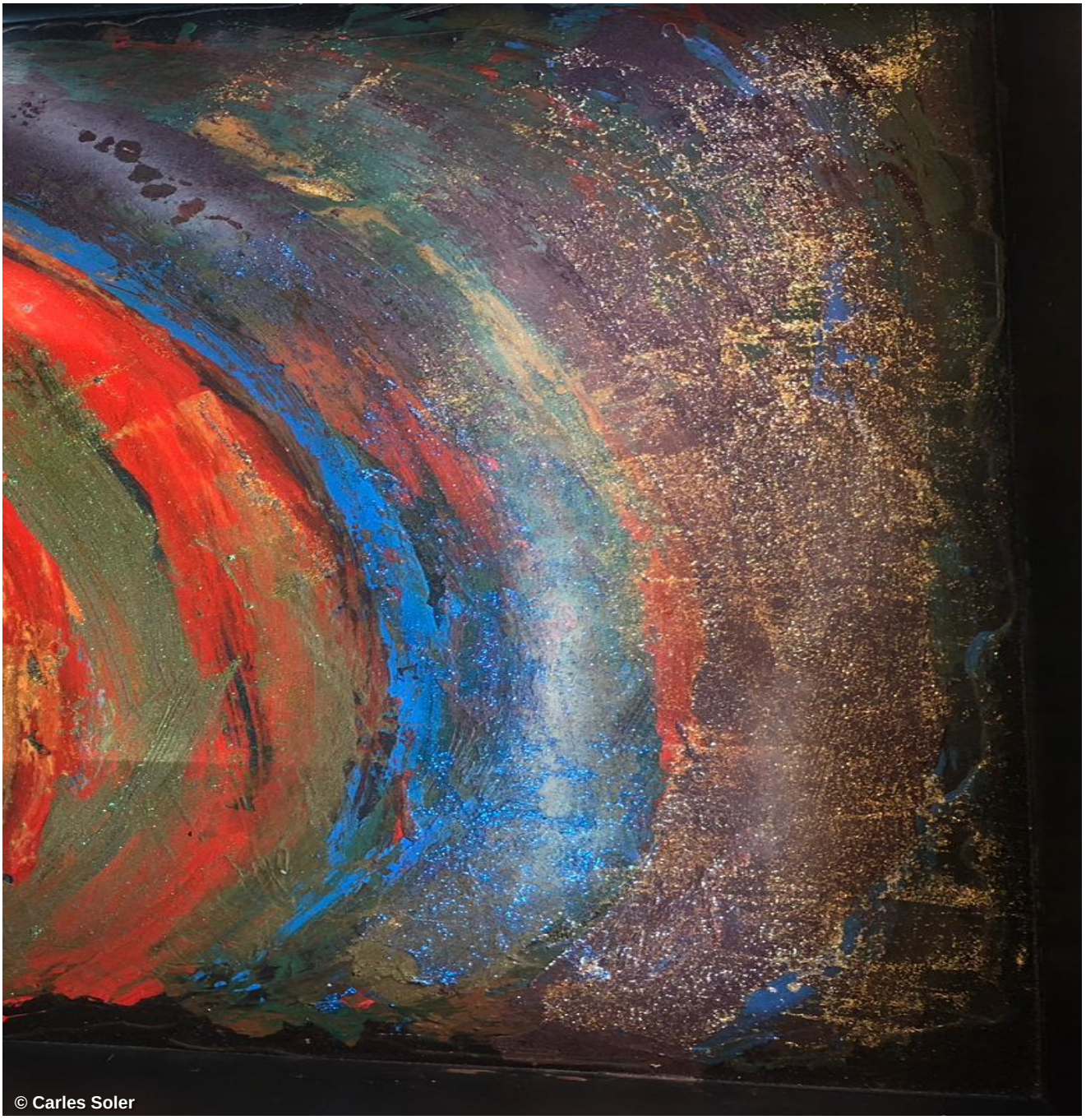
Regarding morphology, the general picture is similar to that of motility. In human two (Vásquez et al., 2016) or three (Santolaria et al., 2016; Yániz et al., 2016) morphometric subpopulations were observed. In other species a different number of subpopulations indicated species-specific subpopulations structures, being three for puma (Cucho et al., 2016) and rooster (García-Herreros, 2016), four for a cat (Gutiérrez-Reinoso & García-Herreros, 2016) and five for guinea fowl (García-

Herreros, 2016). Following that observed regarding motility, each animal inside the same species showed a different subpopulation frequency what can be a consequence of its genetics and physiology.

In the best of our knowledge, only one previous work, on fox semen, comprises the combination of kinematic and morphometric data for defining an integrative subpopulation structure study. Three subpopulations were observed when only kinematic or morphometric were considered, and four alter combining both databases (Soler et al., 2017). This kind of integrative work must be applied in the future, including other parameters (DNA fragmentation, viability and membrane stability) to obtain a better comprehension of what the ejaculate is.

As a corollary, we can indicate that the principal meaning of subpopulation structure analysis is to understand sperm biology better. Nevertheless, until now we have just established the conceptual basis, the future must be devoted to looking for the biological basis on the frame of sperm competition, movement along the female track, environmental effects and, in the end, fertility determination.

CHAPTER II:
GENERAL AND SPECIFIC OBJECTIVES



General objectives

The approach advocated in this thesis is based on the fact that there is a gap of information regarding optimization of semen analysis protocols. Only for human samples there is a general agreement about the protocol to use as it was expressed in the different editions of the WHO manual for semen analysis. However, even in this case, alternative protocols are recognized by different international organizations.

Centered on CASA technology, as it was indicated in the previous introduction, to obtain a reliable quantitative data it is needed to define protocols assuring the consistency and universal application of the results. However, this standardization has never been done following an integrative point of view, what composes the **first general objective** of the present Thesis. There are three main aspects to consider when optimizing automated semen analyses by CASA-Mot technology, namely the type and depth of the counting chamber, the dilution media and the frame rate of image acquisition. The problem that now arises is that high frame rate values bring significant changes in the value of some sperm kinetic parameters and this must now be considered. The technical aspects relatives to morphometric analysis were not included in the present Thesis, is the central objective of other of the Thesis work that is being developed in our research team.

The **second general objective** of the present Thesis was to study sperm subpopulations structure in different species, and how different technical and operative settings can influence them.

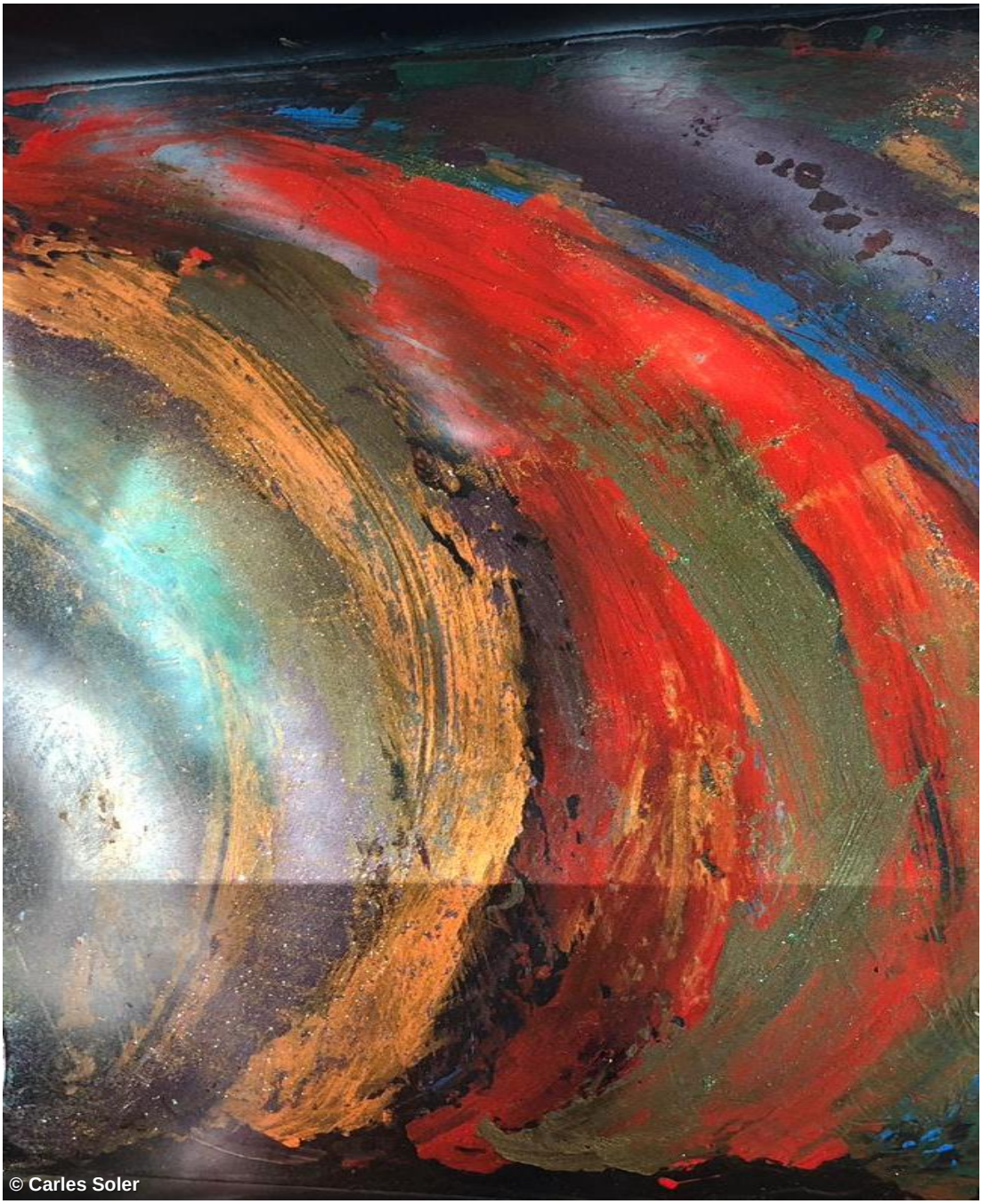
Specific objectives

The specific objectives of this study were:

1. Compare different statistical approaches for the evaluation of bull sperm morphometry, with the aim of defining the most useful and precise method and apply it to evaluate different ejaculates and post-thawed straws from the same individual.
2. Determine the effect of the frame rate in evaluating kinematic parameters in boar spermatozoa and its importance in the correct determination of sperm motility subpopulation structure.
3. Determine the effect of the video capture length in evaluating motility and kinematic parameters in boar sperm for the optimization of the analysis protocol.

4. Collaborate in the definition and establishment of the knowledge about sperm morphometry by using CASA technology.
5. Analyse the differential sperm distribution and motility characteristics in bulls along the counting area in three different commercial counting chambers (CellVu®, Leja® and Makler®) and the effect of two different commercial dilution media (Biladyl® and Andromed®) on this distribution, with a view to optimizing the use of currently available CASA-Mot technology.
6. Assess sperm motility with a commercial CASA-Mot system in a different dog (*Canis familiaris*, L.) breeds, and to analyze whether prolonged artificial selection carried out within the species has led to a significant gamete differentiation process between breeds.
7. Characterize sperm kinematic and morphometric parameters in *Caiman crocodilus fuscus* semen samples and to determine the existence of sperm subpopulations.

CHAPTER III:
GENERAL MATERIALS AND METHODS



3.1 Fieldwork

Fieldwork was conducted in the following areas: Xenética Fontao S.A. (42°58' N, 7°34', W) Lugo, Spain; at the Semen Cardona, S.L. (41°54' N, 1°43', E), Cardona, Barcelona, Spain; Reprovalcan and Clínica Veterinaria Sangüeso (39°29' N, 0°22', W), Valencia, Spain; Mejoramiento Porcino S.A., in the Northwest of Costa Rica, San José de la Montaña (10°05' N, 84°06', W), Heredia, Costa Rica, and in the Scientific Ecotourism Project (EcoTEC, 10°21' N, 84°30', W), Alajuela, Costa Rica, at the School of Agronomy, at Costa Rica Institute of Technology, San Carlos Campus, during the season (started in January until the end of June 2015 in Costa Rica, while in Spain during 2016 and 2017. The domestic species used in this thesis were cattle (*Bos taurus*), mainly Holstein bulls that regularly employed in artificial insemination under a regime of collection of two ejaculates per week; swine (*Sus scrofa*), where the breed utilized was Pietrain and their genetic lines; dog (*Canis familiaris*, L) coming from six breeds with an evident pedigree and genuinely representative of their breeds: Staffordshire Bull Terrier, Labrador Retriever, Spanish Mastiff, Valencian Rat Hunting Dog, British Bulldog, and Chihuahua. The wild species used in this thesis was brown caiman (*Caiman crocodilus fuscus*) from four sexually mature male alligators used as semen donors by digital manipulation without sedation.

3.2 Semen collection and processing

In the cattle experiments, within 5 to 10 min of semen collection using an artificial vagina, samples were assessed for volume in a conical tube graduated at 0.1 mL and gross motility determined by placing 20 µL of fresh semen on a pre-warmed slide at 37 ºC. The refrigerated samples were packaged in 0.25 mL straws (IMV Technologies, L´Aigle, France) with an automatic filling and sealing machine (MRS 1, IMV Technologies) and were immediately frozen by using a programmable freezer (Digitcool 5300, IMV, Technologies) with the following curve: 4 °C to -10 °C at -5 °C/min; -10 °C to -100 °C at -40 °C/min; -110 °C to -140 °C at -20 °C/min, and then plunged into liquid nitrogen for storage. Two straws per sample were thawed in a water bath at 37 °C for 30 s, and then the contents of the straws were emptied in a test tube kept at the same temperature in a dry bath. In order to collect uniform sperm subsamples and avoid inaccuracies, the semen was mixed gently before collecting aliquots for further analyses. Ejaculates were processed with a commercial egg yolk extender (Biladyl®) and the other with a soy lecithin-based extender (Andromed®), both from Minitube GmbH, (Tiefenbach, Germany).

From boar experiments at Costa Rica, semen samples were collected in the morning, once per week, using the "gloved-hand" technique (Hancock & Hovell, 1959) and immediately placed in a

water bath at 38 °C at the farm laboratory. In all cases, the sperm-rich fractions were collected, diluted with a commercial extender (Androstar Plus®; Minitube). The boar experiment in Spain eighteen seminal commercial doses were used. Samples were transported to the lab. in the same refrigerated conditions (17 °C) used for commercial distribution. Upon arrival in the lab. The samples were mixed, and 1mL placed in an Eppendorf tube and maintained at 37 °C for 30 min before use.

In dog experiment, manual stimulation was used to collect semen samples into sterile sample cups (Soler et al., 2017). Since a high sperm concentration can have a confounding effect on sperm velocity and linearity (Günzel-Apel, Günther, Terhaer, & Bader, 1993; Rijsselaere et al., 2003) all samples with >50 x 10^6 sperm/mL were diluted with CaniPlus Chill® sperm extender (Minitub Ibérica S.L., Tarragona Spain) before motility analyses.

In the assessment from brown caiman, semen collection was conducted by digital manipulation as previously described by (Johnston et al., 2014). A gloved hand was introduced into cloaca to gently exteriorize the phallus; once the phallus 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 presented in less than five minutes. Following massage semen flowed down the sulcus of the phallus and 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 a micropipette fitted with a 10-100 µL pipette tip. Semen was lavaged from the sulcus with approximately 100 µL of buffered Dulbecco's phosphate-buffered saline (DPBS, pH = 6.8, Sigma-Aldrich Inc.).

3.3 Assessment of sperm variables

In the first cattle experiments (see chapter IV), microscope slides were analyzed for sperm head morphometry by the ISAS®v1 (Integrated Semen Analysis System, Proiser R+D, Paterna, Spain). The equipment comprised a microscope (Olympus BH 2; Tokyo, Japan) equipped with a 100x bright field objective and a 3.3x photo‑ocular. A video digital camera (A312, Basler, Ahrensburg, Germany) 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 442368 pixels and 256 gray levels. Resolution of images was 0.08 µm per pixel in both the horizontal and vertical axes. In other cattle (see paper VIII) experiment, samples were analyzed for kinematics by the CASA-Mot system ISAS®v1 (Integrated Semen Analysis System, Proiser

R+D, S.L., Paterna, Spain). The equipment consisted of a microscope (Nikon Eclipse E600; Tokyo, Japan) equipped with a heated stage set at 38 °C and a 10x negative phase-contrast objective. A video digital camera (Proiser 782M) was mounted on the microscope to capture images and transmit them to a computer. The array size of the video frame grabber was 768 x 576 x 8 bits and 256 grey levels. Resolution of images was 0.84 µm per pixel in both the horizontal and vertical axes. The frame rate used was 30 fps, capture time one second, with the tail detection facility activated for ignoring non-sperm particles, with particle area between 14-80 μm^2 and connectivity of 14 µm. After dilution, each sample was analyzed using three different chambers: Leja® 4 chambers (L4; 20-µm depth; prod. code SC-20-01-04-B; Leja®, IMV technologies, L'Aigle, France), CellVu® sperm counting chamber (CVD; 20-µm depth; prod. code DRM-600; Millennium Sciences, Inc., NY, EEUU), and Makler® counting chamber (10-µm depth; Sefi-Medical Instrument, Haifa, Israel).

In the boar experiments (chapter V and VI), for analysis of motility, kinetics and concentration, ISAS®D4C20 disposable counting chambers (Proiser R+D) were used after being pre-warmed to 37°C. After thorough mixing of the samples, a volume of 3 µL was distributed along the counting chamber tracks by capillarity, filling them. Analyses were conducted with the CASA-Mot system ISAS®v1 (Proiser R+D). The video-cameras were Proiser HS640m (Proiser R+D), with a frame rate of 200 fps and a final resolution of 640 x 478 pixels and Proiser 782m (Proiser R+D) with a frame rate of 50 fps and a final resolution of 746 x 578 pixels. The cameras were attached to a microscope UB203 (UOP/Proiser R+D) with a 1x eyepiece and a 10x negative-phase contrast objective (AN 0.25) with a resolution images of 0.84 µm/pixel on both axes, and an integrated heated stage maintained at 37 ± 0.5°C. The same technical conditions were used for brown caiman experiment (chapter X), except analysis temperature that it was 25 ± 0.5 °C.

In dog experiment (chapter IX), throughout the process, the samples were kept at 24 ºC using a PC-12T (Proiser R+D) heating system. Kinematic analyses were carried out using the ISAS®v1 CASA-mot system (Proiser R+D). The video camera employed was a Proiser 782 m attached to a microscope UB203 (UOP/Proiser) and equipped with a 10x negative phase contrast objective. Resolution of analyzed images was 0.84 µm/pixel on both axes. Samples were captured at 25 fps following the set-up of the manufacturer for dog semen.

3.4 Sperm motility and kinematic analyses

The CASA-Mot parameters considered in the study were: straight line velocity (VSL, $\mu m \cdot s^{-1}$), corresponding to the straight line from the beginning to the end of the track; curvilinear velocity

(VCL, $\mu m \cdot s^{-1}$), measured over the actual point-to-point track followed by the cell; average path velocity (VAP, $\mu m \cdot s^{-1}$), the average velocity over the smoothed cell path. Other parameters of oscillation were amplitude of lateral head displacement (ALH, μm), defined as the maximum of the measured width of the head oscillation as the sperm cells swim; beat-cross frequency (BCF, Hz), defined as the frequency with which the actual track crossed the smoothed track in either direction. Furthermore, motility (%), the percentage of the total motile cells; and progressive motility (%), corresponding to spermatozoa swimming forward quickly in a straight line. Three progression ratios, expressed as percentages, were calculated from the velocity measurements described above: linearity of forwarding progression (LIN=VSL/VCL·100), straightness (STR=VSL/VAP·100), and wobble (WOB=VAP/VCL·100).

3.5 Sperm morphometry measurements

Sperm heads were captured 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 system. When it was not possible to obtain a correct boundary, the sperm head was deleted from the analysis. Following the criteria of (Boersma, Braun, & Stolla, 1999) at least sixty sperm heads were measured on each slide for four primary parameters of head size (length [L, μm], width [W, μm], area [A, μm^2], and perimeter [P, μm]) and four derived dimensionless parameters of head shape (ellipticity [L/W], rugosity [$4\pi A/P^2$], elongation [(L − W)/ (L + W)], and regularity [$\pi LW/4A$]). Data from each sperm cell were saved in an Excel® (Microsoft Corporation, Redmond, Washington, USA) -compatible database by the software for further analysis.

3.6 Statistical analysis

The data obtained from the analysis of all sperm parameters were first tested for normality and homoscedasticity by using Shapiro-Wilks and Levene tests. A normal probability plot was used to check for a normal distribution. In trying to obtain a normal distribution, data were transformed using arcsine square root (arcsin $\sqrt{x}$) before a repeated measures ANOVA was conducted. Even after arcsine transformation, the kinematic variables of the sperm were not normally distributed, as determined by the Shapiro-Wilks test. The kinematics sperm variables did not satisfy the normality requirement for parametric analysis of variance. Therefore, non-parametric analyses were performed with 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.

Discriminant analyses were performed to test the predictive power of using combined sperm kinematic parameters to classify dog breeds correctly. Canonical discriminant analysis creates multiple orthogonal functions that are formed so that in each breed implications on the resulting canonical variable are as different as possible due to maximizing between-class variance and at the same time minimizing within-class variance.

Clustering procedures were performed to identify sperm subpopulations from the set of motility data. All the kinematic and morphometric parameters were standardized in order to avoid any scale influence. The first step was to perform a principal component analysis (PCA) of these data (each variable was weighed with their variances extracted for that principal component, known as eigenvectors) was performed to derive a small number of linear combinations that retained the information in the original variables as much as possible. The number of principal components (PC) used in the next step of the analysis was determined using the Kaiser criterion, namely selecting only those with an eigenvalue (variance extracted of each PC) >1 (Spencer, 2013). As a rotation method, the varimax method with Kaiser normalization was used (Kaiser, 1958). The second step was to perform a non-hierarchical analysis using the k-means model that uses Euclidean distances from the quantitative variables after standardization of these data, so the cluster centers were the means of the observations assigned to each cluster (Kaufman & Rousseeuw, 1990). The multivariate k-means cluster analysis was conducted to classify the spermatozoa into a reduced number of subpopulations (clusters) according to their kinematic and morphometric parameters. In the final step, to determine the optimal number of clusters, the final centroids were clustered hierarchically using the Ward method (Murtagh & Legendre, 2014). Thus, every cluster provided a final cluster formed by the spermatozoa linked to its centroids. ANOVA and χ^2-test procedures were applied to evaluate statistical differences in the distributions of observations 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 their 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 parameters defining the different sperm subpopulations. Differences between means were then analyzed by Bonferroni test. Results are presented as the mean ± standard error of the mean (SEM). Statistical significance was considered at $P< 0.05$. All data were analyzed using InfoStat Software (v. 2008) for Windows, IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

CHAPTER IV:

MORPHOMETRY AND SUBPOPULATION STRUCTURE OF HOLSTEIN BULL SPERMATOZOA: VARIATIONS IN EJACULATES AND CRYOPRESERVATION STRAWS

CHAPTER IV:

Morphometry and subpopulation structure of Holstein bull spermatozoa: variations in ejaculates and cryopreservation straws

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INVITED ORIGINAL ARTICLE

Semen Analysis

Morphometry and subpopulation structure of Holstein bull spermatozoa: variations in ejaculates and cryopreservation straws

Anthony Valverde[1], Héctor Arenán[2], María Sancho[3], Jesús Contell[3], Jesús Yániz[4], Alejandro Fernández[2], Carles Soler[3,5]

Sperm quality is evaluated for the calculation of sperm dosage in artificial reproductive programs. The most common parameter used is motility, but morphology has a higher potential as a predictor of genetic quality. Morphometry calculations from CASA-Morph technology improve morphological evaluation and allow mathematical approaches to the problem. Semen from 28 Holstein bulls was collected by artificial vagina, and several ejaculates were studied. After general evaluation, samples were diluted, packaged in 0.25 ml straws, and stored in liquid nitrogen. Two straws per sample were thawed, and slides were processed and stained with Diff-Quik. Samples were analyzed by a CASA-Morph system for eight morphometric parameters. In addition to the "classical" statistical approach, based on variance analysis (revealing differences between animals, ejaculates, and straws), principal component (PC) analysis showed that the variables were grouped into PC1, related to size, and PC2 to shape. Subpopulation structure analysis showed four groups, namely, big, small, short, and narrow from their dominant characteristics, representing 31.0%, 27.3%, 24.1%, and 17.7% of the total population, respectively. The distributions varied between animals and ejaculates, but between straws, there were no differences in only four animals. This modern approach of considering an ejaculate sperm population as divided into subpopulations reflecting quantifiable parameters generated by CASA-Morph systems technology opens a new view on sperm function. This is the first study applying this approach to evaluate different ejaculates and straws from the same individual. More work must be done to improve seminal dose calculations in assisted reproductive programs.

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Keywords: cryopreservation; Holstein bull; sperm morphometry; subpopulations

INTRODUCTION

The appropriate characterization of male fertility is of highest importance because a bad selection of dairy sires will compromise animal production planning for long periods. The way to evaluate the fertility potential of a male is semen quality evaluation, which is the principal tool for the calculation of sperm doses needed for artificial insemination in most farm animals.

Traditionally, the most commonly used seminal parameter was sperm motility,[1] while morphology analysis had a secondary place because it takes much more time, the definition of universal and clear patterns of normal morphology is highly complex, and the process lacks precision.[2–4] Nevertheless, morphological characteristics are genetically defined, making its analysis reliable and informative on the genetic quality of the ejaculate.[5] Obviously, one sample without motility will not be able to fertilize, but motility is affected by many environmental factors,[6] while morphology is more related to spermatogenesis and epididymal sperm maturation processes.[7,8]

Despite the presence of extreme forms (perfectly round or greatly elongated heads), the principal problem of morphological analysis is that it is not easy to discriminate subjectively between similar forms. This fact explains the great coefficients of variation of both intra- and inter-observer, reducing the feasibility of the obtained results.[9] In heteromorphic sperm species, different efforts have been made to define morphology with universal morphological classifications (bull,[10–12] cat,[13] human,[14] llama,[15] and stallion[16–18]).

The development of CASA-Morph (Computer-Assisted Semen Analysis for Morphology) technology for the study of sperm morphometrical has opened new possibilities for the morphology evaluation of spermatozoa.[19,20] In the beginning, this technique was used with low-power statistics to perform comparisons following ANOVA, assuming a normal distribution of data or some nonparametric analysis in more accurate work.[21–23] Since then, multivariate analysis has been introduced for the simultaneous consideration of all the parameters, including their relationships.[24] During recent decades, new efforts have been made to define the best analytical approaches by using subpopulation analysis[25] and morphological sperm subpopulation structure, based on morphometric data, which has been observed in a great variety of species: dogs,[26,27] boars,[28,29] bulls,[30,31] foxes,[32] humans,[33,34]

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llamas,[15] marmosets,[35] pumas,[36] rabbits,[37] rams,[38–40] red deer,[41] and stallions.[42]

The purpose of the present work was to compare different approaches for the evaluation of bull sperm morphometry for the establishment of the most useful and precise method. Moreover, the effect of animal, ejaculate, and straw on both morphometric parameters and subpopulation structure was analyzed.

MATERIALS AND METHODS

Semen collection and processing

Semen was collected, during spring, from 28 Holstein bulls by artificial vagina, under an extraction program of two ejaculates per week. Animals were housed in Xenética Fontao S.A. (Lugo, Spain) installations. For the study, five ejaculates from each animal and two straws from each ejaculate were used, for a total of 280 samples.

Within 5 to 10 min of collection, the semen samples were assessed for volume by conical tube graduated at 0.1 ml, gross motility by placing 20 μl fresh semen on a prewarmed slide at 37°C, concentration by a bovine photometer Accucell (IMV, L'Aigle, France) at 530 nm wavelength, and sperm viability by flow cytometry with SYBR 14 and PI from a commercially available Live/Dead Sperm Viability Kit (Life Technologies, Carlsbad, CA, USA).

The raw semen was diluted with a commercial egg yolk extender (Optidyl® -IMV, L'Aigle, France) to a final live sperm concentration of 25×10^6 cells/straw. Diluted semen was cooled slowly to 4°C at a linear rate of −0.3°C min^{-1} in a refrigerator. After cooling of semen, equilibration occurred over 4–5 h at the same temperature.

The semen was then packaged in 0.25 ml straws, which were sealed via automatic filling and sealing machine (MRS 1, IMV Technologies, L'Aigle, France) and frozen by a programmable freezer, Digitcool 5300 (IMV, L'Aigle, France) with the following curve: 4°C to −10°C at 5°C min^{-1}, −10°C to −100°C at 40°C min^{-1}, −110°C to −140°C at 20°C min^{-1}, and then plunged into liquid nitrogen for storage. All samples were coded in such a way that the technician who performed the morphometric analysis could not deduce the number of the bull, the number of the ejaculate, or which ejaculate belonged to a particular bull.

Sample preparation for morphometric analysis

The semen straws were sent by courier to Proiser R+D, S.L. (Paterna, Spain) in a Dry shipper 3.0 (ST Reproduction Technologies LLC, Navasota, TX, USA). Duplicate samples for morphometric analysis were prepared from two straws per frozen ejaculate. After being thawed at 37°C for 30 s in a water bath, 150 μl per straw was diluted with 450 μl emCare (Bodinco, Alkmaar, The Netherlands). After being mixed, 5 μl of each sample was spread on a glass slide and subsequently air-dried.

The slides were stained by using the Diff-Quik kit (Medion Diagnostics, Düdingen, 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 analyzed in a double-blind scheme.

Computerized morphometric analysis

Microscope slides were analyzed for sperm head morphometry by the ISAS® v1 (Integrated Semen Analysis System, Proiser R+D, Paterna, Spain). The equipment comprised a microscope (Olympus BH-2; Tokyo, Japan) equipped with a 100× bright-field objective and a 3.3 × photo-ocular. A video digital camera (A312, Basler, Ahrensburg, Germany) 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 442368 pixels and 256 gray levels. Resolution of images was 0.08 μm per pixel in both the horizontal and vertical axes.

Sperm heads were captured 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 system. When it was not possible to obtain a correct boundary, the sperm head was deleted from the analysis.

Following the criteria of Boersma *et al*.,[43] at least sixty sperm heads were measured on each slide for four primary parameters of head size (length [L, μm], width [W, μm], area [A, μm^2], and perimeter [P, μm]) and four derived dimensionless parameters of head shape (ellipticity [L/W], rugosity [$4\pi A/P^2$], elongation [(L – W)/(L + W)], and regularity [$\pi LW/4A$]). Data from each individual sperm cell were saved in an Excel® (Microsoft Corporation, Redmond, Washington, USA)-compatible database by the software for further analysis.

Statistical analysis

The data obtained from the analysis of all sperm parameters were first tested for normality and homoscedasticity by using Shapiro–Wilk and Kolmogorov–Smirnov tests, respectively. To evaluate the classical statistical analysis approach, repeated measures ANOVA was performed, assuming normal distribution and homogeneity of variances, following the classical approach to the problem. Nevertheless, as morphometric sperm variables did not satisfy the normality requirements, nonparametric Kruskal–Wallis test was performed followed by the Mann–Whitney paired U-test when significant differences were found. The statistical model used was: ($x_{ijk} = \mu + A_i + E_j + S_k + \varepsilon_{ijkl}$), where: x_{ijk} = measured sperm morphometry variable, μ = overall mean of variable x, A_i = effect of animal, E_j = effect of ejaculate, S_k = effect of straw, and ε_{ijkl} = residual.

Multivariate analysis of variance (MANOVA), based on Wilk's lambda criterion, was performed.[44] The test used ejaculate and straw as within-bull factors. The multivariate linear model was: ($y_{ij} = \mu + \alpha_i + \beta_j + e_{ijk}$,… i = 1,…, a; j = 1,…, b,) where μ is the overall mean, α_i is the additive effect of the level i of factor ejaculate, β_j is the additive effect of the level j of factor, and e_{ijk} is the residual.

Clustering procedures were performed to identify sperm subpopulations from the complete set of morphometric data.[45] The first step was to perform a principal component analysis (PCA). To select the number of principal components that should be used in the next step of analysis, the criterion of selecting only those with an eigenvalue (variance extracted for that particular principal component) >1 (Kaiser criterion) was followed. The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA. All the sperm morphometric measurements within each ejaculate and straw were clustered by shape and size parameters using a nonhierarchical clustering procedure (k-means model and Euclidean distance). This classifies the spermatozoa of the data set into a small number of subpopulations according to their head dimensions, as has been described previously.[29] This analysis allowed the identification of sperm subpopulations and the detection of outliers.

The effects of clusters within and between treatments for the measurements of morphometric parameters were analyzed by the generalized linear model. The influence of each ejaculate within bulls on the relative distribution frequency of spermatozoa belonging to each subpopulation was analyzed by Chi-square and Mantel–Haenszel Chi-square tests. After characterizing sperm subpopulations, ANOVA was performed to explore the relationships between the proportions of

each sperm subpopulation in the sample. The results are presented as mean ± standard deviation (s.d.). Statistical significance was considered as $P < 0.05$. All data were analyzed using InfoStat Software (v. 2008) for Windows.[46]

RESULTS

Traditional analysis

Here, we present a resume of the obtained results. After performing ANOVA analysis (assuming normality and homogeneity of the samples) for each independent morphometric variable, there were statistically significant differences ($P < 0.05$) between 12 (Length), 14 (Width and Regularity), 16 (Ellipticity, Rugosity, and Elongation), and 17 (Area and Perimeter) animals from the total number of 28 animals studied. Following the criterion to evaluate the most useful parameter to differentiate between animals, Perimeter and Area should be considered.

Looking for differences between ejaculates from the same animal, only in one case, there were no differences between the five ejaculates observed. In the other animals, most of the parameters showed differences in at least two of the ejaculates. The most sensitive parameter able to distinguish the ejaculates was the Area, showing differences between all the ejaculates in two animals and between four ejaculates in 11 animals. The less informative parameter was Regularity, with 11 animals not showing differences between ejaculates.

Regarding the differences between straws for one ejaculate, only four animals showed no differences for any straw in the five ejaculates, while for the remaining animals, differences between straws in at least one of the ejaculates were recorded. In this case, the parameter with more differences was Rugosity, showing variation in 16 animals, followed by Area in 11 animals.

The analysis by MANOVA of all the variables showed significant differences between all the animals. Only in one animal (the same as that after ANOVA), differences between ejaculates were not observed, while the remainder showed differences between three (five animals), four (thirteen animals), or even between the five ejaculates (nine animals). In reference to straws, only eight animals presented no differences between straws.

Principal component analysis and subpopulation structure analysis

The PC analysis produced two components, explaining 75.6% of the variance. PC1 was represented by Length, Area, and Perimeter, and called the "size" component; PC2 referred to Width, and negatively, Ellipticity and Elongation, called the "elongation" component (**Table 1**).

The analysis of subpopulations revealed four well-defined groupings (**Figure 1**). The characteristics of SP1 showed the lowest size, named "small," comprised 27.3% of the total cells; SP2 comprised low Length with high Rugosity, named "short" cells, and represented 24.1%; SP3 included the cells with higher Area, named "big" cells, being 31.0%; and SP4 was characterized by high Length, Ellipticity, and Elongation, and were named "narrow" cells with a 17.7% of the total (**Table 2**).

The distribution of subpopulations among animals showed differences after Chi-square analysis. In 18 animals, one of the subpopulations was clearly the most representative: SP1 in five, SP2 in four, SP3 in five, and SP4 in two; in nine animals, two subpopulations presented equivalent values and only one animal showed a similar distribution between three subpopulations (SP2, SP3, and SP4) (**Table 3**).

Among ejaculates, there were differences in some subpopulations in all animals except one; another showed differences only for SP1, four for two subpopulations, fifteen for three, and seven for all the subpopulations. The most variable subpopulations were SP1 and SP3 (different among ejaculates for 25 animals), followed by SP2 (for 19 animals), and SP4 (only for 13). Different ejaculates showing different patterns among them were common, but in some cases, there was no difference between ejaculates (**Figure 2**).

Regarding the differences of subpopulation distributions between straws of the same ejaculate, four animals showed no differences, five differed in only one subpopulation, ten in two, six in three, and three

Table 1: Eigenvalues of each parameter in both PCs for bull sperm head morphometry found in frozen-thawed samples

	PC1 (size)	*PC2 (elongation)*
Head length	0.53*	
Head width	0.27	0.56*
Head area	0.45*	0.33
Head perimeter	0.44*	
Ellipticity	0.31	–0.53*
Rugosity		0.11
Elongation	0.31	–0.53*
Regularity		–0.05
Explained variation (%)	42.90	32.70

*Expresses the more important variable in each PC. Only eigenvalues >0.3 are presented. PCs: principal components

Table 2: Mean values (±s.d.) of each morphometric parameter corresponding to different SPs from frozen/thawed bull spermatozoa

Variable	*SP1*	*SP2*	*SP3*	*SP4*
n/%	4791/27.28	4233/24.10	5440/30.97	3100/17.65
Head length (μm)	8.54±0.35	8.39±0.32	9.16±0.29	9.40±0.46
Head width (μm)	4.37±0.18	4.75±0.19	4.90±0.18	4.52±0.20
Head area (μm^2)	32.46±2.14	34.40±2.05	38.31±1.97	36.23±2.44
Head perimeter (μm)	24.33±0.89	24.36±0.83	26.07±0.88	26.34±1.46
Ellipticity	1.96±0.09	1.77±0.08	1.87±0.08	2.08±0.14
Rugosity	0.69±0.04	0.73±0.03	0.71±0.03	0.66±0.05
Elongation	0.32±0.02	0.28±0.02	0.30±0.02	0.35±0.02
Regularity	0.90±0.03	0.91±0.03	0.92±0.03	0.92±0.03

SPs: subpopulations; s.d.: standard deviation

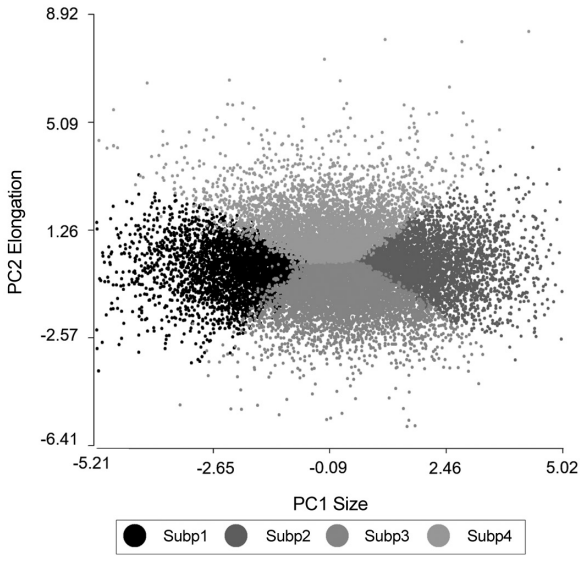

Figure 1: Distribution of subpopulations according to their PC values.

Table 3: Percentage of cells assigned to each SP per animal

Animal	*SP1*	*SP2*	*SP3*	*SP4*
1	22.50	2.32	28.75	46.43*
2	3.88	4.35	58.39*	33.39
3	40.00*	20.34	18.47	21.19
4	21.78	55.17*	21.14	1.91
5	44.28*	35.95	15.36	4.41
6	18.84	9.89	52.75*	18.52
7	38.52	37.29	17.72	6.47
8	34.72	40.57*	19.70	5.01
9	12.06	3.65	36.19	48.10*
10	23.13	32.57	36.64	7.65
11	26.68	17.94	36.51	18.88
12	26.92	26.38	29.74	16.96
13	14.29	10.05	50.71*	24.96
14	18.71	5.16	39.68	36.45
15	36.38	2.87	25.45	35.30
16	32.01	19.97	32.33	15.69
17	43.56*	19.63	20.25	16.56
18	39.22	45.80*	9.94	5.04
19	34.72	21.04	33.64	10.60
20	23.26	50.95*	23.89	1.90
21	18.66	25.68	44.69*	10.96
22	30.07	22.55	32.84	14.54
23	44.26*	27.05	14.43	14.26
24	15.36	10.08	44.64*	29.92
25	15.41	23.56	38.22	22.81
26	42.70*	16.21	20.55	20.55
27	25.70	31.91	35.68	6.71
28	15.89	51.82*	28.81	3.48

*The most relevant SP in each animal. SP: subpopulation

in four. The most variable subpopulation was SP3, followed by SP1, SP4, and SP2, the latter being the less variable one.

DISCUSSION

The relationship between sperm head morphometry and fertility in the bull is clearly established, indicating that bulls with high fertility produce more elongated and tapered spermatozoa[47,48] (perhaps our SP1, see below). In addition, some of the differences observed in sperm nuclear shape could be related to the various levels of chromatin stability.[49,50]

Cryopreservation is a common technique in some species including the bull, but considerable variation in post-thaw semen viability exists.[51] Independent of sperm quality before freezing, the semen of certain individuals will consistently freeze/thaw badly, resulting in poor motility, disrupted acrosomal and plasma membrane,[52] and thus reduced fertilizing ability, indicating the existence of variation in membrane properties and their response to freeze-thawing between animals. Recent studies suggest that there is a genetic basis for variation in post-thaw semen quality.[53] Previous work has analyzed the morphometric characteristics of bull spermatozoa before and after cryopreservation, observing that the results of raw semen differ from that obtained post-thawing, indicating that the cryopreservation process can affect the different types of cells in different ways or modify their previous morphology.[30]

Osmotic stress is related to differences in osmolality across the plasma membrane, its hydraulic conductivity, and also the cell's volume and surface area. It is likely that subtle differences among spermatozoa in shape and volume or area are responsible for differences in the speed of water exchanges across the plasmalemma, which could be the origin of different subpopulations of sperm morphology.[26] In any case, the sampling processes of smearing and air drying the samples for morphology assessment cause high osmotic stress and membrane damage that can overlap any inherent morphological details in the morphometry analysis. In trying to solve this problem, the use of a new technique for morphological analysis, based on the observation of cells directly on seminal plasma, has been proposed.[54–56]

In the present work, we have not studied the effect of cryopreservation as such centering the work on frozen samples and comparing different statistical methods for the comparison of morphometric characteristics of spermatozoa. The traditional concept of a sperm population in an ejaculate was to look for "normal cells," those with supposed high-fertility potential. It assumed a more or less unimodal morphological distribution, and so statistical calculations were based on ANOVA (frequently without previous normality and homogeneity analysis) or, in the best case, after this analysis using nonparametric tests such as the Kruskal–Wallis.[57] This approach has several limitations: (i) each variable is considered independently; (ii) it assumes a uniformity in the population that is not real in heteromorphic species, as the bull; (iii) it cannot be applied to complicated studies such as the present one with a high number of animals, five ejaculates per animal and two straws per ejaculate, as the presentation of the results requires too many tables or graphs.

In the present study, many partial differences were found by following this approach in all the comparisons (individuals, ejaculates, and straws). The use of the MANOVA improved some of the previous limitations because all the variables are considered in a multivariate way. This approach is much better because the morphometric data for each cell are considered simultaneously, increasing the statistical power and reducing the data to work with. Nevertheless, the idea of a homogeneous sperm population remains inherent in this approach.

It has also been used as a new approach to a nonhomogeneous population in species, in which different morphologies have previously been described, following multivariate discriminant analysis.[15] The major limitation of this *a priori* approach is that it is based on subjective classification, even if it is mathematically categorized and provides a mathematical classification matrix to be used for subsequent analysis. The subjectivity is limited to the definition of the canonical cells defined for the matrix calculation.

During the last decade, *a posteriori* subpopulation structure based on principal components and cluster analysis has been introduced. This is the best approximation to the real sperm population in an ejaculate. In a previous attempt, traditional statistics and the new approach to subpopulation structure in stallion were compared.[42] In future, this kind of work is needed to translate the former results to the new approach to include all the background data.

Taking kinematic data into consideration, four sperm subpopulations were established in Asturiana de los Valles bulls,[58] suggesting that the presence of four subpopulations could be a common feature of bovine ejaculates. In these studies, differences between animals were only related to the subpopulation with highest velocity and progressiveness, even after cryopreservation.[58]

Sperm morphometric subpopulation structure in the bull has also been described.[45] In that study, with nuclear fluorescence staining, three PC and four subpopulations were observed. Different animals showed clearly different subpopulations, but only one ejaculate from each bull was analyzed.[45] Here, we have also found four subpopulations defined by two PCs. Another work has provided evidence of three bull sperm morphometric subpopulations, but these were from mixed data from

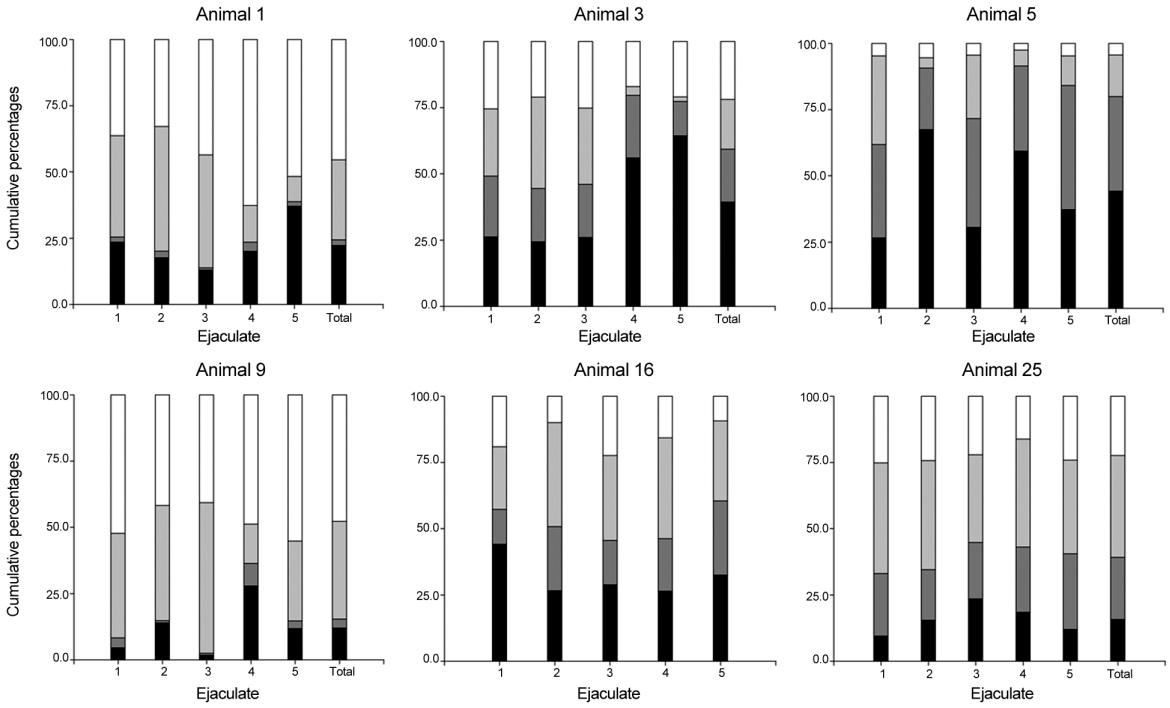

Figure 2: Distribution of subpopulations (SP) per ejaculate in some representative animals. SP1 (black), SP2 (dark gray), SP3 (light gray), and SP4 (white) bars. Animal 1 presents differences for subpopulations SP1, 3 and 4; Animal 3 for SP1 and 3, but the first three ejaculates present equivalent number of cells for each SP; Animal 5 for SP1 and 3, but the distribution of SP in each ejaculate was different; Animal 9 for SP1, 2 and 3; Animal 16 for SP3 and 4; and Animal 25 showed no differences among ejaculates.

five Holstein and five Brahman bulls, not taking into consideration the possible inter-breeding variations.[30] Mixing different breeds is common in other species, such as the dog, but it has been demonstrated that different breeds have different sperm subpopulations, so more work is needed on the evaluation of these results.[27]

Most of the subpopulation studies have shown different distribution between animals, indicating that endogenous factors (genetic, physiological, etc.) are involved.[35] The combination of the genetic and physiological status of an individual must be translated into different gamete strategies that reflect the sperm competition context in a particular species.[59]

In a variety of species, a subpopulation structure based on both kinematic and morphometric parameters has been established,[60] and the differences between animals were considered to be an individual animal strategy. In this work, we have analyzed, for the first time, the sperm morphometry of different ejaculates from the same animal. If the strategy was based on an individual's genetics, the differences between ejaculates should be insignificant or do not exist, but we have found clear differences among ejaculates. In the same direction, mid-piece length is different between bull breeds and also between ejaculates of the same bull.[61] This may indicate that the idea of a subpopulation strategy being just related to the animal strategy on the basis of genetic characters is incorrect or incomplete. More work is needed to understand the real meaning of these results, but we can hypothesize that a combination of genetics and physiological status must be responsible for the final subpopulation strategy of an individual.

As the ejaculate content reflects the sperm reserves available in the distal cauda epididymis at that time, variations in sperm quality in any ejaculate are likely to reflect the balance between distal caudal emptying (depending on the frequency of copulation or program of semen collection), caudal filling from the proximal cauda (depending on the extent of prior sperm depletion), and mixing of the spermatozoa during seminal emission before ejaculation. In addition, variations in the accessory gland fluid composition could have an effect on the final sperm morphometry.

The result obtained on the differences in sperm subpopulations between straws can be related to the fact that the ejaculate is not homogeneous and it is not possible to take reproducible aliquots, even with thorough mixing before removing a portion. The differences could also be explained by each straw being produced at different times from semen dilution. Either way this needs more study, perhaps by increasing the number of analyzed cells per sample, because if confirmed, these results could have consequences for reproductive success.

CONCLUSIONS

The former approaches to the study of sperm morphometry based on the differences analysis (ANOVA or MANOVA) are not good enough to define the true sperm populations, and it is necessary to use multivariate statistics based on principal component analysis to define subpopulations structure. Differences among ejaculates from the same animal challenge the former idea that the subpopulation structure is an individual characteristic, it could be also related to a physiological response to changes in the environment, even if based on the genetic basis.

AUTHOR CONTRIBUTIONS

AV, HA, AF and CS conceived and designed the experiments; HA, MS, JC, and JY performed the experiments; AV and CS analyzed the data; and CS wrote the paper.

COMPETING INTERESTS

CS is Professor at Valencia University and acts as Scientific Director of Proiser R+D S.L Research and Development Laboratory. Neither he nor the other authors have interests that influenced the results presented in this paper.

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CHAPTER V: EFFECT OF FRAME RATE CAPTURE FREQUENCY ON SPERM KINEMATIC PARAMETERS AND SUBPOPULATION STRUCTURE DEFINITION IN BOARS, ANALYZED WITH A CASA-MOT SYSTEM

CHAPTER V:

Effect of frame rate capture frequency on sperm kinematic parameters and subpopulation structure definition in boars, analyzed with a CASA-Mot system

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Effect of frame rate capture frequency on sperm kinematic parameters and subpopulation structure definition in boars, analysed with a CASA-Mot system

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Contents

Motility is the most widely used indicator of sperm quality. Computer-Assisted Semen Analysis (CASA) allows the objective evaluation of sperm motility parameters. CASA technology is a common tool to predict semen doses in farm animal reproduction. The kinds of video cameras used until now for image acquisition have presented limited frame rates (FR), which have a negative influence on the quality of the obtained data. The aim of the present work was to define the optimal frame rate for a correct evaluation of boar sperm motility and its subpopulation structure. Eighteen ejaculates from nine mature boars of the Pietrain breed were used. Using the ISAS®v1 CASA-Mot system, with a video camera working up to 200 Hz, six FRs (25, 50, 75, 100, 150 and 200 fps) were compared. ISAS®D4C20 counting chambers, warmed to 37°C, were used. FR affected all the kinematic parameters, with curvilinear velocity (VCL) and BCF the most sensitive ones. All the parameters showed differences among animals. Non-linear correlation showed the asymptotic level for VCL at 212 fps, being the highest FR for all the parameters. For future studies based just on progressive motility, almost 100 fps FR for 0.5 s must be used, while when kinematics must be considered, almost 212 fps for one-second should be analysed. Three principal components were obtained (velocity, progressivity and oscillation), being similar at 50 and 200 fps. Cells were grouped in four subpopulations but with different kinematic and cellular distribution at both FRs.

KEYWORDS

CASA-Mot, computer-assisted semen analysis, frame rate, pig, sperm motility, subpopulations

1 | INTRODUCTION

Potential boar fertility is assessed by semen analysis. Sperm motility is the most widely used indicator of semen quality and is the key parameter that is used for acceptance or rejection of ejaculates for artificial insemination in farms. In assessing sperm motility, significant correlations with fertility have been reported for bovine (Budworth, Amann, & Chapman, 1988), equine (Samper, Hellander, & Crabo, 1991), ovine (Santolaria et al., 2015), rabbit (Lavara, Mocé, Lavara, Viudes de Castro, & Vicente, 2005) and swine (Broekhuijse, Šoštarić, Feitsma, & Gadella, 2012a) spermatozoa.

The development of the swine industry by artificial insemination (AI) has brought improvements in the quantitative analysis of the quality of spermatozoa produced by boars to predict the fertility

potential of seminal doses (Waberski, Petrunkina, & Töpfer-Petersen, 2008). Subjective analysis, based on technician experience or "manual" estimation motility methods, has shown a high variance in results. To address this problem, from the 1980s, Computer-Assisted Semen Analysis (CASA) technology was developed to improve accuracy and precision on semen analysis (Bompart et al., 2018; Gil et al., 2009; Tardif, Laforest, Cormier, & Bailey, 1999). Currently, more than twelve different CASA systems brands are available for semen motility evaluation in laboratories and AI centres (Amann & Waberski, 2014); therefore, it is necessary to update the standardization of the methods for the objective evaluation of sperm quality (Amann & Katz, 2004; Gil et al., 2009; Verstegen, Iguer-Ouada, & Onclin, 2002).

The result of motility analysis by CASA technology (CASA-Mot; Soler, García, Contell, Segervall, & Sancho, 2014) is associated with software and hardware capabilities, in addition to biological variation associated with sperm samples within animals and the process of seminal dose elaboration. The evaluation of semen motility and other kinetic parameters such as curvilinear, straight line and average path velocities is an essential part of sperm quality evaluation in livestock species (Amann & Katz, 2004). From the beginning of CASA-Mot technology development, it has been pointed out that one of the more determining parameters is the frequency of image acquisition (Morris, Coutts, & Robertson, 1996). At the beginning of CASA-Mot system use, the most common frame rates were 25/30 frames per second (fps), reflecting European or US standards. This limitation was basically related to the kind of applicable video cameras, but image capture technology is being improved very fast and much more efficient cameras are affordable now. The problem that now arises is that high fps values bring significant changes in the value of some sperm kinetic parameters and this must now be considered (Bompart et al., 2018; Castellini, Dal Bosco, Ruggeri, & Collodel, 2011; Mortimer & Swan, 1999).

Another aspect is that the correct determination of sperm tracks results in a fundamental shift in the determination of motility and morphology subpopulation structure. The fact that an ejaculate is constituted of different subpopulations of spermatozoa has been well established in a great variety of species (boar (Abaigar, Holt, Harrison, & del Barrio, 1999; Flores et al., 2008; Holt, Holt, & Moore, 1996; Soler et al., 2018), bull (Valverde et al., 2016; Yániz et al., 2018), donkey (Flores et al., 2008), eel (Gallego et al., 2015), fox (Soler et al., 2014, 2017), gazelle (Abaigar et al., 1999), goat (Vázquez et al., 2015), ram (Luna et al., 2015), stallion (Ortega-Ferrusola et al., 2009), human (Vásquez, Soler, Camps, Valverde, & García-Molina, 2016; Yániz et al., 2016), salmon (Caldeira et al., 2018)), but most of these studies were not designed for considering the effect of the FR.

The aim of this study was to determine the effect of the frame rate in evaluating kinematic parameters in boar spermatozoa and its importance in the correct determination of sperm motility subpopulation structure.

2 | MATERIALS AND METHODS

2.1 | Animals

Eighteen commercial seminal doses, obtained from nine healthy boars of the Pietrain breed, 2–3 years old, were used. These animals were housed in climate-controlled buildings (Semen Cardona, S.L., Cardona, Barcelona, Spain), without any requirement of approval from the animal research review board of the University of Valencia. Boars were fed a standard boar adjusted diet (2.3 kg/d) and provided with water ad libitum.

Samples were transported to the laboratory in the same refrigerated conditions (17°C) used for commercial distribution. Upon arrival in the laboratory, the samples were mixed and 1 ml placed in an Eppendorf tube and maintained at 37°C for 30 min before use.

2.2 | Assessment of sperm variables

For the analysis of motility, kinetics and concentration, ISAS®D4C20 disposable counting chambers (Proiser R+D, S.L., Paterna, Spain) were used after being pre-warmed to 37°C. After thorough mixing of the samples, a volume of 3 μl was distributed along the counting chamber tracks by capillarity, filling them. Analyses were conducted with the CASA-Mot system ISAS®v1 (Proiser R+D). The video camera was Proiser HS640m (Proiser R+D), with a frame rate of 200 fps and a final resolution of 640 × 478 pixels. The camera was attached to a microscope UB203 (UOP/Proiser R+D) with a 1× eyepiece and a 10× negative-phase contrast objective (AN 0.25) and an integrated heated stage maintained at 37 ± 0.5°C.

2.3 | Kinematic analysis

Analyses were performed in seven microscope fields on a total of at least 600 cells per sample. The CASA-Mot parameters considered in the study were as follows: straight line velocity (VSL, μ/ms), corresponding to the straight line from the beginning to the end of the track; curvilinear velocity (VCL, μm/s), measured over 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 sperm cells swim; beat cross-frequency (BCF, Hz), defined as the frequency with which the actual track crossed the smoothed track in either direction; motility (%), the percentage of the total motile cells; and progressive motility (%), corresponding to spermatozoa swimming forward quickly in a straight line, (STR ≥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).

2.4 | Experimental design

Eighteen ejaculates (two ejaculates for each boar) and three replicates by ejaculate were analysed. All semen samples were recorded at 200 fps frame rate (FR) for 1 s. This video was segmented into 25, 50, 75, 100 and 150 FR videos. The command used was as follows: [*echo off: set fps = 25, 50, 75, 100, 150: for %%i in (.*.avi) do (set fname=%%˜ni) & call: encodeVideo; goto eof :encodeVideo: ffmpeg.exe -i %fname%.avi -r %fps% -c libx264 -preset slow -qp 0 "%fname%_(%fps%fps).avi"; goto eof*].

2.5 | Statistical analysis

These data obtained from the analysis of all sperm parameters were first tested for normality and homoscedasticity by using Shapiro–Wilks and Kolmogorov–Smirnov tests. A normal probability plot was used to check for a normal distribution. In trying to obtain a normal distribution, data were transformed using arcsine square root (arcsin $\sqrt{x}$) before repeated-measures ANOVA was run. Even after arcsine transformation, the kinematics variables of the sperm were not normally distributed, as determined by the Shapiro–Wilks test. The kinematics sperm variables did not satisfy the normality requirement for a parametric analysis of variance. Therefore, non-parametric analyses were performed with 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. The statistical model used was as follows:

$$X_{ij} = \mu + A_i + \varepsilon_{ij}$$

where x_{ij} = Measured sperm kinematic variable; μ = Overall mean of variable *x*; A_i = Effect of group; ε_{ij} = Residual.

For correlation and regression analyses, the effects of FR were tested in an exponential model, in the form $y = \beta \times \alpha$ exp $(-\beta/x)$, where *y* is VCL and *x* is FR, *a* is the asymptotic level, *b* is the rate of increase to the asymptote, exp is the base of natural logarithms. The biological significance of the equation is that the asymptotic values (*a*) represent the maximum achievable when the FR is above the threshold level. The threshold level is conventionally calculated as the FR needed to obtain 95% of the maximum value. The rate of the approach to the asymptote represents the dependence of the curve on the FR; that is, a high value of *b* indicates high growth of VCL as FR increases and vice versa.

Clustering procedures were performed to identify sperm subpopulations from the complete set of motility data. The first step was to perform a principal component analysis (PCA). The number of principal components (PCs) that should be used in the next step of the analysis was determined from the Kaiser criterion, namely selecting only those with an eigenvalue (variance extracted for that PC) >1. The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA. All sperm cells within a FR of 50 and 200 fps were clustered by using a non-hierarchical clustering procedure (k-means model and Euclidean distance). This analysis enabled the identification of sperm subpopulations and the detection of outliers.

The results are presented as mean ± standard deviation (*SD*). Statistical significance was considered at $p < 0.05$. All data were analysed with IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

3 | RESULTS

The frame rate had no effect ($p > 0.05$) on the total sperm motility, and progressive motility was different only at 150 and 200 fps in reference to the lower FRs (Table 1). Regarding the kinematic parameters, only VSL was shown to be quite independent of the FR,

TABLE 1 Effect of frame rate (FR) on boar sperm motility and kinematic measurements (means ± *SD*, *n* = 14,653, percentage variation with respect to 25 fps in brackets

	FR (fps)					
	25	50	75	100	150	200
TMOT (%)	90.5 ± 4.3	90.8 ± 4.5 (0.3)	90.6 ± 4.4 (0.1)	90.2 ± 4.2 (−0.3)	88.4 ± 4.7 (−2.4)	89.5 ± 5.1 (−1.1)
PMOT (%)	82.4 ± 6.3^{a}	83.8 ± 5.7^{a} (1.7)	84.2 ± 4.8^{a} (2.1)	82.8 ± 5.8^{a} (0.5)	79.1 ± 4.4^{b} (−4.1)	78.6 ± 5.9^{b} (−4.7)
VCL (μm/s)	67.9 ± 22.6^{a}	87.8 ± 33.6^{b} (32.7)	114.2 ± 44.9^{c} (41.5)	137.8 ± 52.2^{d} (51.3)	162.0 ± 54.2^{e} (58.9)	187.9 ± 63.0^{f} (63.9)
VSL (μm/s)	60.8 ± 23.0^{a}	66.9 ± 31.3^{b} (9.2)	68.0 ± 31.8^{b} (10.6)	66.1 ± 31.4^{b} (8.0)	66.5 ± 30.0^{b} (8.6)	67.3 ± 31.4^{b} (9.7)
VAP (μm/s)	64.1 ± 23.0^{a}	76.1 ± 30.3^{b} (15.8)	81.3 ± 31.5^{c} (21.2)	83.4 ± 32.5^{d} (23.1)	91.5 ± 33.4^{e} (30.0)	98.9 ± 37.2^{f} (35.2)
LIN (%)	88.0 ± 16.0^{a}	76.0 ± 19.^{b} (−15.6)	60.14 ± 16.3^{c} (−46.4)	47.0 ± 14.0^{d} (−87.2)	41.2 ± 12.2^{e} (−113.6)	35.2 ± 11.3^{f} (−149.8)
STR (%)	93.2 ± 12.1^{a}	86.4 ± 16.1^{b} (−7.9)	82.1 ± 16.2^{c} (−13.5)	78.1 ± 16.2^{d} (−19.3)	72.4 ± 16.4^{e} (−28.7)	67.0 ± 16.0^{f} (−39.1)
WOB (%)	93.2 ± 9.2^{a}	86.1 ± 12.1^{b} (−8.3)	72.2 ± 12.0^{c} (−29.1)	60.2 ± 11.1^{d} (−54.8)	56.4 ± 9.1^{e} (−65.3)	52.2 ± 9.1^{f} (−78.6)
ALH (μm)	1.53 ± 0.39^{a}	1.51 ± 0.45^{a} (−1.3)	1.63 ± 0.46^{b} (6.1)	1.64 ± 0.56^{b} (6.7)	1.68 ± 0.52^{b} (8.9)	1.58 ± 0.43^{ab} (3.2)
BCF (Hz)	7.9 ± 2.9^{a}	15.5 ± 5.9^{b} (49.1)	26.8 ± 10.2^{c} (70.6)	33.9 ± 13.6^{d} (76.8)	37.4 ± 16.8^{e} (78.9)	37.2 ± 16.9^{e} (78.8)

Notes. TMOT: total motility (%); PMOT: progressive motility (%); VCL: curvilinear velocity (μm/s); VSL: straight line velocity (μm/s); VAP: average path velocity (μm/s); LIN: linearity of forward progression (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat cross-frequency (Hz); FR: frame rate; *SD*: standard deviation.

$^{a-f}$Different superscripts indicate significant differences among frame rates. $p < 0.05$.

being different only for the results obtained at 25 fps, with all the other values of the FR not showing differences among them. Both VCL and VAP increased with all FR, arriving at an increment from 25 to 200 fps of 176.8% and 53.9%, respectively. The three indices of motility (LIN, STR and WOB) decreased with increasing FR, with LIN being the most affected. Finally, ALH showed no significant differences and BCF increased the most between 25 and 200 fps arriving at 374.2% (Table 1). These results were very similar when the analysis was done animal-by-animal (Figure 1 for VCL as an example).

The regression model showed that the most sensitive parameter to FR was VCL (Figure 2), with an alpha value of 212.20, while the other parameters presented a value of alpha lower than 100 (Table 2).

Principal component analysis showed three PCs (named velocity, progressivity and oscillation) that were similar at 50 and 200 Hz, but some interesting differences were observed. The weight of ALH and BCF was much higher at 200 Hz, being included in the first PC, indicating that they are much better determined at high FR (Table 3).

When compared the results obtained with 50 and 200 fps, four subpopulations were present at both FRs, namely, Rapid progressive, Medium non-progressive, Medium progressive and Slow slight progressive. The number of tracks included in the SP1 was similar at both FRs, but the other subpopulations varied in their number with the FR. As was noted above, most kinematic values were much higher at 200 fps in each equivalent subpopulation (Table 4).

4 | DISCUSSION

Currently, most porcine artificial insemination centres evaluate sperm motility by using CASA-Mot systems (Kime et al., 2001; Verstegen et al., 2002). Each commercial system CASA-Mot has its individual design and manufacturing standards that must be considered before its routine use. The CASA system provides an objective and repeatable assessment of the number of motile sperm cells in a sample, as well as for measuring several kinematic variables. This can allow determination of the "poorer" samples or the samples with poor sperm motility but can also be a useful technique in predicting the most desirable boars for AI based on sperm motility and kinetic variables.

Several authors have indicated that the results are dependent on the set-up of the system used, such as the field of observation location (Nöthling & dos Santos, 2012), frame rate of recordings (Wilson-Leedy & Ingermann, 2007), the kind and depth of the counting chamber used, the volume of the drop and other factors (Bompart et al., 2018; Soler et al., 2018).

The possible bias of the results depending on these elements and intrinsic factors makes it necessary to define well the protocol used in each case, optimizing all the components of the analytical process for analysis in a correct manner to obtain real sperm kinetic values (Björndahl, 2011; Bompart et al., 2018; Palacios et al., 2012; Simonik et al., 2015; Verstegen et al., 2002).

Until recently, the CASA-Mot frame rate used was limited by hardware restrictions from 16 to 60 Hz (Contri, Valorz, Faustini, Wegher, & Carluccio, 2010; Holt, O'Brien, & Abaigar, 2007). Nevertheless, as has been previously indicated, the rate at which images are captured and the length of video recording both affect the distance that a spermatozoon might move between successive frames (Mortimer, Serres, Mortimer, & Jouannet, 1988). This has a direct effect on the estimated trajectory for each sperm cell, deviations from the recorded path of a spermatozoon's centroid over successive frames, and other output values for sperm motion (Amann & Waberski, 2014).

Mortimer and Swan (Mortimer & Swan, 1999) have shown that by using a low FR (<50 fps), it is not possible to obtain some characteristics of the trajectory that occur at intervals shorter than the time elapsed between images. As a result, the information of the "real" trajectory is not obtained, leading to inaccuracies in the kinematic evaluation (Bompart et al., 2018).

Here, a new camera of higher image capture frequency that allowed analysis of the effect of frame rate on sperm kinematic results was used. Logically, the total motility was not affected by the

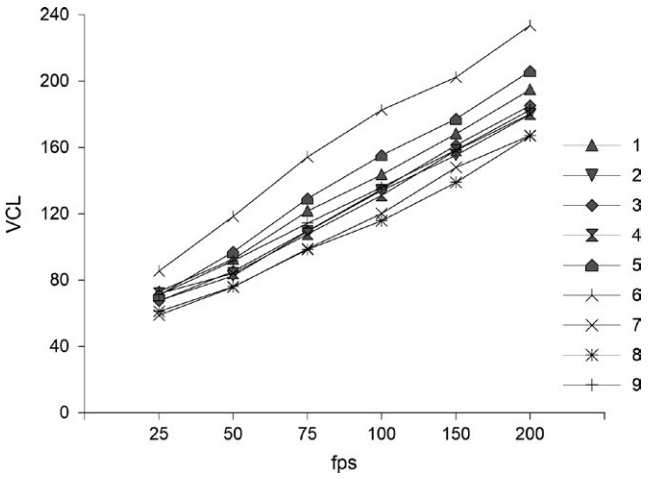

FIGURE 1 Effect of frame rate (fps) on curvilinear velocity (VLC, μm/s) for boars 1–9

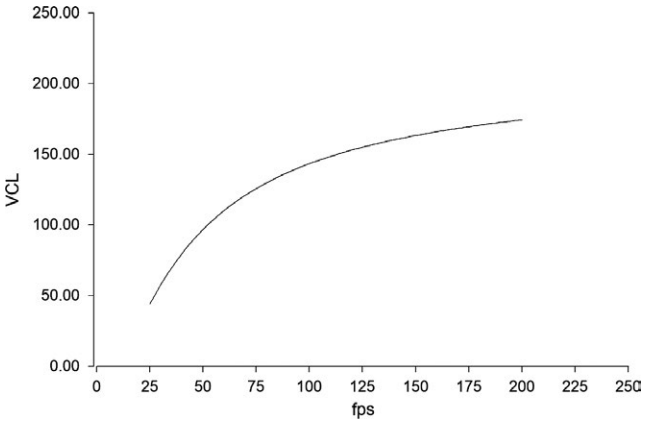

FIGURE 2 Effect of frame rate on boar spermatozoa curvilinear velocity (VLC, μm/s)

TABLE 2 Asymptotic level (α), rate of increase (β), correlation α/β ($\rho^{\alpha,\beta}$), and estimate (up to a frame rate of 250 fps) of boar sperm kinematic variables needed to obtain the threshold level (95% of the maximum)

						Kinematic estimated values at different fps			
	α	*SE*	β	*SE*	$\rho^{\alpha,\beta}$	50	100	200	250
VCL	212.2	1.4	39.2	0.6	0.86	96.9	143.4	174.4	181.4
VSL	68.3	0.5	1.8	0.4	0.80	65.9	67.1	67.7	67.8
VAP	98.6	0.6	11.6	0.4	0.81	78.2	87.8	93.1	94.1
LIN	39.8	0.2	−21.5	0.2	0.83	61.2	49.4	44.3	43.4
STR	69.9	0.2	−8.0	0.2	0.81	82.0	75.7	72.7	72.1
WOB	54.3	0.2	−15.1	0.1	0.82	73.5	63.1	58.5	57.7
ALH	51.6	0.1	1.5	0.2	0.80	50.0	50.8	51.2	51.3
BCF	50.4	0.4	49.0	0.8	0.87	18.9	30.9	39.5	41.5

VCL: curvilinear velocity (μm/s); VSL: straight line velocity (μm/s); VAP: average path velocity (μm/s); LIN: linearity of forward progression (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat cross-frequency (Hz); *SE*: standard error.
$p < 0.05$.

TABLE 3 Eigenvectors of principal components (PCs) for boar sperm kinetic parameters in boars at 50 and 200 frames per second

	50 fps			200 fps		
Principal component[a/b]	Velocity	Progressivity	Oscillation	Velocity	Progressivity	Oscillation
VCL	0.960			0.987		
VSL	0.826	0.499		0.755	0.566	
VAP	0.978			0.904		0.384
LIN		0.924	−0.337		0.844	0.517
STR		0.971			0.992	
WOB		0.490	−0.705			0.961
ALH	0.671		0.574	0.970		
BCF	0.477		0.626	0.792		0.302
Variance explained%	41.6	29.9	17.3	49.7	26.2	18.7

Total variance explained = 88.8% for 50 fps; 94.6%. for 200 fps.
VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; LIN: linearity of forward progression; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat cross-frequency.
[a]Expresses the more important variables in each PC. Only eigenvectors >0.4 are presented. [b]Rotated component matrix.

increase in the FR. The percentage of progressivity (LIN) decreased at the highest FR (200 fps) because of the increment of VCL and VAP regarding VSL. This implies that a new definition is needed when higher speed cameras are used in the calculation of seminal dose sizes.

Regarding the sperm kinematic parameters, VSL was not changed from 50 Hz in accordance with Mortimer et al. (1988), who observed that VSL was not affected by video frame rates of 25, 30 and 60 fps. On the other hand, VCL and VAP showed a statistically significant increase, rising to more than 175% higher than the values corresponding to 25 and 200 fps. These results confirm and extend the observations of previous work dealing with the effect of FR on sperm kinematics in different species (carp (Ravinder, Nasaruddin, Majumdar, & Shivaji, 1997); dog (Rijsselaere, Van Soom, Maes, & de Kruif, 2004); eel (Gallego et al., 2013); human (Morris et al., 1996; Mortimer et al., 1988)). Only one study had been designed in a similar way as the present, obtaining the asymptotic points of 128, 153, 253 and 302 fps for human, bull, ram and rabbit spermatozoa, respectively (Castellini et al., 2011). In comparison, the results for boar showed and intermediate value (212 fps, indicating that their motility pattern is not so variable or spermatozoa not so fast as other mammalian species).

The indices LIN, STR and WOB showed a clear decrease with increasing FR, as a consequence of the constancy of VSL and that VCL increased much more than VAP. This knowledge implies that a new definition of progressivity will be needed when high FR are used.

The lateral head displacement, measured by the ALH, was not significantly changed from 50 Hz, indeed a slight increase was maintained at higher FRs. This result was different from that obtained previously showing a reduction in ALH at higher FRs, because, in that paper, greater sampling frequency reduced the distance of sperm head deviation from the average path (Kraemer, Fillion, Martin-Pont, & Auger, 1998; Zhu, Pacey, Barrett, & Cooke, 1994). That each study was done with a different commercial CASA-Mot system should

TABLE 4 Boar sperm subpopulations for kinematic parameters at 50 and 200 fps

Parameter/Subpopulation	Rapid progressive	Medium non-progressive	Medium progressive	Slow slight progressive
50 fps				
VCL	125.8 ± 1.1[a]	91.6 ± 1.9[b]	79.8 ± 0.8[c]	58.9 ± 1.3[d]
VSL	96.2 ± 1.0[a]	33.2 ± 1.8[b]	69.2 ± 0.7[c]	36.9 ± 1.3[b]
VAP	104.1 ± 1.0[a]	75.2 ± 1.8[b]	74.8 ± 0.7[b]	43.3 ± 1.3[c]
LIN	76.2 ± 0.5[a]	34.4 ± 0.9[b]	86.1 ± 0.4[c]	62.2 ± 0.7[d]
STR	91.4 ± 0.4[a]	42.2 ± 0.7[b]	91.6 ± 0.3[a]	83.4 ± 0.5[c]
WOB	82.7 ± 0.4[a]	79.0 ± 0.7[b]	93.5 ± 0.3[c]	73.5 ± 0.5[d]
ALH	2.0 ± 0.01[a]	1.7 ± 0.02[b]	1.3 ± 0.01[c]	1.4 ± 0.02[d]
BCF	21.5 ± 0.2[a]	15.0 ± 0.4[b]	13.6 ± 0.1[c]	13.3 ± 0.3[c]
n	481	156	987	317
200 fps				
VCL	285.0 ± 2.1[a]	188.9 ± 2.4[b]	171.3 ± 1.4[c]	144.1 ± 1.8[d]
VSL	108.5 ± 1.0[a]	37.6 ± 1.2[b]	73.5 ± 0.7[c]	43.1 ± 0.8[d]
VAP	146.7 ± 1.3[a]	99.9 ± 1.5[b]	100.1 ± 0.8[b]	61.3 ± 1.1[c]
LIN	38.0 ± 0.4[a]	19.1 ± 0.4[b]	42.9 ± 0.2[c]	29.2 ± 0.3[d]
STR	73.4 ± 0.5[a]	36.2 ± 0.6[b]	73.4 ± 0.3[a]	68.7 ± 0.4[c]
WOB	51.6 ± 0.3[a]	51.9 ± 0.4[a]	58.4 ± 0.2[b]	41.9 ± 0.3[c]
ALH	2.1 ± 0.01[a]	1.6 ± 0.01[b]	1.5 ± 0.01[c]	1.3 ± 0.01[d]
BCF	57.0 ± 0.7[a]	33.9 ± 0.8[b]	37.9 ± 0.4[c]	23.3 ± 0.5[d]
n	391	296	928	559

VCL: curvilinear velocity (μm/s); VSL: straight line velocity (μm/s); VAP: average path velocity (μm/s); LIN: linearity of forward progression (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat cross-frequency (Hz).
[a–d]Within row, different superscripts indicate significant differences among sperm subpopulations. $p < 0.05$.

be considered, and that ALH was the most sensitive parameter to manufacturer-specific algorithms should be noted (Boryshpolets, Kowalski, Dietrich, Dzyuba, & Ciereszko, 2013).

The most changed parameter was BCF (total increase of 374%) because the sensitivity to the changes in the direction of the head movement (a consequence of tail beat) was dramatically increased. This result is in agreement with previous work (Sellés, Gadea, Romar, Matás, & Ruiz, 2003) showing that variations are more considerable at low FR. This variable is associated with the frequency of head sperm oscillations about the average path. The most accurate prediction of this parameter can probably be estimated in spermatozoa whose progression is similar to or equivalent to that of a sinusoidal wave (Davis, Niswander, & Katz, 1992; Davis, Rothmann, & Overstreet, 1992). Furthermore, significant increases in BCF at higher FR suggest that a more accurate analysis of this variable is provided at higher video frames.

Previously, the whole population of spermatozoa in an ejaculate has been considered; as it was described by a normal distribution model, spermatozoa were considered as equivalent, with the same chances of being "selected" for fertilization. Nevertheless, by considering the quantitative data obtained from CASA systems, different authors have proposed that the actual distribution of sperm cells is not uniform, not normally distributed but structured in well-defined subpopulations, (Amann & Hammerstedt, 1993; Caldeira et al., 2018; Gallego et al., 2015; Hirai et al., 2001; Soler et al., 2017; Thurston, Watson, Mileham, & Holt, 2001; Valverde et al., 2016; Vásquez et al., 2016; Yániz et al., 2016, 2018). Even today, in the boar, a relationship between these subpopulations and the fertilizing capacity of the ejaculates has been established (Abaigar et al., 1999; Holt et al., 1996; Vyt et al., 2008).

Unfortunately, all the studies developed until now have been done with FRs commonly lower than that defined in the present work. We have observed that, even maintaining the subpopulation structure 50 and 200 fps capture frequencies introduced sensitive variations, both in the PC analysis and its subsequent subpopulation values.

In previous papers done at 25 fps, only three subpopulations were observed with kinematic values lower than here, what is logical at this low FR (Quintero-Moreno, Rigau, & Rodríguez-Gil, 2004), while with the same FR, other groups also find four subpopulations, equivalent to those observed here (Flores et al., 2008; Ramió et al., 2008). In addition to the different FR, the different algorithms used for different CASA-Mot brands implies that kinematic values for each subpopulation can vary even at the same FR (Ibănescu, Leiding, & Bollwein, 2018). At 60 fps, four subpopulations have also been observed but with higher values than those corresponding to 50 fps in the present study. In any case, it is necessary to indicate that no reference to boar breed was indicated in previous works dealing with

this topic (Bucci et al., 2018; Ibănescu et al., 2018) which can limit possible comparisons between results.

Therefore, a future revision of the subpopulation structure species-by-species at an adequate FR in each case will need to be developed. This, added with the individual variation, indicates that future work must be done to optimize the production of seminal doses for artificial insemination programmes (Broekhuijse, Šoštarić, Feitsma, & Gadella, 2012b; Didion, 2008).

As a conclusion and taking BCF and VCL as the most sensitive parameters to FR, it is recommended to use a capture frequency as close as possible to 225 fps in the evaluation of boar ejaculates for the study of their kinetics, while it can be considered enough at 50 fps for the evaluation of general motility, and 150 fps progressive motility of the samples. Any future work must be developed on the basis of these results, both in the research and in the production fields.

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AUTHOR CONTRIBUTIONS

It was previously indicated and no change was being produced.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CHAPTER VI: EFFECT OF VIDEO CAPTURE TIME ON SPERM KINEMATIC PARAMETERS IN BREEDING BOARS

CHAPTER VI:

Effect of video capture time on sperm kinematic parameters in breeding boars

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Abstract

Computer Assisted Semen Analysis (CASA) technology is being a common tool in the seminal analysis for seminal doses production in farm animals. The aim of the present work was to define the optimal capturing time for a correct evaluation of boar sperm motility. Six sexually mature boars from the Topigs Lines, were used as semen donors. ISAS®v1 CASA-Mot system was used with an image acquisition rate of 50 Hz for two seconds capturing. Obtained video frames were fragmented into four segments corresponding to 0.5, 1.0, 1.5 and 2.0 s. ISAS®D4C20 counting chambers, warmed at 37 °C were used. Total and progressive motility were not affected by capture time. Capture time had a significant effect on velocity and indexes values ($P< 0.05$) but not on lateral head displacement (ALH) and beat cross frequency (BCF). All the parameters showed animal differences. The results demonstrate that sperm motility is affected by the video recording time length ($P< 0.05$). This effect is evident both in motility parameters and in single cell kinetics, studied by cluster analysis that showed a short time of video recording can to overestimate the percentage of rapid non-progressive cells in a subpopulation ($P< 0.05$). Studies based just on motility can use one-half second, while when kinematics must to be considered two seconds must be analyzed.

Keywords: computer-assisted semen analysis, CASA-Mot, sperm motility, swine, reproduction

Introduction

Semen analysis is the test for fertility potential evaluation of an animal. When sperm motility was evaluated, significant correlations with fertility have been reported for bovine (Budworth et al., 1988), equine (Samper et al., 1991), rabbit (Lavara et al., 2005) and swine (Tardif et al., 1999; Didion, 2008; Broekhuijse et al., 2012b).

Traditional subjective analysis based on technician experience have shown a high degree of variation in the final results independent of the species (Knuth et al., 1989; Amman and Waberski, 2014; Gallego et al., 2018). To overpass this problem, from the 80' years of past century Computer Assisted Semen Analysis (CASA) technology was developed to improve accuracy and precision on semen analysis. However, the result of the motility analysis using CASA-Mot technology is associated with software and hardware capabilities, in addition with other biological variation sources as semen samples within animal (Bompart et al., 2018). The development of the swine industry by artificial insemination (AI) have led to improvements on quantitative analysis of boar semen samples to predict the fertility potential of seminal doses. Even the number of using CASA systems in continuously increased in AI centers, it is necessary to update the standardization of the methods for the objective evaluation of sperm quality (Verstegen et al., 2002; Amann and Katz, 2004).

CASA systems determine several sperm parameters, mainly by detecting and analyzing the sperm head positions in successive frames by video images (Davis and Katz, 1993). In some works, in other species as dog (Rijsselaere et al., 2003) or human (Owen and Katz, 1993), a lower frame rate affected the curvilinear path that occur over shorter intervals than the elapsed time between two consequent frames (i.e. 1/30 or 1/60 s; Owen and Katz, 1993). Consequently, the sperm cell's actual trajectory may be lost, resulting in significant errors for various semen motility characteristics (Owen and Katz, 1993).

In consequence, the aim of this study was to determine the effect of the video capture length in evaluating motility and kinematic parameters in boar sperm for the optimization of the analysis protocol.

Materials and Methods

Animals

The experiment was conducted at a commercial swine farm (Mejoramiento Porcino S.A., Heredia, Costa Rica) during 2015 in the Northwest of Costa Rica (San José de la Montaña, 10°3'0" N, 84°7'0" W, Barva, Heredia, Costa Rica, Central America) following 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. Six sexually mature and healthy boars from the Topigs Lines, 2-3 years of age and known fertility, were used as semen donors in this study. Breeding boars were housed individually in well-ventilated pens with average temperature range of 16-22 °C, during the time of the experiment. Animals were feed with the standard breeder mixture containing maize, soybean meal, mineral mixture and common salt, as ingredients to fulfill the nutrient requirements (Nutrient Requirements of Swine, 1998), and provided with water *ad libitum*.

Collection and examination of semen

Semen samples were collected in the morning, once per week, using the “gloved-hand” technique (Hancock and Hovell, 1959) and immediately placed in a water bath at 38 °C at the farm laboratory. In all cases, the sperm-rich fractions were collected, diluted with a commercial extender (Androstar Plus®; Minitube, GmbH, Tiefenbach, Germany). Samples from each ejaculate were evaluated for subjective motility, morphology and concentration parameters (Spermacue, Minitube International, Costa Rica) following established protocols (Martín Rillo et al., 1996), and only ejaculates with at least 85% motile spermatozoa, 90% morphologically normal spermatozoa and greater than 10×10^9 total spermatozoa per ejaculate were used. Immediately after collection, semen was diluted in the appropriate medium (BTS, Minitube, Mejoramiento Porcino S.A., Heredia, Costa Rica) and dilution ratio to result in a concentration of approximately $30x10^6$ sperm/mL.

Assessment of sperm variables

For the analysis of motility, kinetics and concentration, ISAS®D4C20 disposable counting chambers (Proiser R+D, S.L., Paterna, Spain) were used after pre-warmed at 37 °C. After homogenization of the samples, a volume of 3 µL was distributed along the counting chamber race by capillarity to fill them completely. Analyses were conducted using the CASA-Mot system ISAS®v1 (Proiser R+D S.L., Paterna, Spain). The video-camera used was a Proiser 782m (Proiser R+D). Frame rate used was 50 Hz, with a final resolution of the images of 746x578 pixels. The camera was attached to a microscope UB203 (UOP/Proiser R+D) with an eyepiece 1x and a 10x negative phase contrast objective (AN 0.25) and an integrated heated stage maintained at a constant temperature of 37±0.5 °C.

Kinematic analysis

The CASA-Mot parameters included in the study were: 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 over 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 sperm cells swam; beat-cross frequency (BCF, Hz), defined as the frequency with which the actual track crossed the smoothed track in either direction; motility (%), the percentage of the total motile cells; and progressive motility (%), corresponding with spermatozoa swimming forward quickly in a straight line, (STR ≥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).

Experimental design

To assess the influence of video recording time, twelve ejaculates (two ejaculates of each boar) and six replicates by ejaculate were analyzed at image acquisition rate of 50 Hz and the capture time was two seconds. Subsequently, the video frames were fragmented into four segments corresponding to 0.5 s, 1.0 s, 1.5 s, and 2.0 s; according with: [*@echo off: for %%i in (\ * avi) do (set fname = %% ~ ni) & call: divideVideo: goto eof*].

Statistical analyses

The data obtained from the analysis of all sperm parameters were first tested for normality and homoscedasticity using Shapiro-Wilks and Kolmogorov-Smirnov tests. Q–Q plots were used to check for departures from the normal distribution. Trying to adjust to a normal distribution, data were transformed using arcsine square root (arcsin $\sqrt{x}$) before repeated measures ANOVA was run. Even after arcsine transformation, the kinematics variables of the sperm were not normally distributed, as determined by the Shapiro-Wilks test. The kinematics sperm variables did not satisfy the normality requirement for a parametric analysis of variance. Therefore, non-parametric analyses 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 motility data. The first step was to perform a principal component analysis (PCA). The number of principal components (PC) that should be used in the next step of the analysis was determined using the Kaiser criterion, namely selecting only those with an eigenvalue (variance extracted of each PC) >1. Furthermore, was found the factorial analysis feasibility, using Bartlett's sphericity test to test the null hypothesis that the correlation matrix is an identity matrix and the KMO index (Kaiser-

Meyer-Olkin), which determines the calculation of the correlations between two variables once the influence that the remaining variables have on them has been eliminated (Spencer, 2013). The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA. All sperm cells within each generation and time after activation were clustered using a non-hierarchical clustering procedure (*k*-means model and Euclidean distance). This analysis enabled the identification of sperm subpopulations and the detection of outliers.

The effects of clusters between treatments for measuring motility parameters were analysed using the Kruskal–Wallis test, followed by the Mann–Whitney paired non-parametric *U*-test when significant differences were found. In total, 103 306 cells were analysed. The results are presented as mean ± standard deviation (SD). Statistical significance was considered at $P< 0.05$. All data were analyzed using IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Considering all the cells, total and progressive motility as well as ALH and BCF were not affected for the capturing time while the other kinematic parameters were affected in different degree ($P<0.05$). The highest increase was found for VCL with a total difference between the time of 0.5 s and 2.0 s of 12.4 %, followed by VAP with a difference between these two times of 10.8 %. VSL showed significant differences between times even the highest difference implies only a 6.2 % of between the values corresponding to 0.5 s and 1.5 s, what we consider no presenting biological significance. Therefore, the derived parameters LIN and STR presented a considerable decrease between values observed at 0.5 s and 2.0 s of 17.2 % and 13.1 %, respectively. About WOB, the decrease was no so high, even being significant (Table 1).

The kinematic results boar by boar for VCL, VAP and ALH presented a similar pattern with significant increase regarding capture time. It was observed a decrease in the values of VSL, LIN and STR. WOB and BCF, indicating that these parameters are less influenced by the time of capture, and not showing differences in some of the animals (data not shown).

The principal component analysis generated three principal components with a total variance explained of 92.0 %, that were used for subsequent cluster analysis (Table 2). The cluster analysis showed three sperm subpopulations. These subpopulations showed different kinetics characteristics that allowed to identify them as: 1) rapid progressive; 2) rapid non- progressive; 3) slow non- progressive. All kinematic parameters showed significant differences between sperm subpopulations cluster (Table 3). Sperm cells were assigned to the different clusters and significant

differences in the percentages of spermatozoa belonging to each subpopulation were found between the video recording times length (Table 4).

Discussion

Currently, different livestock species farms and artificial insemination centers evaluate sperm motility by using CASA-Mot systems (Kime et al., 2001; Verstegen et al. 2002). Several authors have indicated that the results are dependent on the settings of the system used, such as the field of observation location (Nothling and dos Santos, 2012), frame rate of recordings (Wilson-Leedy and Ingermann, 2007), the kind and depth of the counting chamber used, the volume of the drop and other factors (Bompart et al., 2018; Soler et al., 2018).

The possible bias on the results depending on these factors makes necessary to well define the protocol used in each case, optimizing all the components of the analysis process to analyze in a correct way for obtaining the real sperm kinematics values (Verstegen et al., 2002; Björndahl, 2011; Palacios et al., 2012; Simonik et al., 2015; Bompart et al., 2018).

First to consider the results obtained the present study it is convenient to make a comment about the statistics used in the present work. It is too much common to overpass the not normal distribution of data by some mathematical transformation, as arcsine square root used here, if it converts the data distribution in a normal one, without any more analysis. We have observed that even after this kind of transformation, the distribution remains being not normal and for this another statistical no based on normal distribution model approach must be used.

Until recent time, the frame rate to be used was limited by hardware constrictions from 16 to 60 Hz (Holt et al., 2007; Contri et al., 2010). Nevertheless, as it was previously indicated, the rate at which images are captured and the duration of image affect the distance that a spermatozoon might move between successive frames (Mortimer et al., 1988). This has a direct effect on the estimated trajectory for each sperm cell, deviations from the recorded path of a spermatozoon's centroid over successive frames, and other output values for sperm motion (Amann and Waberski, 2014).

Using a low frame rate (lower than 50 Hz) is not possible to obtain some characteristics of the trajectory that occur at intervals shorter than the time elapsed between images (Mortimer and Swan, 1999). As a result, the information of the "real" trajectory is not obtained leading to inaccuracies in the kinematic characteristics evaluation (Bompart et al., 2018).

The percentage of total motile and progressive spermatozoa was not affected by the video recording time. Regarding kinematics variables, the VSL, LIN, STR, and WOB decreased with the

increase in the capturing time, while the rest of the variables increased. The highest variation was observed for LIN, STR and VCL, and the lowest for VSL.

When analyzing the effect of the number of images (at the same frame rate, more time implies more analyzed images) on sperm kinetics, it was relevant that the increase in the amount of available information for the analysis of the spermatic trajectory allows to a more precise reconstruction. These results imply that it is needed to define conveniently the video time length to guarantee equivalent results, even by using the same commercial CASA-Mot system. (Holt et al., 1994). Even more, capture time has much more importance when real sperm trajectories are irregular than regular.

The lateral head displacement, measured by the ALH, was not significant changed, even a slight increase was maintained with higher times video recording. This result was similar with that obtained previously showing an increase of ALH at higher video time length because in that papers greater sampling frequency reduces the distance of sperm head deviation from the average path (Zhu et al., 1994; Kraemer et al., 1998). It is needed to consider that each work was done with different commercial CASA-Mot systems and that ALH is the most sensible parameter to manufacturer specific algorithms (Boryshpolets et al., 2013).

The beat-cross frequency was not significant changed because the sensitivity to the changes in the direction of the head movement (consequence of tail beat) was dramatically unimproved. This variable is associated with the frequency of head sperm oscillations about the average path. The most accurate prediction of this parameter can probably be estimated in spermatozoa whose progression is like or equivalent to a sinusoidal wave (Davis et al., 1992 a, b). Furthermore, no significant increases in BCF suggest that the more accurate sperm analysis of this variable can be better at higher video frames.

In the past, the whole population of spermatozoa in an ejaculate was considered being distributed following a normal distribution function, it means they were considered as equivalents looking for the same objective: to be the “selected” for fertilization. Nevertheless, in the recent years considering the quantitative data obtained with CASA technology, different authors proposed that the actual distribution of sperm cells is not uniform neither normal, but structured in well-defined subpopulations (Amman and Hammerstedt, 1993; Hirai et al., 2001; Thurston et al., 2001; Soler et al., 2014; Valverde et al., 2016; Vásquez et al., 2016; Yániz et al., 2016; Soler et al., 2017; Caldeira et al., 2018; Yániz et al., 2018). Even more, almost in boar, it was established a relationship between these subpopulations and the fertilizing capacity of the ejaculates (Holt et al., 1996; Abaigar et al., 1999; Vyt et al., 2008).

To better understand the spermatozoa kinetic depending upon the times of video recording length, we performed cluster analysis using kinematics parameters of single sperm cells (Abaigar et al., 1999). Based on cluster analysis, sperm were classified into four clusters, with the following characteristics: one included sperm cells with low VSL, VAP and linearity and was considered as “slow non-progressive”; the second showed high velocity and highly linear cells (high VSL, LIN and STR) and was considered “rapid progressive”; and the third showed high velocities (VCL, VSL and VAP) but low linearity and was defined as “rapid non-progressive”. The effects of video recording length on the proportions of each sperm subpopulation were significative, as those a longer video time recording length showed a significant increase in the percentage of slow non-progressive cells, which was accompanied by a decrease in the percentage of “rapid non-progressive” cells. In addition, the percentage of “rapid progressive” cells, was not significantly different at the video recording times length. If we consider “rapid non-progressive” sperm cells as hyperactivated-like (Schmidt and Kamp, 2004), this could provide a proof of the effect of video recording time length. Therefore, take two seconds of video recording length may prevent to overestimate number of “rapid non-progressive” cells.

In conclusion and taking the VCL as the most sensible parameter it is recommended to use a capture for two seconds in the evaluation of boar ejaculates for the study of their kinetics, while it can be considered enough with one-half second for the evaluation of general motility and progressive motility of the samples.

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Conflict of interest

The authors declare no conflicts of interest.

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Table 1. Boar sperm motility and kinematics at four video-recording time lengths (CASA-Mot, mean ± SD, change regarding lowest time in percentage, n=139 473)

	Time video recording (s)			
	0.5	1.0	1.5	2.0
TMOT (%)	70.5±13.3	69.7±13.5 (-1.2)	70.3±12.8 (-0.3)	69.5±13.5 (-1.4)
PMOT (%)	67.5±12.5	66.8±12.6 (-1.1)	67.3±11.9 (-0.3)	66.7±12.3 (-1.2)
VCL (μms^{-1})	81.2±21.5[a]	86.9±32.5[b] (7.4)	88.0±33.7[c] (8.4)	91.2±35.2[d] (12.4)
VSL (μms^{-1})	42.1±16.3[a]	41.4±22.9[b] (-1.7)	39.4±23.8[c] (-6.4)	40.3±26.[c] (-4.1)
VAP (μms^{-1})	48.4±14.3[a]	51.3±21.6[b] (6.0)	51.4±22.2[b] (6.2)	53.63±23.9[c] (10.8)
LIN (%)	52.1± 6.2[a]	47.2±17.0[b] (-9.4)	44.1±18.2[c] (-15.5)	43.1±19.3[d] (-17.2)
STR (%)	85.8±16.1[a]	77.5±20.1[b] (-9.6)	73.0±22.1[c] (-14.8)	71.0±24.0[d] (-13.1)
WOB (%)	60.9±12.4[a]	59.1±12.1[b] (-2.9)	58.1±12.2[c] (-4.5)	57.2±11.9[d] (-5.9)
ALH (μm)	1.62±0.42	1.77±0.48 (8.5)	1.81±0.49 (10.5)	1.85± 049 (12.4)
BCF (Hz)	16.5±6.0	17.1±6.9 (5.5)	17.6±7.0 (6.2)	18.1±7.0 (8.9)

SD: standard deviation; TMOT: total motility; PMOT: progressive motility; VCL: curvilinear velocity (μms^{-1}); VSL: straight line velocity (μms^{-1}); VAP: average path velocity (μms^{-1}); LIN: linearity of forward progression (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat-cross frequency (Hz). In brackets is indicated the percentage of variation regarding time 0.5 s. [a-d] Within rows, values with different superscripts letters indicates significant differences between recording times. P <0.05.

Table 2. Eigenvectors of principal components (PCs) for boar sperm kinetics parameters at two second of video-recording time

Principal component[*/a]	PC1	PC2	PC3
VCL	0.962		
VAP	0.867		
ALH	0.857		
BCF	0.750		
STR		0.981	
LIN		0.822	0.553
VSL	0.656	0.663	
WOB			0.933
Variance explained (%)	52.8	29.6	8.9

Total variance explained = 91.3%. [*]Expresses the more important variables in each PC. Only eigenvectors > 0.4 are presented. [a] Rotated component matrix, rotation method: Varimax with Kaiser normalization. VCL: curvilinear velocity (μms^{-1}); VSL: straight line velocity (μms^{-1}); VAP: average path velocity (μms^{-1}); LIN: linearity of forward progression (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat-cross frequency (Hz).

Table 3. Boar sperm subpopulations for kinetics parameters (mean±SD) at two second of video-recording time

Parameter/Subpopulation	Rapid progressive	slow non- progressive	Rapid non-progressive
Number of cells	46 309	19 947	37 050
VCL	69.6±23.6[a]	83.9±25.7[b]	109.2±28.2[c]
VSL	41.5±19.6[a]	20.0±12.4[b]	50.9±22.5[c]
VAP	46.8±19.3[a]	46.1±18.0[b]	58.9±21.4[c]
LIN	57.4±13.6[a]	23.2±11.2[b]	45.4±13.0[c]
STR	86.0±10.8[a]	42.0±16.2[b]	83.9±12.4[c]
WOB	65.9±10.5[a]	54.2±10.7[b]	53.0±10.2[c]
ALH	1.42±0.30[a]	1.87±0.41[b]	2.11±0.41[c]
BCF	15.8±6.78[a]	16.4±6.58[b]	19.1±6.24[c]

SD: standard deviation; VCL: curvilinear velocity (μms^{-1}); VSL: straight line velocity (μms^{-1}); VAP: average path velocity (μms^{-1}); LIN: linearity of forward progression (%); STR: straightness (%); WOB: wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat-cross frequency (Hz). [a-c] Within rows, values with different superscripts letters indicates significant differences between sperm subpopulations. P <0.05.

Table 4. Subpopulation distribution of spermatozoa in percentage depending on video time recording length

Time (s)/Subpopulation	Rapid progressive	Rapid non-progressive	slow non-progressive
0.5	42.9	44.1[a]	13.0[a]
1.0	42.7	39.2[a]	18.1[a]
1.5	43.6	34.0[b]	22.4[b]
2.0	43.0	34.3[b]	22.7[b]

Each row indicates the percentage of spermatozoa belonging to the different cluster (sum of percentage for each time = 100). Different superscripts indicate significant differences within column regarding to times, chi square (χ^2) test, P <0.05.

CHAPTER VII: AFTERWORD TO SPERM MORPHOMETRICS TODAY AND TOMORROW SPECIAL ISSUE IN ASIAN JOURNAL OF ANDROLOGY

CHAPTER VII:

Afterword to Sperm morphometrics today and tomorrow special issue in Asian Journal of Andrology

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INVITED COMMENTARY

Semen Analysis

Afterword to *Sperm morphometrics today and tomorrow* special issue in *Asian Journal of Andrology*

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The problems associated with the subjective assessment of human sperm morphology have been well aired in another *Asian Journal of Andrology* Special Issue[1] that marked the publication of the 5th edition of the WHO Semen analysis manual, and contrary views have subsequently been presented.[2] However, the vagaries of the eye-brain system in assessing whether a sperm head is large or small can be eliminated by objective assessment where definitive structures are defined by their dimensions. These can then be classified automatically into as many categories as the data permit, conventionally on the basis of preset upper and lower limits, but also by more comprehensive analysis as discussed here.

This Special Issue on computer-aided sperm morphology assessment comprises four reviews (on sperm transport in mammals,[3] the current status of sperm morphometry in mammals[4] and birds,[5] and the relevant statistical methods to assess the morphometric results[6]); three clinical research papers (on sperm subpopulations in split ejaculates from adult men with normozoospermia[7] and in ejaculates from adolescents with or without varicocoele,[8] including the use of computer-assisted sperm analysis in assessing sperm nuclear DNA fragmentation[9]); and six veterinary research papers on sperm populations in the epididymis (in normozoospermic and teratozoospermic domestic cats[10]) and in ejaculated spermatozoa (from an endangered puma species,[11] roosters and guinea fowls,[12] rams, bulls and boars,[13] and on cryopreserved bovine semen[14]).

Although most of these studies examined the dimensions of the sperm head on air-dried, fixed, and stained cells, others have taken advantage of the objective method to determine the extent of organelle-specific fluorescent dye binding. DNA-binding dyes were used to examine sperm nuclear morphology in fixed smears of split human ejaculate fractions,[7] and together with chromosome-specific probes to determine the morphometrics of X- and Y-bearing bovine spermatozoa.[15] A combination of a nuclear dye with a fluorescent dye specific for the acrosome-specific dye permitted the simultaneous assessment of the whole head as well as its nuclear and acrosomal components.[13] These are novel approaches that should lead to rapid descriptive and diagnostic advances in both veterinary and clinical fields.

That there were more submissions from the veterinary than clinical field probably indicates the financial importance given to improving sperm diagnosis and selection in commercial industries. In fact, from the scientific point of view, much more work on sperm morphology and morphometry significance has been developed on other animal species than the human. The irruption of ICSI could also explain this difference. The fact that only one spermatozoon is enough to achieve a pregnancy has delayed spermatology research in human. On the other hand, the high variability of human infertility cases may make it seem an unattractive investment whereas the opposite is the case: the papers presented here show that investment in the equipment, and the researchers, to correlate clinical data with the morphometric results, would generate a range of observations on sperm subpopulations in fertile and infertile men that could explain currently unexplained causes of infertility.

THE SIGNIFICANCE OF THE MORPHOMETRIC ANALYSIS OF SPERM CELLS

The application of principal component (PC) and discriminant analysis to reveal subpopulations of spermatozoa is a powerful tool to evaluate raw semen and processed sperm cell suspensions, but not many clinicians are aware of the technique. As described in several papers here, PC analysis is a multivariate statistical method that reduces the number of variables used in subsequent calculations used to describe the data. By integrating the original variables according to their coherence in a database into a new complex mathematical variable, clearly defined homogeneous subpopulations of spermatozoa can be defined. In support of the theory above, the papers presented here showed that most of the variance from up to 13 morphometric variables could be explained by only two or three PCs: two in bulls,[16] adolescent humans,[8] adult human sperm head DNA,[9] domestic cats,[10] puma,[11] roosters, and guinea fowls[12] and three PCs in adult human split ejaculate samples.[7]

From these PCs, discriminant analysis was used to generate clearly separable homogeneous subpopulations of morphological forms. Here, the number of subpopulations ranged from two to five: two (for the X-/Y-bearing bovine sperm heads,[15] for large+elongated/small+elongated sperm heads in human adolescents[8]), three (for large+round/elongated/small spermatozoa in human sperm heads in split ejaculate fractions,[7] for elongated+intermediate/large+high acrosome/short+small sperm heads in the puma,[11] for small, wide and slightly elliptical/average size, long, narrow and very elliptical/very large, wide and elliptical sperm heads in the rooster[12]), four (for large/high medium/low medium/small in human sperm head DNA,[9] for small/short/large/narrow sperm heads in the bull,[14] for shape-related sperm heads in both normo- and terato-zoospermic cats[10]) to five (for very small, wide, very short and slightly elliptical/small, very short, very wide and slightly elliptical/very large, very wide, short and slightly elliptical/average size, very long, very narrow and very elliptical/average size, long, narrow and elliptical sperm heads in the guinea fowl[12]).

These awkward, convoluted, and very subjective descriptions of the nature of the sperm clusters generated by this technique highlight very well the difficulty in getting agreement (be it intra- or inter-laboratory, national or international) between observers on the definitions of normal sperm morphology, let alone abnormal forms. In contrast, the ability not only to detect, but also unambiguously define, subpopulations of spermatozoa by objective measurements derived from CASA-Morph is an important advance in morphological analysis. From this first step, advantage has to be taken of this knowledge for diagnosis of infertility, or promotion of reproductive performance in conservation biology, animal husbandry, or in the clinic. In other

words, subpopulations generated by this method could, and in future should, replace the previous approach of an artificial "*a priori*" classification of spermatozoa based on subjective evaluations. For example, in future, a new named and objective subpopulation, based on three PCs (e.g., SP2), could replace the subjective term "small." This paper opens the door to an integrated and holistic approach to sperm function. Until now, all the sperm parameters have been evaluated independently, diminishing the global power prediction. The more integrated the different interactive variables become, the better the evaluation of semen quality will be.

The significance of the different numbers of sperm populations in the species examined and seminal fractions obtained in remains to be followed up by studies investigating whether the presence or extent of certain sperm populations is associated with indicators of fertility or infertility. For example, if the changes in sperm populations upon maturation in the cat epididymis[10] are indicative of the epididymal maturation process, aberrant populations in the ejaculate could be indicative of epididymal malfunction in endangered feline species; the rapid and automated assessment of human sperm DNA damage[9] could be useful in optimizing selection methods that enrich populations in the less damaged cells required for ART; likewise, it would be interesting to find out whether differences in bovine sperm subpopulations between bulls, ejaculates and thawed straws[16] are present in the native semen or introduced by the cryopreservation protocol, and to use these subpopulations to monitor the development of methods to select the sperm subpopulation (of elongated and tapered spermatozoa) previously associated with fertility for AI.

THE FUTURE

New techniques of sperm morphometry have recently been developed, e.g., for the analysis of sperm nuclear morphology by the use of fluorescent stains, providing additional information on cell function,[16,17] as presented here,[13] and similar developments with new dyes are to be expected. The effect of preparative interventions on the final morphology and morphometry of sperm cells is well documented,[18–23] but eliminating the problem is a better option than attempting to take into account the preparative artifacts produced in the cells examined.

In this regard, the novel Trumorph® method dispenses altogether with air-drying, fixation, and staining, together with their artifacts, and involves the direct morphological examination of living, immobilized cells in raw semen.[24,25] Rapid, automated morphometric evaluation of such cells will provide the first approach to real-time analysis of sperm morphology that could precede the selection and removal of an unadulterated sperm cell, or spermatozoa, for ART. Perhaps observations could be extended to three dimensions in scanning confocal microscopy.

A NOTE ON CASA TERMINOLOGY

In preparing this Special Issue, it became apparent that the conventional CASA terminology was inadequate to describe the different uses to which the technology is now being put. In the papers initially submitted to this Special Issue, authors used several acronyms to describe the method they were using, including CASMA (Computer-Aided Sperm Morphology Analysis), CASMA-F (when fluorescent dyes were assessed) and ASMA (Automated Sperm Morphology Analysis). With these terms, neither the nature of the automation (with ASMA) nor the morphology examined (with CASMA-F) is clear from the abbreviation. In this Special Issue, for example, for spatulate spermatozoa, the sperm head itself, its acrosome, or its nucleus can each be analyzed by the system,[13] and filiform spermatozoa permit additional values on the length of the head and tail.[5] Thus, a change in terminology to one that indicates which sperm feature the system is measuring is needed.

The acronym CASA itself (computer-aided/assisted sperm analysis) is uninformative since the analysis could refer to any aspect of spermatozoa: their concentration, motility, kinematic parameters or morphology, or combinations of these. Indeed, the early papers used this blanket term to cover them all although the term CASA today is generally used in association with sperm kinematics.[26] For this use, the acronym would be more informative by the simple addition of M for motility (CASMA), but this letter could also be taken to stand for morphology. Using K for kinematics (CASKA) would be an alternative although not signifying that the percentage of moving cells is also recorded.

Any abbreviation must be informative, not only as to whether motility or morphology is being assessed, but also for the latter which organelles (whole head, acrosome, nucleus, midpiece, tail), sperm status (DNA fragmentation) or other features, are being analyzed. We suggest the following hyphenated compound terminology: the generic use of CASA for any kind of sperm computer-aided sperm analysis, followed by an abbreviation indicating the analysis performed, i.e., CASA-Conc (for concentration), CASA-Mot (for motility, including kinematics), CASA-Morph (for morphology, including morphometry), and CASA-DNA (when DNA is being studied). These could be extended if necessary to indicate when fluorescent dyes are used for morphology (CASA-Morph-F) or when DNA fragmentation is being assessed (CASA-DNAf).

In the revised manuscripts presented in this Special Issue, all authors agreed to use this terminology in their papers, and we hope others will also find it more informative and useful both for the authors and the readers.

AUTHOR CONTRIBUTIONS

CS, TGC, AV, and JLY contributed to different aspects of the review; CS and TGC wrote the manuscript.

COMPETING INTERESTS

CS developed Trumorph®. Neither he nor the other authors have interests that influenced the views presented in this paper.

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CHAPTER VIII: COMBINED EFFECT OF TYPE AND CAPTURE AREA OF COUNTING CHAMBER AND DILUENT ON HOLSTEIN BULL SPERM KINEMATICS

CHAPTER VIII:

Combined effect of type and capture area of counting chamber and diluent on Holstein bull sperm kinematics

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Combined effect of type and capture area of counting chamber and diluent on Holstein bull sperm kinematics

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Summary

The evaluation of sperm motion is crucial for processing of seminal doses for artificial insemination. Here the combined effect on sperm motility of the type, capture area of three counting chambers, together with the type of diluent employed, was analysed. Ejaculates from thirteen Holstein bulls were used for sperm kinematic analysis with the ISAS®v1 CASA-Mot system, using two capillary loaded counting chambers (Leja® and CellVu®) and one drop displacement chamber (Makler®). Nine fixed positions were analysed per chamber type, considering central and lateral and three longitudinal fields. Independent of the diluent used, differences were found between the three chambers. Independently on the extender, no differences in x-axis were observed with CellVu®, while using Leja®, some parameters showed lower values in the centre than in lateral areas. In both counting chambers the lowest values were observed in the distal area. Results obtained with the two diluents were highly different with a very low correlation between them. In conclusion, the capture area inside the chambers leads to significant changes in sperm kinematic parameters and different dilution media introduce considerable differences in the motility patterns. It is necessary to optimize sampling methods and specific set-ups to be used with CASA-Mot technology.

Keywords: bull sperm kinematics, counting chamber, sampling, CASA-Mot system

1. INTRODUCTION

Cattle are a fundamental resource in human economy, basically because of their use for milk and meat products. This has led to an intensive artificial selection throughout human history to obtain high quality animals in order to meet different purposes (Felius et al., 2014). Currently, cattle breeding involves the use of various forms of assisted reproductive techniques (ART) (Velazquez, 2018). The use of cryopreserved seminal doses for artificial insemination is, right now, the most widely used ART in this and other species (Correa, Pace, & Zavos, 1997; Waberski, Petrunika, & Töpfer-Petersen, 2008; Lyashenko, 2015). Other, more sophisticated techniques, such as intracytoplasmatic sperm injection (ICSI) or embryo selection, are also used although to a lesser extent (Skrzyszowska et al., 2002; Ohlweiler et al., 2013).

The accurate evaluation of semen quality is fundamental to maximize the efficient use of seminal doses. However, even today, subjective semen analysis is commonly performed in many bull stud farms, which reduces the number of doses produced. In this context it is not an uncommon practice to make approximations to the closest 5% value when analysing both sperm concentration and sperm motility (Naib et al., 2011). The progressive introduction of computer assisted semen analysis (CASA) systems in the production lines has considerably improved the processing of semen samples, offering higher consistency in results (Vyt et al., 2004; Broekhuijse et al., 2011; Yániz et al., 2018). In any case, the correct use of CASA technology must be associated with optimized protocols to provide valuable and reliable information for the final calculations in dose production (Amman & Waberski, 2014; Bompart et al., 2018; Yeste et al., 2018). There are three main aspects to consider when optimizing automated semen analyses, namely the type and depth of the counting chamber (Soler et al., 2012; Gloria et al., 2013; del Gallego et al., 2017), the dilution media (Awad, 2011; Büyükleblebici et al., 2014) and the frame rate of image acquisition (Castellini et al., 2011; Valverde et al., 2018).

The aim of the present work was to analyse the differential sperm distribution and motility characteristics within the capture area in three different commercial counting chambers (CellVu®, Leja® and Makler®) and the effect of two different commercial dilution media (Biladyl® and Andromed®) on this distribution, with a view to optimizing the use of currently available CASA-Mot technology.

2. MATERIAL AND METHODS

2.1. Semen collection and processing

This study was performed on Holstein bulls (n=13, 1.5–7 years old), regularly employed in artificial insemination (AI) under a regime in which two ejaculates collected per week. Animals were

housed in Xenética Fontao AI Centre, S.A. (Lugo, Spain), following all European Union regulations for animal husbandry.

Within 5 to 10 min of semen collection by artificial vagina, samples were assessed for volume in a conical tube graduated in 0.1 mL subdivisions and gross motility determined by placing 20 µL of fresh semen on a pre-warmed slide at 37 ºC, using a cover-slide of 20x20 mm. All ejaculates were split into two aliquots, one processed with a commercial egg yolk extender (Biladyl®, referred to as BLD) and the other with a soy lecithin-based extender (Andromed®, referred to as ADM), both from Minitube GmbH, (Tiefenbach, Germany). The semen aliquots were diluted in a two-step procedure when using the BLD extender, and in one step when the ADM extender was used, to a final concentration of about 100 × 10^6 spermatozoa/mL, using as a reference value that estimated during gross motility analysis. After dilution samples were slowly cooled to 4°C at a linear rate of –0.3°C min^{-1} in a refrigerator and maintained at this temperature during 4-5 h for equilibration.

The refrigerated samples were packaged in 0.25 mL straws (IMV Technologies, L´Aigle, France) with an automatic straw filling and sealing machine (MRS1, IMV Technologies) and they were immediately frozen by using a programmable freezer (Digitcool 5300, IMV, L´Aigle, France) with the following curve: 4°C to -10°C at -5°C/min; -10°C to -100°C at -40°C/min; -110°C to -140°C at -20°C/min, and then plunged into liquid nitrogen for storage.

For the assessment of motility, two straws per sample were thawed in a water bath at 37°C for 30 s and then the contents of the straws were emptied in a test tube kept at the same temperature in a dry bath. In order to collect uniform sperm subsamples and avoid inaccuracies the semen was mixed gently before collecting aliquots for further analyses.

2.2. Sperm motility evaluation

Samples were analysed for kinematics by using the CASA-Mot system ISAS®v1 (Integrated Semen Analysis System, Proiser R+D, S.L., Paterna, Spain). The equipment consisted of a microscope (Nikon Eclipse E600; Tokyo, Japan) equipped with a heated stage set at 38°C and a 10× negative phase-contrast objective. A video digital camera (Proiser 782M) was mounted on the microscope to capture images and transmit them to a computer. The array size of the video frame grabber was 768x576x8 bits and 256 grey levels. Resolution of images was 0.84 µm per pixel in both the horizontal and vertical axes. The frame rate used was 30 fps, capture time one second, with the tail detection facility activated for ignoring non-sperm particles, with a particle area between 14-80 μm^2 and a connectivity value of 14 µm.

Sperm parameters analysed were concentration and total motility (%), whereas the kinematic parameters were average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat/cross frequency (BCF, Hz,), wobble (WOB, %), straightness (STR, %) and linearity (LIN, %).

After dilution, each sample was analysed in three different chambers: Leja® 4 chamber (L4; 20-µm depth; prod. code SC-20-01-04-B; Leja®, IMV technologies, L'Aigle, France), CellVu® sperm counting chamber (CVD; 20-µm depth; prod. code DRM-600; Millennium Sciences, Inc., NY, EEUU), and Makler® counting chamber (10-µm depth; Sefi-Medical Instrument, Haifa, Israel). All chambers were prewarmed at 38°C, and each was loaded with the amount of diluted semen and using the loading procedures recommended by the manufacturer. Each slide was maintained on the heated stage of the microscope for 30 s before analysis to prevent possible passive movement of liquid in the chamber. Nine fields were captured for each analysis of the samples and all the assessments were completed within 2 min. All the captures followed the same pattern, recording the position in the microscope stage (Fig. 1). The order of analysis among counting chambers was randomised.

2.3. Statistical analyses

Data were examined for normality of distribution, homogeneity of variance and analysed using the general linear model (GLM) repeated-measures procedure to determine if there were differences among mean values of the 3 counting chambers and the 2 extenders for each kinematic variable, which were tested independently. Mathematically, the model may be expressed as follows: $Y_{ijklm} = \mu + A_i + C_j + E_k + P_l + CE_{(jk)} + \varepsilon_{ijkl}$. Here, Y_{ijklm} is the "m"th value of individual `i' measured with counting chamber `j', on the extender "k" and the (x_i,y_i) position "l"; "µ" is the overall mean, "A_i" is a random effect describing variation between individuals, "C_j" is a fixed effect of counting chamber, "E_k" is a fixed effect of the extender, "P_l" is a fixed effect of the x_i,y_i position on counting chamber area describing variation between x_i,y_i positions, "$CE_{(jk)}$" is a interaction effect between counting chamber-extender, and "ε_{ijkl}" is the residual variation. If differences were detected among factors for each kinematic variable, Bonferroni post-hoc tests were used to determine the pairwise directional differences between counting chambers and extenders. Results are reported as the mean ± the standard error of the mean (SEM). Data were considered to differ at $P < 0.05$ (i.e., Type I error was set at $\alpha = 0.05$). Pearson correlation was calculated for VCL values between diluents for counting chamber and between counting chambers for dilution media. All statistical analyses were performed using IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. RESULTS

Although the total sperm count in each bull sperm straw was, in theory, 25 x 10^6 spermatozoa, we found departures from this expected value when different chambers were used to estimate concentration of straws after thawing. The actual concentration was significantly higher with the CellVu® chamber and lower with the Makler® one. Total sperm motility was significantly higher with the Makler® chamber and lower with the CellVu®, whereas the Leja® showed no differences with regards to the other two chambers (Table 1). When comparing the fields positions inside the chambers (for Leja® and CellVu®), concentration and total motility showed no differences between positions, both in vertical and horizontal axis.

All the kinematic parameters were significantly lower, but with higher coefficient of variation (CV), when the BLD diluent was used, independently of the counting chamber used (Table 2). After dilution with the ADM diluent, the highest values for VCL and BCF and the lowest for LIN and WOB were observed when using the Makler® chamber, indicating an increment in the oscillatory movement with regards to the chambers loaded by capillarity. Other parameters showed no differences between counting chambers (Table 2). The use of BLD introduced much more variability in the three counting chambers. In this case, the highest VCL was observed with the Leja®, while all the other parameters were higher in the Makler® and lower in the CellVu® chambers (Table 2). The effect of the interaction extender x counting chamber was significant for all kinematic parameters ($P < 0.05$) except for ALH ($P > 0.05$).

Regarding the location for the analysis in the chambers loaded by capillarity (Fig. 1), independently of the dilution medium used, CellVu® showed no differences between lateral and central areas, even though all the values were slightly higher in the lateral than in the central positions. Most of the kinematic parameters (VCL, VSL, VAP, LIN, WOB and BCF for ADM, and VCL, VSL, VAP for BLD) were significantly higher in the area closest to the site of drop deposition and lower in the place far away from where the drop was placed. In the case of BLD, BCF was higher in the central position than in both the proximal and the distal ones (Tables 3 and 4, Fig. 2).

Similar results were obtained regarding central and lateral positions when the Leja® chamber was used. Nevertheless, when the ADM diluent was employed, VAP was significantly higher in the central position, whereas when using BLD diluent, VCL and ALH were higher in the central position and WOB was higher in the lateral one. Concerning the direction in which the drop progresses, highest values for VCL, VSL and VAP were observed in the proximal area with the lowest values in the distal one. For LIN and STR the highest values were found in the central area and the lowest ones in the proximal area. A different pattern was observed with BDL diluent, with

which the highest values for VCL, VSL, VAP, LIN, WOB and BCF were observed in the proximal areas and the lowest in the distal ones. Only STR had the highest values in the central area and the lowest ones in the distal area (Tables 5 and 6, Fig. 2).

Correlation values for VCL when assessed using different counting chambers were higher for the use of ADM diluent (0.99-0.96) than with BLD diluent (0.82-0.73). The highest correlation values were observed between the Leja® and the Makler® chambers (Fig. 3). Values of VCL correlated poorly between two diluents (0.23-0.10), independently of the counting chamber used (Fig. 4).

4. DISCUSSION

Although fertility is multifactorial and involves not only male effects, but others related to females, such as oocyte quality, oviductal environment, or time of insemination/fertilization, among others (Utt, 2016), the correct evaluation of seminal characteristics is the first essential step in the preparation of seminal doses for ART (Broekhuijse et al., 2012; Amann and Wabersky, 2014). Two basic parameters have been considered as the best indicators of semen fertility, concentration and motility of spermatozoa. For a long time, the most popular technique for sperm counting involved the use of a haemocytometer (Eliasson, 1971). The improved Neubauer chamber has been accepted as the gold standard for the estimation of sperm concentration (Tomlinson et al., 2001; World Health Organization, WHO, 2010). On the other hand, it is still common to use wet preparations, placing sperm suspensions between a slide and a coverslip, for the assessment of motility (Gloria et al., 2013; Del Gallego et al., 2017). The introduction of the Makler® chamber resulted in the opportunity for faster sperm counting (Makler, 1978), even if there are discrepancies about its reliability (Matson et al., 1999; Cardona-Maya, Berdugo, & Cadavid, 2008; Bompart et al., 2018), which has led WHO to recommend the use of the hemocytometer for the estimation of sperm counts (WHO, 2010).

The subjective analysis of motility in semen samples generates considerable variability in results (Naib et al., 2012), which has been largely overcome by the development of CASA technology during the 1980's (see Yániz, Soler & Santolaria, 2015, Soler, et al., 2016, Bompart et al., 2018, Gallagher, Smith & Kirkman-Brown, 2018, and Yániz et al., 2018, for reviews of CASA history). CASA systems afford high accuracy, repeatability and large amounts of quantitative data but, nonetheless, they require a strict setting to achieve reliable and comparable results (Verstegen, Iguer-Ouada, & Onclin, 2002; Contri et al., 2010). The effect of repeated collection fom the same animal on semen quality has been well documented in previous work and is not considered here (Valverde et al., 2016).

Regarding the evaluation of sperm quality, two of the main factors affecting concentration, motility and kinematic parameters are the counting chamber and the dilution media used. With regards to counting chambers, there are two main physical principles that are relevant for their use, capillarity (CellVu® and Leja®, in the present study) and drop displacement (Makler®, as used here) (Bompart et al., 2018). Another factor, particularly important in chambers loaded by capillarity, is related to the shape of the counting area, because some of them can introduce turbulence in the fluid, due to their design, as in the case of the Leja® chamber (Bompart et al., 2018). In addition, chambers vary in their depths, being 10 µm (Makler®) or 20 µm (CellVu® and Leja®), with greater depths being unavailable because of the optical limitations of microscopes. Both aspects, loading principle and depth, can thus affect the final results of motility analysis, including some of the differences we have found here (Gloria et al., 2013; Del Gallego et al., 2017; Bompart et al., 2018).

Concerning concentration, in a previous work on bull semen using a different CASA system, no differences were observed between the three chambers used (Gloria et al., 2013). This is in contrast with the differences observed here and in other previous studies (Hansen et al., 2006; Bailey et al., 2007; Hoogewijs, et al., 2012), that can be attributable to the dissimilar sampling areas considered in the different studies.

With regards to total motility, other studies in diverse CASA systems agree with our results, showing higher motility with the Makler® chamber than with the Leja® slides (Contri et al., 2010; Lenz et al., 2011; Gloria et al., 2013). Furthermore, other studies in goat (Del Gallego et al., 2017), human (Soler et al., 2012), ram (Palacín et al., 2013), and stallion (Hoogewijs et al., 2012) spermatozoa, based on different CASA-Mot systems and counting chambers, showed that the motility and kinematic parameters observed in capillary chambers presented lower values than those observed in their drop displacement counterparts. These results appear to indicate that the drop distribution principle is more important than species differences, or the actual brand of the counting chambers or the CASA-Mot system. In this sense it is possible that loading by capillarity disrupts in some way sperm motility as a consequence of the resultant fluid flow, because capillary action may damage the sperm tail and thus affect sperm movement (Lenz et al., 2011; Palacín et al., 2013) and vitality (Gloria et al., 2013) in comparison with drop displacement counting chambers (Hoogewijs et al, 2012; del Gallego et al., 2017). Nevertheless, the highly significant regression of VCL values observed here between capillary and droplet displacement chambers suggests that a possible toxic effect of the adhesive or the paint used for the serigraphy of the chambers is not a likely explanation for the differences in kinematic parameters as was previously proposed (Gloria et al., 2013). Furthermore, it was interesting that different depths of the counting chambers (10 µm for Makler®, and 20 µm for Leja® and CellVu®) showed high correlation for VCL values, which is not in

agreement with results obtained with the use of a 3D lensless microscopy and CASA-Mot for boar semen (Soler et al., 2018).

It is necessary to point out that most of the earlier work developed on potential effects of counting chambers has not considered the area in which counts were performed. In the work of Gloria et al. (2013), only the centre and the edges of the Leja® chamber were taken into consideration, but what exactly these positions refer to is not clear. Another study using slides and coverslips, and analysing sperm motility along the equatorial area of the preparation with another CASA system, showed differences just in the fields close to the border, but not in the other sampling areas (Nöthling and dos Santos, 2012). In a study considering differences in ram sperm motility between central and peripheral areas, when a slide and a coverslip were used, revealed higher values in the central area for total and progressive motility, VCL and VAP (Palacín et al., 2013). In the present work, the coordinates of the microscope stage were well defined and used repeatedly to obtain a strict sampling model for analyses, revealing no differences in motility and kinematics between the edge and the centre but showing variation along the length of the chamber capture area. The highest values were obtained close to the place where the drop was deposited and the lowest at the end of the fluid movement, which cannot be completely explained by Poiseuille flow and the consequent Segre-Silderberg effect (Kuster, 2005) or by the possible effect of surface tension on the perimeter of the coverslip (Lenz et al., 2011), thus requiring alternative explanations. When counting chamber design allowed a defined linear sampling, some species showed no motility differences along the counting area (human, Soler et al., 2012), but others agreed with the results observed here (goat, Del Gallego et al., 2017; fox, Soler et al., 2014), indicating that these differences are species-specific and require a biological explanation.

Regarding the effect of diluents on sperm kinematics, previous studies have shown that the increase in the percentage of egg yolk in the diluent elevates the viscosity inducing a decrease in sperm velocity and progressive motility (Hirai et al., 1997; Aires et al., 2003). Our results agree with these observations because the use of Byladil (egg-based medium) showed lower velocity and linearity than when Andromed (lecithin-based medium) was used. In an apparent contradiction, it has been reported that VCL is higher with the use of egg yolk (Tryladil) than with egg yolk-free media (TCM-199 and Ham's F10), but these results were obtained using a different bull breed (Raseona et al., 2017).

In conclusion, under the conditions used in the present work, the use of different counting chambers leads to significant changes in estimation of sperm kinematic parameters. In addition, the use of different dilution media introduces differences in the motility patterns. All these results indicate the necessity of repeatable and representative sampling and to define specific set-ups to

be used with CASA-Mot technology when different counting chambers or dilution media are used for obtaining reliable results in the calculation of seminal doses for artificial insemination programs.

Conflict of interest

The authors have no conflicts of interest to declare.

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Table 1. Effect of sperm counting chamber on total motility and concentration of bull sperm as assessed by computer assisted semen analysis (mean ± SD).

	CellVu®	Leja®	Makler®
Total motility (%)	60.1±14.9^{a}	62.2±14.2^{ab}	67.1±14.1^{b}
Concentration (x10^{6})	30.6±7.3^{a}	26.9±5.7^{ab}	21.3±7.2^{b}

^{ab}Values with different superscripts differ significantly within row ($P < 0.05$).

Table 2. Effect of semen diluent and three sperm counting chambers on kinematic parameters of bull sperm as assessed by computer assisted semen analysis (mean ± SD).

	Andromed®			Biladyl®		
	CellVu®	Leja®	Makler®	CellVu®	Leja®	Makler®
n	3862	4269	4079	4073	4032	3963
VCL	105.8±41.7^{ab}	103.8±43.5^{b}	107.2±39.2^{a}	92.6±40.9^{b}*	101.0±42.1^{a}*	98.8±39.2^{a}*
VSL	52.3±24.9	51.6±26.3	51.3±22.9	43.3±25.6^{b}*	49.2±25.7^{a}*	50.4±24.6^{a}
VAP	62.8±23.5	62.6±25.6	62.7±21.6	55.0±24.6^{b}*	60.6±24.5^{a}*	61.6±23.6^{a}*
LIN	49.7±18.6^{ab}	50.0±19.0^{a}	48.8±18.0^{b}	46.1±19.8^{c}*	48.9±19.2^{b}*	51.8±20.3^{a}*
STR	80.0±20.8	79.4±21.0	79.6±20.5	74.2±23.3^{c}*	77.6±21.8^{b}*	79.1±20.5^{a}
WOB	60.7±12.8^{b}	61.4±12.9^{a}	59.8±12.2^{c}	60.2±13.2^{c}*	61.2±13.0^{b}	63.6±14.2^{a}*
ALH	3.5±1.4^{a}	3.3±1.4^{b}	3.5±1.3^{a}	3.4±1.5^{b}*	3.5±1.5^{a}*	3.3±1.4^{b}*
BCF	13.1±5.5^{b}	13.8±5.4^{a}	14.0±5.3^{a}	10.8±5.1^{c}*	11.7±5.1^{b}*	12.3±5.1^{a}*

^{abc}Values with different superscripts differ significantly within row and sperm diluent; *Indicates differences between diluents for chamber ($P < 0.05$). VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 3. Mean (±SD) values for the kinematic sperm motility variables from Holstein bulls on planar (x_i, y_i) coordinates of the CellVu® chamber when using Andromed® diluent.

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
n	2367	1495	1324	1387	1151
VCL	106.0±42.1	105.2±41.1	109.0±43.3[a]	106.1±40.7[a]	101.7±40.8[b]
VSL	52.5±24.7	51.5±25.1	56.1±27.0[a]	52.1±23.7[b]	47.8±23.1[c]
VAP	62.9±23.5	62.4±23.6	66.9±25.6[a]	62.5±22.3[b]	58.5±21.8[c]
LIN	49.9±18.2	49.3±19.1	51.2±18.4[a]	49.8±18.7[a]	47.8±18.6[b]
STR	80.4±20.1	79.3±21.9	80.2±20.6	80.5±20.9	78.8±21.0
WOB	60.8±12.7	60.5±13.0	62.4±12.3[a]	60.3±13.1[b]	59.1±13.0[b]
ALH	3.5±1.4	3.4±1.3	3.4±1.3	3.5±1.4	3.5±1.5
BCF	12.9±5.6	13.2±5.5	13.5±5.6[a]	13.2±5.5[a]	12.6±5.3[b]

[abc]Values with different superscripts differ significantly within row and X_i or Y_i coordinates ($P < 0.05$; values are mean ± SE). VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 4. Mean (±SD) values for the kinematic sperm motility variables from Holstein bulls on planar (x_i, y_i) coordinates of the CellVu® chamber when using Biladyl® diluent.

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
n	2639	1434	1331	1547	1195
VCL	93.5±40.8	92.5±41.3	94.4±42.6[a]	94.1±39.6[a]	90.5±40.6[b]
VSL	44.0±25.7	43.1±25.5	44.5±27.0[a]	44.3±25.1[a]	41.9±24.5[b]
VAP	55.5±24.5	54.7±24.7	56.4±26.2[a]	55.9±23.8[a]	53.1±23.8[b]
LIN	46.5±20.1	46.1±19.5	46.1±20.3	46.7±19.9	46.0±19.2
STR	74.7±23.3	74.6±23.3	73.9±23.3	75.3±23.6	74.6±22.9
WOB	60.3±13.3	59.9±13.0	60.3±13.5	60.2±13.1	59.8±12.9
ALH	3.4±1.5	3.4±1.4	3.4±1.5	3.4±1.4	3.4±1.5
BCF	10.8±5.1	10.7±5.1	10.7±5.0[b]	11.0±5.1[a]	10.4±5.2[b]

[ab]Values with different superscripts differ significantly within row and X_i or Y_i coordinates ($P < 0.05$; values are mean ± SE). VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 5. Mean (±SD) values for the kinematic sperm motility variables from Holstein bulls on planar (x_i, y_i) coordinates of a Leja® chamber when using Andromed® diluent.

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
n	2680	1589	1842	1317	1110
VCL	104.0±43.5	102.5±43.5	107.3±43.9[a]	104.8±44.0[a]	97.6±41.7[b]
VSL	52.1±26.3	50.7±26.3	53.2±28.3[a]	52.5±24.6[a]	48.4±24.7[b]
VAP	61.3±25.4[b]	63.1±25.7[a]	65.7±27.4[a]	62.9±24.0[b]	58.0±23.7[c]
LIN	50.6±19.1	49.8±19.0	49.2±19.6[b]	51.5±18.5[a]	49.9±18.8[ab]
STR	79.5±21.0	79.9±20.9	77.6±21.9[b]	81.3±19.7[a]	80.2±20.8[a]
WOB	61.0±13.0	61.8±12.7	61.6±12.9	61.9±12.8	60.7±13.1
ALH	3.3±1.4	3.3±1.3	3.4±1.3	3.4±1.5	3.3±1.4
BCF	13.7±5.4	13.9±5.2	13.7±5.3	14.0±5.4	13.6±5.5

[ab]Values with different superscripts differ significantly within row and X_i or Y_i coordinates ($P < 0.05$; values are mean ± SE). VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 6. Mean (±SD) values for the kinematic sperm motility variables from Holstein bulls on planar (x_i, y_i) coordinates of a Leja® chamber when using Biladyl® diluent.

	X_i coordinate		Y_i coordinate		
	1	2	1	2	3
n	2651	1381	1427	1347	1258
VCL	100.1±42.0[b]	102.2±42.2[a]	104.2±43.8[a]	99.8±41.7[b]	99.5±40.3[b]
VSL	49.1±25.9	48.9±25.4	51.4±27.7[a]	48.9±24.7[b]	46.5±24.5[c]
VAP	60.9±24.5	60.2±24.3	63.0±25.9[a]	59.9±23.9[b]	58.6±23.2[b]
LIN	49.0±19.3	48.2±18.9	49.4±19.8[a]	49.3±18.2[a]	47.2±19.5[b]
STR	77.5±21.9	77.2±21.5	77.8±22.3[a]	78.3±20.4[a]	76.0±22.6[b]
WOB	61.4±13.2[a]	60.7±12.8[b]	61.6±13.2[a]	61.4±12.6[a]	60.3±13.3[b]
ALH	3.5±1.5[b]	3.6±1.5[a]	3.5±1.5	3.5±1.5	3.6±1.5
BCF	11.8±5.1	11.6±5.2	12.1±5.3[a]	11.8±5.0[a]	11.3±5.1[b]

[ab]Values with different superscripts differ significantly within row and X_i or Y_i coordinates ($P < 0.05$; values are mean ± SE). VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

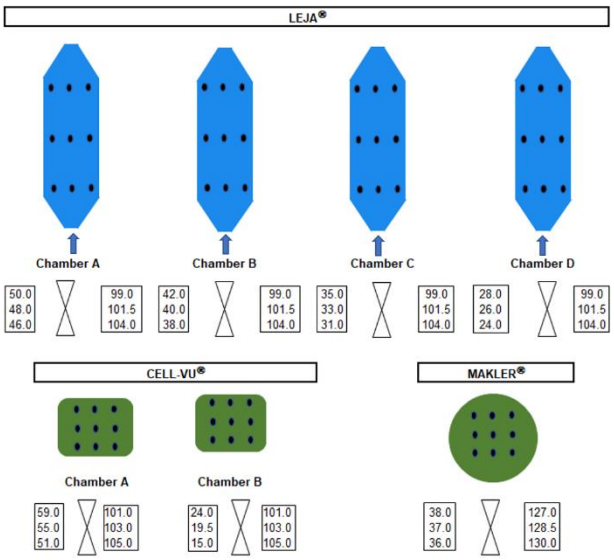

Figure 1. Microscope stage micrometric positions analysed in each of the counting chambers used in this study. Left column for each counting chamber indicates the x-position and the right the left-position of the stage. A total of nine fields were analysed per sample, named in the tables 1 for lateral and 2 for central x-positions and 1, 2, and 3 for proximal, central and distal regarding the drop deposition for the y-positions.

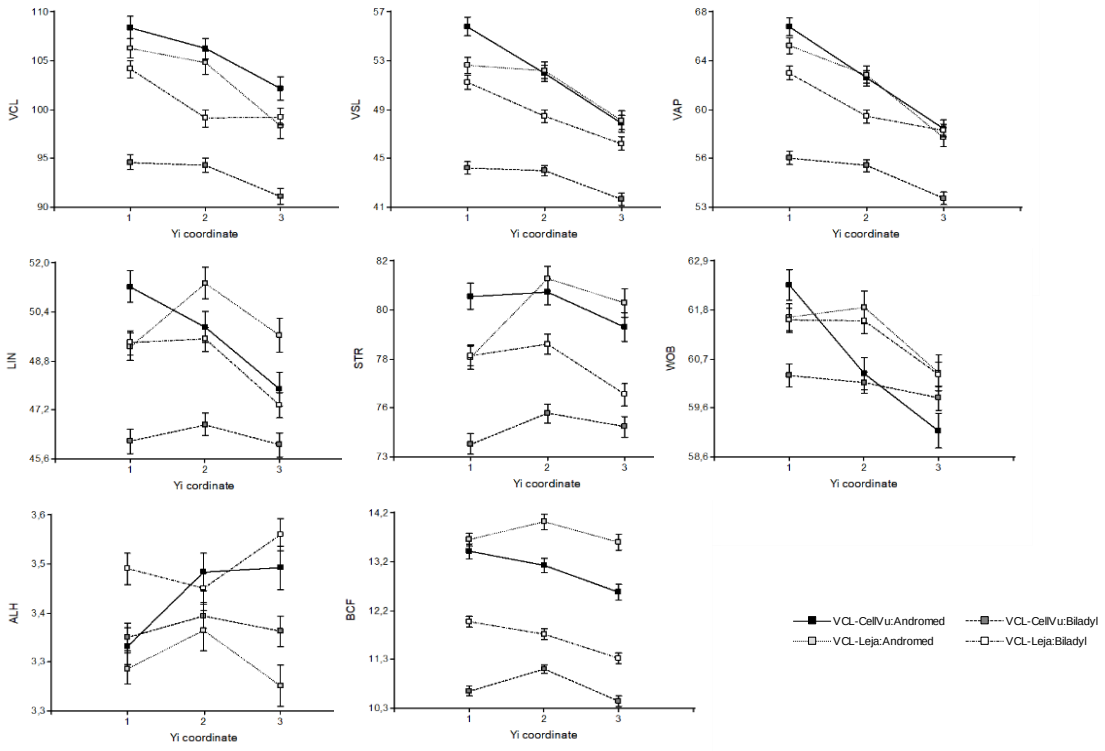

Figure 2. Kinematic parameters along a longitudinal distribution in the counting chambers loaded by capillarity.

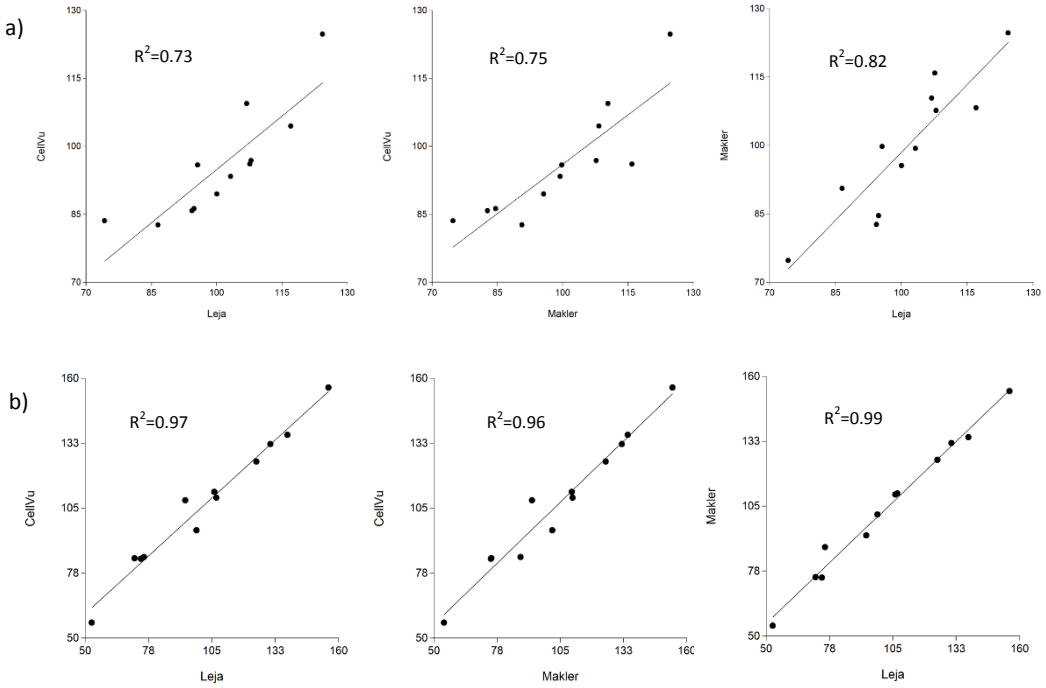

Figure 3. Correlation and regression analysis of VCL values between chambers. Upper row corresponds to Biladyl medium, lower row to Andromed

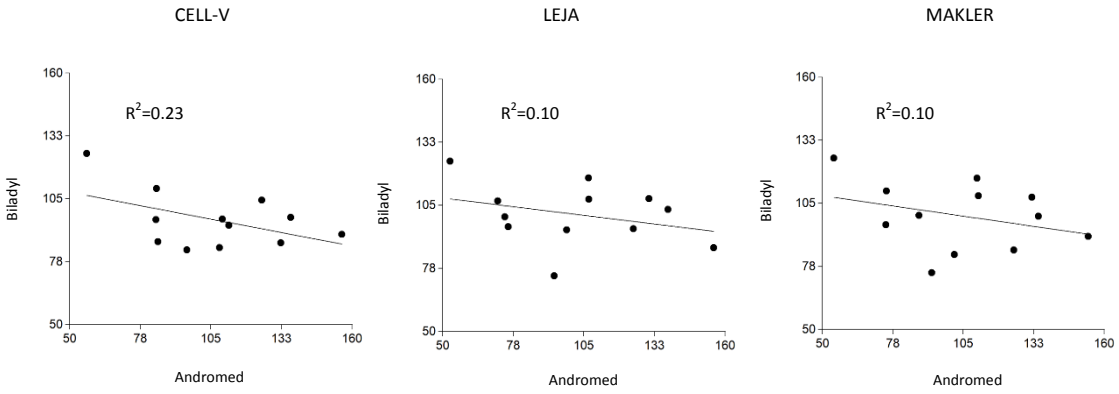

Figure 4. Correlation and regression analysis of VCL values between media (Biladyl and Andromed) in the different counting chambers.

CHAPTER IX: DOG SPERM SWIMMING PARAMETERS ANALYSED BY THE CASA-MOT SYSTEM REVEAL MAJOR BREED DIFFERENCES

CHAPTER IX:

Dog sperm swimming parameters analysed by the CASA-Mot system reveal major breed differences

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Dog sperm swimming parameters analysed by the CASA-Mot system reveal major breed differences

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Running Title: Dog sperm motility

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Abstract

Dogs have undergone an intensive artificial selection process ever since the beginning of their relationship with humans. As a consequence, a wide variety of well-defined breeds exist today. Due to the enormous variation in dog phenotypes and the unlikely chance of gene exchange between them, the question arises as to whether they could still be regarded as a single species or, perhaps, they could be considered as different taxa that possess different reproductive traits. The aim of this study was therefore to characterise some male reproductive traits, focusing on kinematic characteristics of dog spermatozoa from several breeds. Thirty-seven dogs from the following breeds were used: Staffordshire Bull Terrier, Labrador Retriever, Spanish Mastiff, Valencian Rat Hunting Dog, British Bulldog and Chihuahua. Samples were obtained via manual stimulation and diluted to a final concentration of 50 million/mL, and they were subsequently analysed using the Computer Assisted Sperm Analysis (CASA-Mot) ISAS v1 system. Eight kinematic parameters were evaluated automatically. All parameters showed significant differences among breeds and among individuals within each breed. The fastest sperm cells were those of Staffordshire Bull Terriers and the slowest were recorded in Chihuahuas. The intra-male coefficient of variation (CV) was higher than the inter-male one for all breeds with the Staffordshire Bull Terrier showing the lowest values. When taking into consideration the cells by animal and breed, discriminant analyses showed a high capability to predict the breed source. Cluster analyses showed a hierarchical classification very close to that obtained after phylogenetic studies using genome markers. In conclusion, future work on dog spermatozoa should bear in mind major differences between breeds and that results cannot be extrapolated from one to another. Because sperm characteristics are associated to breed diversity, dogs could represent a good model to examine changes in reproductive parameters associated to selection processes.

Keywords: dog breeds, CASA-Mot, sperm kinematics, evolution, artificial insemination

1. Introduction

The dog is one of the oldest domestic mammals and is the only carnivore species belonging to the *Canidae* family that has become completely domesticated from wild wolves (Vilà et al., 1997) once (Europe: (Leonard et al., 2002; Thalmann et al., 2013); Asia: (Savolainen, Zhang, Luo, Lundeberg, & Leitner, 2002) or twice (Frantz et al., 2016) during human history. Due to a great intensity of artificial selection, the species has given rise to a wide variety of breeds. However, and despite their wide differences, from the Chihuahua to the Great Dane, they are still regarded as the same species (vonHoldt et al., 2010). The species is one of the basic units of biological classification and a taxonomic rank. However, the issue of what is a species is controversial because its understanding varies with the biological definition used (Mallet, 2007; Soler, Sancho, et al., 2014). In any case, and based on the major differences observed between breeds, the question arises as to whether it is still possible when examining phenotypic traits to consider them as a unity within the concept of “dog species”, or if it is necessary to differentiate clearly between breeds when undertaking studies of this species.

Comparative analyses in a phylogenetic framework have identified important selective forces that could explain differences in reproductive traits, including sperm form and function. One important selective force is sperm competition (a form of post-copulatory sexual selection) that is known to have greatly influenced morphology of spermatozoa and performance of the sperm cells (Gomendio & Roldan, 2008; Soler, Cooper, Valverde, & Yániz, 2016; Tourmente, Gomendio, & Roldan, 2011). It is not clear whether this selective force has had a major impact in the evolution of dog reproductive traits; neither relative testes mass nor sperm dimensions appear to be under the influence of sperm competition (Iossa, Soulsbury, Baker, & Harris, 2008). Differences in sperm traits may also appear as a result of strong selection in the female tract, because sperm have to overcome several barriers to reach the site of fertilization. In dogs, in particular, there is potentially a big-time gap between mating and fertilization and spermatozoa have to survive many days in the female tract while maintaining their ability to move and retain their fertilizing capacity. Alternatively, differences in sperm traits may arise as a result of drift after the onset of reproductive barriers, with the potential for a subsequent appearance of incompatibilities in gametes. In any case, sperm traits, especially morphology, show considerable variation between species and it is usually possible to distinguish species based on this phenotypic trait (Liana & Witaliński, 2005).

The environment in which fertilisation occurs could vary between breeds as it occurs between species. Increased sperm swimming speed is often associated with higher sperm competition to

reach the site of fertilisation when the sperm of more than one male coexists in the female tract, as occurs in birds (Lüpold, Calhim, Immler, & Birkhead, 2009), some fishes (Fitzpatrick et al., 2009) and mammals (Tourmente et al., 2011). Other studies have suggested associations between body mass and sperm size, so that the bigger the animal the smaller its spermatozoa (Cummins & Woodall, 1985; Soler, Sancho, et al., 2014). However, this was not observed in the case of dog breeds (Soler et al., 2017b).

Quantitative motility and kinematic sperm analysis rely on the use of CASA-Mot (Computer Assisted Semen Analysis) systems which allow for the collection of a great number of parameters to be used for appropriate statistical analysis (Bompart et al., 2018; Schäfer-Somi & Aurich, 2007; Yániz, Silvestre, Santolaria, & Soler, 2018). In the present study various characteristics based on sperm motility and kinematics were compared among six dog breeds: Staffordshire Bull Terrier, Labrador Retriever, Spanish Mastiff, Valencian Rat Hunting Dog, British Bulldog and Chihuahua. These breeds were chosen for this study based on their considerable phenotypic variability amongst them and also because they represent the major phylogenetic lines (vonHoldt et al., 2010). This diversity in breed characteristics could reveal differences in sperm performance that could be the result of selection processes.

The aim of this study was to assess sperm motility with a commercial CASA-Mot system in different dog (*Canis familiaris,* L.) breeds to analyse whether prolonged artificial selection carried out within the species has led to a significant gamete differentiation process between breeds.

2. Materials and Methods

2.1 Collection and preparation of samples

Samples were obtained from 37 *Canis familiaris*, L. individuals encompassing seven Staffordshire Bull Terrier, five Labrador Retriever, eight Spanish Mastiff, seven Valencian Rat Hunting Dog, four British Bulldog and six Chihuahua. Sampling and analysis were carried out at the Reprovalcan and Clínica Veterinaria Sangüeso veterinary clinics, located in Valencia (Spain). Only animals with a clear pedigree and truly representative of their breeds were included in the study. All the samples were obtained for routine artificial insemination (AI) programs and the requirement of approval from the animal research review board of the University of Valencia was not required.

Manual stimulation was used to collect semen samples into sterile sample cups (Soler et al. 2017). Since a high sperm concentration can have a confounding effect on sperm velocity and linearity (Günzel-Apel, Günther, Terhaer, & Bader, 1993; Rijsselaere, Van Soom, Maes, & Kruif, 2003) all samples with >50 x 10^6 sperm/mL were diluted with CaniPlus Chill® sperm extender (Minitub Ibérica S.L., Tarragona Spain) before motility analyses. Throughout the process the samples were kept at 24 ºC using a PC-12T (Proiser R+D, S.L., Paterna, Spain) heating system.

2.2 Kinematic analysis of breed and individual differences

ISAS®D4C20 counting chambers (Proiser R+D, S.L.) were used to analyze motility, kinematics and concentration. The chamber comprises four "strips", each with seven printed squares and with a constant depth of 20 µm between slide and coverslip. A sample volume of 3 µL was distributed along the strip by capillarity. For a homogeneous sampling method, one field was captured in the centre of each printed square, numbered from 1 to 7, where 1 was the closest to where the sample was deposited and 7 the one at the opposite end (Del Gallego et al., 2017; Soler et al., 2012). The design of the counting chambers allows for immediate analysis since no drift takes place. Possible dissimilarities along the counted fields were evaluated. Kinematic analyses were carried out using the ISAS®v1 CASA-mot system (Proiser R+D, S.L.). The video camera employed was a Proiser 782 m attached to a microscope UB203 (UOP/Proiser) and equipped with a 10x negative phase contrast objective. Resolution of analysed images was 0.84 µm/pixel on both axes. Samples were captured at 25 fps following the set-up of the manufacturer for dog semen.

Only tracks corresponding to cells present in all 25 images were used for calculations. This allows for standardisation of kinematic calculations since incomplete tracks can introduce bias. A total of 84,647 sperm cells were analyzed. Kinematic parameters (VCL: curvilinear velocity, VSL: linear velocity, VAP: average path velocity, LIN: linearity, STR: straightness, WOB: wobble, ALH: lateral head displacement and BCF: beat cross frequency; see (Bompart et al., 2018) for definitions) were calculated automatically by the CASA-Mot system.

2.3 Statistical analysis

The normality of distributions and variance homogeneities were assessed using Kolmogorov-Smirnov and Levene tests, respectively. ANOVA was used with normal distributions. When distribution was not normal Kruskal-Wallis was employed to make comparisons among animals within and between breeds. After the Kruskal-Wallis test, a *posteriori* Bonferroni test was used to compare between counting chamber fields.

Discriminant analyses were performed to test the predictive power of using combined sperm kinematic parameters to classify dog breeds correctly. Canonical discriminant analysis creates multiple orthogonal functions that are formed so that in each breed implications on the resulting canonical variable are as different as possible due to maximising between-class variance and at the same time minimising within-class variance. In this way, maximum separation is achieved between classes. The Box's M test of equality of covariance matrices was performed for variables grouped by breeds. As a result, the covariance matrices of the dependent variables were not equal across the groups. Thus, one discriminant function was created, from which the canonical variable was obtained for each spermatozoon. Such new variables were calculated considering all kinematic parameters while optimising the capacity for correct classification. Canonical discriminant analysis establishes a discriminant criterion, or a canonical variable value that makes it possible to separate the sperm cells into six breeds, Staffordshire Bull Terrier, Labrador Retriever, Spanish Mastiff, Valencian Rat Hunting Dog, British Bulldog and Chihuahua. Canonical structure coefficients were assessed in order to determine which kinematic parameter was most important to distinguishing breeds. These measures indicate the correlation between the kinematic parameters and the canonical variable. When the absolute magnitude of the coefficient is large, either the feature is very important in the formation of the discriminant function, or this feature is probably reasonable for the separation of the six breeds. The number of functions is equal to the number of groups and take the form (Spencer, 2013):

$K_i = c_{i0} + c_{i1} X_1 + c_{i2} X_2 + \ldots + c_{ik} X_k$

K_i: classification functions, $i = 1, 2, \ldots, g$

C_{ij}: coefficients of classification functions (weights); $i = 1, 2, \ldots, g$; $j = 0, 1, \ldots, k$

X_j: discriminant variables taken into consideration in the discriminant functions, $j = 1, \ldots, k$

After obtaining the discriminant function, error and posterior error were calculated. Simple error corresponds to the percentage of sperm cells that was correctly classified by using the canonical variables. Posterior error was calculated by cross-validation. Finally, hierarchical clustering by Euclidean distance matrix was carried out to define breed relations from kinematic variables. All statistical analyses were carried out using SPSS 23 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $P<0.05$.

3. Results

3.1 Effect of counting zone in the ISAS®D4C20 chamber

Even though some differences between analyzed fields were observed, no trend was found,

indicating that these variations were occurring at random (data not shown). In any case, this reinforces the benefit of analyzing all seven proposed fields in the chamber to achieve a stable and representative result which is not affected by sampling.

3.2 Kinematic differences among breeds and among individuals within breed

Most of the parameters showed significant differences among all the breeds, with the highest velocities (VCL, VSL, VAP) and BCF found in Staffordshire Bull Terrier, the highest indexes (LIN, STR and WOB) observed in the British Bulldog and the highest ALH seen in the Labrador Retriever and Spanish Mastiff. The lowest values for all parameters were obtained in the Chihuahua (Table 1).

In all breeds equivalent significant differences between animals were observed for all the kinetic variables analyzed (data not shown). Assessing male variations within breeds revealed that VCL and WOB exhibited the highest degree of difference, while STR, VSL and BCF showed the lowest. In general terms, intra-male CV was higher than inter-male CV, with the Staffordshire Bull Terrier showing the lowest values (both intra- and inter-male), and the Valencian Rat Hunting Dog and the Chihuahua revealing high intra-male CVs. The Spanish Mastiff and the British Bulldog showed the highest values for inter-male CV (Table 2).

3.3. Multivariate classification matrix

A classification matrix was obtained taking into account breeds and animals (Table 3) and was applied to the total population of spermatozoa (84,647 cells), obtaining a global correct classification of 95.7%. The best classified corresponded to Labrador Retriever and Spanish Mastiff (99.95% and 99.4%, respectively), whilst Staffordshire Bull Terrier and Valencian Rat Hunting Dog were the worst ones (94.4% and 91.9%, respectively, Table 4).

3.4. Hierarchical classification of breeds

Cluster analysis was done to obtain the hierarchical classification of breeds (Fig. 1). The Chihuahua breed was separated into an independent branch of the hierarchical tree, followed by British Bulldog in the next step. The third level separated Staffordshire Bull Terrier, the fourth step separated Valencian Rat Hunting Dog, with Spanish Mastiff and Labrador Retriever being the closest breeds. Distances between cluster steps were very similar in all cases (Fig. 1).

4. Discussion

The results of this study revealed clear differences between dog breeds in sperm kinematic parameters and also intra- and inter-male variations in these parameters that were different among breeds. Discriminant analyses were able to distinguish breeds according to their sperm swimming parameters. The diversity identified in dog swimming patterns underscores the different evolutionary history of dog breeds that have undergone a long process of artificial selection and also highlights the need to bear such differences in mind when using dogs as models for reproductive studies or monitor the impact of environmental factors.

In our attempt to characterize dog sperm kinematics, we first examined whether there were variations due to the characteristics of the chambers used to capture information for sperm motility analyses. We observed that there were different motility distributions along the counting chambers employed. This distribution seems to be species dependent, being almost non-existent (i.e., homogeneous) in some species (human, (Soler et al., 2012)) whereas there is a trend for skewed distribution in others (fox, (Soler, García, Contell, Segervall, & Sancho, 2014)). Each of the various reusable and disposable counting chambers currently available have shown a different kind of distribution stability which needs to be taken into account when assessing motility data (Bompart et al., 2018; Del Gallego et al., 2017). For this reason, all the scored fields were included in our analyses to reduce the random effect related to non-homogeneous distribution.

Discriminant analyses showed a very good classification of cell tracks according to breed, indicating that the characteristics of sperm movement are breed-dependent. In addition, cluster analyses grouped dog breeds with a close agreement to that seen in phylogenetic analyses based on a genome-wide characterization (vonHoldt et al., 2010). Chihuahua was the breed that split first from the other breeds and the British Bulldog, which has been under heavy selective pressure and requires assisted reproduction to breed, showed a big cladistics distance from the rest. It is likely that these distances between breeds are the product of the selective pressures and the time they have been under selection for desired traits, and it is possible that differences in gamete biology may, at least in part, respond to differences in changes taking place in their reproductive biology during evolution of the different breeds.

Previous studies have identified associations between sperm form or morphometrics and performance, with a now well-established association between form and dimensions of several sperm components with sperm velocity, both at the interspecific and at the intraspecific levels (Gomendio & Roldan, 2008; Malo et al., 2006; Tourmente et al., 2011). In particular, associations between sperm head dimensions and swimming parameters has been recorded in birds and

mammals (Humphries, Evans, & Simmons, 2008; Lüpold et al., 2009; Malo et al., 2006). In a previous study, we examined dimensions of the sperm head from different dog breeds (Soler et al., 2017b) and a preliminary comparison between them and those of the present study suggest a trend for a positive link between spermatozoa, with bigger sperm heads exhibiting higher velocities. This apparent association requires further examination using a larger number of animals and breeds.

Dogs, which have undergone an intense artificial selection by humans, show dramatic differences in phenotype among breeds, including sperm traits such as sperm kinematic parameters, as revealed in this study. This observation has two major implications. On the one hand, there is the question of whether the phenotypic sperm parameters found in these breeds can be regarded as representative of one and the same species. The issue then is what, if anything, is a dog? There are many studies which have reported parameters of dog semen but taking this as representative of the species could be misleading if a clear appreciation of breed or inter-male variability is not made explicit in each study, leading to a serious bias in the final results (Soler et al., 2017b). It is thus necessary to identify which is the relative weight of the various breeds considered in each study and, ideally, studies should be carried out on defined breeds if they are to offer reliable and repeatable conclusions. There is the additional issue of whether dogs should still be regarded as a single species. Clearly, some breeds cannot mate (e.g., the extremes represented by the Chihuahua and the Great Dane) and this, which implies a lack of gene flow, may be sufficient to argue for species splitting, according to some species concepts (Mallet, 2007; Mayr, 1942; Soler, Sancho, et al., 2014). Thus, although dogs descend from a common ancestor, the grey wolf, from which they diverged about 12,000-14,000 years ago (Vilà, 2000; vonHoldt et al., 2010) they have been subjected to artificial selection within isolated populations ever since humans engaged in a selection process for the most appropriate features, resulting in the wide variety of breeds existing today.

Another implication relates to the observation that species differences usually involve differences in male gametes, to such an extent that sperm morphology can be used in many occasions for taxonomic purposes and for species identity. And, following from this, is the question about what has driven such enormous diversity in male gametes. There are significant variations in both morphometric (Roldan, Gomendio, & Vitullo, 1992; Soler et al., 2017a; Soler, Sancho, et al., 2014; Tourmente et al., 2011) and kinematics parameters of spermatozoa (Fitzpatrick et al., 2009; Tourmente et al., 2011). In recent decades, sperm characteristics have been successfully used for taxonomic studies in many animal groups including annelids

(Ferraguti & Erséus, 1999; Ferraguti, Erséus, Kaygorodova, & Martin, 1999), insects (Dias, Oliveira, & Lino-Neto, 2013; Jamieson, 1987), crustacean (Jamieson, 1991b), arachnids (Liana & Witaliński, 2005), amphibian (Selmi, Brizzi, & Bigliardi, 1997), fishes (Jamieson, 1991a), birds (Jamieson, 2007) and mammals (Roldan et al., 1992; Soler et al., 2017b; Soler, Sancho, et al., 2014). However, it should be borne in mind that similarities have been found in sperm swimming kinematic patterns between distantly-related organisms, which could not be explained solely by phylogenetic relationships (Guasto, Burton, Zimmer, Hosoi, & Stocker, 2013). Regarding the possible mechanisms that have given rise to differences in sperm traits (such as kinematics) between species and breeds, it has been argued that post copulatory sexual selection may have had a major role. Therefore, one major selective force leading to evolutionary changes in spermatozoa is sperm competition, a process in which sperm from rival males compete to gain fertilizations (Engqvist, 2012; Gage et al., 2004; Tourmente et al., 2011). Another crucial evolutionary force is selection of sperm in the female tract (Eberhard, 1996) through which females may exert skews on the sperm that reach the site of fertilization and interact with the female gametes, based on either spatial or temporal factors, or both (Almiñana et al., 2014; Anderson, Dixson, & Dixson, 2006; Dixson & Anderson, 2001; Gomendio & Roldan, 1993; Malo et al., 2005). Sperm traits that have been found to evolve under the influence of post copulatory sexual selection include sperm numbers, viability motility and morphology (Donoghue, Sonstegard, King, Smith, & Burt, 1999; Gage et al., 2004; Holt et al., 1989; Jamieson, 2007; Malo et al., 2006). Overall, selection pressures have thus resulted in major evolution of male and female reproductive traits (Dixson, 1998; Dixson & Anderson, 2004). Interestingly, we observed a trend for males from larger breeds (hence, with longer female tracts) to have higher values of kinematic parameters, which suggests some adaptations of motility patterns to selection in the female tract. This deserves future investigation to clarify the possible associations between female tract biology and evolution of sperm traits. In any case, it is not yet clear whether dog breeds have evolved under these selective pressures, particularly since artificially selected dogs may have had few opportunities to engage in sperm competition. Nevertheless, there is the possibility that selection in the female tract may have had a more important role in evolution, particularly if the long interval between mating and ovulation is taken into account, and which could have given rise to the need for dog sperm to develop the ability to survive for long periods of time with males evolving several adaptations in form and function of spermatozoa. This could have also led to the development of divergence in dog sperm kinematics and, in addition, to evolution of different bioenergetic strategies, because the latter is essential to ensure cell viability, and the maintenance of fertilizing capacity (Palomo et al., 2003; Tourmente, Villar-

Moya, Varea-Sánchez, et al., 2015; Tourmente, Villar-Moya, Rial, & Roldan, 2015).

In conclusion, based on the major differences observed between breeds in sperm kinematic traits, which agree with diversity in other sperm traits already described, it may be advisable to refrain from a generalization for dogs when carrying out reproductive studies in this species (Hewitt, Leahy, Sheldon, & England, 2001; Peña, Barrio, Quintela, & Herradón, 1998; Sánchez-Calabuig et al., 2017). On the other hand, dog breeds could constitute a very useful model to examine a diversity of responses in reproductive studies, including the effect of environmental contaminants on sperm traits (Lea et al., 2016) and also to understand different aspects of female reproductive biology that may influence the evolution of sperm traits, as carried out in other taxa focusing on intra- and interspecific comparisons (Anderson et al., 2006; Roldan et al., 1992; Soler, García, et al., 2014).

Conflict of interest

The authors have no conflicts of interest to declare.

Contributors

SA was involved with all laboratory experiments and experimental design together with AGM and DB. SA was involved in the semen collection procedures with MC. ERSR contributing 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 and CS 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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Table 1. Sperm kinematic parameters (mean ± SD) in different dog breeds.

	SBT	LR	SM	VRH	BB	CHI
VCL	158.4 ± 54.4[a]	150.1 ± 48.4[b]	130.3 ± 50.0[c]	124.7 ± 50.0[d]	93.9 ±42 .1[e]	81.5 ± 53.5[f]
VSL	66.2 ± 34.7[a]	57.0 ± 33.1[b]	49.8 ± 31.9[c]	47.4 ± 32.0[d]	53.0 ± 35.9[e]	27.5 ±26.4[f]
VAP	95.1 ± 31.8[a]	83.7 ± 29.9[b]	76.6 ± 32.4[c]	66.7 ± 30.1[d]	67.3 ± 35.1[d]	44.0 ± 29.9[e]
LIN	41.9 ± 18.4[a]	37.1 ± 18.1[b]	37.3 ± 18.4[c]	36.5 ± 19.1[c]	51.5± 24.3[d]	30.7 ± 16.1[e]
STR	67.0 ± 23.4[a]	64.4 ± 24.2[b]	61.9 ± 24.0[c]	65.8 ± 26.1[d]	71.9 ± 26.4[e]	55.6 ± 23.7[f]
WOB	61.1 ± 11.5[a]	55.9 ± 11.4[b]	58.5 ± 11.5[c]	53.3 ± 12.5[d]	68.8 ± 14.2[e]	53.7 ± 10.8[f]
ALH	3.6 ± 1.2[a]	3.7 ± 1.2[b]	3.7 ± 1.4[b]	3.1 ± 1.1[c]	3.3 ± 1.3[d]	2.8 ± 1.2[e]
BCF	18.3 ± 5.9[a]	16.3 ± 5.5[b]	14.5 ± 6.7[c]	14.5 ± 6.0[c]	10.3 ± 4.5[d]	9.5 ± 6.2[e]

SBT: Staffordshire Bull Terrier; LR: Labrador Retriever; SM: Spanish Mastiff; VRH: Valencian Rat Hunting; BB: British Bulldog; CHI: Chihuahua. VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency. Different superscripts mean significant statistical differences among breeds, $P<0.05$

Table 2. Intra- and inter-male coefficients of variation in sperm kinematic parameters for different dog breed.

	SBT		LR		SM		VRH		BB		CHI	
	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
VCL	31.9	15.6	32.8	21.3	31.1	32.6	38.6	18.5	43.4	24.2	45.5	16.7
VSL	51.5	7.7	56.3	12.6	59.5	18.5	68.4	12.9	64.6	20.6	65.2	31.3
VAP	32.0	14.9	34.6	18.3	37.7	33.7	44.9	18.1	46.2	26.5	45.2	24.9
LIN	42.7	6.6	45.2	8.0	47.9	8.4	50.9	7.9	47.7	27.5	45.1	18.5
STR	34.9	5.2	37.4	5.5	37.9	7.4	38.6	9.3	36.5	35.6	34.1	28.0
WOB	16.7	10.4	17.7	17.9	18.9	15.2	22.0	12.3	22.3	21.0	19.3	10.8
ALH	31.7	11.2	32.2	16.7	33.4	25.1	34.6	11.4	38.7	13.2	41.0	10.9
BCF	31.7	12.9	32.5	17.5	35.7	22.0	41.8	16.5	45.9	24.5	47.4	20.1

SBT: Staffordshire Bull Terrier; LR: Labrador Retriever; SM: Spanish Mastiff; VRH: Valencian Rat Hunting; BB: British Bulldog; CHI: Chihuahua. VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 3. Fisher's linear discriminant functions of the matrix classifications from breed dog kinematic parameters.

	SBT	LR	SM	VRH	BB	CHI
VCL	0.750	0.813	0.704	0.792	0.620	0.670
VSL	0.347	0.290	0.312	0.293	0.370	0.395
VAP	-1.431	-1.286	-1.341	-1.342	-1.494	-1.495
LIN	-3.785	-3.896	-3.795	-3.829	-3.490	-3.809
STR	1.860	1.960	1.891	1.937	1.706	1853
WOB	4.210	4.015	4.116	4.031	4.250	4.267
ALH	-3.921	-9.170	-3.829	-7.623	2.365	-0.791
BCF	0.490	0.643	0.555	0.601	0.288	0.420
Constant	-146.523	-316.286	-176.694	-242.855	-113.883	-120.920

SBT: Staffordshire Bull Terrier; LR: Labrador Retriever; SM: Spanish Mastiff; VRH: Valencian Rat Hunting; BB: British Bulldog; CHI: Chihuahua. VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 4. Percentage of spermatozoa of the reference population by breed assigned to each breed after discriminant analysis

Breed	SBT	LR	SM	VRH	BB	CHI	Total
SBT	94.4	0.0	1.4	0.0	0.0	4.2	17024
LR	0.0	99.95	0.0	0.05	0.0	0.0	10596
SM	0.1	0.0	99.4	0.5	0.0	0.0	151236
VRH	0.0	8.0	0.1	91.9	0.0	0.0	28058
BB	0.0	0.0	0.0	0.0	98.1	1.9	2650
CHI	0.5	0.0	0.0	0.0	1.7	97.8	11193

The 95.7% of the reference animal by breed respectively, was classified correctly. SBT: Staffordshire Bull Terrier; LR: Labrador Retriever; SM: Spanish Mastiff; VRH: Valencian Rat Hunting; BB: British Bulldog; CHI: Chihuahua.

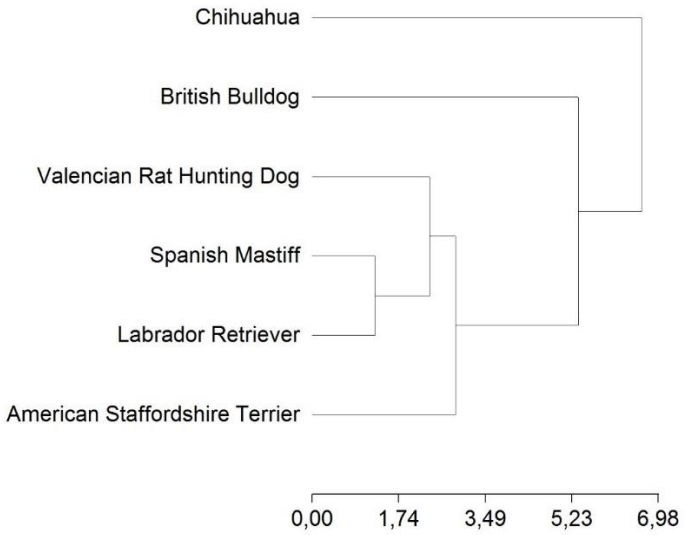

Figure 1. Hierarchical classification of dog breeds after cluster analysis.

CHAPTER X: KINEMATIC AND HEAD MORPHOMETRIC CHARACTERIZATION OF SPERMATOZOA FROM THE BROWN CAIMAN (*Caiman crocodilus fuscus*)

CHAPTER X:

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

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Running Title: Brown caiman sperm analyses in the tropics

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Abstract

Background: 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 tool in the seminal analysis for animals but there has been no application of this technique to reptilian spermatozoa.

Objective: The aim of this study was to analyze sperm kinematic and morphometric parameters in *Caiman crocodilus fuscus* semen samples and to determine the existence of sperm subpopulations.

Materials and methods: Four ejaculates from four sexually mature captive alligators in were used for this study. Integrated Semen Analyses Systems (ISAS®v1) CASA-Mot and CASA-Morph system was used with an image acquisition rate of 50 Hz for two seconds of capture. ISAS®D4C20 counting chambers were used and sperm were incubated at 25 °C for analysis. ***Results:*** Total and progressive motility were not affected ($P > 0.05$) by animal effect. There was a significant animal effect with respect to sperm morphometry, motility velocity and indexes values including linearity (LIN) and straightness (STR) ($P < 0.05$). Principal components (PCs) analysis showed that the variables were grouped into four components: PC_1, related to progressivity, PC_2 to velocity, PC_3 to oscillation and PC_4 to sperm head size. Subpopulation (SP) structure analysis showed four groups, namely, small medium non-progressive (SP_1), medium size slow non-progressive (SP_2), big medium progressive (SP_3), and small rapid progressive (SP_4), representing 23.0%, 52.0%, 9.7%, and 15.3% of the total population, respectively.

Discussion: The distributions of each sperm subpopulation varied between animals potentially representing a relationship with fertilization ability.

Conclusion: This study represents the first computer-assisted semen analysis of Crocodylia spermatozoa. Our findings demonstrate the importance of continuing development of reliable protocols regarding the standardization of computer-based semen analyses in reptilian species.

Keywords: andrology, caiman, crocodilia, sperm analysis, reptile reproduction

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 the 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 Yucatan peninsula (Charruau *et al*., 2015) to the northwest of Venezuela (Goombridge, 1982; Meden, 1983, 1981). The *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 of the reproduction of the caiman is a key element for understanding their conservation and in the development of captive breeding expertise for other closely related endangered species of Crocodylia. To this end, there 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 a semen collection protocols and characterization of seminal parameters (Fitri *et al*., 2018; Johnston *et al*., 2014b; Romero-Solórzano *et al*., 2010). There have also been studies determining sperm physio-chemo diluent requirements (Johnston *et al*., 2014c), the assessment of sperm DNA quality (Gosálvez *et al*., 2016; Johnston *et al*., 2015) and preliminary attempts of sperm cryopreservation (Johnston *et al*., 2017); all examining the spermatozoa of the salt water crocodile (*Crocodylus porosus*).

Assessment of sperm motility and morphometric is now commonly performed by means of computer assisted semen analysis (CASA) technologies that allow for objective and accurate assessment of sperm parameters 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). CASA systems provide information based on values of thousands of individual sperm tracks of the sample (Amann and Waberski, 2014). CASA analysis also allows identification of motile subpopulations of spermatozoa that show characteristic kinematic and morphometric patterns, but the biological meaning of these different sperm subpopulations and their interactions is not always clear. Studies of sperm subpopulations studies have been conducted for bovine (Valverde *et al*., 2016; Yániz *et al*., 2018), ovine (Yániz *et al*., 2015), swine (Gil *et al*., 2009; Valverde *et al*., 2018), feline

(Gutiérrez-Reinoso and García-Herreros, 2016), poultry (García-Herreros, 2016), salmon (Caldeira *et al*., 2018), fox (Soler *et al*., 2014, 2017) 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 our understanding of male crocodilian breeding soundness and assisted breeding technology, but also help to quantify and further characterize reptile sperm metabolism and physiology, such as capacitation (Nixon *et al*., 2016). Consequently, the aim of this study was to analyze kinematic sperm and morphometric parameters using CASA and to determine the existence of sperm subpopulations in *Caiman crocodilus fuscus*.

Materials and methods

Study site

This study was conducted as part of the crocodile management and exhibition facilities associated with the Scientific Ecotourism Project (EcoTEC) based in 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 m above sea level, in a tropical wet forest with a basal altitudinal floor, in accordance with Holdridge life zones system (Holdridge, 1967). According to the data recorded at the closest whether station (069567, St Clara, University Campus), the crocodile facility has annual minimum and maximum temperature of 21.7 °C and 30.7 °C respectively and a relative humidity of 88.5 %, with the rainfall rate of 3321.1 mm per year.

Animal husbandry

The experiment was conducted following 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 between 14 and 16 years of age. The EcoTEC crocodile facility has been 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. They are

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

Timing of semen collection

From observations of caiman behavior at the EcoTEC crocodile facility since 2008, it was concluded that the *C. c. fuscus* show courtship and mating behavior from February to June, the females deposit eggs and incubate during 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).

Animal restraint

The semen collection procedure was carried out and restrained caiman without the need for sedation or drugs for immobilization. Briefly, this included the removal of the animal from the fresh-water pond and using 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 and caiman (Fig. 1E). All animal restraint procedures were conducted without incident.

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 exteriorize the phallus; once the phallus 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 presented in less than five minutes. Following massage semen flowed down the sulcus of the phallus and 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 a micropipette fitted with a 10-100 µL pipette tip. Semen was lavaged from the sulcus with approximately 100 µL of buffered Dulbecco's phosphate-buffered saline (DPBS, pH = 6.8, Sigma-Aldrich, Inc.). The total time handling the animal for semen collection typically did not exceed 30 minutes. All semen collection procedures were conducted without incident.

Assessment of sperm parameters

The pH of undiluted semen was determined using narrow range pH paper strips (±0.3-0.4 pH unit; Sigma-Aldrich, Inc.). For the analysis of motility and kinetic parameters, 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 semen samples, 3 µL of diluted semen was dispensed along the counting chamber tracks by capillarity. A further dilution 1:10 in DPBS extender was used for motility and kinematic assessment before loaded the sperm in the counting chamber. Analyses were conducted with 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^{-1}) was estimated using a CASA-Mot system after accounting for the initial dilution of the semen sample.

The morphology of live spermatozoa was observed using the Trumorph® device (Proiser R+D) that briefly, increases the temperature of the sample to 45 ºC for stopping motility and applies a light pressure of 6 kP for extending the volume of the sample (2 µL) in a conventional slide and 22x22 mm cover slide. The depth between slide and coverslide is of ~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.

CASA analyses were performed over seven microscope fields on a total of at least 600 cells per sample (Video 1; see supplementary material). A mean of the seven fields was used for statistical analyses. CASA-Mot parameters assessed in this study included straight line velocity (VSL, $\mu m \cdot s^{-1}$) corresponding to the straight line from the beginning to the end of the track; curvilinear velocity (VCL, $\mu m \cdot s^{-1}$) - measured over the actual point-to-point track followed by the cell; average path velocity (VAP, $\mu m \cdot s^{-1}$) - 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 sperm cells swim, beat-cross frequency (BCF, Hz) - defined as the frequency with which the actual track crossed 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^{-1}). 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).

Computerized morphometric analysis

For morphometric analysis, semen smears were prepared, and slides stained using 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 analyzed in a double‐blind scheme. Microscope slides were analyzed for sperm head morphometry by 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 442368 pixels and 256 gray levels. Resolution of images was 0.21 μm/pixel in both the horizontal and vertical axes. Sperm heads were captured 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 one hundred sperm heads were measured on each slide for two primary parameters of head size (area [A, μm^2] and perimeter [P, μm]).

Statistical analysis

The data obtained from the analysis of all sperm parameters were first tested for normality and homoscedasticity by using Shapiro-Wilks and Levene tests. A normal probability plot was used to check for a normal distribution. The kinematics sperm variables did not satisfy the normality requirement for a parametric analysis of variance. Therefore, non-parametric analyses were performed with 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 motility data. All the kinematic (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) and morphometric

(area-head, perimeter-head) parameters were standardized in order to avoid any scale influence. The first step was to perform a principal component analysis (PCA) of these data (each variable was weighed 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 step of the analysis was determined using 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 conducted (Spencer, 2013). As a rotation method, the varimax method with Kaiser normalization 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 & Hothorn, 2011). The second step was to perform a non-hierarchical analysis using the k-means model that uses Euclidean distances from the quantitative variables after standardization of these data, so the cluster centers were the means of the observations assigned to each cluster (Kaufman & Rousseeuw, 1990). The multivariate k-means cluster analysis was conducted to classify the spermatozoa into a reduced number of subpopulations (clusters) according to their kinematic and morphometric parameters. In the final step, to determine the optimal number of clusters, the final centroids were clustered hierarchically using the Ward method (Murtagh & Legendre, 2014). Thus, every cluster provided a final cluster formed by the spermatozoa linked to its centroids. 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 their 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 parameters defining the different sperm subpopulations (i.e. the cluster centers). Differences between means were then analysed by the Bonferroni test. Results are presented as mean ± standard error of the mean (SEM). Statistical significance was considered at $P< 0.05$. All data were analyzed using IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

There was no animal effect ($P >0.05$) with respect to the total sperm motility (%) and/or progressive motility (%). The range of least squares mean (LSM) 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^{-1}) of the samples was 3.80 ± 0.4 with a range of 1.3 - 6.6. Mean (± SE) pH of the samples was 6.4 ± 0.1. The kinematic parameters corresponding to the whole analysed spermatozoa population are shown in the Table 1. The majority of the kinematic parameters showed differences between animals, with VCL, VAP, LIN, STR and ALH being the most variable. Only the WOB values were not significantly different between animals (Table 2).

Normal sperm morphologies in alive cells, using the Trumorph® technique, showed the typical filiform head and a tail that was approximate three times as long (Fig. 3). The area of the sperm head was significantly different among animals but there was difference in the perimeter of the sperm heads between caiman (Table 2).

Principal component analysis showed four PCs (named progressivity (PC1), velocity (PC2), oscillation (PC3) and head size (PC4) with a total variance explained of 86.85 %. These results indicated that sperm kinematics have stronger influence on the total variance than that of size (Table 3). The subsequent cluster analysis rendered four subpopulations. The kinetic and morphometric values corresponding to each subpopulation which were characterised as: small medium non-progressive (SP1), medium size slow non-progressive (SP2), big medium progressive (SP3) and small rapid progressive (SP4) (Table 4, Fig. 4). The distribution of subpopulations was significantly different between animals, with the medium size slow non-progressive spermatozoa being the most frequent in two of the animals (Table 5).

Discussion

Following the same principals used for breeding soundness in mammalian species (Valverde *et al*., 2018, 2016; Soler *et al*., 2017), it follows that a better understanding of caiman male reproductive physiology will consequently lead to improvements in their reproductive management both in wild and in captivity. While 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 revealed that sperm movement speed is directly correlated with fertilization success, this current study represents the first computer assisted sperm assessment in a crocodilian species.

The mean total motility reported in this study (49.6 ± 10.7%) was similar to that reported previously after post-mortem epididymal recovery (Larsen *et al*., 1982; 1992). Depending on the study and recovery technique, the motility described for *Crocodylus porosus* was also in the same range or slightly lower (Fitri *et al*., 2018): 45.0 ± 17.56 %, (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 fertilization (Birkhead *et al*., 1999). A raised sperm motility could be favored under situations of sperm competition, lower motility could be presented in species with sperm storage (Gist *et al*., 2000). In ectotherms, female body temperature has the potential to adjust sperm motility after mating (Uller *et al*., 2010). In turtles there are increase of the sperm motility at low temperatures thus suggesting that local adaptation of sperm motility in relation to the environment temperature during the timing of copulation (Gist *et al*., 2000). In the case of Leopard tortoise the total motility showed a high degree of variability ranging from 10 to 80%, what indicate a possible adaptation to the sperm motility under situations of sperm competition (Gist *et al*., 2000; Zimmerman & Mitchell, 2017) as also could be related to collection success or technique. Motility and kinematic parameters 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 lizard (*Tupinambis merianae*) (Young *et al*., 2017) but the number of frames per second used in some works were lowest (30 fps; Gist *et al*., 2000).

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

The curvilinear velocity (VCL) (54.16 ± 0.48 $\mu m \cdot s^{-1}$) reported in this study for the Caiman was similar to that documented for the *Boa constrictor* (58.97 $\mu m \cdot s^{-1}$; Tourmente *et al*., 2007), whereas straight-line velocity (VSL) in the *Boa constrictor* (50.22 $\mu m \cdot s^{-1}$) was significantly higher than that for the Caiman of (14.15 ± 0.21 $\mu m \cdot s^{-1}$). The high motility percentage exhibited by the snake could be an adaptation to sperm competition pressures (Snook, 2005; Tourmente *et al*., 2007) or 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 was

substantially higher than that found in the Caiman (28%), despite the analyses being carried out at the same temperature (25 °C).

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 in the crocodile by Jamieson *et al*. (1997), Gribbins *et al*. (2011) and Johnston *et al*. (2014b). Assumpção *et al.* (2017) reported spectacled caiman spermatozoa to have 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 our study, the CASA-Morph system used only is able to reports morphometric values of head area and head perimeter with range of 4.95-6.50 ± 0.11 μm^2 and 14.82-15.15 ±0.19 µm respectively. Even more, it is necessary to evaluate that the measurements doing after Diff-Quik staining implies the introduction of some level of artifacts. This fact is common for all the species (Soler *et al*., 2016), but particularly important in crocodilian sperm which do not contain cysteine in the protamine, having a special tendency to decondense when the smears are dried (Cummins, 1980). The use of Trumorph® technique showed the real morphology of the caiman sperm morphology, but, unfortunately, no CASA-Morph system is now available for the measurement of this image source.

While most studies of sperm motility typically describe parameters for whole sperm population in the ejaculate (normal distribution model), the increasing use of the CASA analytical systems has encouraged and promoted the concept of reporting and identifying sperm subpopulations, (Amann & Hammerstedt, 1993; Caldeira *et al*., 2018; Gallego *et al*., 2015; Hirai *et al*., 2001; Soler *et al*., 2017; 2014; Thurston *et al*., 2001; Valverde *et al*., 2016; Vásquez *et al*., 2016; Yániz *et al*., 2016; 2018). Based on cluster analysis, caiman sperm cells in this study were classified into four clusters with the following characteristics: sperm cells moderate velocities (VSL and VAP) but low linearity and was defined as SP_1 or “medium non-progressive”; the second showed low VSL, VAP and linearity and was considered as SP_2 or “slow non-progressive”; the third showed moderate velocities and high linearity (LIN, STR) and designated as SP_3 or “medium progressive”; finally, the last one was related with high velocity and highly linear cells (high VSL, LIN and STR) and was considered SP_4 or “rapid progressive” subpopulation. Subpopulations with higher head area sperm were SP_2 and SP_3 while the smaller sperm cells were distributed into SP_1 and SP_4. These observations prompt the question as to whether reptilian sperm cell size, more specifically head area, may be associated with progressivity? Furthermore, sperm head size

should be attributed to differences in chromatin (Gosálvez *et al*., 2016). The animal effect 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 their fertilization potential. Future studies will explore the use of CASA based systems to identify sperm abnormalities in crocodilian ejaculates.

Conclusion

The evaluation of seminal quality constitutes a critical parameter in species conservation so that the results of this work will contribute to the development of reliable protocols regarding the standardization of computer-based semen analyses in reptilian species with respect to the establishment of kinematic and morphometric parameter values that can be used to evaluate the reproductive potential of the crocodilian populations and allow more objective measures of semen evaluation. CASA based systems of crocodilian sperm 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 this study provide a basis for the standardization of reptilian sperm parameters.

Conflicts of interest

The authors declared no conflict of interest in this study.

Contributors

MMV was involved with all field and laboratory experiments and experimental design together with OCM and AGR. SJ was involved in the training of semen collection procedures to MMV, contributing ideas for this article and manuscript editing together with AV. AV contributed to interpretation of the data and was responsible for the data analysis. AV and CS 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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Table 1. Sperm kinematics (mean ± SEM) parameters found in four ejaculates of four Brown Caiman (*C. c. fuscus*)

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

Number of cells = 3 136. VCL = curvilinear velocity (μm·s^{-1}); VSL = straight line velocity (μm·s^{-1}); VAP = average path velocity (μm·s^{-1}); LIN = linearity of forward progression (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz); SEM = standard error of the mean. SD: standard deviation; Min-Max = minimum and maximum values. Q1: lower quartile; Q3: upper quartile.

Table 2. Sperm kinematics and morphometric (mean ± SEM) parameters in alligator (*C. c. fuscus*) (n = 4).

Male	1	2	3	4
VCL	50.02±0.95^{a}	54.99±1.03^{bc}	57.25±0.88^{c}	52.95±0.98^{b}
VSL	12.48±0.42^{a}	14.07±0.46^{b}	15.24±0.39^{b}	14.02±0.44^{b}
VAP	22.23±0.43^{a}	23.80±0.46^{b}	25.11±0.40^{c}	22.54±0.44^{a}
LIN	26.01±0.70^{a}	26.69±0.76^{ab}	28.23±0.65^{bc}	29.41±0.72^{c}
STR	52.99±1.04^{a}	55.38±1.12^{ab}	57.07±0.96^{b}	61.03±1.08^{c}
WOB	46.28±0.60^{a}	44.60±0.65^{a}	46.09±0.55^{a}	46.20±0.62^{a}
ALH	2.15±0.04^{a}	2.69±0.05^{bc}	2.81±0.04^{c}	2.62±0.04^{b}
BCF	4.49±0.09^{a}	4.43±0.10^{a}	4.41±0.09^{a}	4.13±0.10^{b}
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}

VCL = curvilinear velocity (μm·s^{-1}); VSL = straight line velocity (μm·s^{-1}); VAP = average path velocity (μm·s^{-1}); LIN = linearity of forward progression (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz); SEM = standard error of the mean. $^{a-d}$ Different superscripts within row indicate significant differences among subpopulations. P< 0.05.

Table 3. Eigenvectors of principal components (PCs) for alligator (*C. c. fuscus*) sperm kinematic and morphometric parameters

Principal component[*/a]	PC1	PC2	PC3	PC4
VCL			0.911	
VSL	0.815	0.466		
VAP		0.815	0.435	
LIN	0.930			
STR	0.949			
WOB	0.418	0.761		
ALH			0.889	
BCF		0.744		
Head perimeter				0.940
Head area				0.888
Variance explained (%)	27.18	22.43	20.18	17.06

Total variance explained = 86.85%. [*]Expresses the more important variables in each PC. Only eigenvectors > 0.4 are presented. [a] Rotated component matrix. VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; LIN: linearity of forward progression; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

Table 4. Sperm subpopulations for kinematic and morphometric parameters (means ± SEM) in alligator (*C. c. fuscus*) (n = 4)

	SP1	SP2	SP3	SP4
n/%	720/23.0	1 632/52.0	304/9.7	480/15.3
VCL	75.28±1.35[a]	38.59±0.90[b]	45.81±2.08[c]	59.27±1.65[d]
VSL	10.26±0.76[a]	7.53±0.51[b]	13.80±1.18[a]	28.39±0.94[c]
VAP	26.55±0.80[a]	15.84±0.53[b]	23.53±1.23[a]	42.36±0.98[c]
LIN	13.50±1.48[a]	19.98±0.98[b]	30.91±2.28[c]	49.98±1.81[d]
STR	38.23±2.252[a]	47.15±1.68[b]	55.93±3.88[bc]	68.89±3.09[c]
WOB	36.08±1.26[a]	41.86±0.84[b]	51.57±1.94[c]	72.32±1.54[d]
ALH	3.15±0.08[a]	1.54±0.05[b]	1.97±0.12[c]	1.85±0.10[c]
BCF	4.16±0.23[a]	4.07±0.15[a]	4.42±0.35[a]	7.35±0.28[b]
Head area (μm^2)	4.81±0.10[a]	5.69±0.07[b]	8.06±0.16[c]	4.96±0.13[a]
Head perimeter (μm)	14.76±0.15[a]	14.55±0.10[a]	18.67±0.23[b]	14.39±0.18[a]

VCL = curvilinear velocity ($\mu m \cdot s^{-1}$); VSL = straight line velocity ($\mu m \cdot s^{-1}$); VAP = average path velocity ($\mu m \cdot s^{-1}$); LIN = linearity of forward progression (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz); SEM = standard error of the mean. [a-d] Different superscripts within row indicate significant differences among subpopulations. $P < 0.05$.

Table 5. Subpopulation distribution of alligator spermatozoa (*C. c. fuscus*) in percentage depending on animal

Alligator/Subpopulation	Medium non-progressive	Slow non-progressive	Medium progressive	Rapid progressive
1	1.7	88.8*	8.4	1.1
2	2.3	74.2*	17.3*	6.2
3	57.5*	26.3	6.1	10.1
4	33.0*	17.0	7.0	43.0*

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

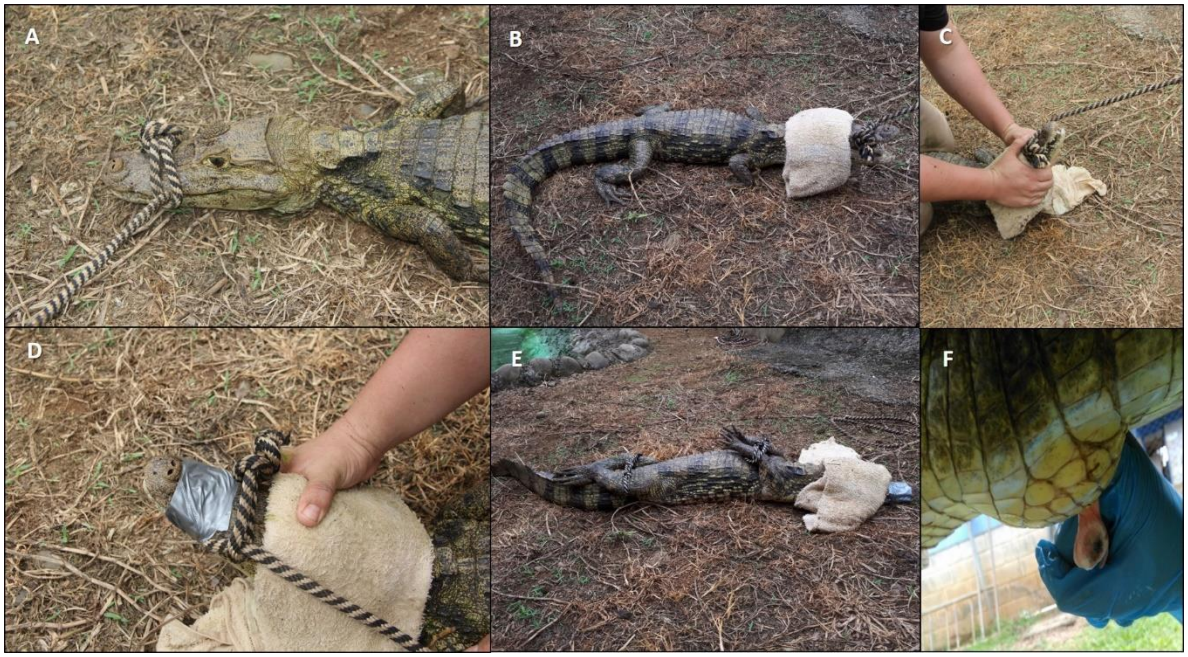


Figure 1. Caiman restraint by removal of the animal from the fresh-water pond (A). Moistened cotton cloth was then placed directly over the animal's eyes (B). Two expert handlers physically restrained the animal (C). Secure the top and bottom jaw with vinyl tape (D). Front and hind legs were then tied 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*).

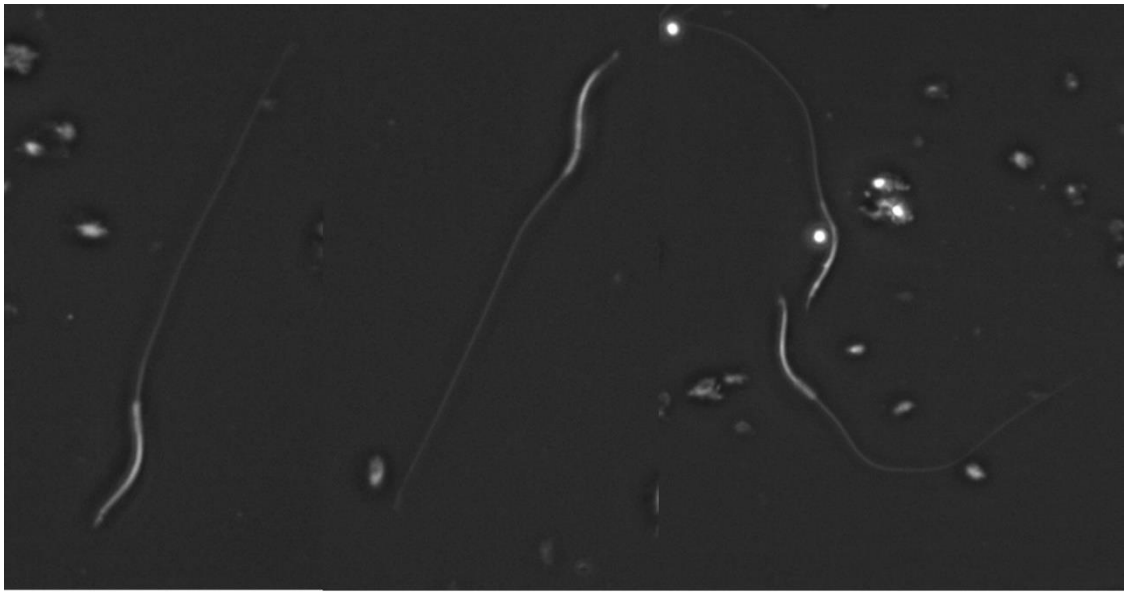


Figure 2. Normal alligator (*C. c. fuscus*) spermatozoa captured with Trumorph® system. Scale bar — 10 µm.

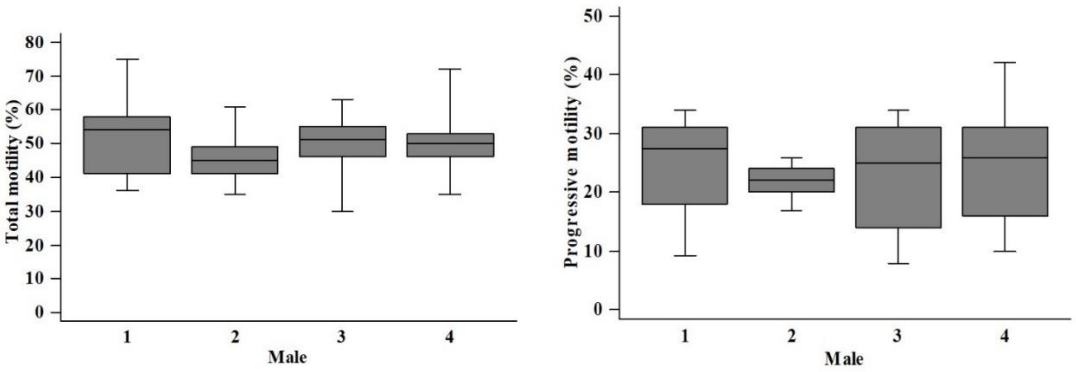


Figure 3. Boxplot (25th/75th percentiles, -: median; ⊥ ⊤: Minimum and maximum values considering three standard deviation) graphics of *Caiman crocodilus fuscus* total and progressive (STR >45%) motility.

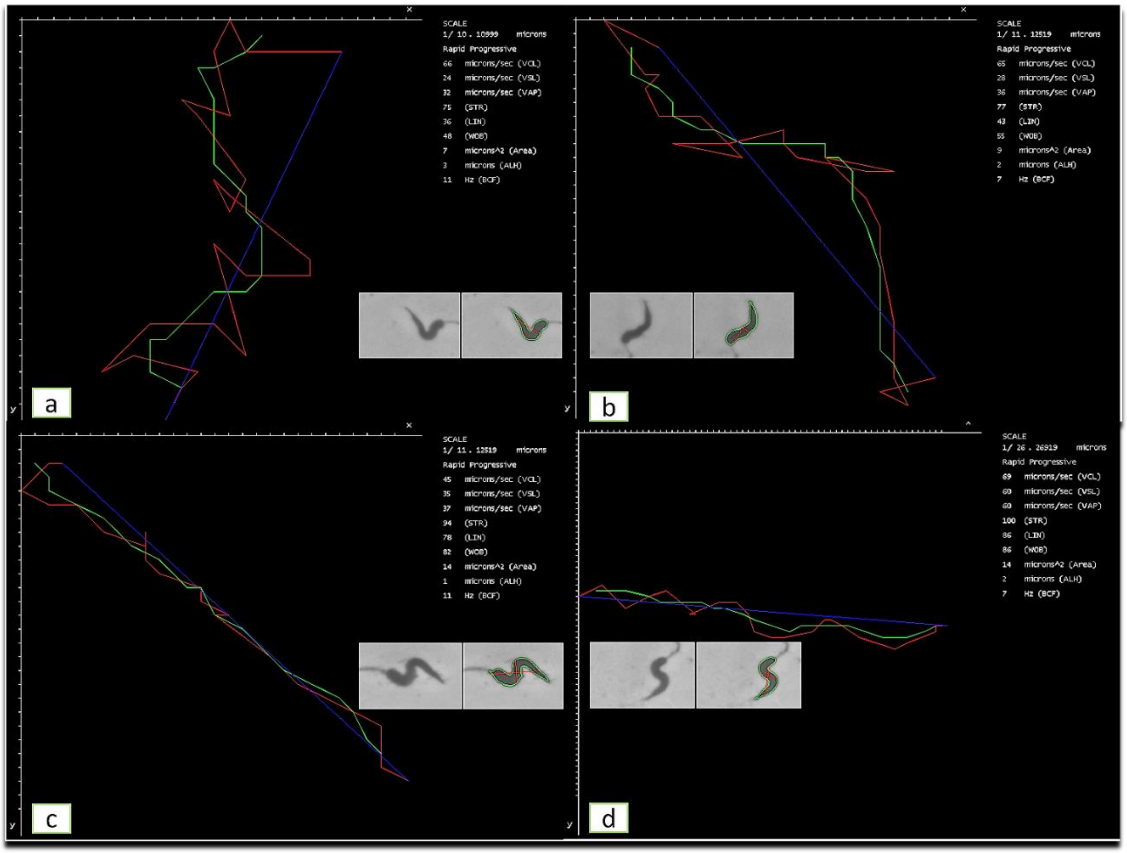
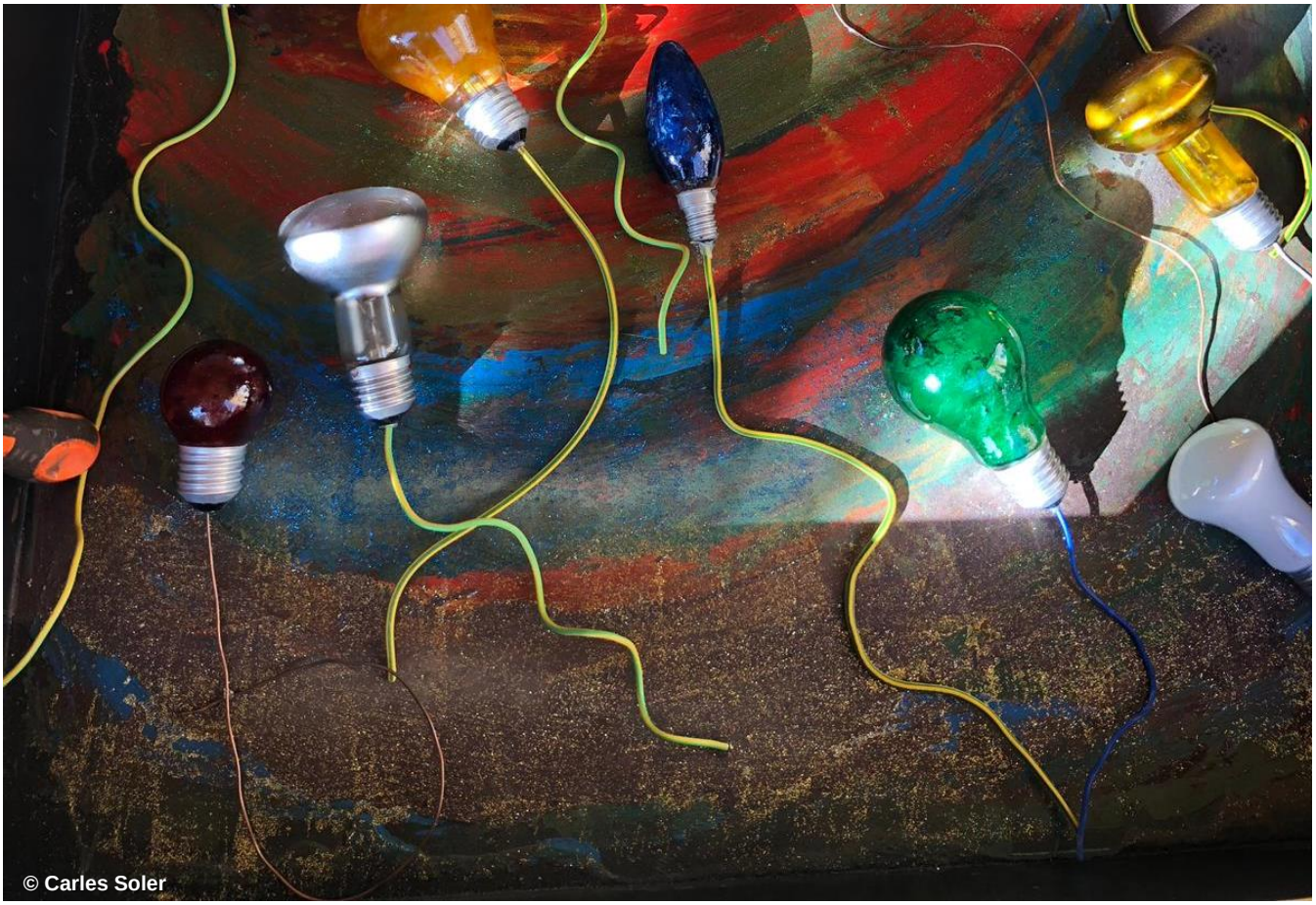


Figure 4. Representative trajectories by subpopulation of alligator sperm (*C. c. fuscus*) analyzed with ISAS®v1 CASA-Mot system. **a:** small medium non-progressive (SP1); **b:** medium size, slow non-progressive (SP2); **c:** big medium progressive (SP3); **d:** small rapid progressive (SP4). Lines: Blue = VSL; Red = VCL; Green = VAP. VCL = curvilinear velocity (μm·s^{-1}); VSL = straight line velocity (μm·s^{-1}); VAP = average path velocity (μm·s^{-1}). SP: subpopulation.

CHAPTER XI:
RESULTS AND GENERAL DISCUSSION



Results and general discussion

In the first work (chapter IV), the "classical" statistical approach, based on variance analysis (revealing differences between animals, ejaculates, and straws), principal component (PC) analysis showed that the variables were grouped into PC1, related to size, and PC2 to shape. Subpopulation structure analysis showed four groups, namely, big, small, short, and narrow from their dominant characteristics, representing 31.0 %, 27.3 %, 24.1 %, and 17.7 % of the total population, respectively. The distributions varied between animals and ejaculates, but between straws, there were no differences in only four animals. This modern approach of considering an ejaculate sperm population as divided into subpopulations reflecting quantifiable parameters generated by CASA-Morph systems technology opens a new view on sperm function.

The second work (chapter V), showed that FR affected all the kinematic parameters, with curvilinear velocity (VCL) and BCF the most sensitive ones. All the parameters showed differences among animals. Non-linear correlation showed the asymptotic level for VCL at 212 fps, being the highest FR for all the parameters. For future studies based just on progressive motility, almost 100 fps FR for 0.5 s must be used, while when kinematics must be considered, almost 212 fps for one-second should be analyzed. Three principal components were obtained (velocity, progressivity, and oscillation), being similar at 50 and 200 fps. Cells were grouped in four subpopulations but with different kinematic and cellular distribution at both FRs. The kinds of video cameras used until now for image acquisition have presented limited frame rates (FR), which have a negative influence on the quality of the obtained data.

In the third work (chapter VI), total and progressive motility were not affected by capture time. Capture time had a significant effect on velocity and indexes values ($P< 0.05$) but not on lateral head displacement (ALH) and beat cross frequency (BCF). All the parameters showed animal differences. The results demonstrate that sperm motility is affected by the video recording time length ($P< 0.05$). This effect is evident both in motility parameters and in single cell kinetics, studied by cluster analysis that showed a short time of video recording can to overestimate the percentage of rapid non-progressive cells in a subpopulation ($P< 0.05$). Studies based just on motility can use a one-half second, while when kinematics must be considered two seconds must be analyzed.

The fourth work (chapter VII), indicated that acronym CASA itself (computer-aided/assisted sperm analysis) is uninformative since the analysis could refer to any aspect of spermatozoa: their concentration, motility, kinematic parameters or morphology, or combinations of these. We suggest the following hyphenated compound terminology: the generic use of CASA for any sperm computer-aided sperm analysis, followed by an abbreviation indicating the analysis performed, i.e., CASA-Conc (for concentration), CASA-Mot (for motility, including kinematics) and CASA-Morph (for morphology, including morphometry).

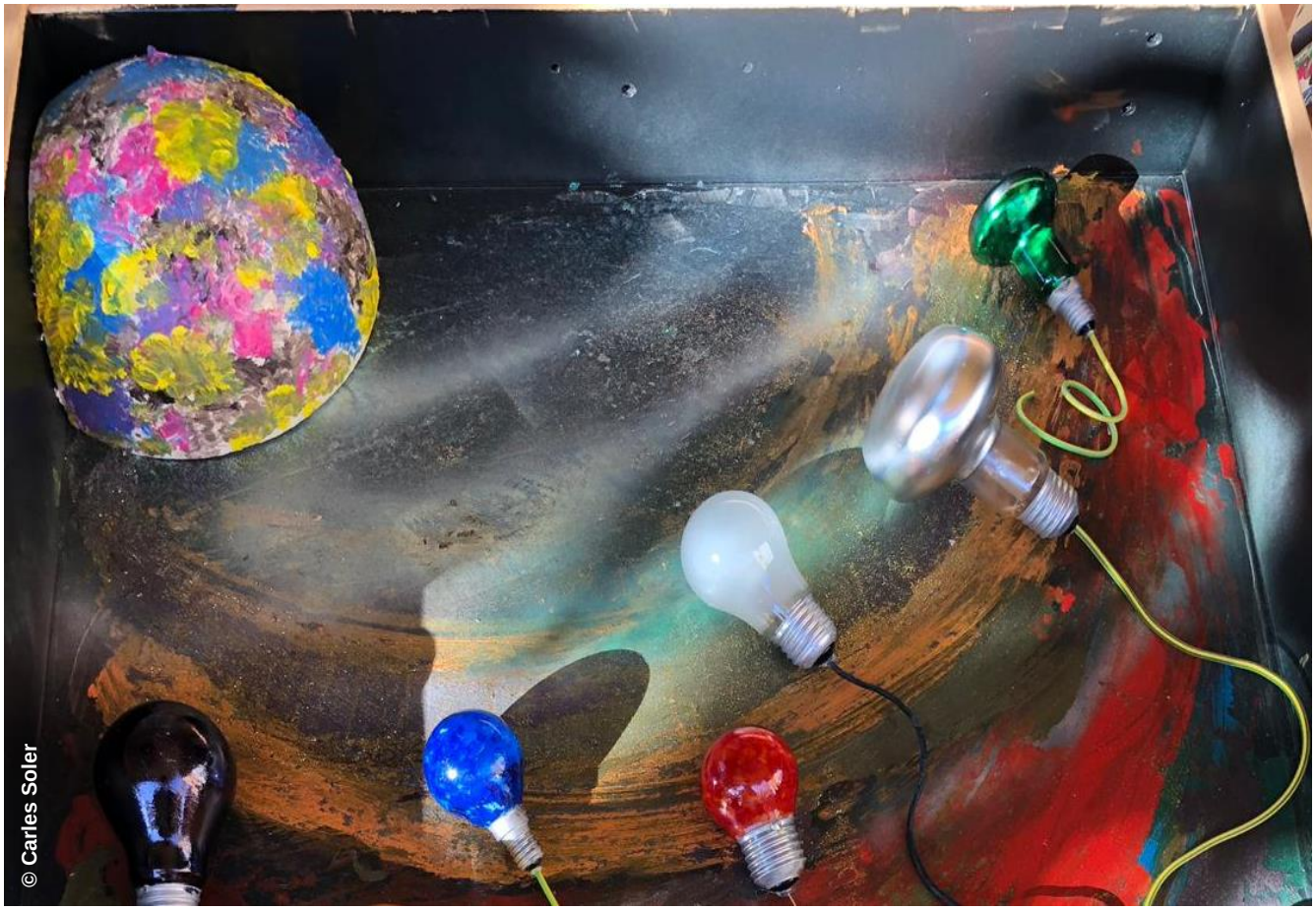
In the fifth work (chapter VIII), nine fixed positions were analyzed per camera type, considering central and lateral (summing both sides) and three longitudinal fields. Independently on the diluent used, differences were found between the three chambers and inside capillary loaded. Results obtained with the two diluents were significantly different with a very low correlation between them. In conclusion, the counting area inside the chambers leads to significant changes in sperm kinematic parameters and different dilution media introduce considerable differences in the motility patterns. Thus, it is necessary to define representative sampling methods and to define specific set-ups to be used with CASA-Mot technology.

The sixth work (chapter IX), showed that all kinematic parameters showed significant differences among breeds and individuals within each breed. The fastest sperm cells were those of Staffordshire Bull Terriers, and the slowest sperm cells were associated with Chihuahuas. The intra-male coefficient of variation (CV) was higher than the inter-male one for all breeds with the Staffordshire Bull Terrier showing the lowest values. When taking into consideration the cells by animal and breed, discriminant analyses showed a high capability to predict the breed source. Cluster analyses showed a hierarchical classification very close to that obtained after phylogenetic studies using genome markers. In conclusion, future work on dog spermatozoa should bear in mind significant differences between breeds and that results cannot be extrapolated from one to another. Because sperm characteristics are associated to breed diversity, dogs could represent a good model to examine changes in reproductive parameters associated with selection processes.

Finally, in our seventh work (chapter X), in *Caiman crocodilus fuscus* semen samples, total and progressive motility were not affected ($P > 0.05$) by the animal effect. There was a significant animal effect concerning sperm morphometry, motility velocity, and indexes values including linearity (LIN) and straightness (STR) ($P < 0.05$). Principal components (PCs) analysis showed that the variables were grouped into four components: PC1, related to progressivity, PC2 to velocity, PC3 to oscillation

and PC4 to sperm head size. Subpopulation (SP) structure analysis showed four groups, namely, small medium non-progressive (SP1), medium size slow non-progressive (SP2), big medium progressive (SP3), and small rapid progressive (SP4), representing 23.0 %, 52.0 %, 9.7 %, and 15.3 % of the total population, respectively. The distributions of each sperm subpopulation varied between animals potentially representing a relationship with fertilization ability. This study represents the first computer-assisted semen analysis of Crocodylia spermatozoa. Our findings demonstrate the importance of continuing development of reliable protocols regarding the standardization of computer-based semen analyses in reptilian species.

CONCLUSIONS



Conclusions

The present Ph.D. Thesis analyzed the application of CASA technology and multivariate analysis to optimize the semen evaluation in domestics and wild species. The following section summarizes the main findings and conclusions.

1. At least in the case of bull species, the former approaches for sperm morphometry study, based on the ANOVA, are not good enough to define the true sperm subpopulations, being necessary the use multivariate statistics based on the principal component analysis. Differences among post-thawed ejaculates from the same animal challenge the former idea that the subpopulation structure is an individual characteristic, indicating that can also be related to a physiological response to the environment.

2. Taking BCF and VCL as the most sensitive parameters to FR, it is recommended to use a capture frequency as close as possible to 225 fps in the evaluation of boar ejaculates for the study of their kinematics, while it can be considered enough at 50 fps for the assessment of general motility, and 150 fps progressive motility of the samples. Any future work must be developed from these results, both in the research and in the production fields.

3. It is recommended to use capture for two seconds in the evaluation of boar ejaculates for the study of their kinetics, while it can be considered enough with a one-half second for the assessment of general motility and progressive motility of the samples.

4. We suggested the following hyphenated compound terminology: the generic use of CASA for any sperm computer-aided sperm analysis, followed by an abbreviation indicating the analysis performed, i.e., CASA-Mot (for motility, including kinematics) and CASA-Morph (for morphology, including morphometry).

5. At least in the case of bull samples, the use of both different counting chambers and dilution media leads to significant changes in the estimation of sperm kinematic parameters. Attention regarding the selected area inside the counting chamber must be done.

6. Based on the significant differences observed between breeds in kinematic sperm traits, it may be advisable to refrain from a generalization for dogs when carrying out reproductive studies in this species. The dog breeds could constitute a handy model to examine a diversity of responses in reproductive studies and also to understand different aspects of female reproductive biology that may influence the evolution of sperm traits.

7. The evaluation of semen quality constitutes a critical parameter in species conservation as it was shown in work with caiman. Results can contribute to the development of reliable protocols regarding semen analyses in reptilian species that can be used to evaluate the reproductive potential. Crocodilian CASA based sperm assessment will also facilitate studies of sperm physiology and preservation.

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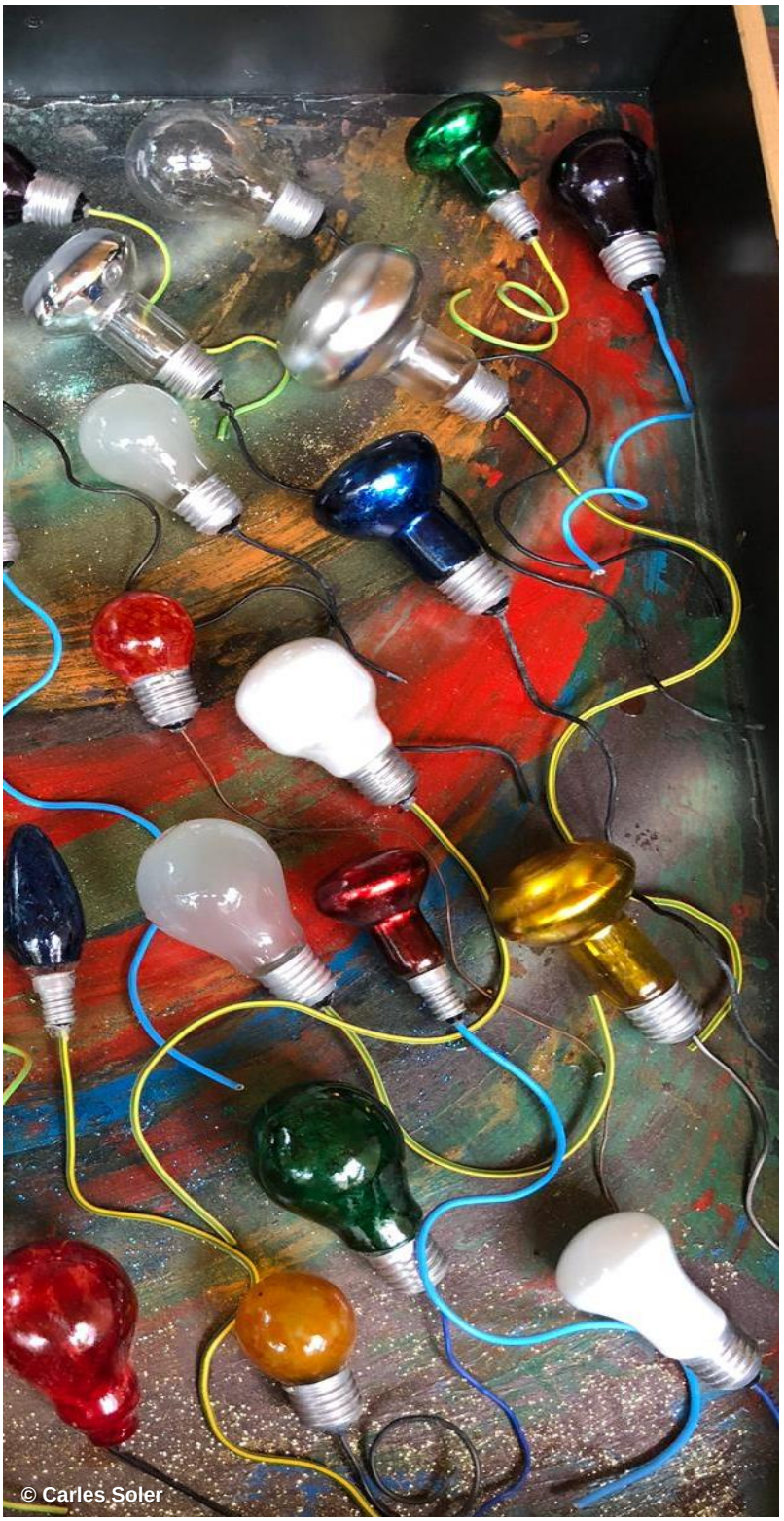

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THIS THESIS IS FOCUSED ON APPLICATION OF CASA TECHNOLOGY AND MULTIVARIATE ANALYSIS TO OPTIMIZE THE SEMEN EVALUATION IN DOMESTIC AND WILD SPECIES, DEFINING PROTOCOLS THAT ASSURING THE CONSISTENCY OF THE RESULTS AND STUDYING SPERM SUBPOPULATION STRUCTURE.

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