Universitat Autònoma de Barcelona

Computerised analysis of semen in equids

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Que la tesis titulada: "Computerised analysis of semen in equids." presentada por **Sabrina Gacem** para optar por el grado de Doctor en Medicina y Sanidad Animales por la Universidad Autónoma de Barcelona, se ha realizado bajo nuestra dirección y, considerándola terminada y cumpliendo los requisitos para poder optar por la Mención Internacional, autorizamos su presentación para ser juzgada por la comisión correspondiente.

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Bellaterra (Cerdanyola del Vallès), 18 de diciembre del 2020.

A mon ange Zahraa

A vous mes très chers parents, et à ma Fatma

« *Le poisson rouge ne peut ramener la complexité des océans à la quiétude de son bocal* ».

Yasmina Khadra,

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Abbreviations

AI	Artificial Insemination
ALH	Amplitude of Lateral Head displacement (μm)
AMP	Ductus deferens ampullae
ANOVA	Analysis of variance
BCF	Beat Cross Frequency (Hz).
BUG	Bulbourethral gland
CA	Capsular artery
CASA	Computer Assisted Sperm Analysis
CLC	Capillary Loaded Chamber
D4C10	ISAS® disposable chamber 10 μm depth
D4C20	ISAS® disposable chamber 20 μm depth
D4C20 L	ISAS® Leja disposable chamber 20 μm-deep
DDC	Drop Displacement Chamber
EDV	End Diastolic Velocity
Fps	Frames Per Second
FR_O	Frame rate optimum
LIN	Linearity (%)
N	Number of spermatozoa analysed
OFR	Optimum Frame Rate
PI	Pulsatility Index
PPP	Proximal Pampeniform Plexus
PPT	Testicular artery of pampiniform plexus
PROS	Prostate gland

PSV	Peak Systolic Velocity
RI	Resistive Index
SD	Standard Deviation
SEM	Standard Error of the Mean
SP	Subpopulation
Spk	Spermtrack® reusable chambers
Spz	Spermatozoids
ST	Supra-testicular area
STR	Straightness of sperm trajectory (%)
TABF	Total Arterial Blood Flow
TAMV	Time Average Maximum Velocity
VAP	Average path velocity (μm/s)
VG	Vesicular gland
VLC	Curvi-Linear Velocity (μm/s)
VSL	Straight-Linear Velocity (μm/s)
WOB	Percentage of Wobble coefficient (%)

Abstract

The breeding soundness evaluation in equids consists of the overall analysis of those characteristics that influences his ability to achieve a high pregnancy rate and produce normal and healthy foals. The evaluation of the stallion or donkey for potential breeding soundness consists of 4 parts: general physical examination, the examination of external reproductive organs, the examination of internal reproductive organs, semen collection, and evaluation. When the traditional BSE was first outlined, the testis and accessory sex glands (ASG) were prospected by palpation, sperm motility was estimated visually, sperm morphology was examined using either light microscopy on stained semen smears or phase-contrast microscopy on unstained and chemically fixed semen samples. The aim of the present dissertation was to use new technology to improve the stallion and donkey reproductive study. B-mode high-resolution ultrasonography was used to check the ASG and testis and Pulsed-Doppler ultrasonography to analyse the testis vascularization. While, newly performed cameras of a computer-assisted semen analysis (CASA) were used to improve and standardize the sperm motility analysis. As well, new sperm morphology technique was investigated in the present thesis. Fresh ejaculates were collected on different horse breeds (Arabian and Spanish pure breeds) and Catalonian donkey. All animals were semen donors and undergo CEE health condition protocol. Results of the first work regarding the reproductive ultrasonography in donkey showed significantly higher bulbourethral glands, prostate, and ductus deferens ampullae than stallion. Those high sizes of ASG result in high seminal plasma production and was correlated to CASA sperm motility. Regarding Pulsed-Doppler ultrasonography indices of testicular artery showed a correlation with semen parameters (concentration, CASA kinematics, and sperm viability) therefore, any disturbance in the blood flow could impair spermatogenesis and thus semen quality and quantity. These results provide a reference for ASG measures and aspects and testis vascularization indices of donkey male. Also, help in the detection of reproductive dysfunction if taking into consideration the CASA technology used for better data return. The second work was projected to standardize and improve sperm motility analysis. CASA system consists of a microscope, camera for image captures, computer and software for data analysis. Currently, new high-resolution cameras and different chambers are introduced to the system. The optimization of the protocol is required to obtain tracks as closer as possible to the real track. The optimal frame rate (OFR) obtained was 300 fps and 278 fps for stallion and donkey respectively when using CASA for motility analysis. In any case the OFR

varied depending on the counting chamber used, both type, depth and counting area considered. For stallion and donkey, drop displacement chambers (DDC) returned significantly higher curvilinear velocity (VCL), straight line velocity (VSL), straightness (STR), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF) values than the capillary loaded chambers (CLC), However, for the stallion in the 20 µm-deep chambers returned higher kinematic values than for donkey. Regarding chamber area, in donkey and stallion, for DDC 10 µm the sperm velocities (VCL, VSL, VAP) and ALH values decreased significantly from the center to the edges of the counting area, while WOB and BCF increased. The same was reported for donkeys in 20µm while in stallion no difference in motility parameters was observed. For stallion in the CLC 10µm depth, VSL and STR decreased from the sperm deposition point towards the chamber end, while the opposite was seen in the CLC 20 µm chamber. The CLC 20µm also returned higher VCL, WOB, and BCF values in the distal area. No defined behaviour was observed along with the CLC in the donkey. Finally, DDC 10 and 20µm represent the best choice when analysing donkey and stallion sperm respectively and considering the mean of the kinematic variables of 9 fields taken from the central counting area. Sperm concentration in donkey was another factor that affected sperm behaviour since kinematic variables were higher in a highly concentrated sample. It's suggested to use the concentration of 30×10^6 sperms/ml for sperm motility analysis in donkey.

Using a 10 µm chamber and 250 frames per second (the maximum FR disponible with the present software capabilities) a study of the sperm subpopulation (SP) structure in donkey and stallion was performed like the next step in the general study. In both species, thee sperm motility subpopulations were found. The dominant subpopulation comprises spermatozoa with fast, straight, and lineal with a high tail beat. This SP included 38.2% of donkey cells and 41.7% of stallion. While the lowest subpopulation was for the slowest and non-linear sperms. the third SP grouped the fast sperms with high beat and non-linear trajectory. Comparing stallions' breeds, Arabian, and Spanish showed a slight difference in reference to the percentage of subpopulations but showing significant difference in the values of kinematic parameters between subpopulations. It's important to consider those differences when analysing any biological characteristics or treatments on equine sperm.

The final work was focused on the use of the new Trumorph® technique for sperm morphology analysis which allowed a good vision and count of sperm abnormalities on live unstained sperms. Also, Trumorph® technique was compared with the usual eosin-nigrosin stain and showed no

difference in sperm abnormalities counting. Trumorph® can represent a simple and reliable technique and can be a good alternative for sperm morphology analysis.
In conclusion, the new technological advances in both ultrasonography and CASA permitted to define new approaches and new definitions when breeding soundness examination in equids.

Keywords*:* donkey; stallion, accessory sex gland, ultrasonography; CASA, sperm motility, sperm morphology, sperm subpopulation.

Resumen

La evaluación de solidez reproductiva (EEB) en équidos consiste en el análisis general de aquellas características que influyen en su capacidad para lograr una alta tasa de preñez y producir potros normales y sanos. La evaluación del semental o burro para determinar su potencial reproductivo consta de 4 partes: examen físico general, examen de los órganos reproductores externos, examen de los órganos reproductores internos, recolección de semen y evaluación. Cuando se delineó por primera vez la EEB tradicional, los testículos y las glándulas sexuales accesorias (ASG) se prospectaron mediante palpación, se estimó visualmente la motilidad de los espermatozoides, se examinó la morfología de los espermatozoides mediante microscopía óptica en frotis de semen teñidos o microscopía de contraste de fases en frotis de semen teñidos y químicamente fijados muestras de semen. El objetivo de la presente tesis fue utilizar nueva tecnología para mejorar el estudio reproductivo de sementales y burros. La ecografía de alta resolución en modo B se utilizó para comprobar la ASG y los testículos y la ecografía Doppler pulsado para analizar la vascularización testicular. Mientras tanto, se utilizaron cámaras recién realizadas de un análisis de semen asistido por computadora (CASA) para mejorar y estandarizar el análisis de motilidad de los espermatozoides. Asimismo, en la presente tesis se investigó una nueva técnica de morfología espermática. Se recogieron eyaculados frescos de diferentes razas de caballos (razas puras árabes y españolas) y burro catalán. Todos los animales fueron donantes de semen y se sometieron al protocolo de estado de salud de la CEE. Los resultados del primer trabajo con respecto a la ecografía reproductiva en burro mostraron glándulas bulbouretrales, próstata y ductus deferens ampullae significativamente mayores que en sementales. Esos tamaños altos de ASG dan como

resultado una alta producción de plasma seminal y se correlacionaron con la motilidad del esperma CASA. En cuanto a la ecografía Doppler pulsado, los índices de la arteria testicular mostraron una correlación con los parámetros del semen (concentración, cinemática CASA y viabilidad del esperma), por lo que cualquier alteración en el flujo sanguíneo podría afectar la espermatogénesis y por tanto la calidad del semen. Estos resultados proporcionan una referencia para las medidas y aspectos de ASG y los índices de vascularización testicular del macho de burro. Además, ayude en la detección de disfunción reproductiva si se tiene en cuenta la tecnología CASA utilizada para un mejor retorno de datos. El segundo trabajo se proyectó para estandarizar y mejorar el análisis de la motilidad de los espermatozoides. El sistema CASA consta de microscopio, cámara para captura de imágenes, computadora y software para análisis de datos. Actualmente, se introducen en el sistema nuevas cámaras de alta resolución y diferentes cámaras. Se requiere la optimización del protocolo para obtener pistas lo más cercanas posible a la pista real. La frecuencia de imagen óptima (OFR) obtenida fue de 300 fps y 278 fps para sementales y burros, respectivamente, cuando se utilizó CASA para el análisis de motilidad. En cualquier caso, la OFR varió según la cámara de recuento utilizada, tanto el tipo como la profundidad y el área de recuento considerada. Para sementales y burros, las cámaras de desplazamiento de caída (DDC) arrojaron valores de velocidad curvilínea (VCL), velocidad en línea recta (VSL), rectitud (STR), amplitud del desplazamiento lateral de la cabeza (ALH) y frecuencia cruzada de latido (BCF) significativamente más altos que las cámaras de carga capilar (CLC), sin embargo, para el semental en las cámaras de 20 µm de profundidad arrojaron valores cinemáticos más altos que para el burro. Con respecto al área de la cámara, en burro y semental, para DDC 10 µm las velocidades de los espermatozoides (VCL, VSL, VAP) y los valores de ALH disminuyeron significativamente desde el centro hacia los bordes del área de conteo, mientras que WOB y BCF aumentaron. Lo mismo se informó para los burros en 20 µm mientras que en el semental no se observaron diferencias en los parámetros de motilidad. Para el semental en la CLC de 10 µm de profundidad, VSL y STR disminuyeron desde el punto de deposición de esperma hacia el extremo de la cámara, mientras que se observó lo contrario en la cámara de CLC de 20 µm. El CLC de 20 µm también arrojó valores más altos de VCL, WOB y BCF en el área distal. No se observó un comportamiento definido junto con el CLC en el burro. Finalmente, DDC 10 y 20µm representan la mejor opción cuando se analizan los espermatozoides de burro y semental respectivamente y se considera la media de las variables cinemáticas de 9 campos tomados del área central de conteo. La concentración de esperma en burro fue otro factor

que afectó el comportamiento de los espermatozoides, ya que las variables cinemáticas fueron más altas en una muestra altamente concentrada. Se sugiere utilizar la concentración de 30×10^6 espermatozoides / ml para el análisis de la motilidad de los espermatozoides en burros.

Utilizando una cámara de 10 µm y 250 cuadros por segundo (el máximo FR disponible con las capacidades actuales del software) se realizó un estudio de la estructura de la subpoblación de espermatozoides (SP) en burro y semental como el siguiente paso en el estudio general. En ambas especies, se encontraron tres subpoblaciones de motilidad espermática. La subpoblación dominante la componen los espermatozoides con un ritmo rápido, recto y lineal con un latido de la cola alto. Este SP incluyó el 38,2% de células de burro y el 41,7% de semental. de semental. Mientras que la subpoblación más baja fue para los espermatozoides más lentos y no lineales. el tercer SP agrupó los espermatozoides rápidos con latido alto y trayectoria no lineal. La comparación de las razas de sementales, árabes y españoles mostró una ligera diferencia en referencia al porcentaje de subpoblaciones, pero mostró una diferencia significativa en los valores de los parámetros cinemáticos entre subpoblaciones. Es importante tener en cuenta esas diferencias al analizar las características biológicas o los tratamientos en los espermatozoides equinos.

El trabajo final se centró en el uso de la nueva técnica Trumorph® para el análisis de la morfología de los espermatozoides que permitió una buena visión y recuento de las anomalías espermáticas en espermatozoides vivos no teñidos. Además, la técnica Trumorph® se comparó con la tinción habitual de eosina-nigrosina y no mostró diferencias en el recuento de anomalías espermáticas. Trumorph® puede representar una técnica simple y confiable y puede ser una buena alternativa para el análisis de la morfología de los espermatozoides.

En conclusión, los nuevos avances tecnológicos tanto en ecografía como en CASA permitieron definir nuevos enfoques y nuevas definiciones a la hora de criar exámenes de solidez en équidos.

Palabras llave: burro; semental, glándula sexual accesoria, ecografía; CASA, motilidad espermática, morfología espermática, subpoblación espermática.

Resum

L’avaluació de la solidesa reproductiva (ESB) en èquids consisteix en l’anàlisi global de les característiques que influeixen en la seva capacitat per aconseguir una taxa d’embaràs elevada i produir poltres normals i sans. L’avaluació potencial de la solidesa reproductiva de un semental o un ruc consta de 4 parts: exploració física general, examen d’òrgans reproductors externs, examen d’òrgans reproductors interns, recollida i avaluació de semen. Quan es va esbossar per primer cop l’ESB tradicional s’exploraven els testicles i les glàndules sexuals accessòries (ASG) per palpació, es valorava visualment la motilitat de l’esperma a microscopia òptica, s’examinava la morfologia de l’esperma mitjançant microscòpia òptica en frotis de semen tenyits o amb microscopia de contrast de fases en tincions fixades químicament. Posteriorment l’ús de sistemes computeritzats en l’anàlisi de la motilitat espermàtica (CASA) o la morfologia (ASMA) varen aportar objectivitat. L'objectiu de la present Tesi ha estat utilitzar noves tecnologies per millorar l'estudi reproductiu dels sementals i rucs. S’ha utilitzat l’ecografia d’alta resolució en mode B per explorar les ASG i el testicle i la ecografia Doppler Pulsat per analitzar la vascularització del testicle. D’altra banda es varen, per millorar l’anàlisi de la motilitat espermàtica mitjançant sistemes CASA es varen utilitzar sistemes informàtics més potents, videocàmeres d’alta resolució y dispositius (cambres) d’anàlisi específiques. Així mateix, es va introduir una nova tècnica en l’estudi de la morfologia de l'esperma. Es van recollir ejaculats frescos en diferents races pures de cavalls (Arab i Espanyol, PRE) i ruc català. Tots els animals eren donants de semen sota els requisits sanitaris de la CEE.
En el primer treball mitjançant ecografia transrectal en ruc es va observar que les glàndules bulboretrals, la próstata i les ampolles dels conductes deferens son significativament més grans que en un semental. Aquests resultats segurament estan relacionats amb una alta producció de plasma seminal i es van correlacionar amb la motilitat de l'esperma evaluada mitjançant CASA. Pel que fa a la ecografia Doppler polsat, es va observar una disminució significativa del fluxe sanguini conforme ens acostem al testicle. Els índexs que evaluen el flux sanguini de l’artèria testicular van mostrar una correlació important amb els paràmetres del semen (concentració, cinemàtica CASA i viabilitat de l’esperma), per tant, qualsevol pertorbació del flux sanguini podria afectar l’espermatogènesi i, per tant, la qualitat i la quantitat del semen. Els resultats obtinguts son una referència important per la detecció de disfuncions reproductives en ell ruc.

El segon treball es va projectar per estandarditzar i millorar l'anàlisi de la motilitat dels espermatozoides mitjançant un sistema computeritzat (CASA). Varen utilitzar-se noves cambres d'alta resolució (fins a 500 frames per segon) i càmeres d'anàlisi específiques. La freqüència òptima de fotogrames (OFR) obtinguda va ser de 300 fps i 278 fps per a semental i ruc respectivament. En qualsevol cas, l'OFR va variar en funció de la cambra de recompte utilitzada, tant el tipus, la profunditat com la superfície de recompte considerada. Per a sementals i rucs, les cambres de desplaçament de caiguda (DDC) van oferir velocitats curvilínies (VCL), velocitat de línia recta (VSL), rectitud (STR), amplitud del desplaçament lateral lateral del cap (ALH) i valors de freqüència de batuda (BCF) significativament superiors a les les càmeres de cèrrega per capil·laritat (CLC), però, per el semental les cambres de 20 μm de profunditat van aportar valors cinemàtics més elevats que per a l'ase. Pel que fa a l'àrea de la cambra, en ruc i semental, per a DDC de 10 μm, les velocitats dels espermatozoides (VCL, VSL, VAP) i els valors ALH van disminuir significativament del centre a les vores de la zona de recompte, mentre que WOB i BCF augmentaven. El mateix es va observar en rucs a 20 μm, mentre que en un semental no es va observar cap diferència en els paràmetres de motilitat. Per a un semental amb una profunditat de 10 μm de CLC, VSL i STR van disminuir des del punt de deposició d'espermatozoides cap a l'extrem de la cambra, mentre que es va veure el contrari a la cambra de 20 μm de CLC. Les CLC 20μm també va oferir valors més elevats de VCL, WOB i BCF a la zona distal. No es va observar cap comportament definit amb CLC en el ruc. Finalment, les DDC 10 i 20 μm representen la millor opció a l'hora d'analitzar els espermatozoides de ruc i semental respectivament i de considerar la mitjana de les variables cinemàtiques de 9 camps extrets de l'àrea de recompte central. La concentració d'espermatozoides en ruc va ser un altre factor que va afectar el comportament dels espermatozoides, ja que les variables cinemàtiques eren més altes en una mostra molt concentrada. Es recomana utilitzar la concentració de 30×10^6 espermatozoides / ml per a l'anàlisi de la motilitat de l'esperma al ruc.

Utilitzant una càmera de 10 μm i 250 fotogrames per segon (el màxim FR disponible amb les actuals capacitats de programari) es va realitzar un estudi de l'estructura subpoblacional espermàtica (SP) en ruc i semental com el següent pas de l'estudi general. En ambdues espècies, es van trobar subpoblacions mòbils d'espermatozoides. La subpoblació dominant comprèn espermatozoides amb un patró de motilitat ràpida, recta i lineal. Aquesta SP incloïa el 38,2% de les cèl·lules del ruc i el 41,7% dels sementals. La subpoblació més escasa va ser per als

espermatozoides més lents i no lineals. Una tercera SP agrupava els espermatozoides ràpids amb ritme elevat i trajectòria no lineal. La comparació de les races de sementals, àrabs i espanyols va mostrar una lleugera diferència en referència al percentatge de subpoblacions, però va mostrar una diferència significativa en els valors dels paràmetres cinemàtics entre les subpoblacions. És important tenir en compte aquestes diferències a l'hora d'analitzar les característiques biològiques o tractaments de l'esperma equí.

El darrer treball es va centrar en l'ús de la nova tècnica Trumorph®, per a l'anàlisi de morfologia dels espermatozoides. Aquesta tècnica es basa en l'increment de temperatura a 41ºC que i mobilitza els espermatozoides i una lleugera pressió per col.locar-los plans i facilitar-ne la visió i el recompte d'anomalies. La tècnica Trumorph® es va comparar amb la tinció clássica d'eosina-nigrosina i no es varen observar diferències en el recompte d'anomalies de l'esperma. El Trumorph® pot representar una tècnica senzilla i fiable i pot ser una bona alternativa per a l'anàlisi de la morfologia de l'esperma.

En conclusió, els nous avenços tecnològics tant en ultrasonografia com en CASA van permetre definir nous enfocaments i noves definicions a l'hora de realitzar un examen de solidesa reproductiva en èquids.

Paraules clau: ruc; semental, glàndula sexual accessòria, ecografia, CASA, motilitat espermàtica, morfologia espermàtica, subpoblació espermàtica.

Introduction

1.1. How the sperm is generated and shaped?

1.1.1. Development of male gonads

The influx of interstitial cells to the foetal male gonads was seen after 20 days of gestation, these cells are derived from the degenerating mesonephros. After this stage, the characteristic structure of the testis began to differentiate (Barreto et al., 2017).

The testes are made up of two different compartments: the seminiferous tubules (where the germ cells and Sertoli cells are located) and the interstitium (where the Leydig cells are located) (Figure 1). Its functions are the production of male gametes (sperm), as well as the synthesis of hormones (testosterone) (Deurdulian et al., 2007).

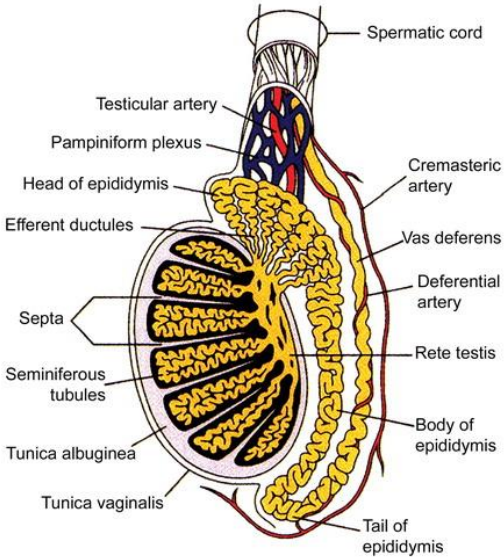

Figure 1 . Internal structure of stallion testis (Sudakoff et al., 2002).

Puberty is the period in which, in males, the differentiation of primordial germ cells begins. In this vital stage, the secretion of testosterone begins the production of sperm cells, the secretion of the adnexal glands and the secondary sexual characteristics (Holstein et al., 2003). The germ cells present in the seminiferous tubule include spermatogonia, primary and secondary spermatocytes, spermatids (in all their maturation stages) and finally spermatozoa. Spermatogonia is found in the basal compartment. Instead, spermatocytes (in the process of meiosis) and spermatids (in the process of maturation) will be in internal positions to the tubules (Hall, 2016) (Figure 2).

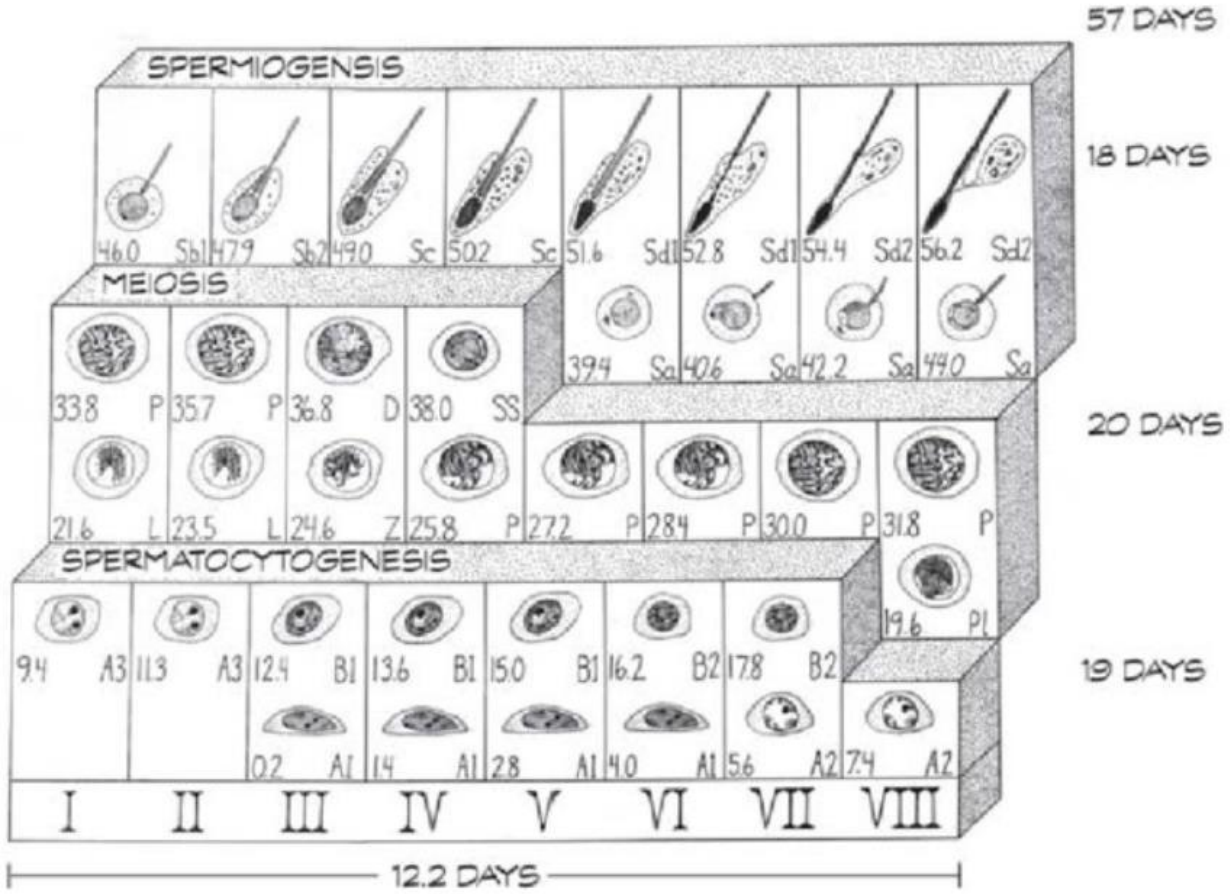

Figure 2: Spermatogenesis in equids (Dickson, 2007)

Three major divisions of spermatogenesis (spermatocytogenesis, meiosis, and spermiogenesis) combine to constitute the stages of the cycle of the equine seminiferous epithelium (I–VIII). During the 19 days of spermatocytogenesis, A1 spermatogonia enter cyclic activity during stage III and undergo mitotic division to produce B1 and B2 spermatogonia, followed by the production of preleptotene primary spermatocytes. During eight the 20 days of meiosis, preleptotene primary spermatocytes differentiate through zygotene meiotic division to produce Sa spermatids (Sa). During the 18 days of spermiogenesis, Sa spermatids differentiate through Sb 1, Sb 2, Sc, Sd 1, and Sd 2 steps of development before spermiation (i.e., release from the seminiferous epithelium) as spermatozoa. The letters indicate the development step; the numbers associated with each germ cell step indicate the developmental age (in days) of each cell type in the middle of each spermatogenic stage. The cycle length is 12.2 days, and the duration of spermatogenesis is 57 days in stallions.

The other cellular components of the seminiferous tubules are the Sertoli cells, columnar-somatic cells located in the germinal epithelium. Its role is to provide structural support and take progressive control of germline cell metabolism (Figure 2). The basal junction of the Sertoli cells, the basal lamina, and other cell types give rise to the blood-testicular barrier, which will isolate the

seminiferous tubule from the individual's immune system. Furthermore, Sertoli cells are capable of secreting endocrine substances such as inhibin or proteins such as transferrin or plasminogen activator, necessary in the development of spermatozoa on formation (Ross and Pawlina, 2003) (Neves et al., 2014). Spermatogonia are undifferentiated, immotile, and diploid cells that are supported by Sertoli cells in the basal area of the seminiferous tubule. Leydig cells represent the interstitial tissue and are the main producers of steroid sex hormones, their function begins with the early differentiation of the male foetus but remain inactive until puberty (Ross and Pawlina, 2003).

1.1.2. Spermatozoa formation

Spermatozoa formation occurs in the seminiferous epithelium of the testis. It begins at puberty, after a long preparatory period, and continues throughout life until old age (Holstein et al., 2003). Spermatozoa formation starts with the mitotic division of spermatogonia during which DNA replicates and cell division results in a continuous source of cells for sperm production through meiosis. The subsequent meiotic divisions consist of two successive cell divisions of spermatocytes, following one round of DNA replication, giving rise to four haploid cells, spermatids (Bruce et al., 1994). Three major stages of spermatozoa formation are spermatocytogenesis, spermatogenesis, and spermiogenesis (Figure 2).

Spermatogenesis is one of the most productive self-renewing systems in the body. It's a process of generating from a stem-cell population of germ cells called spermatogonia. This process begins in peripubertal and normally continues for all life. The entire process takes 47.2 days to occur in donkeys and 54.9 in horses (Čoudková, 1981; Hess and De Franca, 2008). However, it is not established yet which genes regulate the duration of spermatogenesis, recent work has demonstrated that the spermatogenic cycle length is under the control of germ cell genotype (Holstein et al., 2003; Rajender et al., 2010; Neveset al., 2014).

Donkey have higher spermatogenic efficiency than a stallion which match with other studies in mammals showing that a shorter spermatogenic cycle is more efficient is spermatogenesis (Hess and De Franca, 2008; Costa et al., 2010) (Table 1). That could result from the combination of higher Sertoli cell support capacity for germ cells (Sertoli cell efficiency) and greater number of Sertoli cells per gram of testis (Hess and De Franca, 2008; Neves et al., 2014) .

Table 1: Comparison of testis parameters in sexually mature donkeys and horses (a: Neves et al., 2014; b: Swiestra et al., 1974).

Parameters	Donkey[a]	Horse[b]
Testis weight (g)	166-219	117-213
Gonadosomatic index (%)[c]	0.15	0.12
Seminiferous tubule percentage	85	72
Coefficient of efficiency of spermatogonial mitosis[d]	1 : 8.2 (75)	1 : 8.3 (75)
Meiotic index[e]	3.1(25)	3 (25)
Overall rate of spermatogenesis[f]	1 : 26 (80)	1 : 26 (80)
Sertoli cell efficiency (spermatids per Sertoli cell)	15.1	8.7
Sertoli cells per gram of testis (million)	30	28
Spermatogenic cycle length (days)	10.5	12.2
Total duration of spermatogenesis (days)	47.2	54.9
Daily sperm production per gram of testis (million)	42	20

C: calculation of the gonadal mass as a proportion of the total body mass, d: ratio of early primary spermatocytes per type A spermatogonia at stage 1of SEC (percentage of presumptive germ cell loss during the spermatogonial phase in parenthesis). E: Ration of spermatids per primary spermatocytes (percentage of germ cell loss during meiosis in parenthesis). F: Ratio of round spermatid per type A spermatogonia at stage 1 of the SEC.

Spermatogenesis takes place in the convoluted portions of seminiferous tubules and involve three separate, continuous stages: spermatogenesis, meiosis, and spermiogenesis (Holstein et al., 2003):

- Proliferative or spermatogonium phase: spermatogonia are located in the basal compartment of the seminiferous tubules and separated by the blood-testicular barrier. They can be divided into three classes according to their function and the staining of their chromatin: the first are the stem cells of spermatogenesis, the Ad (dark nucleus), these cells will divide to restore the cell population of spermatogonia. Through the successive mitoses, the clear spermatogonia will create and later the type B spermatogonia. The last one give rise to the primary spermatocytes, creating bridges between their cytoplasm, allowing a synchronous division. The spermatogonia remain partially united by cytoplasmic bridges in the previous mitosis cycles, giving incomplete cytokinesis that allows the formation of a syncytium within which, a synchronous development will take place until the end of the spermatogenesis process (De Jonge and Barratt, 2017). Spermatogonia cells pass throw the differentiation process to produce spermatocytes.
- Meiotic Phase: during this phase, the spermatocytes formed in the Proliferative phase undergo meiosis processes in which the reduction of the diploid endowment to haploid spermatids. In this

stage, the cells undergo two meiosis controlled by the pituitary-hypothalamic-gonad axis (McKinnon and Voss, 1993). Furthermore, during prophase one an exchange of chromosomal segments called " crossing-over " allows the creation of cells with genetic diversity unlike prophase two (Ross and Pawlina, 2007).

 Spermiogenesis: the spermatids undergo complex transformation and remodelling processes until maturity, forming the sperm encountered in normal ejaculate. In this phase, large functional and relevant morphological changes occur. Spermiogenesis passes through three phases than undergo maturation (Figure 3):

- Golgi phase: the Golgi apparatus of the Sa spermatid produces an acrosomal vesicle representing the cranial pole of the developing sperm (Amorocho-LLanos, 2012). This vesicle is formed by positive PAS (periodic acid-Schiff's reagent) granules, which are preacrosomic granules composed of glycoproteins (Ross and Pawlina, 2007). In parallel, the centrioles move from the juxtanuclear region towards the opposite pole. The proximal centriole joins the cell nucleus, while the distal centriole forms the axoneme (internal structure flagellum, composed of nine central and two internal microtubule doublets). Morphologically, Sa spermatids have a heteromorphic central nucleus with chromatin clusters and an acrosomal vesicle in the upper part.
- Cap phase: during this stage, the acrosomal sac flattens, forms a cap attached to the nuclear envelope through the acroplaxome and initials its descent along the nucleus. The Sb1 spermatid change the location of the acrosome towards the basal lamina. The Sb2 spermatids have a slightly elongated nucleus compared to Sc, the latter is also remodelled to a conical form in the anterior cell and globular form in the posterior cell part. In addition, the manchette structure acquires her highest length. Sd1, in which another nuclear elongation takes place and the manchette become indistinguishable from the flagellum (Amorocho-Llanos, 2012).
- Acrosomal phase: a period of condensation and elongation of the asymmetric nucleus in which dispersed and compact chromatin structures are created, in turn, there is a loss of most histones. In this stage, the spermatid rotates with the acrosome pointing to he basal lamina and the developing axoneme extending into the lumen of seminiferous. The nuclear structure flattens and extends, moving, together with its overlapping acrosome, toward the anterior part of the plasma membrane. Parallel to the nuclear movement, the cytoplasm moves towards the posterior pole. A microtubules- containing manchette develops (Amorocho-Llanos, 2012), grouping perinuclear cytoplasmic microtubules in a cylindrical sheath, this sheath extends from the caudal part of the

acrosome to the caudal pole of the spermatid. During this phase there is a great advance in the development of the tail and the intermediate piece: consecutively the centrioles return from the back of the cell to the nuclear caudal surface, where they undergo a transformation to develop the neck. Later, nine dense fibers originate from the neck expand inside the tail and unite the cell nucleus with the flagellum. During flagellar growth, the flagella is covered by cytoplasm. Regarding the other organelles of the cell, the mitochondria shuffle around the dense fibers of the neck (and the contiguous region) forming the intermediate piece. The ordered mitochondria will be necessary to strengthen and provide energy that requires the successful execution of the movements that sperm needs to move (Ross and Pawlina, 2007).

- Maturation: it is the final phase of spermiogenesis, the elimination of excess cytoplasm occurs through the formation of residual bodies present in the luminal border of the Sertoli cells. The residual bodies activate Sertoli cells to secrete a series of substances such as tubular fluid, inhibin, and androgen-binding proteins. Once the residual bodies eliminated, a new spermatogenic cycle begin (Ross and Pawlina, 2007). Sperm maturation occurs along the epididymal duct, presenting less mature sperm at the beginning of the epididymis and more mature at the end. The maturation process comes to an end with the evacuation of the sperm into the light of the seminiferous tubule (Spermiation), whereby peristaltic movements the gametes will be directed to the efferent ducts and from there to the epididymis (Olivera et al., 2006).

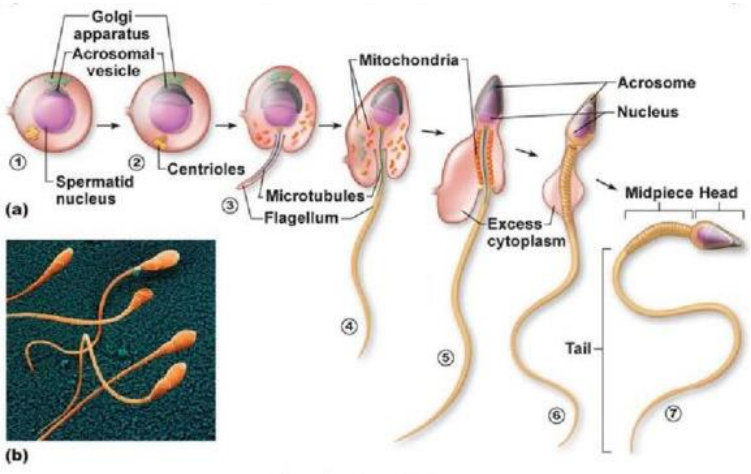

Figure 3 : *Differentiation and maturation of spermatogenic stem cell (Mescher et al., 2010)*

1.1.3. Ejaculate

The various components of the ejaculate originate from different parts of the male reproductive tract (Figure 3) and are released in a concrete order. In equids, emission and ejaculation follow a series of strong and pulsatile contractions of the urethral and bulbospongiosus muscles. As a consequence, ejaculates are the result of six to nine jets of semen (Kareskoski and Katila, 2008). Split ejaculation begins with the watery pre-sperm faction, derived from the urethral glands. The milky, but not gelatinous, sperm-rich fraction follows. This portion comes from the epididymis and the ampulla and possesses a high content of glycerylphosphoryl-choline (epididymal), ergothioneine (ampulla) and some traces of citric acid. The third fraction is the viscous and gelatinous post-sperm fraction, mainly coming from vesicular secretions (Flint et al., 2015).

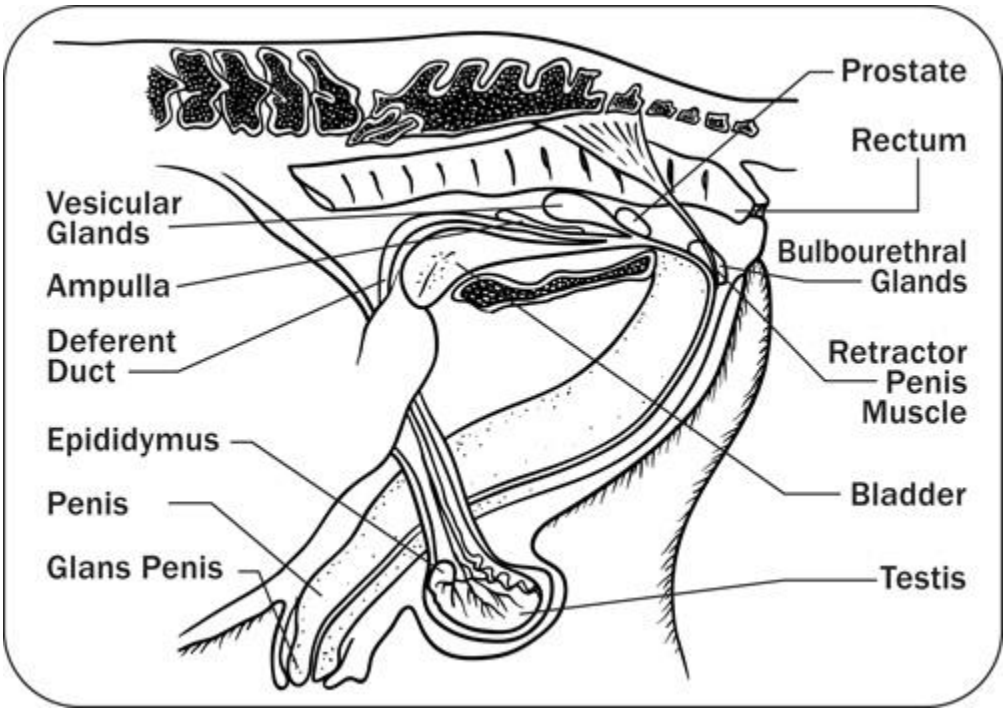

Figure 4: Sperm reproductive tract and annex glands sagittal view (Pickett et al., 1972)

1.1.4. Sperm structure

A spermatozoon has only one role to fill, that of fertilisation of an oocyte. A billions of spermatozoa are generally present in an ejaculate to fulfil that mission but only 0.0007% of spermatozoa deposited into the uterus of the mare gain access into the oviductal luminae where

fertilisation occurs (Rigby et al., 2000). A spermatozoon is an extremely sophisticated and adaptable to achieve the task of fertilisation, requiring a series of highly coordinated cellular-level and molecular-level functions to be successful in its mission.

The spermatozoon is classically divided anatomically into a head and a flagellum (or tail). The head contains the nucleus, an overlying acrosome and a reduced complement of cytosolic elements. The head can be subdivided into an acrosomal region, equatorial segment, post acrosomal region and posterior ring, which demarcates the junction between the head and flagellum. The posterior ring is the site of the plasma membrane anchoring to the nuclear envelope and is thought to produce a tight seal that separates cytosolic components of the head and flagellum. The flagellum can be subdivided into a connecting piece, middle piece (or midpiece), principal piece and end piece (Figure 1). These various parts of the spermatozoon are surrounded by a common plasma membrane; however, the composition of the plasma membrane can be subdivided into regional domains that impact its multiple functions, such as: sperm–oviductal adhesion; penetration of the cumulus–oophorus matrix; sperm–zona adhesion; the acrosome reaction; acquisition of activated motility and hyperactivated motility; and sperm–oocyte adhesion and fusion (Brito, 2007; Varner et al, 2015).

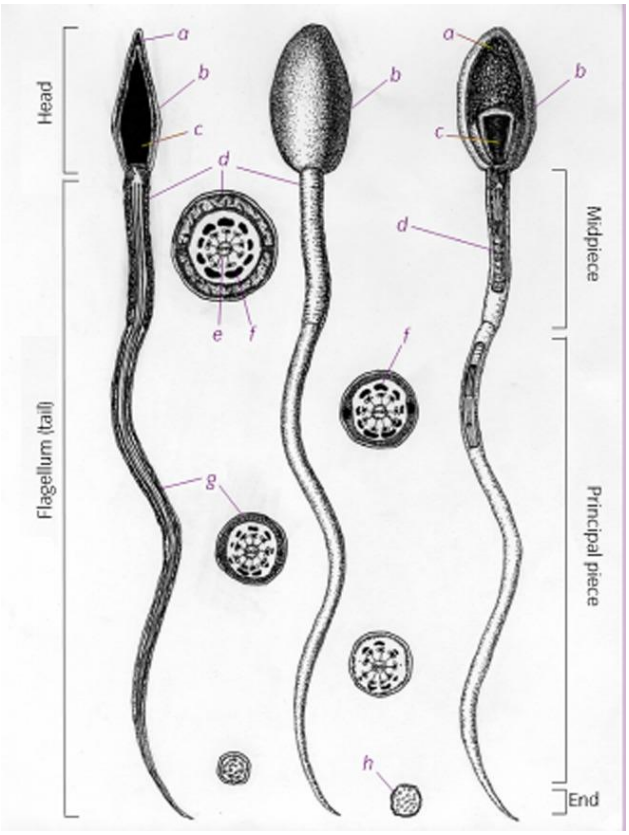

Figure 5: Drawings demonstrating magnified views of an equine spermatozoon

represented by an uncut view (centre), a mid-sagittal view (left), and a partially resected view (right). *The various lengthwise divisions of the spermatozoon are represented as head, flagellum (tail), midpiece, principal piece, and end piece (end). a. acrosome, b. plasma membrane, c. nucleus, d. mitochondria, e. axoneme, f. outer dense fibres, g. fibrous sheath, h. axonemal microtubules (Varner and Johnson, 2007)*

The average length of an equine spermatozoon is 61–86 μm^2. The sperm head is somewhat elliptical, is flattened in one plane (dorsoventrally), and is thicker in the posterior portion of the head than in the apical portion (Figure 2).

The nucleus, which occupies the majority of space within the head, contains the paternal genetic material. The connecting piece of the flagellum consists primarily of a capitulum, segmented columns of fibers, and the proximal and distal centrioles. The connecting piece serves to attach the flagellum to the head, and also functions to produce and stabilise some structural components of the flagellum. The capitulum articulates with the head at the level of the implantation fossa, attaching by fine filaments that connect the capitulum to the basal plate. The segmented columns anchor the dense fibers of the flagellum (Figure 2). The centrioles, oriented at right angles to each other, are involved in the development of the connecting piece and the

axoneme. The proximal centriole remains attached at the implantation fossa, but the distal centriole gives rise to the axoneme during tail development.

The primary structural elements of the flagellum include the axoneme, the outer dense fibers, the fibrous sheath, and the mitochondria. The midpiece is characterised by the presence of an axoneme, an array of outer dense fibers, and an overlying sheath of helically arranged mitochondria. The midpiece joins the principal piece at the annulus, a point where the mitochondrial sheath is replaced with a fibrous sheath. The principal piece thereby consists of a centralised axoneme, outer dense fibers of variable length, and a fibrous sheath. The fibrous sheath terminates at the junction of the principal piece and end piece, with the end piece consisting of a small extension of the axoneme (or individually arranged acrosomal microtubules) past the termination site of the fibrous sheath. The entire flagellum is enveloped by a plasma membrane (Brito, 2007).

1.1.5. Sperm morpho-abnormalities

Defective spermatogenesis, as well as some epididymal pathologies, are related to an increase in the percentage of abnormal spermatozoa. Depending on the anomaly, the fertilizing potential of these cells will be affected to one degree or another (WHO, 2010). Sperm abnormalities may represent physiological alterations that affect not only abnormal sperm but also normal or almost normal sperm from the same ejaculate (Soler et al., 2016a).

Classification systems for sperm defects proposed for bulls have been adapted by some authors for stallions (Jasko et al., 1992). However, Different classifications were proposed for sperm morphology according to their origin (primary sperm defects and secondary) or according to the perceived effects on fertility (major and minor defects). Several other classification systems have been reported in stallions and the nomenclature used for specific defects varies widely. Although the adoption of a standard classification system and standard nomenclature for specific defects would have enormous benefits for both basic and applied research and ultimately for the ability of veterinarians to interpret the findings of the breeding soundness evaluation, this has not happened in any species. The present Society for Theriogenology forms for stallion breeding soundness evaluation has the following categories listed in the differential spermiogram (Figure 6): normal sperm, abnormal acrosomal regions/ heads, detached head, proximal droplets, distal

droplets, abnormal midpieces, and bent/coiled tails. The presence of other cells (round germ cells, WBC, RBC, etc.) should also be indicated. Ideally, the specific defects of different sperm regions should also be enumerated. Acrosome defects include knobbed, roughed, and detached acrosomes. Head defects include microcephalic (small, underdeveloped, or dwarf), macrocephalic (large or giant), pyriform (narrow at the base), tapered (narrow), other shape defects (those usually also micro or macrocephalic), nuclear vacuoles (pouches or craters), and multiple heads. Midpiece defects include midpiece reflex (simple bent or folded midpiece), segmental aplasia of the mitochondrial sheath, fractured, swollen (thick, droplet), roughed (corkscrew), swollen/ roughed/broken (Dag-like), disrupted sheet (filamentous), duplicated and stump tail. Bent or coiled tails refer to those sperm in which both the midpiece and the principal piece are bent or coiled, or the distal part of the principal piece is coiled. The percentage of normal and abnormal detached heads (tailless or separated heads) could be recorded separately, and teratoids ideally should be classified as a completely separate category (Brito, 2007).

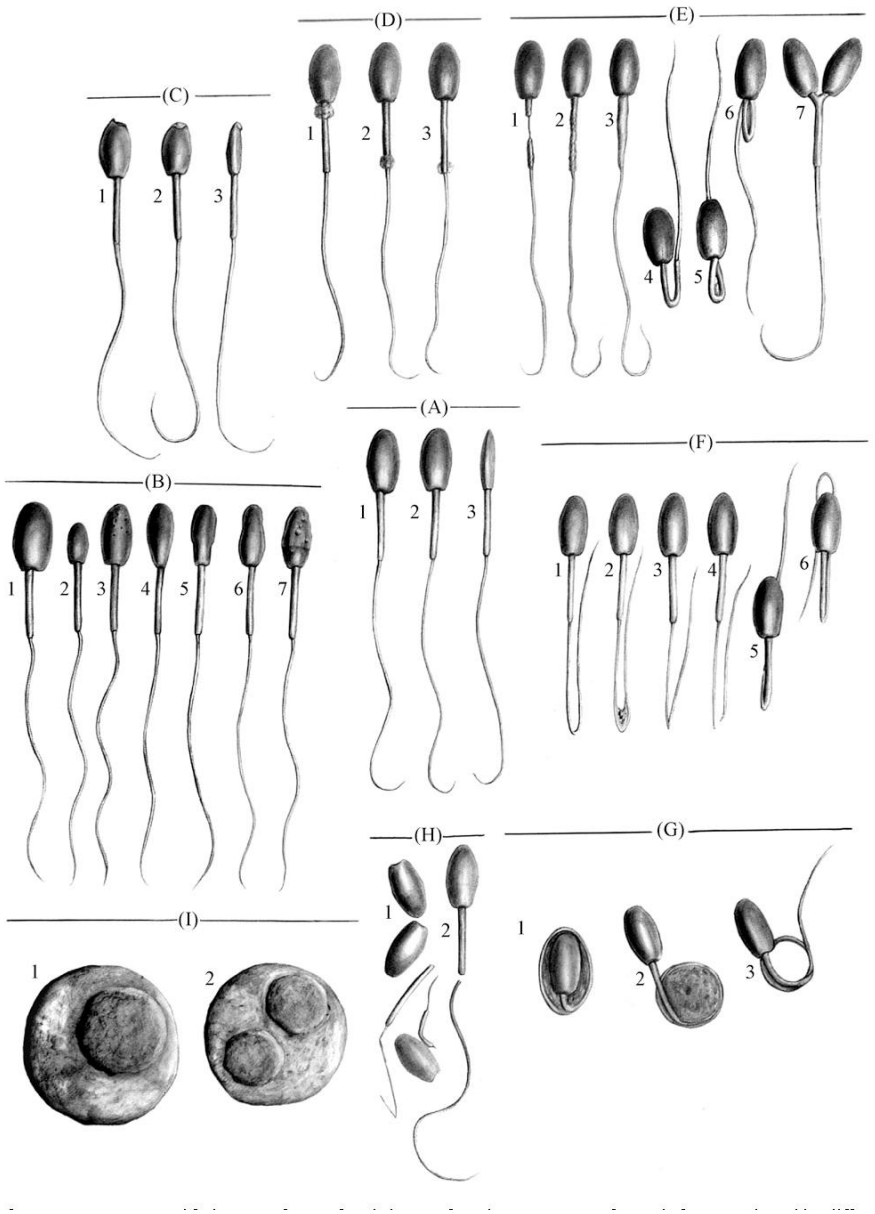

Figure 6: Drawings of spermatozoa to resemble images of normal and abnormal equine spermatozoal morphology, as viewed by differential-interference contrast (DIC) microscopy of a fixed and unstained wet-mount semen specimen. (Varner et al., 2008)

(A) Normal spermatozoal morphology, in dorsoventral (A1, A2) and side (A3) views. Abaxial midpieces (A1) are considered to be morphologically normal. (B) Variations in abnormal head morphology, including macrocephalic (large head) morphology (B1), microcephalic (small head) morphology (B2), nuclear vacuoles (or crater defects; considered by our laboratory to be normal if low in number and size, and located randomly over acrosomal region; B3), tapered head (B4), pyriform head (B5), constricted or hour-glass head (B6), and degenerate head (B7). (C) Acrosomal defect in dorsoventral (C1, C2) and side (C3) views. This morphologic defect is termed ‘‘knobbed acrosome.’’ (D) Proximal (D1) and distal (D2, D3) cytoplasmic droplets. (E) Abnormalities of the midpiece, including segmental aplasia of the mitochondrial sheath (E1), roughed midpiece from uneven distribution of mitochondria (E2), enlarged mitochondrial sheath (E3), bent midpiece (E4, E5, E6), and double midpiece/double head (E7). (F) Bent tail (or hairpin tail), involving the mid-region of the principal piece (F1–F4) or with a singular bend (F5) or proximal bend

(F6) involving the midpiece-principal piece junction. (G) Coiled tail, with the tail tightly encircling the head (G1) or the tail coil not encircling the head (G2, G3). (H) Fragmented sperm, including head detachment (termed detached heads or tailless heads; H1) or fragmentation at the level of the annulus (H2). Fragmentation can also occur at other sites, as demonstrated in F4. (I) Premature germ cells with a single nucleus (I1) or multiple nuclei (I2).

1.2. How do the sperm move?

Sperm leaving the testes are immotile and acquire motility throughout the epididymis journey. Sperm is virtually devoid of transcription and translation due to highly condensed DNA and lack of endoplasmic reticulum (Baldi et al., 2002). The interaction between sperm and the environment created by the epididymis and the female reproductive tract is essential to trigger sperm motility. To be functional, sperm cells must undergo (i) maturation in the epididymis, (ii) capacitation, and (iii) acrosome reaction in the female reproductive system (Abou-haila et al., 2009). These events are co-dependent since acrosome reaction does not occur if capacitation is impaired and capacitation depends on functional maturation of sperm in the epididymis. Motility acquisition is essential for sperm function and ultimately male fertility. In 2011, Paoli defined sperm motility as propagation of transverse waves along the flagellum in a proximal-distal direction producing an impulse that pushes the spermatozoon through the female genital tract (Paoli et al., 20011).

1.2.1. Sperm transport

Whether introduced via natural mating into the vagina or artificial insemination into the body of the uterus, the journey of the sperm in the female tract is long and tortuous. The female reproductive tract is filled with viscous fluid, dead ends and potentially hostile immune cells, such as neutrophils as well as Immunoglobulins (Miller, 2018). Movement is achieved through a combination of the midpiece, which contains spirally arranged mitochondria that produce the energy required for sperm movement, and the flagellum that propagates the propulsive waves initiated at the midpiece (Mortimer, 2000). The direction of travel of the sperm is controlled by a combination of chemotaxis (sperm travel in the direction of secretions from the oocyte e.g. amino acids, peptides, lipids), thermotaxis (sperm travel towards a warmer environment as there is a temperature gradient between the isthmus and the ampulla) and rheotaxis (sperm travel against the flow of mucous in the uterus) (Taymour et al, 2014). The sperm provide most of the motility

required to reach the site of fertilization, but are also helped by the active motions of organs within the female reproductive tract, and inanimate objects can reach the isthmus, (Taymour et al, 2014),

One of the most intriguing aspects of spermatozoon motility is the way in which its detailed form can change to meet the demands imposed by synchronising the arrival of fully capacitated spermatozoa with mature oocytes in the ampullae of the Fallopian tubes in preparation for fertilisation. In all Eutherian mammals, such synchronisation is achieved by creating a post insemination reservoir of spermatozoa in the isthmic region of the oviduct (Hunter et al., 2012). In this location, the spermatozoa remain in a quiescent state until a signal associated with ovulation induces their sudden activation and release. When spermatozoa are released from the isthmic store, their motility changes from the small amplitude symmetrical flagellar beat pattern characteristic of freshly ejaculated spermatozoa to large amplitude, high frequency asymmetrical 'hyperactivated' beat pattern that is capable of generating the kind of propulsive forces needed to break away from the isthmic epithelium and penetrate the investments surrounding the egg. Hyperactivated movement is thus an extremely important aspect of spermatozoon function without which fertilisation cannot occur (Bedfort, 1983).

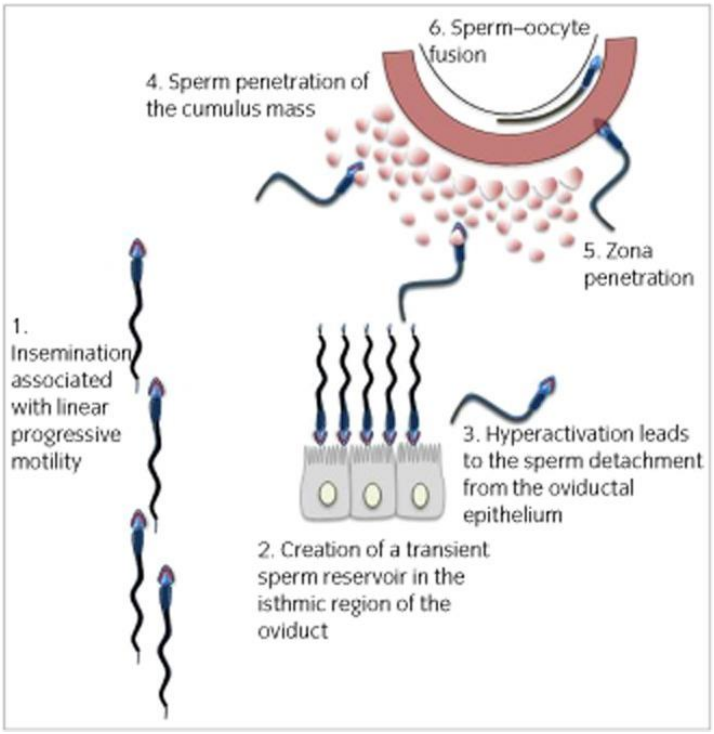

Figure 7: The key components of spermatozoal function in the stallion (Varner, 2014).

At insemination spermatozoa are inseminated into the female reproductive tract and immediately express a vigorous pattern of progressive motility that carries them through the intramural portion of the Fallopian tube and into the isthmic region. 2) In this location, the spermatozoa establish intimate contact with the epithelium and enter a state of relative quiescence forming a transient post insemination sperm store. 3) Signals associated with ovulation lead to the activation of hyperactivated motility in these spermatozoa causing them to break away from the oviductal epithelium and transit towards the ampulla of the Fallopian tube where fertilisation will occur. 4) As hyperactivated spermatozoa approach the oocyte, they respond to the high concentration of progesterone in the immediate vicinity of this cell by initiating the acrosome reaction. The latter may be driven to completion before the spermatozoa reach the zona pellucida and/or may be completed on spermatozoal binding to the zona pellucida. 5) The acrosome reacted spermatozoa then penetrate through the zona pellucida and enter the perivitelline space. 6) In this location, the plasma membrane overlying the equatorial segment of the fertilising spermatozoon binds to the vitelline membrane of the oocyte and initiates fusion.

1.3. Breeding soundness examination and semen analysis.

Breeding Soundness Examination (BSE) of stallions for the purpose of prospective identification of fertile and subfertile individuals was formally introduced in 1975 (Ball, 2008). Criteria for classification of a stallion as a satisfactory prospective breeder for rendering pregnant at least 75% of 40 or more mares bred naturally or 120 mares bred artificially (considered a full book of mares at that time) included that the stallion demonstrates normal libido, have a normal penis, have no evidence of a reproductive tract infection, have normal testes and produce a minimum of one billion progressively motile, morphologically normal sperm in the second of two ejaculates collected 1 h apart (Ball, 2008). It is very important that measurements must be accurate and reproducible. At the same time, the techniques used for assessment must be objective, standardized and sensitive to recognize deviations from the normal ranges (Mortimer, 1994).

When the traditional BSE was first outlined, sperm motility was estimated visually, sperm morphology was examined using either light microscopy on stained semen smears, or phase-contrast microscopy on unstained, fixed semen samples, and concentration of sperm in the ejaculate was most often estimated based on the transmittance of light through raw semen as determined by a spectrophotometer (“Standard” semen analysis techniques). Since then, technological improvements including CASA, differential interference contrast microscopy for evaluation of sperm morphology, plasma membrane intactness, acrosome function, DNA integrity, fluorescent markers in combination with fluorescence microscopy, flow cytometry or genetic markers. These modern techniques have the potential to discriminate different levels of fertility in a group of stallions, as well as to determine the causes of unexplained subfertility/infertility. Other techniques, such as mitochondrial function and ROS production are particularly useful when

different protocols for sperm storage are evaluated. Those methods rely on the assessment between different sperm organelles and functions, which are needed by the spermatozoa during its travel through the male and female reproductive tract, the subsequent oocyte fertilization and early embryo development.

1.3.1. Ultrasonography of reproductive tract

According to The Society of Theriogenology, the recommended protocol for assessing stallion fertility should include an ultrasound examination of the reproductive tract associated with the semen analysis (Ball, 2008). The testicular volume and the daily Sperm output are calculated using ultrasonography. Two ultrasonographic modes are widely used in the assessment of the male reproductive tract.

Firstly, B-mode ultrasonography of the reproductive tract (Little and Woods, 1987; Weber and Woods, 1992; Pozor, 2005; Kumari et al., 2016), permits the visualization and evaluation of the anatomic relationship, physical dimensions and the acoustic characteristics of the stallion accessory sex glands represented by the seminal vesicle, prostate, bulbourethral glands and deferent duct ampulla. 95% of total semen volume in the stallion is secreted by the sexual glands (Pickett et al., 1983). The B-mode ultrasonography is used in the diagnostic of several pathological conditions of prostate, vesicular gland, ductus deferens ampullae, testis...etc. that can affect semen quality and fertility.

On the other hand, pulsed-Doppler ultrasonography recently has been used to assess the vascular perfusion of the testicular artery (Figure 8) and has been demonstrated to be a good early indicator of sperm dysfunction (Ortiz-Rodriguez et al., 2017). Resistive Index (RI), Pulsatility Index (PI), Peak Systolic Velocity (PSV), End Diastolic Velocity (EDV), Time Average Maximum Velocity (TAMV), Total Arterial Blood Flow (TABF) and TABF rate are the main evaluated parameters. The capsular artery is the most reliable location to carry out spectral Doppler assessment and blood flow parameters of this artery, showing an important correlation with sperm quality parameters in the stallion. Nevertheless, a significant difference in all the Doppler parameters were observed between fertile and sub-fertile stallions. The principal components analysis assay determined that fertile stallions are characterized by high EDV, TAMV, TABF and TABF rate values (high vascular perfusion). In contrast, sub-fertile stallions tend to present high values of PI and RI (high vascular resistance). The testicular artery revealed that the best Doppler

parameters to predict sperm quality in stallions were: Doppler velocities (PSV, EDV and TAMV), the diameter of the capsular artery and TABF parameters (tissue perfusion parameters) (Ortiz-Rodriguez et al., 2017).

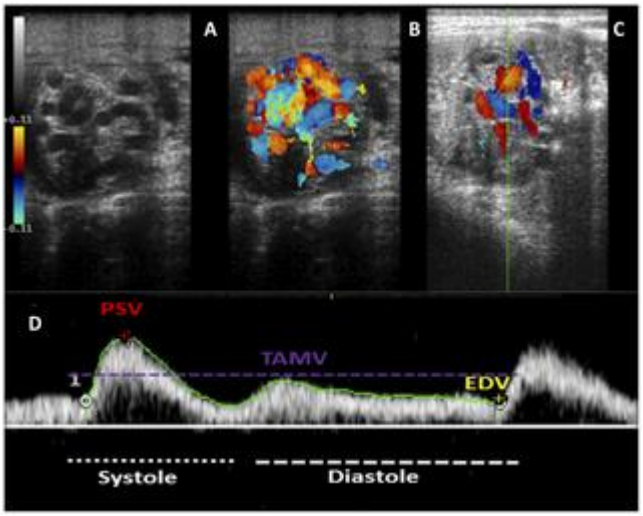

Figure 8: Ultrasonography of spermatic cord (Ortiz-Rodriguez et al., 2018)

B-Mode ultrasound (grey scale); B. Colour Doppler ultrasound of the spermatic cord´s vessels; C. Pulse Doppler ultrasound of the supratesticular artery within the spermatic cord. D. Display of the equipment used to measure a cardiac cycle using pulse Doppler. Three Doppler velocities calculated by the ultrasound equipment's algorithm package

1.3.2. Computer Assisted Semen Analysis of motility (CASA-Mot)

Sperm motility is evaluated manually and subjectively or through a CASA-Mot system (Figure 9). The subjective and conventional evaluation of sperm motility is simple and cheap, but measurements may not be accurate and vary between technicians because of differences in skill, training and experience. In effect, variations of 30–60% have been reported when motility parameters of the same ejaculates are evaluated subjectively (Verstegen et al., 2002). In this context, the introduction of CASA-Mot systems was in the 1980s, more than four decades ago providing a more objective means to evaluate sperm motility (Mortimer et al., 2015). CASA and visual measures of percent motility usually differ because their definitions are not identical. In visual measures, a spermatozoon is usually considered to be motile if its flagellum is twitching even though it may not exhibit forward progression. In CASA, a spermatozoon must achieve a minimum VSL to be motile (Davis and Katz, 1996). Budworth et al., (1987) and Farrell et al.

(1997) set a threshold level of velocity VAP for sperm to be considered motile at ≥ 20 μm/s. CASA has advanced significantly during the past two decades due to major innovations in science and bioengineering, as well as development in software algorithms and increased computational power. This revolutionary effort between industry and science helped establish CASA as reliable and objective research and diagnostic instrument in the medical, veterinary, laboratory animal and wildlife fields (Holt et al., 2007).

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 et al., 2013) and to find differences between males or treatments and interactions inside one experiment (Verstegen et al., 2002).

A computer-aided sperm analysis (CASA) system consists of a phase-contrast microscope, a video camera and a computer (hardware) with specific software (Figure 9). Initially conceived to evaluate human spermatozoa in IVF clinics, the Hamilton Thorne company designed a CASA-Mot system for stallion spermatozoa in 1986, which was subsequently adapted to other animal species (Amann and Katz, 2004). Currently, there are about 12 CASA-Mot commercial systems on the market. Most of these systems are based on the identification of sperm head centroid position (x1, y1) in the first image of the sequence that must be linked with the new (x2, y2) position in the second one, continuing in the same way until the last image (Bompart et al., 2018).

From the early stages in the development of CASA-Mot technology, the accuracy in measurements for some kinematics variables was directly related to the FR used, represented by the number of images taken during a defined time (Mortimer and Swan, 1995; Mortimer, 1999, 2000), because some variables, particularly VCL, have marked changes between relatively lesser and greater FR (Contri et al., 2010; Castellini et al., 2011; Gallego et al., 2013; Caldeira et al., 2019; Valverde et al., 2019). Until recently, the ability of these machines to correctly analyze a semen sample and in turn predict fertility was poor and subject to human error. The most recent CASA machines are still not 100 % objective because of the bias in operators setting limits on what is considered motile, and what is considered debris.

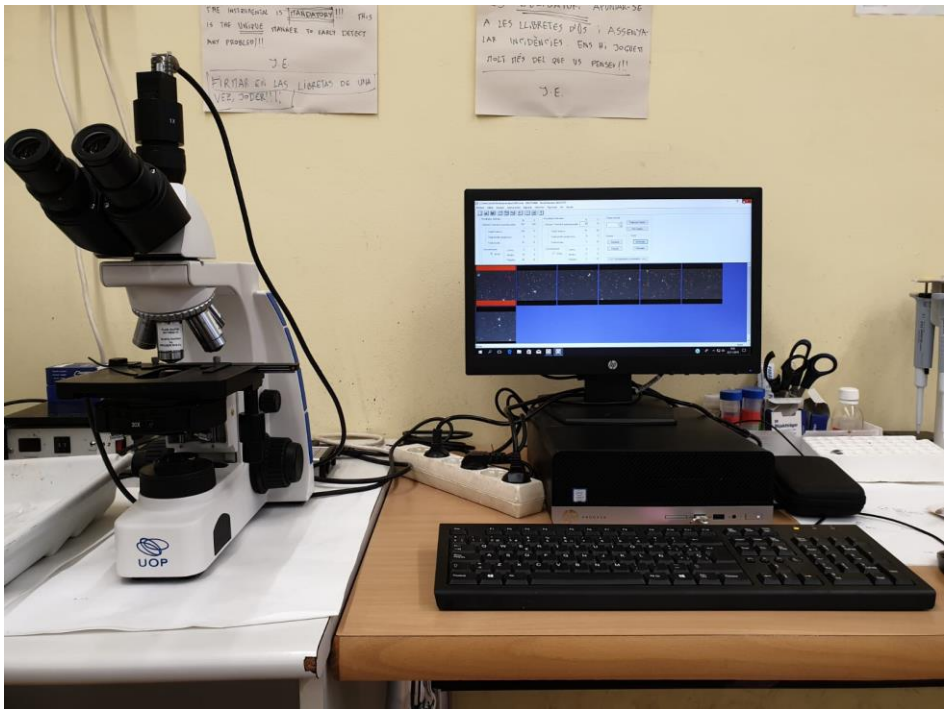

Figure 9: Views of computer-assisted semen analysis (CASA) system.

1.3.2.1. Computer semen analysis and fertility

Semen quality has been considered as one of the major parameters in a male´s reproductive health, even if pregnancy rate and birth respectively are the real indicator of semen fertility of stallion and may be influenced by different independents factors as mare fertility and reproductive technique used (Colebrander and Col., 2003; Samper and Estrada, 2007). Semen parameters could explain only 30% of stallion fertility (Quintero-Montero and Col., 2003) that could represent a few spermatozoids for the whole ejaculate and few sperm structure parts (Wilhem and Col., 1996a; Colebrander and Col., 2003; Kirk and Col., 2005; Samper and Estrada, 2005). Nevertheless, how fertile a sperm population is cannot be determined precisely and only a probability analysis can be done to assess a male's fertility status. In this regard, it is necessary to a) determine the values for certain semen/sperm parameters of fertile males, b) define the normal ranges of these values and c) statistically compare the observed values of a semen sample of unknown fertility with the normal ranges reported for fertile males (Mortimer, 1994).

1.3.2.2. Technical conditions and limitations of CASA technology

The final results can be influenced by many parameters unrelated to the semen samples factors (Ehlers et al, 2011), such as dilution of the semen sample, the 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 and dos Santos, 2012), number of analysed fields (Broekhuijse et al., 2011), recording frame rate (Wilson-Leedy and Ingermann, 2007; Castellini et al., 2011), staining technique for morphological evaluation (Soler et al., 2005), and type of CASA system used (Jasko et al., 1990; Boryshpolets et al., 2013).

There are disadvantages to the CASA system, but many of them are easily overcome by appropriate parameterization of the machine (Broekhuijse et al., 2011; Amann and Waberski, 2014; Simonik et al., 2015). Below is a list presented by Davis and Katz (1996) of factors that can affect CASA results:

- Instrument precision
- Instrument accuracy
- Microscope
- Counting chamber
- Diluents or extender
- Video framing (or digitization) rate
- Physiological state of sperm
- Temperature effect on VCL and motility
- Specimen concentration
- Presence of debris in the sample
- Instrument parameter settings
- Digitization threshold (grey scale)
- Number of points per track
- Number of frames tracked
- Number of fields (or sperm) analysed
- The field analysed
- Computational algorithms for average path and ALH
- Statistical methods

- Laboratory supplies
- Videotaping (inter-tech variation)
- Between-aliquot variation

Many of these can be overcome by simple solutions such as, using pre-warmed slides from the same company, maintaining an average number of cells viewed per frame, setting the minimum and maximum parameters on the machine for clarity, and eliminating cells that aren't alive, moving, or swim off the screen as soon as the analysis starts.

Attention must be devoted to the counting chamber used. The availability of different slide choices on the market 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 the 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, and Li, 2015), ram (Palacín et al., 2013), and stallion (Hoogewijs et al., 2012).

Attending the CASA system design, 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 and Ingermann, 2007), number of consecutive frames analysed, 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 et al., 2006), as well as to define the different morphological components of the cell (Yániz et al., 2016).

Another key factor is the frame rate (FR), and it was conditioned for a long time by the available video cameras. At the begging of the technology, low frames (16 or 25 frames per second) were used. Recently, particularly for human samples an acquisition frequency rate of 50–60 Hz has been recommended (Morris et al., 1996). Nevertheless, when cells display high speed and low linearity, such as the hyperactivated sperm, an increased frame rate is recommended (Mortimer and Swan, 1999). Furthermore, CASA systems can provide high accuracy and repeatability (Davis et al., 1992; Farrell et al., 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. All these factors claim a well-defined inclusive protocol for the standardization of results output, being one of the central objectives of the present work.

1.3.3. Automated sperm morphology analysis (ASMA)

When the traditional BSE was first outlined, sperm morphology was examined using either light microscopy on stained semen smears, or phase-contrast microscopy on unstained, fixed semen samples ("Standard" semen analysis techniques)(Gago et al., 1998; Banaszewska, Czubaszek, et al., 2015; Łacka et al., 2016). Since then, automated methods were introduced and include the use of freely available user-developed plug-ins for image (e.g., the ASMA plugin described within Butts et al., 2011) or flow cytometry, which can be done either conventionally (Gillan et al., 2005) or through a combination of conventional methods with fluorescent microscopy to analyse each cell within a population (Basiji et al., 2007; Blasi et al., 2016). Moreover, computer-aided sperm analysis systems CASA (Steigerwald and Krause, 2009); see also computer-assisted sperm morphometric analysis, referred to as CASMA (World Health Organization, 2010) or CASA-Morph (Maroto-Morales et al., 2016) or ASMA (Sancho et al., 1998), can estimate sperm morphology while tracking cell movement (Luther et al., 2020). This latter option allows for quantifying the morphology of the sperm head and tail (World Health Organization, 2010), with a bias toward measuring spherical sperm heads rather than heads with more irregular shapes (Santiago-Moreno et al., 2016). Dependent on the CASA system, quantitative morphometric parameters that can be measured include the head length, head width, head area, head ellipticity, head elongation, or head regularity, and thus are useful in defining normal sperm for different species (Brito, 2007; Yániz et al., 2015; Van Der Horst et., 2018; Maree and Du Plessis, 2018).

The percentage of morpho-abnormalities present in an ejaculate has shown to be an important indicator of fertility decline in various animal species (Bielanski et al., 1979, 1982; Hurtgen et al., 1982; Jasko et al., 1990; Hellander et al., 1991; Casey et al., 1997). However, the use of subjective methods previously discussed makes their interpretation difficult. Sperm morpho-analysis requires the introduction of objective and precise process that reduce intra- and inter-observer differences as well to achieve reliable and reproducible results between laboratories (Gravance et al., 1996; Ehlers et al., 2011). In order to improve the morphological evaluation of the sperm cells were developed in the 1980s the first systems semi-automatic for image analysis

of sperm morphology (Jagoe et al., 1987). It took until the decade of the 90s for the arrival of the current systems called ASMA (Automatic Semen Morphology Analysis) or CASMA (Computer Automatic Semen Morphology Analysis). This technology, originally validated for the human sperm (Davis et al., 1992 ; Kruger et al., 1993), has been progressively adapted to some animal species such as the horse (Hidalgo et al., 2008), the dog (Verstegen et al., 2002) or the ram (Sancho et al., 1998), among others. With this methodology, the seminal samples, previously fixed and stained, can be analysed individually to determine the morphometric characteristics of the sperm head in terms of size (area, perimeter, length and width) and shape (ellipticity, elongation, regularity and roughness). In addition, it provides data on the proportion of the head that belongs to the acrosome. Some systems even offer information on the intermediate piece (area and width) and data regarding the insertion of the intermediate piece on the head, such as the distance and angle of the insertion (Gil et al., 2009).

In general, these systems consist of a microscope that, via a built-in camera, transmits images from pre-stained sperm samples, onto a capture card located on the computer. The digitized microscopic image is processed by the analysis software, reporting individually, the morphometric characteristics of the captured sperm (Hidalgo et al., 2005). Unlike the variability inherent in subjective analysis (Manet et al., 2000), this technology has made it possible to quantify precisely and repeatable morphometric characteristics of the head and mid-piece of the sperm (Davis et al., 1993a).

The classical techniques involve different fixation, air drying and staining procedures changing the morphology of the examined cells. In fact, the drying and staining of the samples produce what is known as artifacts, these are the consequence of morphological and morphometric changes in the cells. For example, the reduction of the cell area with respect to that observed in fresh semen (Katz et al., 1986). On the other hand, immature sperm can undergo a drastic expansion during the staining process (Soler et al., 2000). Likewise, the loss or elimination of cytoplasmic droplets can be caused, since they are osmotically sensitive (Abraham-Peskir et al., 2002). Also, the use of alcohols (such as ethanol or methanol) can cause clumping and dehydration of cells. It should be noted that not every morphological artifact is subjectively appreciable, while in parallel, a morphometric modification can occur, only evaluable by computer systems.

1.3.3.1. Trumorph® technique

Trumorph® is a new technique for the morphological observation of living spermatozoa (Figure 10), independent of drying, fixing and staining artifacts, allows a clear definition of all sperm characteristics. This supposes the non-formation of artifacts caused by their staining (Soler et al., 2016). The classical techniques involve different fixation, air drying and staining procedures, changing the morphology of the examined cells. This new technique is based on the examination of wet preparations of immobilized sperm after a heat shock in narrow chambers (Soler et al., 2016). The technique includes a system to immobilize the cells, by short temperature shock, and to force the fluid to expand in a very thin layer. An increase in both temperature and pressure could in some way modify sperm morphology, but the work done to date indicates that there are no alterations to morphology created using this technique. Live sperm analysis not only reflects their native form but also allows us to more truly observe the functions they only exhibit in their living environment. (Soler et al., 2016). It is a simple technique that provides ease of preparation that gives it robustness applicable for the analysis of live sperm.

Present works using the Trumorph® technique permitted the quantification of different types of sperm morphologies. However, other works should be developed on the morphometric analysis of the samples, including all the parts, the head (acrosome border, acrosome size, insertion disc, etc.), midpiece (cytoplasmic rests, shape, length, etc.), and principal piece of the tail (length, shape, etc.). All this information must be organized in future classification criteria to be useful in the everyday routine analysis of the fertility of semen samples.

Figure 10: Trumorph® system for morphology analysis (Proiser R+D, S.L., Paterna, España)

1.3.4. Sperm subpopulation concept

The spermatozoon is a dynamic cell: its biochemical processes modify the sperm physiology throughout maturation, ejaculation, transport through the female genital tract and fertilization (Chamberland et al., 2001). These physiological changes are related to flagellar beating; thus, spermatozoa show different swimming patterns in the epididymis, seminal plasma, cervical mucus and oviduct (Hamamah and Gatti, 1998; Tash and Bracho, 1998). Moreover, sperm samples are heterogeneous, implying that spermatozoa with different motility values coexist in the same ejaculate (Katz et al., 1979; Katz and Davis, 1987; Chantler et al., 2004). Also, the morphology is heterogeneous in different levels depending on the species: humans have the most heterogeneous sperm among mammals (Yániz et al., 2015). The historical view has conceived the ejaculate as a conjunct of "equivalent" cells competing for arrival to the oocyte. However, this conceptual approach is in contradiction to the observed heterogeneity. To solve this, during the

21st century, the use of quantitative sperm parameters obtained with CASA technology and multivariate analysis was implemented and the paradigm has shifted to a subpopulation approach of semen (Quintero-Moreno et al., 2003). The recent approach of computing a group of variables by using multivariate statistics that includes principal components and cluster analysis (subpopulations) offers a new comprehension of what is a semen picture and how it is associated with male fertility (Soler et al., 2016; Valverde et al., 2016; Yániz et al., 2016). In horse four sperm motility subpopulations (SP) were identified analysing statistically the CASA parameters by hierarchical clustering (Quintero-Moreno et al., 2003). However, in another study by (Ortega-Ferrusola et al., 2009) using a simple clustering statistical analysis this time, six sperm subpopulations were identified in fresh semen (SP1–SP6). Subpopulations SP1 and SP2 were characterized by low sperm velocities, subpopulations SP3 and S4 corresponded to spermatozoa depicting medium speed values, and finally, subpopulations SP5 and SP6 were those depicting the highest velocities. In donkey it was identified four subpopulations as described by (Miró et al., 2005): SP1 (31.8%) progressive, SP2 (20%) nonlinear trajectory, low VAP and low progressiveness, SP3 (16.8)was the rapid ones with low LIN, SP4 (30.8%) have straight trajectories while low VAP and ALH. These subpopulation frequencies can be used for further statistical analysis, such as regression, to relate those subpopulations to other sperm traits (Quintero-Moreno et al., 2007).

However, it has been recommended that CASA used should be accurate for reliable analysis. The optimization of new methodological approaches, that were commented on previously, suggests that the previous work developed on this topic must be reconsidered. The combination of clearly acquired image-sequences and sophisticated image processing, also a non-staining technique for morphometry allows a reliable and better-quality dataset. This enables a better establishment of real and significant subpopulation structures within and between species (Martínez-Pastor et al., 2011).

Aims and objectives

The approach advocated in this thesis is based on the fact that there is a gap of information regarding optimization of breeding soundness examination along with semen analysis protocols in equids as seen in the previous chapter.

Thus, we hypothesized that:

1. The use of ultrasound analysis in the BSE associated to doppler analysis of testicular artery indices may correlate with sperm analysis (motility, viability, morpho-abnormalities) and can be associated to fertility in donkey.
2. The assessment of sperm motility within CASA using hight frames and different chambers types permit having more information of sperm trajectory and complete insight the results, thus changing the sperm distribution within subpopulation in donkey and stallion.
3. Trumorph® technique do not alter the different sperm parts shape and may provide real sperm morphology compared to staining technique in donkey and stallion.

Considering this hypothesis, the following specific objectives were implemented:

1. Use b-mode and pulsed-Doppler ultrasonography for reproductive evaluation and determine their correlation with semen quality in the donkey.

2. Evaluate the effect of image capture frequency on the evaluation of kinematic parameters in stallion and donkey spermatozoa for the optimization of the analysis protocol by a CASA system with a high-resolution camera.

3. Analyse the differential sperm distribution and the characteristics of mobility and kinetics along with three commercial counting cameras (D4C®, Leja®, and Spermtrack®), that have different loading techniques, depth, and design, intending to optimize the use of currently available. Also, the counting area inside each chamber was considered.

4. Analyse the effect of sperm dilution on the sperm kinematic parameters using the CASA system.

5. Evaluate mobility and kinetics, using a CASA-Mot system, in different stallion breeds (Arabian pure breed and Spanish horse) and their differences with the donkey, to define whether the prolonged artificial selection in this species has led to a significant process of gametic differentiation between breeds.

6. Collaborate in the definition and establishment of knowledge of the meaning of sperm morphology with the use of CASA-Morph technology.

7. Comparing two techniques for sperm morphology analysis, one using dead stained sperms and the second, using alive immobilized sperms.

2.2. Overview of thesis parts

The present thesis is divided into four main chapters, including an introduction, objectives and research articles. Three articles are published and two under a review, all were presented as manuscripts. A general discussion and conclusion and a complete reference list are included at the end of the thesis.

Chapter 1: *Introduction*

An overview of the literature related to sperm production, movement and morphology were described. The different exams for breeding soundness examinations were highlighted.

Chapter 2: *Aim and objectives*

Chapter 3: *Manuscripts*

Examination of a jackass (Equus asinus) accessory sex glands by B-mode ultrasound and of testicular artery blood flow by colour pulsed-wave Doppler ultrasound: Correlations with semen production.

The measures and aspects of accessory sex glands in donkeys are reported, also the measurement of testicular artery blood flow. The results include the correlation between measurements of numerous sperm parameters (motility, viability), accessory sex gland measures, and the pulsed-

Doppler indices of the testicular artery. Possible relationships between the different parameters and sperm quality are discussed.

Optimal frame rate when there were stallion sperm motility evaluations and determinations for kinematic variables using CASA-Mot analysis in different counting chambers

The importance of using standardized analysis techniques and the selection of reliable motility parameters is highlighted. The optimum frame rate was calculated, and the effect of different chamber types, depth, and the field was detailed, and the results discussed.

Optimization of CASA-Mot Analysis of Donkey Sperm: Optimum Frame Rate and Values of Kinematic Variables for Different Counting Chamber and Fields

The same previous experimental design of stallion was used on a donkey. Additionally, it was studied the effect of sperm concentration on sperm motility.

A new approach of sperm motility subpopulation structure in stallion and donkey

Using the protocol defined previously (optimum frame rate, better camera and area) the CASA parameters were calculated and subpopulation using Cluster statistic were defined. We compared the sperm subpopulations distribution between Spanish and Arab stallion breed, also between the stallion and donkey species.

Equine sperm morphology analysis: alive vs dead stained sperms technique

A problem in evaluating the morphology and morphometry of sperm is the lack of standardization of the techniques. The method of staining and evaluating specimens can significantly affect the results of morphologic and morphometric measurements producing artifacts. Two techniques were compared one using staining and the other live sperms.

Atlas of sperm morpho-abnormalities using Trumorph® in equids:

Data from the previous four works were used for the creation of an atlas of equid sperm morpho-abnormalities.

***Chapter 4**: Discussion and Conclusion*

Considering the results of different manuscripts, a definition of ultrasonography measures for ASG in donkey and the use of pulsed Doppler ultrasonography for semen quality prediction. As well, a proposal was made regarding the new setting to be used in both donkey and horse for sperm motility and morphology analysis. Concluding remarks are made and future studies were suggested.

2.2. Research output

The following papers, conference proceedings and conference posters were generated during this study.

2.2.1. Publications

1. **Gazem, S**., Papas, M., Catalan, J., Bonilla, S., and Miró, J. (2018). Jackass accesory glands ultrasonography and testicular artery color doppler evaluation. Chinese donkey industry journals., 4, 216-219.

2. **Gacem, S**.; Papas, M.; Catalan, J.; Miró, J. Examination of jackass (Equus asinus) accessory sex glands by B-mode ultrasound and of testicular artery blood flow by colour pulsed-wave Doppler ultrasound: Correlations with semen production. Reprod. Domest. Anim. 2020, 55, 181–188.

3. **Sabrina Gacem**, Daznia Bompart, Anthony Valverde, Jaime Catalán, Jordi Miró, Carles Soler. Optimal frame rate when there were stallion sperm motility evaluations and determinations for kinematic variables using CASA-Mot analysis in different counting chambers. Animal Reproduction Science, 2020, 106-643.

4. **Sabrina Gacem**, Jaime Catalán, Anthony Valverde, Carles Soler and Jordi Miró. Optimization of CASA-Mot Analysis of Donkey Sperm: Optimum Frame Rate and Values of Kinematic Variables for Different Counting Chamber and Fields. *Animals*. 2020, 10, 1993.

2.2.2. Conference proceedings

1. Poster presentation at the European Society for Domestic Animal Reproduction (ESDAR)

(Cordoba, Spain, September 12-17, 2018) titled “Reproductive ultrasonography in Catalonian Donkey”.

2. Poster presentation at the 19th international congress on animal reproduction (ICAR) (Bologna, Italy, 27th June - 1st July 2021) titled “Effect of frame rate on stallion sperm motility evaluated by CASA-Mot system”.

3. Poster presentation at the 10th International Symposium on Equine Embryo Transfer and Technologies (ISEET) (Pisa, Italy, July 3-5, 2021). titled “Effect of two-chamber type with different depths on stallion sperm kinematic parameters”.

4. Poster presentation at the European Society for Domestic Animal Reproduction (ESDAR) (Thessaloniki, Greece, October 12-17, 2021) titled “Sperm subpopulation variability between stallion breeds”.

5. Poster presentation at the 12th International, 11th European and 32nd German Congress of Andrology (Münster, Germany, December 5–9, 2020) titled “Sperm motility subpopulation structure in stallion: breed differences”.

6. Poster presentation at the 12th International, 11th European and 32nd German Congress of Andrology (Münster, Germany, December 5–9, 2020) titled “. Equine sperm morphology analysis: alive vs dead stained sperms techniques”.

Manuscripts

1. *Examination of a jackass (Equus asinus) accessory sex glands by B-mode ultrasound and of testicular artery blood flow by colour pulsed-wave Doppler ultrasound: Correlations with semen production.*

2. *Optimal frame rate when there were stallion sperm motility evaluations and determinations for kinematic variables using CASA-Mot analysis in different counting chambers*

3. *Optimization of CASA-Mot Analysis of Donkey Sperm: Optimum Frame Rate and Values of Kinematic Variables for Different Counting Chamber and Fields*

4. *A new approach of sperm motility subpopulation structure in stallion and donkey*

5. *Equine sperm morphology analysis: alive vs dead stained sperms technique*

6. *Atlas of sperm morpho-abnormalities in equids using Trumotph®*

Manuscript 1

Examination of a jackass (Equus asinus) accessory sex glands by B-mode ultrasound and of testicular artery blood flow by colour pulsed-wave Doppler ultrasound: Correlations with semen production.

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ORIGINAL ARTICLE

Reproduction in Domestic Animals WILEY

Examination of jackass (*Equus asinus*) accessory sex glands by B-mode ultrasound and of testicular artery blood flow by colour pulsed-wave Doppler ultrasound: Correlations with semen production

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Abstract

The accessory sex glands play a major role in the production of seminal plasma, and testicular artery blood flow seems to strongly influence testicular function. However, very little ultrasound imaging of these organs has been undertaken in donkeys. The present work reports the results of such examinations in five jackasses along the year. The accessory glands were inspected by B-mode ultrasound while the testicular artery blood flow was assessed by colour pulsed-wave Doppler ultrasound. The testicular artery was examined at pampiniform plexus (PPT), supratesticular area (ST) and capsular artery (CA). Values were recorded for the total arterial blood flow (TABF), peak systolic velocity (PSV), end-diastolic velocity (EDV), resistive index (RI), pulsatility index (PI) and time average maximum velocity (TAMV). Semen was obtained and assessed for sperm concentration, viability, abnormalities and motility using a CASA system. The bulbourethral glands, prostate and ductus deferens ampullae were relatively larger than in the stallion. Bulbourethral glands and ampullae sizes were inversely correlated with sperm motility. An reduction in blood flow between the level the PPP and the CA was observed, helping to reduce testis temperature and oxygen pressure. Blood flow at the CA showed the strongest correlation with semen production. PI and RI were positively correlated with the CASA motility variable STR (p = .02, p = .06) and sperm viability (p = .01), while sperm concentration (p = .05) correlated inversely with PSV, EDV, TAMV and TABF. EDV also correlated negatively with the CASA variables VSL, LIN, STR and VAP ($p \leq .05$). PI and RI were also negatively correlated with testis length (p = .0093, p = −.0438).

KEYWORDS
donkey, jackass, semen, ultrasound imaging

1 | INTRODUCTION

The donkey originated in north-east Africa. The world donkey population in 2014 was approximately 43 million, with 42.6% of these animals in Asia, 38.7% in Africa, 17% in the Americas and 1.7% in Europe. In Europe, most donkeys are found in the continent's south and west (FAOSTAT, 2019). Sadly, however, the global donkey population is in decline, especially in China; almost half of its donkeys disappeared between 1998 and 2015, falling from 9.56 to 5.42 million (Wu, 2017). In Asia, Africa and Latin America, the species is

under new pressure as a source of meat and hide for the expanding Chinese market. In Europe, donkey numbers decreased by 80% in the 20th century, a consequence of the mechanization of agriculture (SAVE Foundation, 2013). Most breeds, like the Catalonian donkey, are endangered (Jordana & Folch, 1996). However, new uses for the species are also appearing, such as onotherapy, rural tourism, hobby and competition mules' production, milk production farms or large donkey farms in China to produce meat and skin.

Research into the genetics and reproduction of the donkey has increased over the last 20 years, but if this is to help us preserve endangered breeds or increase fertility in farming systems, a deeper understanding will be required. Ultrasound imaging can provide useful information on the male genital tract and can be used to detect abnormalities (Segabinazzi et al., 2018), including accessory glands affection or testicular dysfunction and subfertility disorders (Ortiz-Rodriguez et al., 2017). Indeed, it is routinely used to monitor the reproductive status of many species (Kahn, 1994; Ortega-Ferrusola, Gracia-Calvo, Ezquerra, & Pena, 2014). The accessory sex glands (ASG), that is, the vesicular glands (VG) (or seminal vesicles), prostate gland (PROS) and bulbourethral glands (BUG) - and in the jackass (i.e. male donkey) the ductus deferens ampullae (AMP) - are involved in the production of seminal plasma. In jackasses, all these glands can be easily examined via transrectal palpation and B-mode (or greyscale) ultrasound imaging (Gazem, Papas, Catalan, Bonilla, & Miro, 2018). The latter can provide information in real time on the anatomical and echogenic structure of the entire male reproductive system. In contrast, colour pulsed-wave Doppler ultrasound can be used to examine the vasculature of organs, including the testes. In stallions, this technique has been used to assess blood flow in the testicular artery, to diagnose testicular dysfunction and subfertility disorders, and to explore their relationship with semen quality (Ortiz-Rodriguez et al., 2017).

Despite these advantages, ultrasound has been little used, however, to study the jackass male reproductive tract. Contri et al. (2008) briefly examined the difference in ASG dimensions before and after ejaculation using B-mode ultrasound, while Kandiel and Shafey (2017) used the same to provide a detailed description of the glands' anatomy and echogenicity in animals of different age. In addition, Rota et al. (2018) used colour pulsed-wave Doppler ultrasound to examine the correlation between testicular artery blood flow and the attainment of puberty. The aim of the present work was to examine the entire reproductive tract of the male jackass, using B-mode ultrasound imaging to describe the ASG, and colour pulsed-wave Doppler ultrasound to record the blood flow to the testes. Correlations between sperm analysis and ASG/testicular blood flow variables were then sought.

2 | MATERIALS AND METHODS

2.1 | Animals

The experimental animals were five Catalonian donkeys in good body condition and of proven fertility, all between 3 and 9 years of age. All were maintained in individual paddocks at the experimental farm belonging to the Veterinary Faculty of the Autonomous University of Barcelona. All were fed grain forage, straw and hay; water was available ad libitum. These animals were all monthly examined, along 1 year, between 10:00 and 12:00 a.m. to avoid the effect of circadian rhythmicity on blood flow (Zaidi, Jurkovic, Campbell, Okokon, & Tan, 1995).

All the procedures on animal exploration and sample collection were approved by the Institutional Ethics Committee for Animal Experimentation.

2.2 | Testis measurements

Both testes were measured (length, width and height) in duplicate using a caliper as described by Pricking et al. (2017), and mean values were recorded.

2.3 | Ultrasound examinations

Ultrasound examinations were performed using a MyLab® Gamma (Esaote) device. The ASG and AMP were assessed transrectally using B-mode ultrasound, employing a 5 MHz linear transducer. Testicular artery blood flow was inspected using colour pulsed-wave Doppler ultrasound employing a 3 MHz transabdominal linear transducer.

The animals were restrained in a quiet stock room and subjected to examination without sedation. Prior to the testicular examinations, the animals were acquainted with the procedure, and the testes slowly palpated to prevent testicular retraction.

2.3.1 | Examination of the accessory sex glands

A gloved, lubricated hand was slowly inserted into the rectum to remove any faeces. The transducer was then introduced. The different ASG were localized, identified by their general appearance and contours, and the BUG height and length, PROS maximum dorso-ventral depth, VG depth and AMP diameter were then recorded.

2.3.2 | Examination of testicular artery blood flow

After covering the probe and the scrotal region with an acoustic gel, the transducer was positioned in contact with the scrotum over the spermatic cord. Blood flow in the testicular artery (previously identified by B-mode ultrasound) was visualized by colour pulsed-wave Doppler ultrasound at three locations: the proximal pampiniform plexus (PPP), the supratesticular area (ST) and capsular artery (CA) (Figure 1). For the PPP, the probe was located horizontally in a latero-medial plane, perpendicular to the spermatic cord and close to the abdomen. The ST was examined with the probe positioned vertically over the spermatic cord near the testis. The examination at the CA site was performed over the testis surface close to the tail of the

epididymis, with the probe in the dorso-ventral plane. When two or three arterial Doppler waveforms with a maximum Doppler shift were displayed, the image was frozen and saved for analysis. The blood flow variables recorded were peak systolic velocity (PSV, m/s), end-diastolic velocity (EDV, m/s), resistive index (RI) (PSV–EDV)/PSV, pulsatility index (PI) (PSV–EDV)/mean velocity, time average maximum velocity (TAMV) and total arterial blood flow (TABF). TABF was calculated for all animals as TABF = TAMV*π*r^2, where r is the diameter of the vessel. All measurements were obtained with an angle of insonation of <60° and with a gate setting of 1 mm (Ginther & Utt, 2004). All measurements were performed in triplet, and the mean was calculated by the software provided with the ultrasound unit.

2.4 | Semen collection and analysis

With the aid of an ovariectomized jenny, semen was collected immediately after the above tests using a Hannover artificial vagina (Minitüb GmbH) equipped with an in-line filter (Minitüb GmbH) to separate the gel fraction,

Immediately after collection, the gel-free semen was diluted 1:5 (v/v) with skimmed milk extender (24 mg/ml skimmed milk and 49 mg/ml glucose) kept at 37°C in a water bath.

Total sperm number and sperm concentration were determined using a Neubauer chamber. Sperm survival and total morphological abnormalities were determined by eosin–nigrosin staining (magnification: 1000×) as described by Bamba (1988), examining a minimum of 200 spermatozoa/sample.

Sperm motion characteristics were examined using an Integrated Sperm Analysis System V1.0 computer-assisted sperm analysis (CASA) system (Proiser SL). Samples were observed using a phase-contrast microscope equipped with a heated stage (37°C). Three fields per drop were analysed. Table 1 shows the CASA sperm motility descriptors and the cut-offs used.

2.5 | Statistical analysis

The normality of the data was examined using the Shapiro–Wilk test. A two-factor Tukey test was used to compare the mean colour pulsed-wave Doppler values recorded at the different testicular artery locations. Kendall correlations were determined for the colour pulsed-wave Doppler values collected and sperm characteristics. Significance was set at $p < .05$. All calculations were performed using JMP SAS software v.14.1.

3 | RESULTS

Transrectal B-mode ultrasound located the BUG caudo-cranially just after the anus. These glands appeared as a pair (right and left)

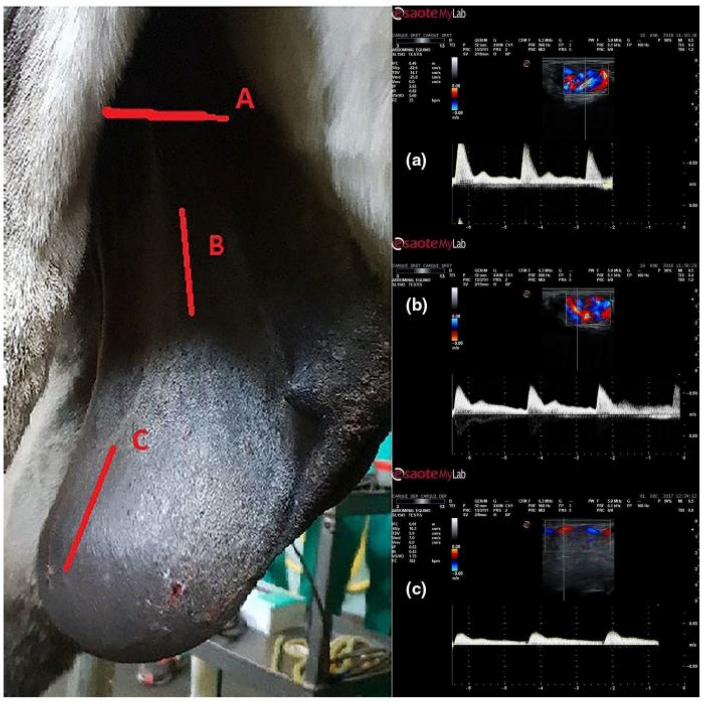

FIGURE 1 Locations where colour pulsed-wave Doppler examinations were made: A. proximal pampiniform plexus (PPP), B. supratesticular area (ST) and C. level of the capsular artery (CA) b. Values for the testicular artery blood flow variables fell between the CA and PPP

TABLE 1 Sperm motility descriptors and the CASA settings used

CASA motility variables	Abbreviation	Definition
Curvilinear velocity	VCL (μm/s)	Total distance the sperm moves in unit time
Straight line velocity	VSL (μm/s)	Distance the sperm moves along a straight line in unit time
Average path velocity	VAP (μm/s)	Mathematically smoothed path along which the sperm moves in unit time
Linearity	LIN (%)	Linearity of the average path
Straightness	STR (%)	Linearity of the average path VSL/VAP
Wobble	WOB (%)	A measure of oscillation of the actual path around the average path, VAP/VCL
Amplitude of lateral head	ALH (μm)	Magnitude of lateral displacement of a sperm head around its average path
Beat cross frequency	BCF (Hz)	Frequency with which the sperm trajectory crosses the average path trajectory
CASA settings		
Frame rate		25 frames/s
Minimum cell size		4 μm^2
Connectivity		6 μm
Magnification		20×
Temperature		37°C
Motile VAP cut-off		≥10 μm/s

and were elliptical in shape. The PROS was detected on the midline just caudal to the AMP and showed large anechogenic spaces. The VG appeared as thin paired glands, slightly caudal and lateral to the AMP; the VG are difficult to palpate because the adjacent AMP are large, and because before teasing they remain collapsed (Figure 2).

The AMP themselves were easily examined transrectally by palpation or ultrasound just cranially to the PROS. They are large in diameter and returned ultrasound images showing anechogenic areas. Table 2 shows the measurements taken of the AMP and other ASG; no significant difference was detected between the left and right sides for any ASG variables. On the other hand, no differences were found between months for ASG and AMP measures of each jackass.

Examining the testicular artery blood flow by colour pulsed-wave Doppler ultrasound was feasible but difficult (especially at the level of the CA) because the animals were not sedated. No difference was found between the left and right testes for any of the

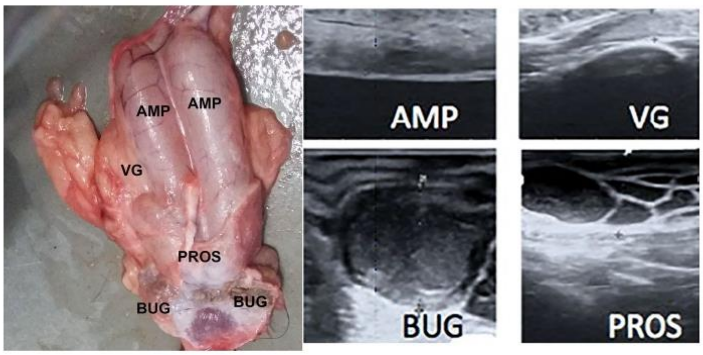

FIGURE 2 Appearance of the accessory sex glands of the jackass; visually and via B-mode ultrasonography. AMP, ductus deferens ampullae; BUG, bulbourethral gland; PROS, prostate gland; VG, vesicular gland

TABLE 2 Ultrasound measurements of the Catalonian donkey accessory sex glands

Accessory gland	Mean (mm) ± standard deviation
BUG L-L	
Left	37.23 ± 4.67
Right	38.7 ± 6.58
BUG D-V	
Left	17.15 ± 4.08
Right	16.73 ± 4.48
AMP (diam)	
Left	30.96 ± 8.08
Right	30.57 ± 10.13
VS (height)	
Left	7.33 ± 1.94
Right	7 ± 1.74
PROS (height)	28.51 ± 4.29

Abbreviations: AMP, ductus deferens ampullae; BUG, bulbourethral gland; D-V, dorso-ventral section; L-L, latero-lateral section; PROS, height of prostate; VS, vesicular gland.

variables examined. Table 3 shows the mean ± standard deviation for the blood flow variables monitored at the three different locations and highlights a significant reduction in PI, RI, PSV and TAMV towards the testis. The testicular artery blood flow showed no significant variations along the year.

The mean semen volume was 75.25 ± 20.49 ml. Sperm viability rates were high (81.6 ± 8.96%) and the abnormal sperms percentage is poor (12.77 ± 8.28%). Table 4 shows the sperm motion results returned by the CASA system.

Table 5 shows the correlation between the sperm variables and the ASG and testicular blood artery blood flow variables. Semen volume and BUG diameter were strongly and positively correlated (p = .02), while all CASA variables correlated inversely with the AMP and BUG dimensions. No correlation was seen between any sperm variable and any testicular artery blood flow variable at the level of the PPT, while at the level of the ST only the percentage of viable sperm correlated with EDV. However, PI and RI correlated strongly with the CASA motility variable STR (see Table 1) (p = .02 and p = .06, respectively) and sperm viability (p = .01), and sperm concentration (spermatozoa/ml) (p = .05) correlated inversely with PSV, EDV, TAMV and TABF. EDV also correlated inversely with the CASA motility variables VSL, LIN, STR and VAP (see Table 1) ($p \leq .05$). PI and RI showed a notably strong inverse correlation with testis length (p = .0093, p = −.0438).

No correlation was found between percentage semen abnormalities and testicular flow variables at any location.

4 | DISCUSSION

Ultrasonography provides an easy, non-invasive means of assessing the ASG and detecting different pathologies (de Souza, Barbosa, et al., 2015). However, to detect pathologies, normal reference ultrasound data must be available. Over the last two decades, there have been many studies that have provided ultrasound-based knowledge of the stallion reproductive tract, but this cannot be said for the jackass. The results of the present work provide clinicians much useful information in this respect.

In the present work, the different glands were more easily detected than earlier described for the jackass (Contri et al., 2008; Kandiel & Shafey, 2017), perhaps due to the relatively large size of the Catalonian donkey breed. The PROS, BUG and AMP were significantly larger in the present animals than in similar sized stallions (Pozor & McDonnell, 2002), but the VG were thinner with no visible lumen. On the other hand, PROS showed anechogenic spaces without sexual stimulation, while in stallion are not normal. The jackass AMP had a glandular appearance with anechogenic spaces, whereas in the stallion they are echogenically homogenous and thinner (Pozor & McDonnell, 2002). The AMP of the jackass may contribute significantly to seminal plasma production, resulting in jackasses ejaculating a larger volume of semen than stallions.

Contri et al. (2008), who examined the Martina Franca donkey, and Kandiel and Shafey (2017), who worked with a local Egyptian breed, reported smaller sizes for all the ASG (except for the VG) than those reported here. In contrast, a study of jackass ASG obtained from a slaughterhouse (Mai, 2013) returned slightly greater mean widths for the BUG and PROS, and slightly smaller values for the AMP and VG, than those reported here. The differences between these results may lie in different breeds having been examined.

TABLE 3 Testicular artery blood flow at the pampiniform plexus (PPP), in the supratesticular area (ST) and at the level of the capsular artery (CA)

	PI	RI	PSV (cm/s)	EDV (cm/s)	TAMV	TABF
PPP	2.87^{a} ± 0.34	0.87^{a} ± 0.03	72.43^{a} ± 11.17	8.24^{a} ± 1.57	22.59^{a} ± 2.52	0.52^{a} ± 0.06
ST	$2{,}36^{b}$ ± 0,36	$0{,}82^{b}$ ± 0,04	$56{,}51^{b}$ ± 14,06	$8{,}56^{a}$ ± 1,72	$20{,}4^{b}$ ± 3,93	0.55^{b} ± 0.06
CA	1.27^{c} ± 0.2	0.67^{c} ± 0.06	29.14^{c} ± 7.24	8.87^{a} ± 2.51	16.29^{c} ± 3.44	0.23^{c} ± 0.05

Note: Values are means ± *SD*. Different superscripts in the same column indicate significant differences (p < .05).

Abbreviations: EDV, end-diastolic velocity; PI, pulsatility index (PSV-EDV/TAMV); PSV, peak systolic velocity; RI, resistive index (PSV-EDV/PSV); TABF, total arterial blood flow (TAMV × A; A: πr^2); TAMV, time average maximum velocity.

TABLE 4 Results for CASA variables

CASA variables	Mean ± *SD*
VCL (μm/s)	118.59 ± 13.78
VSL (μm/s)	69.93 ± 14.05
VAP (μm/s)	92 ± 6.25
LIN %	7.99 ± 22.45
STR %	9.37 ± 25.93
WOB %	9.67 ± 26.78
ALH (μm)	3.75 ± 0.43
BCF (Hz)	9.74 ± 1.83

Note: Abbreviations as for Table 1.

The inverse correlation between the size of the BUG/AMP and the CASA variables might be explained in that seminal plasma—for the production of which the jackass has large glands—negatively affects sperm preservation and motility (Miró et al., 2009).

To our knowledge, this is the first study to compare testicular artery blood flow at the level of the PPP with that at other sites. It was, however, difficult to measure, even though the testes were not retracted during the examination; the capture of good colour pulsed-wave Doppler images demands is easier when an experienced and patient technician (working with a good ultrasound device) is available.

A progressive reduction was seen in the values of the blood flow variables towards the testis. This organ receives its arterial blood supply mainly via the testicular artery, with contributions from the vasal and cremasteric arteries. These arteries coil in the PPP before entering the testes, reducing the amount of blood that enters the organ, thus helping to reduce its temperature some 4-5°C below normal body temperature (Lloyd-Jones, Purohit, Boyle, & Shepherd, 2015) and to reduce the tissue oxygen pressure. As a result, the testis survives on the edge of hypoxia (Bergh, Collin, & Lissbrant, 2001)—perhaps to protect sperm DNA from the damage caused by oxygen radicals. Any disturbance in the blood flow could negatively affect spermatogenesis and therefore fertility (Ortiz-Rodriguez et al., 2017).

As already reported in stallions (Ortiz-Rodriguez et al.., 2017), bulls (Gloria et al., 2018) and dogs (Zelli, Troisi, Elad Ngonput, Cardinali, & Polisca, 2013), the values of the testicular artery blood flow variables were similar in the right and left testis. The present blood flow results are also comparable to those described in stallions (Ortiz-Rodriguez et al., 2017), bulls (Gloria et al., 2018), dogs (de Souza, Barbosa, et al., 2015) and camels (Kutzler, Tyson, Grimes, & Timm, 2011). Certainly, the RI and PI in the stallion are similar to those recorded here. However, the present PSV, EDV and TAMV values were twice those found in stallions and camels, perhaps because of the relatively larger volume of donkey testes and therefore their spermatogenetic requirements (Noronha, Neto, & Borelli, 2001). Rota et al. (2018) described the blood flow in the testicular artery of prepubertal jackasses and found a pronounced difference with the values recorded on their reaching puberty (24 months). The same has been reported in dogs (de Souza, Barbosa, et al., 2015). However, both these studies involved measurements being taken only at a single point.

Several authors report a correlation between testicular artery blood flow variables and semen quality in humans and certain animal species (Gloria et al., 2018; Ortiz-Rodriguez et al., 2017; Pinggera et al., 2008; Zelli et al., 2013). To the best of our knowledge, this is the first study to describe a correlation between sperm viability/semen concentration and Doppler-measured testicular artery blood flow variables in the jackass. As in the dog (Zelli et al., 2013) and stallion (Ortiz-Rodriguez et al., 2017), the strongest correlation with semen quality was recorded at the level of the CA. However, Bollwein, Scheibenzuber, Stolla, Echte, and Sieme (2006) found no correlations at all in the stallion, perhaps because of the location used to take the Doppler measurements or the technology used.

The RI at the level of the CA showed a notable inverse correlation with testis volume. Donkey testes are big, and the diameter of

TABLE 5 Kendal correlations τ ($p < .05$) between testicular artery blood flow variables and those of the accessory sex glands/sperm analysis variables at different points along the artery

		Blood flow variables						Glands	
		PI	RI	PSV	EDV	TAMV	TABF	BUG	AMP
PPP	STR	0.3186							
ST	% viability				0.5962				
CA	LIN				−0.3235			−0.3118	−0.472
	VSL				−0.4647			−0.3824	−0.3776
	VAP				−0.3471			−0.3118	
	STR	0.3658	0.3068		−0.3471				−0.3304
	sperm/ml			0.3103	0.3198	0.3048	0.3048	0.381	
	% viability			0.3501	0.4748	0.3732	0.3732		
	Testicular length	−0.3298	−0.4255						

Note: Abbreviations for CASA variables are as in Table 1.

Abbreviations: AMP, ductus deferens ampullae; BUG, bulbourethral gland; EDV, end-diastolic velocity; PI, pulsatility index (PSV-EDV/TAMV); PSV, peak systolic velocity; RI, resistive index (PSV-EDV/PSV); TABF, total arterial blood flow (TAMV × A; A: πr^2); TAMV, time average maximum velocity.

the testicular artery at the level of the CA is large, and there are branches running in the longitudinal plane towards the caudal pole of the testis.

No correlation was seen between any testicular artery blood flow variable and sperm abnormalities. Since spermatogenesis involves three major divisions, which take 57 days to complete in stallions, any disturbance in sperm production would not be noticeable until 2 months after it had occurred. Any disturbance in the blood flow could impair spermatogenesis and thus semen quality and quantity (Bergh et al., 2001).

In summary, the AMP and ASG (except for the VG) of the jackass are relatively larger than those of the stallion, with a particular ultrasonographic image for each gland. BUG and AMP sizes are inversely correlated with sperm motility, possibly related to these large organs' helping to produce large amounts of seminal plasma—which negatively affects sperm quality. Colour pulsed-wave Doppler ultrasound imaging showed an important reduction in testicular artery blood flow at the level of the PPA compared to that of the CA, which would result in a reduced testis temperature and tissue oxygen pressure, and influence sperm quality. These results help provide a better understanding of the ultrasound findings that might be expected for the reproductive system of healthy jackass males. They therefore provide a reference for the detection of dysfunction.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Jordi Miró designed the study, developed experiences, and drafted and reviewed the paper. Sabrina Gacem developed experiences, analysed the data and drafted the paper. Marion Papas developed experiences and analysed the data. Jaime Catalan developed experiences and analysed the data.

DATA AVAILABILITY

The data that support the findings of this study are available on request from the corresponding author.

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Manuscript 2

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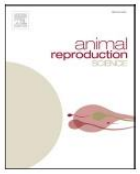

Optimal frame rate when there were stallion sperm motility evaluations and determinations for kinematic variables using CASA-Mot analysis in different counting chambers

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Field

ABSTRACT

This study was conducted to determine optimum image capture frame rates (FRO) when there was evaluation of different types of counting chambers used for CASA-Mot determinations of stallion sperm motility. Sperm VCL was determined at frame rates of 25–250 f/s in: 1) Sperm-track® (Spk) 10 and 20 chambers (drop displacement-type chambers 10 and 20 µm-deep respectively; and 2) ISAS®D4C10, ISAS®D4C20 (10 and 20 µm-deep respectively) and ISAS®D4C20 L (20 µm-deep) capillary loaded chambers. Values for different sperm kinematic variables were determined using each chamber at 250 f/s, which is the maximum frame rate that the software can be used for analyses. With evaluation of Spk chambers, there was a greater curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) values ($P < 0.05$) than with capillary loaded chambers, with there being greatest values with 20 µm-deep chambers. With the Spk10 chamber, VCL and ALH were greater at the chamber centre than periphery. There were no such differences for the Spk20 chamber. With evaluation of the D4C10 chamber, VSL and STR were less when there was a sperm deposition point towards the chamber end, while there were the opposite for the D4C20 chamber. When there was evaluation of the D4C20 chamber, there were also greater VCL, WOB and BCF values in distal areas. With use of most of these chambers, data should be collected from different fields and means determined, however, this is not necessary with Spk20 chambers.

1. Introduction

The motility of sperm collected for assisted reproduction/artificial insemination has been associated with fertility rates (Love, 2011). While lesser-than-optimal motility is a likely indicator of sub-optimal fertility, relatively greater sperm motility is not indicative

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of greater fertilising capacity (Varner, 2008). Fertilising capacity is also affected by sperm morphometry, acrosome integrity and DNA fragmentation as well as other factors (Yániz et al., 2018). Nonetheless, sperm motility, along with sperm concentration and morphology, are the most frequently used indicators of sperm quality (Katila, 2001).

The assessment of sperm motility was subjective until the advent of the CASA-Mot (Computer Assisted Semen Analysis for Motility) system, which is now widely used in clinical and research laboratories (Giaretta et al., 2017; Gallagher et al., 2018; Holt et al., 2018). This technology can be used to rapidly assess total and progressive motility, as well as determine values for other kinematic variables. Different software settings and image capture frame rates (FR), however, can be selected. Options are also available regarding the microscope optics used, the resolution of the video camera, the type of counting chamber utilised, and its depth. The lack of standardised settings for these factors makes it difficult to compare results among experiments (Mortimer, 2002; Holt et al., 2018; Bompart et al., 2018, 2019; Valverde et al., 2019c; Buchelly et al., 2020). Frame rate is the most important variable because it permits the reconstitution of the sperm trajectory, and therefore, the correct calculation of values for the different kinematic variables, particularly the VCL which is the most sensitive (Bompart et al., 2018). Mortimer (2002) recommended using >50 frames per second (f/s) when analysing mammalian sperm, however, there is very little previously published results to validate why this frame speed might be superior for sperm evaluations (Bompart et al., 2018). Current video cameras allow for frame rates as great as 500 f/s, and the optimal rates (FR_O) for use with different species are presently being determined (Castellini et al., 2011; Bompart et al., 2019; Caldeira et al., 2019; Valverde et al., 2019a, 2019b, 2019c, 2019d).

The type of counting chamber because of differences in design and depth also affects the kinematic results when using CAS-Mot (Lannou et al., 1992; Hoogewijs et al., 2012; Gloria et al., 2013; Del Gallego et al., 2017; Soler et al., 2018; Valverde et al., 2018; Basioura et al., 2019). Different chamber types are available, including reusable drop displacement-type chambers, and disposable capillary loaded-type chambers (Bompart et al., 2018). With use of the drop displacement chamber, the drop of semen is deposited in the centre than by the force of the coverslip weight, the drop will diffuse in the total chamber area. While in the capillary loaded chambers (longiline), the drop is deposited at the edge of the first field, then the contents of the drop spread longitudinally by capillarity. Certainly, the design of the capillary loaded-type chambers affects how sperm are distributed within the chamber, as well as the values for the kinematic variables that are recorded (Bompart et al., 2019). Sperm velocity is greater in 20 than 10 μm-deep chambers, irrespective of other factors (Lannou et al., 1992; Hoogewijs et al., 2012; Gloria et al., 2013; Caldeira et al., 2018; Soler et al., 2018; Valverde et al., 2018, 2019a).

The present study was conducted to determine the optimal image capture frame rate (FR_O) when there are assessments with different counting chamber types. The chambers were used for CASA-Mot determinations of stallion sperm motility, and there was determination of the values for sperm kinematic variables obtained with these chambers, and different fields within these chambers, when used at the FR_O.

2. Materials and methods

2.1. Semen collection

There was one ejaculate collected from each stallion ($n = 11$) in the present study. All stallions were housed in individual box stalls at the Equine Reproduction Service of the Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). This is an EU-approved semen collection centre (Authorisation code: ES09RS01E) that operates in ways consistent with the strict protocols for animal welfare and health control. All animals were free of equine arteritis, infectious anaemia and contagious metritis. Because the semen collection centre is operated using procedures approved by the Regional Government of Catalonia (Spain), and considering

Fig. 1. Images of counting chambers used for CASA-Mot analysis of stallion sperm in the present investigation; (1) disposable chamber D4C, (2) disposable chamber Leja chamber D4CL20, (3) Reusible chamber Spermtrack.

there was no imposing of procedures in the present study other than semen collection, no further ethical approval was required for conducting this study. All animals were fed oats and hay three times daily and water was provided ad libitum.

Semen was collected using a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-liner nylon filter to eliminate the gel fraction. Samples were immediately diluted 1:5 (v/v) in Kenney extender (Kenney, 1975), transferred into Corning 50 mL tubes, and transported in a hermetic box to the laboratory within 30 min. In the research laboratory, the tubes were placed in a water bath at 20 °C. The initial sperm concentration was determined using a Neubauer Chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany); counting was performed in triplicate using a phase contrast microscope (mag. × 200).

2.2. Counting chambers and determination of their respective optical frame rates

The counting chambers evaluated were: 1) reusable drop displacement 10 µm- and 20 µm-deep Spermtrack® (Spk10 and 20) chambers; and 2) disposable, capillary loaded 10 µm- and 20 µm-deep ISAS® D4C (subsequently D4C10 and 20) and 20 µm-deep ISAS® D4CL (subsequently D4CL20) chambers (all from Proiser R + D S.L., Paterna, Spain) (Fig. 1). The data collections were made from seven consecutive fields for disposable chambers and nine fields for reusable chambers (Fig. 2).

Samples in the Corning tubes were diluted to a final concentration of 50 × 10^6 spermatozoa/mL, maintained at 37 °C, and 2 and 3 µL of these samples were loaded into the different 10 and 20 µm-deep chambers, respectively. All chambers were maintained at 37 °C on a UB203 heated microscope stage throughout the time period evaluations were conducted to prevent heat shock of the sperm. When using the Spermtrack chambers, the coverslips were rapidly but gently placed to achieve a homogenous distribution of the sample and to avoid the formation of air bubbles. The other chambers were loaded by depositing the sperm sample in the loading area; the sperm then spreads across the surface by capillarity into the different areas of the chamber. Possible post-loading passive movement of the liquid in the chambers was allowed to cease by waiting for 15 s before making observations.

Video images were obtained during a 3 s period from different fields across the chambers (Fig.2), with an average of 500 sperm being evaluated per sample, using an ISAS v.1.2 CASA-Mot system (Proiser R + D S.L.) and a Proiser HS640 m digital camera (Proiser R + D S.L.) - capable of recording 500 f/s - attached to a UOP microscope (Proiser R + D) with a 10x negative phase contrast objective. The final resolution was 0.48 µm/pixel for both the x and y axes. Videos were segmented into FRs of 25, 50, 100, 150, 200, and 250 f/s using the sequence: echo off: set f/s = 25, 50, 75, 100, 150: for %%i in (.Ä*.avi) do (set fname = %%~ni) & call: encodeVideo; goto eof :encodeVideo: ffmpeg.exe -i %fname%.avi -r %f/s% - clibx264 -preset slow -qp 0 "%fname%_(%f/s%f/s).avi"; goto eof].

Optimal frame rates FR_O were determined from the curvilinear velocity (VCL, µm/s) of the spermatozoa using point-to-point reconstructions of the trajectories when there was assessment at each frame rate for which there were evaluations. Data were only used when there were evaluations of tracks for which there were collections at about 60 field locations (20 for second at 25 fps sequences). The results, therefore, were subjected to exponential regression analysis, in which $y = \beta.\alpha \exp(-\beta/x)$, where y is the VCL, x is the frame rate, α is the asymptotic level, β is the rate of increase to the asymptote; and the exp function is technically defined as the inverse of the natural logarithm, such that $\exp(x) = e^x$. The asymptotic value of VCL (VCLα) represents the VCL at which no substantial increase is achieved with increasing FR (i.e., at least 95 % of the maximum VCL has been achieved). The rate of approach to the asymptote represents the dependence of the curve on the FR, thus, a relatively greater β value indicates an increase in VCL with increasing FR and vice versa.

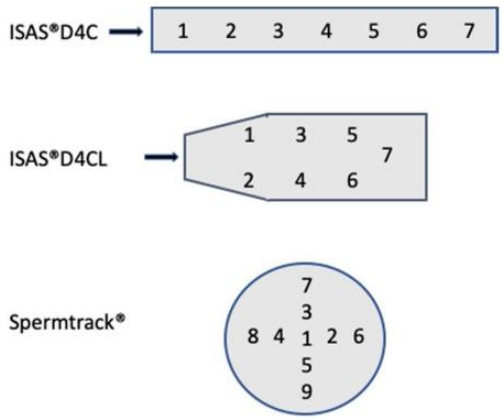

Fig. 2. Different counting chamber types used. Numbers indicate the position of the fields analyzed. Arrows indicate the location of sample deposition. In Spermtrack® semen was deposited in the middle of the circle and gently covered with the coverslip.

2.3. Determination of values for sperm kinematic variables in the different chambers when obtaining images at the closest possible frame rate to the optimal

Although the maximum image for determining FR of the camera was 500 f/s, there is no software that can be used to analyse the large amount of data generated when frame rates are greater than 250 f/s. Considering the FR_O for all the chambers evaluated was either very similar to 250 f/s or >250 f/s, all subsequent experiments were conducted at 250 f/s for each chamber.

Assessments were made of straight line velocity (VSL, μ/ms), determined using the straight line between the first and last point of the sperm trajectory; average path velocity (VAP, μm/s), i.e., the average velocity over the cell path; amplitude of lateral head displacement (ALH, μm), corresponding to the maximum of the measured width of the head oscillation during swimming; and beat-cross frequency (BCF, Hz), i.e., the frequency with which the actual trajectory crossed the smoothed trajectory in either direction. In addition, three indices were calculated: the linearity of forward progression (LIN = VSL/VCL·100), straightness (STR = VSL/VAP·100), and wobble (WOB = VAP/VCL·100). The CASA setting used was for a particle area between 4 and 75 μm^2; connectivity was set at of 6 μm. The advanced tail analysis setting was activated to optimise sperm selection.

2.4. Statistical analysis

Results are presented as mean ± standard deviation (SD). Data for all sperm variables were assessed for normality and homoscedasticity using the Shapiro-Wilks and Levene tests respectively. Normally distributed variables were analysed using an ANOVA followed by conducting a *post hoc* Tukey test for multiple comparisons. Sperm motility variables that were not normally distributed were analysed using the non-parametric Kruskal–Wallis test. When statistically significant differences were detected using the latter test, the non-parametric Mann–Whitney *U* test was used to compare pairs of values directly. The statistical model used was:

$$Xijk = \mu + Ai + Bj + AB\ (ij) + \varepsilon ijk$$

where X_{ijk} = the measured sperm motility variable, μ = the overall mean of variable x, A_i = the effect of depth, B_j = the effect of the counting chamber; $AB_{(ij)}$ = the effect of the interaction *depth*counting chamber*; and ε_{ijk} = the residual.

There were considered to mean differences when there was a $P < 0.05$. All calculations were performed using the IBM SPSS v.23.0 package for Windows (IBM Inc., Chicago, Il, USA).

3. Results

3.1. Optimal frame rates

The FR_O was calculated independently for each counting chamber. The FR_O was greater the deeper the chamber, and greater for the Spermtrack than for the other chambers (Table 1, Figs. 3 and 4). There was the greatest value (309.52 f/s) when there were evaluations using the Spk reusable chamber at the 20 μm depth and the least (248.81 f/s) for disposable chamber D4C10. Note that the FR_O for all chambers was similar or greater than the 250 f/s software limit.

3.2. Effect of chamber depth on values for sperm kinematic variables

The VCL and ALH were less in the 10 μm-deep chambers. The VSL was the same in both the Spk10 and 20 chambers. The LIN and STR were greater in the 10 than 20 μm-deep chambers irrespective of other factors. The BCF was the same in the two Spermtrack chambers, but slightly less in the D4C10 chamber than D4C20 chamber (Table 2). In the D4CL20 chamber, the VCL, VSL, VAP LIN and ALH were all less than in the D4C 20 chamber.

Table 1
Determining the optimal frame rate to be used for stallion sperm when there were evaluations using different counting chamber types and depths; One ejaculate from 11 stallions was analysed using each chamber; Curvilineal velocity of stallion sperm for the optimal frame rate (VCLα) and with different frames rates 25, 50,100,150, 200 and 250 were calculated based on α and β values.

Chamber	Depth	*n*	α*	SEα	β*	SEβ	VCLα	VCL25	VCL50	VCL100	VCL150	VCL200	VCL250
D4C													
	10	25429	248.8	0.9	20.7	0.3	228.9	108.6	164.3	202.2	216.7	224.3	229.0
	20	44887	282.5	0.9	27.2	0.3	256.5	95.1	163.9	215.2	235.6	246.6	253.4
D4CL													
	20	42845	268.0	0.8	22.9	0.3	246.0	107.1	169.5	213.1	230.1	239.0	244.5
Spk													
	10	46534	287.0	0.8	26.1	0.3	262.1	101.2	170.4	221.1	241.2	252.0	258.6
	20	92536	309.5	0.7	31.2	0.2	279.8	88.7	165.7	226.4	251.3	264.8	258.6

n: total number of spermatozoa analysed; VCL = curvilinear velocity (μm/s); α = asymptotic value corresponding to optimal frame rate; β = rate of increase; SE = standard error; VCLα = VCL at asymptotic frame rate value.

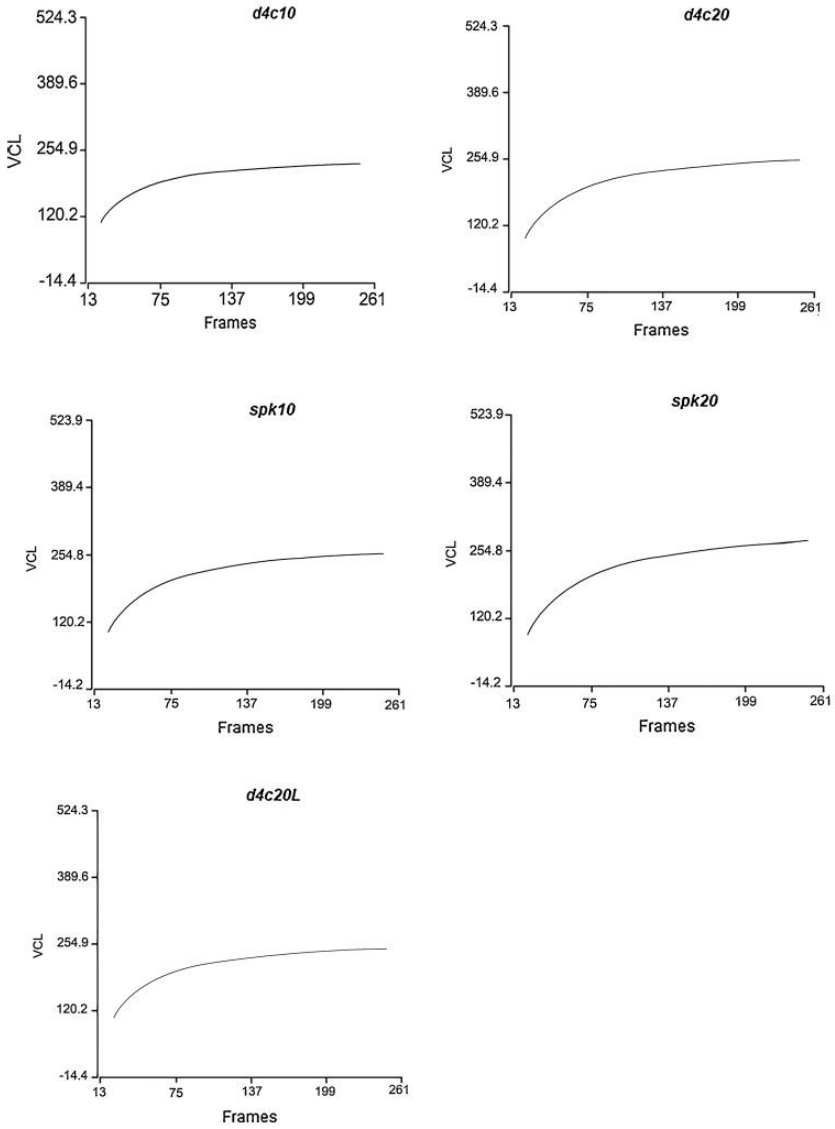

Fig. 3. Effect of frame rate on sperm curvilinear velocity (VCL. μm/s) in the different counting chambers. Disposable chambers ISAS® D4C: d4c10; 10 μm depth, d4c20: 20 μm depth, Leja chamber d4c20L: 20 μm depth and reusable chambers Spermtrack®: SpK 10: 10 μm depth and SpK 20: 20 μm depth.

3.3. Effect of chamber type on values for sperm kinematic variables

When there was evaluation of the Spermtrack chambers, there were greater VCL, VSL, STR, ALH and BCF values than with any of the other chambers. In contrast, WOB was less in the Spermtrack chambers than the other chambers. There was the greatest WOB value when there was evaluation using the D4CL20 chamber (Table 2).

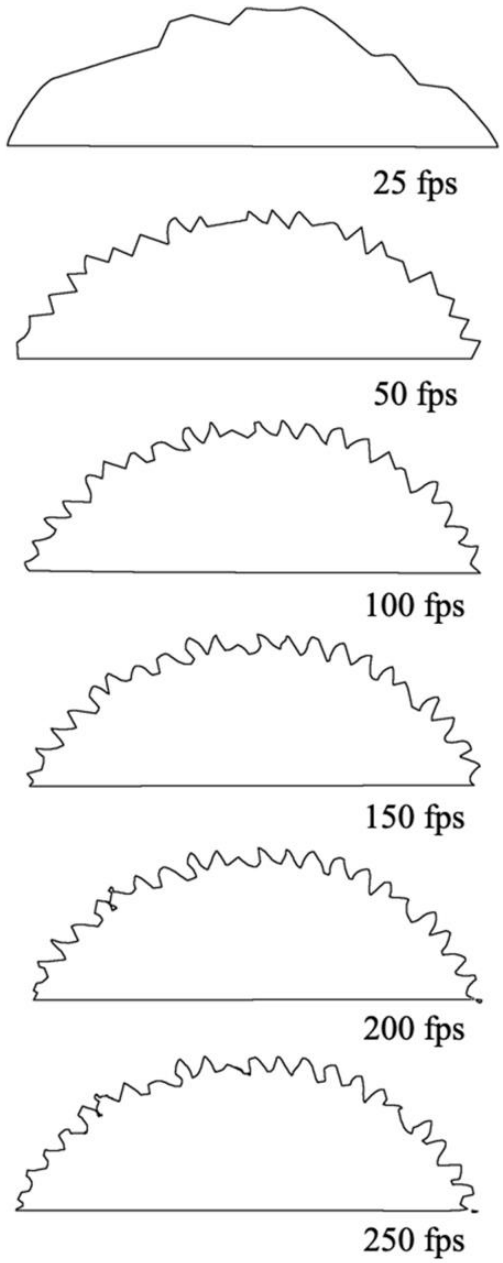

Fig. 4. Sperm trajectory observed at different frame rates.

3.4. Effect of capture chamber field on values for sperm kinematic variables

With the Spk10 chamber, VCL, VSL, VAP and ALH were greater in the centre of the chamber and less towards the peripheral fields (differences for VCL though not VSL or VAP). The LIN, STR and WOB, however, were less in the centre than in the peripheral fields. There were no differences in values in the different fields in terms of BCF (Table 3).

Table 2
Motility characteristics (mean ± SD) of stallion spermatozoa determined using the CASA system utilising the different counting chambers at 250 f/s.

Chamber	Depth	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
D4C									
	10	225.2 ± 84.34^{f}	56.8 ± 36.41^{e}	172.4 ± 71.96^{e}	24.8 ± 12.43^{a}	33.6 ± 16.83^{e}	75.0 ± 10.98^{b}	1.2 ± 0.32^{d}	38.2 ± 15.91^{c}
	20	255.6 ± 100.29^{g}	60.4 ± 42.45^{b}	187.2 ± 79.93^{a}	22.9 ± 12.58^{c}	32.1 ± 17.18^{d}	71.8 ± 9.92^{c}	1.3 ± 0.35^{e}	39.5 ± 16.31^{b}
D4CL									
	20	242.2 ± 89.21^{cl}	59.0 ± 39.99^{bc}	186.8 ± 75.55^{a}	23.6 ± 12.79^{b}	31.3 ± 16.55^{e}	75.7 ± 9.59^{a}	1.2 ± 0.29^{d}	39.5 ± 16.11^{b}
Spk									
	10	261.0 ± 97.22^{b}	66.8 ± 39.16^{a}	182.7 ± 74.16^{b}	25.0 ± 11.23^{a}	36.4 ± 16.06^{a}	68.9 ± 9.23^{d}	1.3 ± 0.35^{b}	41.4 ± 16.11^{a}
	20	279.8 ± 101.43^{a}	66.5 ± 44.14^{a}	186.7 ± 74.63^{a}	22.8 ± 12.03^{c}	34.5 ± 17.46^{b}	65.9 ± 8.93^{e}	1.4 ± 0.38^{a}	41.2 ± 16.09^{a}

VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP (μm/s) = average path velocity; LIN = linearity (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz); Different letters between rows are indicative of mean differences between different chambers and depths ($P < 0.05$).

Table 3
Values for stallion sperm kinematic variables in different chamber fields of the Spermtrack chambers at 250 f/s (mean ± SD).

Field *n*	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Spk10								
I 1085	274.8 ± 102.62^{a}	68.1 ± 42.63^{a}	188.9 ± 77.66^{a}	24.1 ± 11.78^{a}	35.7 ± 17.18^{b}	67.5 ± 9.24^{a}	1.4 ± 0.38^{a}	41.6 ± 16.66
II 4344	261.7 ± 99.64^{b}	66.7 ± 40.01^{a}	181.7 ± 75.26^{b}	24.7 ± 11.23^{a}	36.3 ± 16.13^{a}	68.4 ± 9.31^{b}	1.4 ± 0.36^{b}	41.3 ± 16.18
III 2185	252.9 ± 88.42^{c}	66.6 ± 35.48^{a}	181.5 ± 69.94^{b}	26.0 ± 10.86^{b}	37.0 ± 15.32^{a}	70.6 ± 8.82^{c}	1.3 ± 0.31^{c}	41.79 ± 15.72
Spk20								
I 2566	285.5 ± 103.78^{a}	66.02 ± 43.96^{a}	186.0 ± 74.15	22.1 ± 11.79^{a}	34.4 ± 17.75^{a}	64.3 ± 8.81^{a}	1.5 ± 0.40^{a}	41.3 ± 16.00^{ab}
II 8034	279.0 ± 99.34^{b}	67.86 ± 43.96^{a}	186.7 ± 73.43	23.4 ± 12.03^{b}	35.2 ± 17.27^{ab}	66.1 ± 8.94^{b}	1.4 ± 0.37^{b}	41.3 ± 15.94^{a}
III 2020	275.7 ± 106.27^{b}	62.04 ± 44.71^{b}	187.9 ± 79.84	21.4 ± 12.19^{c}	33.3 ± 17.58^{b}	67.1 ± 8.75^{c}	1.4 ± 0.38^{c}	40.4 ± 16.78^{b}

n: total number of spermatozoa analysed, VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP (μm/s) = average path velocity; LIN = linearity (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz). Numerals I, II and III, indicate different counting areas: I, central position; II, fields 2, 3, 4 and 5; and III, fields 6, 7, 8 and 9 (see Fig. 2); Different letters are indicative of mean differences between different fields in the same chamber ($P < 0.05$).

Table 4
Values for stallion sperm kinematic variables in different chamber fields of the D4C chambers at 250 f/s (mean ± SD).

	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
D4C10								
1	226.57 ± 101.44^{b}	58.72 ± 41.32^{abc}	167.0 ± 86.21^{b}	25.2 ± 13.07^{ab}	36.8 ± 19.42^{a}	70.6 ± 13.47^{a}	1.2 ± 0.40^{bc}	36.5 ± 18.19^{b}
2	248.61 ± 85.47^{a}	62.45 ± 38.32^{a}	187.34 ± 71.01^{a}	24.5 ± 11.75^{ab}	33.7 ± 16.59^{bc}	74.1 ± 10.99^{d}	1.3 ± 0.37^{a}	38.8 ± 14.71^{ab}
3	225.60 ± 82.00^{b}	60.09 ± 37.84^{ab}	176.1 ± 71.37^{ab}	26.4 ± 12.92^{a}	34.5 ± 12.92^{ab}	76.8 ± 10.16^{b}	1.2 ± 0.31^{bc}	38.7 ± 15.85^{ab}
FIELDS 4	218.00 ± 85.12^{b}	54.41 ± 35.68^{c}	166.2 ± 72.28^{b}	24.5 ± 12.37^{ab}	33.3 ± 16.80^{bc}	74.7 ± 10.74^{d}	1.2 ± 0.33^{bc}	37.4 ± 16.25^{b}
5	226.63 ± 78.50^{b}	53.34 ± 32.03^{c}	173.3 ± 69.25^{b}	23.4 ± 11.74^{b}	31.7 ± 15.83^{cd}	74.8 ± 10.70^{cd}	1.2 ± 0.29^{b}	38.7 ± 15.34^{ab}
6	214.54 ± 77.02^{b}	55.8 ± 33.93b^{c}	167.2 ± 66.72^{b}	25.7 ± 12.41^{a}	33.7 ± 15.97^{bc}	76.5 ± 9.37^{bc}	1.1 ± 0.25^{c}	38.0 ± 15.59^{ab}
7	214.45 ± 66.21^{b}	50.91 ± 33.50^{c}	169.8 ± 52.69^{b}	22.8 ± 12.37^{b}	29.0 ± 16.19^{d}	79.2 ± 8.44^{a}	1.1 ± 0.23^{c}	41.4 ± 41.46^{a}
R^2	0.46	0.68	0.12	0.24	0.70	0.68	0.50	0.43
D4C20								
1	253.1 ± 112.09^{ab}	58.1 ± 43.42^{ab}	176.2 ± 84.14^{a}	22.2 ± 12.29^{ab}	33.0 ± 17.78^{ab}	68.0 ± 10.48^{a}	1.3 ± 0.41^{c}	37.5 ± 16.74^{a}
2	246.2 ± 105.99^{a}	56.6 ± 42.55^{a}	177.7 ± 81.70^{a}	21.9 ± 12.23^{a}	31.2 ± 16.59^{a}	70.6 ± 10.34^{b}	1.3 ± 0.38^{ab}	38.2 ± 17.06^{ab}
3	261.7 ± 102.46^{bc}	60.5 ± 42.59^{ab}	192.3 ± 80.89^{bc}	22.4 ± 12.45^{ab}	31.3 ± 16.91^{ab}	72.1 ± 9.46^{c}	1.3 ± 0.36^{bc}	40.0 ± 16.24^{bc}
FIELDS 4	250.8 ± 98.77^{ab}	61.6 ± 42.63^{b}	184.9 ± 79.49^{ab}	23.9 ± 12.88^{b}	33.3 ± 17.60^{b}	72.3 ± 9.56^{c}	1.3 ± 0.34^{ab}	39.7 ± 16.20^{bc}
5	257.2 ± 88.46^{abc}	63.2 ± 41.65^{b}	192.7 ± 73.21^{bc}	23.8 ± 13.07^{b}	32.5 ± 17.62^{ab}	73.8 ± 9.05^{cl}	1.3 ± 0.30^{ab}	40.5 ± 15.78^{c}
6	266.5 ± 91.09^{c}	62.3 ± 41.99^{b}	199.1 ± 77.91^{c}	22.8 ± 12.36^{ab}	31.4 ± 16.71^{ab}	73.2 ± 9.27^{nl}	1.3 ± 0.28^{abc}	40.8 ± 15.96^{c}
7	258.9 ± 84.62^{abc}	64.2 ± 39.84^{b}	200.9 ± 71.28^{c}	24.2 ± 12.98^{b}	31.5 ± 16.35^{ab}	76.8 ± 8.32^{e}	1.2 ± 0.27^{a}	42.1 ± 14.25^{c}
R^2	0.36	0.83	0.85	0.59	0.06	0.89	0.44	0.92

VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP (μm/s) = average path velocity; LIN = linearity (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz); Different letters in columns are indicative of mean differences between different fields of the same chamber ($P < 0.05$).

Table 5
Values for stallion sperm kinematic variables in different chamber fields of the D4CL20 chamber at 250 f/s (mean ± SD).

Fields *n*	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
I 2316	242.4 ± 101.44^{a}	59.8 ± 40.76^{a}	184.8 ± 86.21^{a}	23.6 ± 13.07^{ab}	31.7 ± 19.42^{a}	74.9 ± 13.47^{a}	1.2 ± 0.40^{a}	38.7 ± 18.19^{a}
II 2202	236.6 ± 85.47^{b}	58.5 ± 39.31^{a}	181.7 ± 71.01^{a}	24.0 ± 11.75^{b}	31.9 ± 16.59^{a}	75.4 ± 10.99^{a}	1.2 ± 0.37^{b}	39.2 ± 14.71^{a}
III 1987	248.3 ± 82.00^{c}	58.8 ± 39.80^{a}	194.7 ± 71.37^{b}	23.0 ± 12.92^{a}	30.1 ± 12.92^{b}	77.1 ± 10.16^{b}	1.2 ± 0.31^{ab}	40.6 ± 15.85^{b}

n: total number of spermatozoa analysed, VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP (μm/s)= average path velocity; LIN = linearity(%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency(Hz). Numerals I, II and III indicates counting areas: I, fields 1–2; II, fields 3–4; and III fields 5–7 (see Fig. 2); Different letters are indicative of mean differences between different counting areas in the same chamber ($P < 0.05$).

With the Spk20 chamber, there were no differences in values for sperm variables in the different chamber fields (Table 3). This was particularly evident for LIN, WOB and ALH.

With the D4C10 chamber, there was a lesser VSL (R^2 = 0.68) and STR (R^2 = 0.70) between the sperm deposition point and the fields towards the end of the chamber, while there was a greater WOB in the fields toward the end of the chamber (R^2 = 0.68). With the D4C20 chamber, there was a greater VSL (0.83), VAP (0.85) and BCF (0.92) when there was evaluation of values at the site of sperm deposition and the fields towards end of the chamber (Table 4). With the D4CL20 chamber, there were the greatest values for VCL, VAP, WOB and BCF in the distal chamber fields, and the least values for LIN and STR in the proximal fields (Table 5).

4. Discussion

Artificial insemination and in vitro fertilisation require the use of high-quality semen. Accurate assessments of semen quality, and in particular of sperm kinematic variables, are essential. Improvements in sperm assessment, including the use of the FR_O for different counting chambers, could reduce the use of resources (including labour investment) and help in calculating optimal sperm dosages for maximising fertility - an important factor to consider when using stallion sperm, which can be very expensive (Campbell and Sandøe, 2015).

The CASA technologies are used to assess sperm quality by correlating values for sperm variables such as motility with values for fertilising capacity (Whitesell et al., 2020); new CASA-Mot systems, therefore, are of particular interest because these allow for the fully objective assessment of sperm kinematic variables (Gallagher et al., 2018; van der Horst et al., 2018). These systems, however, need to be optimised if there are going to be reliable results from these evaluations (Bompart et al., 2018). Certainly, spermatozoa move in three dimensions, with trajectories and rapidity of movement being dependent on sperm size and metabolism (Tourmente et al., 2019, 2015), and be affected by the volume capacity of the counting chamber. Because counting chambers differ in design and depth (and therefore volume), the optimal technical conditions need to be defined (Bompart et al., 2018). Furthermore, the direction of a spermatozoa trajectory can appear different depending on the image capture FR used, with greater FRs indicating trajectories more similar to the true course of the cell (Mortimer, 2002). Because values for some kinematic variables are determined using the spermatozoa trajectory, the FR used, therefore, must affect the values obtained (Castellini et al., 2011). Knowing the FR_O for each type of chamber, therefore, would be advantageous. The present results clearly indicate the different frame rates are required when using different chambers. It is not yet possible, however, to use the exact FR_O for many chambers because there is no software available that can accommodate the data generated at FRs of greater than 250 f/s. In the present study, analyses could only be performed at this maximum manageable rate.

The FR_O has been previously defined for different combinations of species and chamber type using the same method as conducted in the present study (e.g., 256 f/s for bull sperm in Spk10 chambers; Bompart et al., 2019), and 212 f/s for boar sperm in DC420 chambers (Valverde et al., 2019a, 2019b, 2019c, 2019d). In a similarly-conducted study, Caldeira et al. (2019) reported values of 189.04 f/s for eel sperm, 260.18 f/s for salmon sperm, and 210.55 for sturgeon sperm in Spk20 chambers. Using a different method and the Makler chamber, Castellini et al. (2011) reported a FR_O of 97 f/s for human sperm, 200 f/s for ram sperm, and 292 f/s for rabbit sperm. The results from the present study are the first where FR_o for stallion semen are reported for different chambers.

For bull sperm, Bompart et al. (2019) also reported a different FR_O for different counting chambers, and as detected in the present study, there was a greater FR_O for drop displacement chambers than for capillary loaded chambers. This apparently confirms that capillary loaded chambers restrict sperm motility in some way. There were similar findings also reported for goat semen (Del Gallego et al., 2017) using the same kind of counting chambers as those used in the present study, although the differences in FR_O were not as large.

In other studies, where there were evaluations of different drop displacement and capillary loaded chambers to those evaluated in the present study, there were reported to be smaller differences in motility values for bull (Valverde et al., 2018b) and stallion (Hoogewijs et al., 2012) sperm. When conducting these studies, however, there were suboptimal FRs.

At 250 f/s, the values recorded for VCL, VAP and ALH were always less in the 10 than the 20 μm-deep chambers irrespective of other factors, while those for LIN, STR and WOB were always less in the 20 μm-deep chambers than in the 10 μm-deep chambers. This indicates that sperm cell movement is restricted by the smaller volume of sample placed in the 10 μm-deep chambers (Palacín et al., 2013; Peng et al., 2015). Furthermore, in the shallower chambers, the sperm cells are in a space with less available oxygen, which likely affects the metabolism and consequently motility in a shorter timeframe than in deeper chambers. In the 20 μm-deep chambers, which provide more space for sperm motility, the values for LIN and STR and ALH were less, which might reflect to a greater extent the motility of sperm cells when inside the female tract (Lannou et al., 1992). These results are inconsistent with those for boar sperm, in which the values for all these variables were greater when using a 100 μm-deep chamber compared to those with smaller depths (Soler et al., 2018). This again highlights the need to optimise for the technical conditions when there are evaluations of different species (and perhaps even each breed).

In the drop displacement chambers (i.e., both Spermtrack chambers), the sperm sample is deposited at the centre of the counting area. The content of the sample, therefore, spreads across the chamber under the weight of the coverslip. In the disposable capillary loaded chambers (i.e., all others evaluated), the drop is deposited at the interface between the slide and the attached cover, and then spreads across the chamber by capillary action (Bompart et al., 2018). The present results from the present study indicate VSL, LIN, STR, ALH and BCF to be greater when there are evaluations with the drop displacement chambers than with capillary loaded chambers, independent of depth. The VAP, however, was greater in the D4C20 chamber than in either of the Spermtrack drop displacement chambers, and values for WOB was greater when evaluations were in both capillary loaded chambers than in the drop displacement chambers. There were similar differences reported in studies with stallion sperm, although not at the FRo (the figures recorded,

therefore, were different from those in the present study) (Hoogewijs et al., 2012). In bull semen, sperm VCL, ALH and WOB have also been reported to be greater in drop displacement chambers, although there were no differences with respect to the chamber loading method in terms of LIN, STR and BCF (Bompart et al., 2018). For goat semen, there is a greater VCL, VSL and VAP and lesser LIN values in drop displacement chambers than in capillary loaded chambers (Del Gallego et al., 2017). Relatively greater VCL, VSL and VAP values have been reported for ram semen in drop displacement chambers, however, there were no differences between differently loaded chambers in terms of LIN and STR (Palacín et al., 2013). With boar semen, however, VCL, VSL, VAP, ALH and BCF have been reported to be greater, and LIN and STR less, in capillary loaded than in drop displacement chambers (Gączarzewicz, 2015). In drop displacement chambers, the honey bee spermatozoa is reported to adhere to the glass, requiring BSA (a Kb diluent) be added (Yániz et al., 2019) for reliable analyses to occur. These sperm cells may interact with ions present on the surface of the glass. In capillary loaded chambers this is less likely to occur because the surface is coated with an anti-surfactant. Furthermore, the values for kinematics of eel sperm appear to not be different in chambers when there is either of the loading types (Gallego et al., 2013). Together, these results indicate that chamber loading type affects the final motility of the cells differently in different species.

Observations made at 250 f/s for each chamber indicated the values for kinematic results were affected by the part of the chamber (or field) where measurements were evaluated. In the Spk10 chamber, which has a circular design, the values for some variables were less when there were evaluations in the outer fields than those collected in the central field (where the sperm are deposited), perhaps because of changes in the surface tension and greater contact with the air (Bompart et al., 2018). There, however, were no marked differences in the central and outer fields when evaluations were made in the Spk20 chambers. With the rectangular D4C 10 chamber, there was a reduction for VSL and STR, while there was a greater WOB at the sperm deposition point and the fields towards end of the chamber. With the D4C20 chamber, were similarly greater values for VSL, VAP and BCF. In other studies there have been inconsistent results when there were evaluations of fox sperm in the same chamber type (Soler et al., 2014). For human sperm samples, however, the values were similar when there were evaluations using different chamber types (Soler et al., 2012). Again, these results indicate that relative values are species-specific for sperm motility when evaluations occur in different types of chambers.

In the present study, there were also differences in the results when there were evaluations in different fields of the coffin-shaped D4CL20 chamber, with vibrating motility being greater and progressivity being less in the outer fields. With the use of these chambers, there are refluxes in the fluid due to differences in amount of the chamber space, which together alter the capillary forces that exist. With bull sperm, however, all the values for all kinematic variables (except BCF) were less towards the outer fields when this type of chamber was used to evaluate sperm (Valverde et al., 2018b), once again highlighting how sperm motility characteristics vary in different chambers, and even different parts of the same type of chamber.

5. Conclusion

The results from the present study indicate that when there are sperm motility evaluations with different counting chamber types, there are different FRo that should be used when examining stallion sperm motility. In all of the chambers in which there were evaluations in the present study, the FRo was virtually identical to or greater than 250 f/s - the limit that can be used with the analytical software presently available. Until there is development of new software, the FR therefore, should be set to 250 f/s when using all the chambers in which there were evaluations in the present study. The results obtained may also vary depending on the field of the chamber where there are evaluations, requiring means be calculated in various fields. With the Spk20 chamber, however, there was no such variation because, there were similar values when there were evaluations at various locations, reducing the need for multiple observations to make accurate assessment of sperm motility. The Spk20 chamber, used at 250 f/s (until the optimum 309.82 f/s can be used), therefore, is an excellent option for examining the motility and determining values for kinematics of stallion sperm.

Authors' contributions

SG contributed to the study design, study execution, data analysis, interpretation and preparation of the manuscript, DB to the study design, AV to the statistical analysis, JC to the study design and its execution, JM and CS to data curation, funding acquisition, project administration, supervision and writing/editing. All authors gave their final approval of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest. Some of the authors (CS and DB) are employees for the company that manufacture the CASA-Mot system and the counting chambers used or collaborate through a collaborative agreement between the València University and the company (CS) that manufacture some of the components used here. In any case, it does not imply any conflict of interest, because no publicity (direct or indirect) is done in the manuscript. These components were just used in the same way we could use from other brands and the elements studied are not related with the brand but with physic and biological topics.

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Data accessibility

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Manuscript 3

Optimization of CASA-Mot Analysis of Donkey Sperm: Optimum Frame Rate and Values of Kinematic Variables for Different Counting Chamber and Fields

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Article

Optimization of CASA-Mot Analysis of Donkey Sperm: Optimum Frame Rate and Values of Kinematic Variables for Different Counting Chamber and Fields

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Simple Summary: A reliable sperm motility exam is important for semen analysis and breeding soundness examination. Different parameters can affect the Computer Assisted Sperm Analysis (CASA) motility results. Today, new high-resolution cameras and different chambers are introduced to CASA systems, and protocol optimization is required to render the estimation results for donkey sperm. The objective of this study is the optimization of the conditions used for donkey semen motility analysis with CASA-Mot by defining the optimum frame rate for different chamber types. Additionally, to study the effect of different chamber types, chamber field and sperm dilution on the sperm kinematic parameters with higher frame rates are examined.

Abstract: In order to optimize the donkey sperm motility analysis by the CASA (Computer Assisted Sperm Analysis)-Mot system, twelve ejaculates were collected from six jackasses. Capillary loaded chamber (CLC), ISAS®D4C depths 10 and 20 µm, ISAS®D4C Leja 20 and drop displacement chamber (DDC), Spermtrack® (Spk) depths 10 and 20 µm were used. Sperm kinematic variables were evaluated using each chamber and a high-resolution camera capable of capturing a maximum of 500 frames/second (fps). The optimum frame rate (OFR) (defined according to curvilinear velocity—VCL) was dependent on chamber type. The highest OFR obtained was 278.46 fps by Spk20. Values for VCL, straight-line velocity (VSL), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) were high in DDC and 10 µm depth. In both DDC 10 and 20 µm, the sperm velocities (VCL, VSL, VAP) and ALH values decreased significantly from the centre to the edges, while Wobble and BCF increased. No defined behavior was observed along the CLC. However, all the kinematic variables had a higher value in a highly concentrated sample, in both chamber types. In conclusion, analyzing a minimum of nine fields at 250 fps from the centre to the edges in Spk10 chamber using a dilution of 30×10^6 sperm/mL offers the best choice for donkey computerised sperm motility analysis.

Keywords: frame rate; drop displacement chambers; capillary loaded chambers; chamber depth; field; sperm dilution

1. Introduction

The domestic donkey (*Equus asinus*) is one of the two domestic species of the genus Equus along with the horse (*Equus caballus*) [1]. In developed zones, donkeys have suffered a significant decrease due to industrialization and mechanization of agriculture. However, in recent years, there has been an increase in donkey product interest: milk, meat or skin [2]. Donkey farming is expanding and the research interest about donkey production and reproduction optimization is increasing [3,4]. This implies a more complete knowledge of their semen quality and general reproductive characteristics especially for achieving a productive-assisted reproduction [5].

Semen quality can be defined upon certain criteria performing certain tests, such as motility, concentration and morphology [5–7]. Nonetheless, from all those tests, sperm motility is commonly considered the most significant parameter for breeding soundness examination [8,9]. Usually, motility is analysed subjectively looking for just the total and progressive motility, but in the last few decades, an objective method—CASA (Computer Assisted Sperm Analysis)—was introduced and is now available and used widely by veterinarians and laboratories [10]. CASA-Mot technology is based on the computational reconstitution of sperm trajectory from image sequences; the last ones are captured by a video camera mounted on a microscope. Then, the sperm sequences are automatically analysed by the computer in a concise time [11]. Moreover, CASA-Mot systems provide a battery of kinematic quantitative parameters that define the sperm cell motility rather than the progressivity [12].

A critical review of the literature revealed that no standard practices have been embraced or recommended by professional societies in the case of donkey samples and thus, no defined protocols are followed within or across CASA-Mot instruments [13]. In fact, in other species, it has been proved that the accuracy and the sensitivity of the measurements obtained with CASA-Mot systems can be affected by different factors, such as the mathematical algorithms, suspending medium, sample concentration, frame rate, chamber type and depth, hardware, and instrument settings [14]. Usually, for the donkey semen assessment within CASA, we use the same setting and protocol as horses. However, comparative studies in both species have identified differences in reproductive strategy as well as in the sperm form and function [15,16]. Effectively, the donkey testis is bigger and has been proved to have more efficient spermatogenesis than a stallion [17–20]. In effect, donkey sperm heads are smaller with a larger mid-piece than the stallion resulting in differences in motility patterns [21]. It was reported that donkey spermatozoon is faster than the horse when using the same CASA set up [22]. However, we need to consider those differences in the definition of the most adequate setting when analysing donkey sperm.

On the other hand, in recent years, the development of high-resolution cameras, the improvement of informatic systems and the development of specific chambers to analyze sperm motility have changed the published data completely on sperm motility patterns in different species by CASA-Mot systems.

The objective of this study was to standardise the method of sperm motility assessment in the donkey, by first defining the optimal frame rate (OFR) based on VCL (curvilinear velocity) data for different counting chambers for the use in CASA-Mot system. Secondly, we analyse the kinematic variables at OFR in different chamber types and depths (including the considered counting area). Finally, an investigation of the effect of semen dilution on donkey sperm motility parameters is carried out.

2. Materials and Methods

2.1. Animals Used and Ethics Statement

The experiment was carried out on six Catalan donkeys with two ejaculates for each one. All males were aged between 3 and 20 years old and they are known for their successful fertility. Animals involved in the study were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Valles, Spain) at a Europe-approved equine semen collection centre (authorization number: ES09RS01E). The centre operates under strict protocols of animal welfare

and health control. All jackasses were semen donors, which were housed in an individual paddock at the centre. Semen has been collected under CEE health conditions (free of Equine Arteritis, Infectious Anaemia and Contagious Metritis). It is important to note that the service runs under the Catalonia Regional Government's approval (located in Spain) and no manipulations to the animals other than semen collection were carried out. The Ethics committee of this institution indicated that no further ethical approval was required. Additionally, all the animals received a standard diet (with mixed hay and basic concentrate) and were provided with water ad libitum. Three times a week, donkeys underwent regular semen collection once a day under the same conditions and samples were collected throughout the year.

2.2. Semen Preparation

The semen was collected manually using an artificial Hannover vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-liner nylon filter to eliminate the gel fraction. Once semen collected, the volume was recorded, then was immediately diluted 1:5 (*v*/*v*) in a skim-milk-based semen extender (Kenney) [23] and allowed in 50 mL conical tubes. Morphological abnormalities and viability were determined by bright field microscopy (mag. ×1000) when examining 200 cells after smear staining with Eosin–Nigrosin. Total sperm concentrations were determined using a Neubauer Chamber (Paul Marienfeld GmbH and Co. KG, Lauda-Königshofen, Germany); counting was performed in triplicate using a phase-contrast microscope (mag. ×20).

2.3. Semen Dilution

The sperm concentration of semen diluted previously was reevaluated and adjusted to obtain two groups: the first one with a high concentration (80×10^6 spz/mL) and the second, low concentrated, (30×10^6 spz/mL) in order to analyze the effect of concentration on sperm motility.

2.4. Counting Chambers and Loading Technique

Five commercial counting chambers (all from Proiser R + D S.L., Paterna, Spain) were used: (1) three disposable, capillary loaded chambers ISAS®D4C having a fixed cover-slide attached by glue, ISAS®D4C10, ISAS®D4C20 (hereafter D4C10 and 20) of 10 and 20 µm depth (Figure 1a) and ISAS®D4C20L (hereafter D4CL20) of 20 µm depth (Figure 1b). (2) two reusable drops displacement chambers Spermtrack® (Proiser R + D, Paterna, Spain) having a separate cover slide, Spermtrack®10 and Spermtrack®20 (hereafter Spk10 and 20) of 10 and 20 µm depth (Figure 1c).

The samples were well homogenized just before being charged in the chambers. All chambers were loaded with the adequate technique and quantity (Figure 1) of semen as recommended by the manufacturer. When using the Spermtrack® chambers, the covers were rapidly but gently put in place to achieve a homogenous distribution of the sample. The other chambers were loaded by depositing the sperm sample in the loading area. Then, the sperm travels by capillarity into the different areas of the chamber. Chambers were maintained on a thermo-plate for 15 s at 37 °C to prevent heat shock and allowing the fluid to cease permitting correct observation. For the analysis, the order of chambers used was randomized to avoid the effect of incubation time.

To study the effect of the field location on sperm motility, 7 fields were identified longitudinally in D4C10 and 20 (Figure 1c). However, in D4C20L two captures were made in each zone A, B and C from the proximal position to the distal position as presented in Figure 1b. Finally, in Spermtrack® chamber different fields were captured from the center to the edges as shown in Figure 1c. An average of 500 sperm was captured per field.

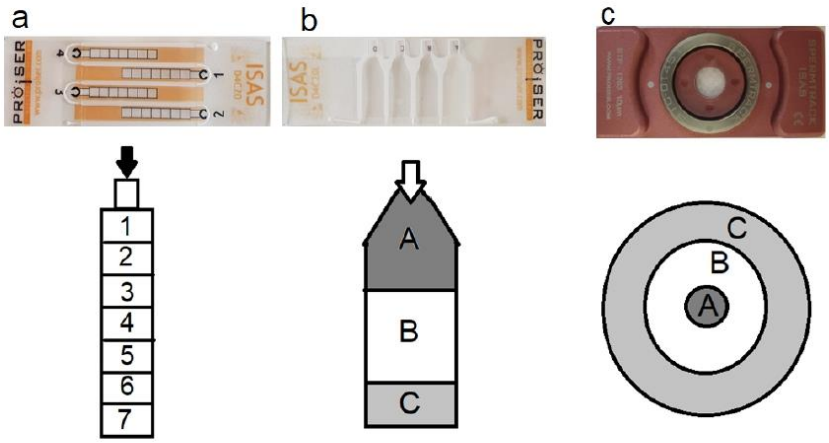


Figure 1. The different chambers types and forms used and the filed analyzed. (**a**): Disposable chamber D4C10, D4C20. (**b**): Disposable chamber D4C20L and (**c**): Reusable chambers SpK10, SpK 20. The arrow demonstrates the place of drop deposition. Numbers (1–7) and characters (A, B, C) inside the chambers demonstrate different capture fields.

2.5. CASA-Mot Analysis

Objective motility assessment was performed using ISAS® v1.2 CASA-Mot system (Proiser R + D, Paterna, Spain) combined with a UOP200i microscope (Proiser R + D) equipped with a negative phase contrast 10× objective (AN 0.25) and an MQ003MG- CM digital camera (Proiser R + D S.L.) which was capable of capturing a maximum frame rate of 500 per second. The final resolution was 0.48 μm/pixel in both x- and y-axis. The system was set with particle area between 4 and 70 μm^2 and connectivity set value of 6 μm. All samples analysis was performed by the same technician to avoid errors related and biases.

Sequences were captured at 500 fps and recorded during 3 s in different fields. For the study, these original videos were later segmented into 25, 50, 100, 150, 200 and 250 fps working videos, using the following command: [echo off: set fps = 25, 50, 75, 100, 150: for %%i in (.Ä*.avi) do (set fname = %%~ni) & call: encodeVideo; goto eof: encodeVideo: ffmpeg.exe -i%fname%.avi -r %fps% -clibx264 -preset slow -qp 0"%fname%_(%fps%fps).avi"; goto eof].

The following kinematic parameters were considered for this study: the sum of the distances between each measured sperm position divided by the analysis time (VCL, μm/s), the straight-line distance between the first and last sperm position divided by the analysis time (VSL, μm/s), the average path velocity is the time-averaged velocity of a sperm head along its average path (VAP, μm/s), the wobble is a measure of oscillation of the actual path about the average path (WOB = VAP/VCL, dimensionless), the straightness is a measure of the linearity of the average path (STR = VSL/VAP, dimensionless), the linearity of forward progression, the linearity of the curvilinear path (LIN = VSL/VCL, dimensionless), the average distance of the sperm head from the average sperm-swimming path where the average path (ALH, μm) and the beat cross frequency the number of lateral oscillatory movements of the sperm head around the mean trajectory (BCF, Hz).

2.6. Calculating the Optimum Frame Rate

Optimal frame rates were determined from the VCL of the sperms using point-to-point reconstructions of their trajectories at each tested FR. For this, the results were subjected to exponential regression analysis:

$$y = \beta.\alpha \exp(-\beta/x) \quad (1)$$

where y is the VCL, x is the FR, α is the asymptotic level, β is the rate of increase to the asymptote and exp based on the natural logarithm. The biological meaning of the formulae is that the asymptotic level (α) represents the maximum achievable when the FR is above the threshold value. 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 on the curve on the FR; a higher value of β indicates high growth of the VCL as FR increases and vice versa. There is no substantial increase in the VCL with the increase of FR which represents α the asymptotic value (at least 95% of the maximum VCL has been achieved). The rate of approach to the asymptote represents the dependence of the curve on the FR, thus, a high β value indicates an increase in VCL with increasing FR and vice versa.

Although, the maximum image captured by the camera was 500 f/s. There is no software that can analyse the huge amount of data generated by capture frequency higher than 250 f/s. Therefore, given the OFR for all the studied chambers was either virtually identical to 250 f/s or >250 f/s. The following experiments were performed at 250 f/s for every chamber.

2.7. Statistical Analysis

The data obtained for the analysis of all sperm variables were first assessed for normality and homoscedasticity by using Shapiro–Wilk and Levene tests respectively. A normal probability plot was used to assess normal distribution. In trying to obtain a normal distribution, data were transformed using acrsine square root (acrsin$\sqrt{x}$) before repeated-measures ANOVA was run. An ANOVA was applied to evaluate statistical differences in the distributions of observation (individual spermatozoa) within disposable and reusable counting chambers and then a generalized linear model (GLM) procedure was used to determine the effects on the mean kinematic values defining the different fields of disposable counting chambers ISAS®D4C depth. Differences between means were analyzed by a Bonferroni test. The statistical model used was:

$$Xijk = \mu + Ai + Bj + AB\ (ij) + \varepsilon ijk \quad (2)$$

where Xijk = the measured sperm motility variable, μ = the overall mean of variable x, Ai = the effect of depth, Bj = the effect of the counting chamber; AB (ij) = the effect of the interaction depth-counting chamber; and εijk = the residual.

Results are presented as mean ± standard error of the mean (SEM) Statistical significance was considered at $p < 0.05$. All calculations were performed using the IBM SPSS V.23.0 package for Windows (IBM Inc., Chicago, IL, USA).

3. Results

3.1. Optimum Frame Rate in Different Chambers

The OFR was calculated for each chamber type (Figure 2). The higher OFR found was obtained by means of reusable chambers. However, OFR was higher in the chamber of 20 μm in depth compared to 10 μm in depth whatever the chamber type. Therefore, the highest value of the OFR was in Spk20 (278.4 fps) while the lowest was in D4C20L (225.3 fps) as presented in Table 1.

The OFR of all the chambers was between 225 f/s and 278 f/s. Thus, all the subsequent experiments were performed at 250 f/s for every chamber assuring a reliable measurement for sperm kinematic parameters in donkey semen.

Sperm trajectory showed a different form as the FR increase. At a higher FR, the trajectory showed a much higher oscillation that was not possible to appreciate at lower FR as shown in Figure 3.

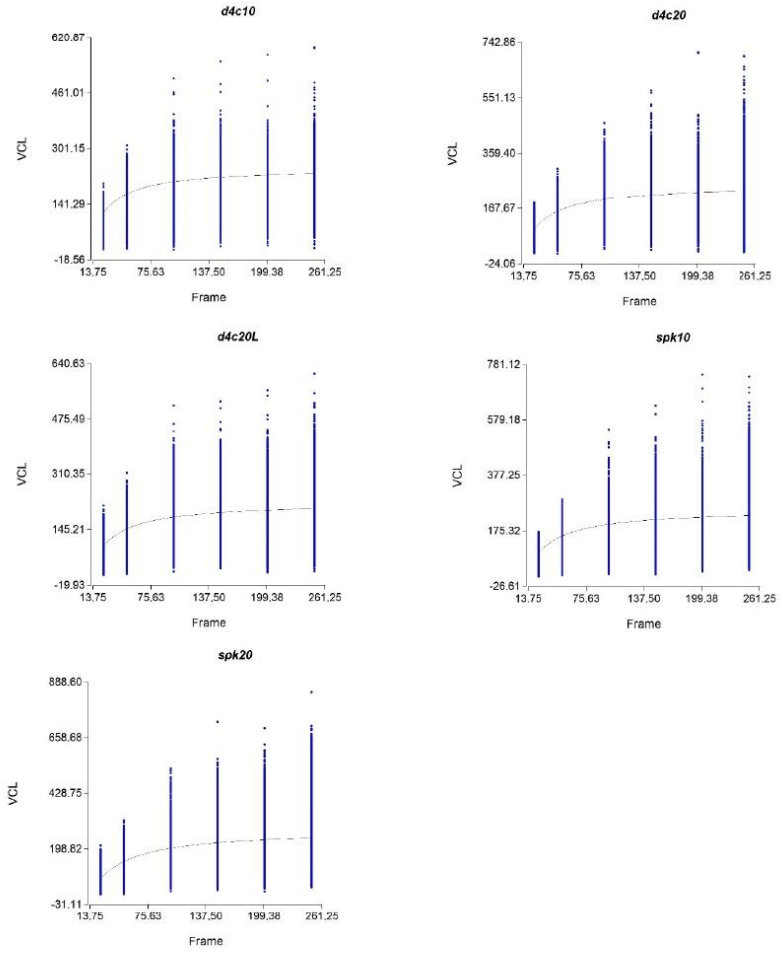
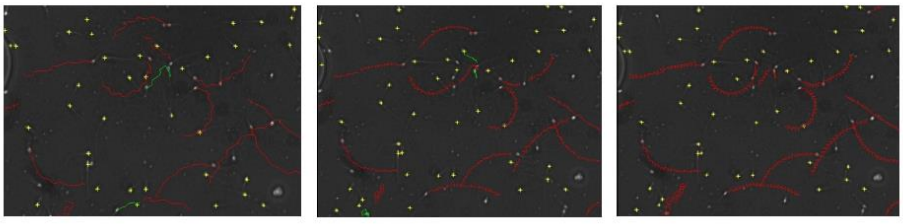


Figure 2. Sperm curvilinear velocity (VCL. µm/s) obtained at different frame rates in the different counting chambers.



25 fps 50 fps 150 fps

Figure 3. Donkey sperm motility tracks at different frames (25, 50 and 150), exhibiting 4 groups of spermatozoa velocity: rapid (red), medium (green), slow (blue) and static (yellow) in D4C10 chamber, fps = frames per second.

Table 1. Optimum frame rate calculated to have the threshold level for different chambers types and depths. Curvilineal velocity of donkey sperm for the optimal frame rate (VCLα) and with different frames rates (25–250) calculated based on α and β values.

Chamber Type	Chamber	*n*	α	SEMα	β	SEMβ	VCLα	VCL25	VCL50	VCL100	VCL150	VCL200	VCL250
Disposable													
	D4C10	14,451	247.6	0.97	18.43	0.31	229.79	118.46	171.26	205.92	218.97	225.8	230.00
	D4C20	64,732	252.96	0.62	23.64	0.25	230.30	98.26	157.65	199.7	216.07	224.75	230.13
	D4C20L	76,812	225.71	0.49	21.18	0.18	207.72	96.74	147.76	182.62	195.98	203.02	207.37
Reusable													
	Spk10	105,679	255.01	0.55	24.23	0.19	231.89	96.74	157.07	200.13	216.97	225.91	231.45
	Spk20	172,918	278.46	0.46	31.80	0.15	248.36	78.04	147.41	202.60	225.26	237.52	245.20

n: number of spermatozoa analyzed; α = asymptote of curvilinear velocity; β = rate of increase; SEM = standard error of the mean; VCL = curvilinear velocity (µm/s). D4C10, D4C20: Disposable chambers 10 µm and 20 µm depth. D4C20L: Leja disposable chamber 20 µm depth. Spk10, Spk20: Reusable chambers (Spermtrack®) 10 µm and 20 µm depth.

3.2. Effect of Chamber Type and Depth on Sperm Kinematic Parameters

The analysis of sperm kinematic parameters (VCL, VSL, VAP, LIN, STR, ALH) was generally characterized by a significantly higher value in reusable chambers compared to disposable chambers ($p < 0.05$). On the contrary, WOB and BCF were lower in reusable chamber Spk.

Then, when comparing the motility parameters in different depths for the same type and design chamber, a disposable D4C chamber of 10 µm depth reveals a higher value for the VAP, LIN, WOB and BCF rather than the D4C 20 µm depth chamber. Nevertheless, ISAS®D4C20L reusable chamber had the lowest value for all kinematic parameters except for WOB, STR and LIN which showed a higher value compared to D4C 20 µm (Table 2).

Table 2. Motility parameters (mean ± SEM) of donkey spermatozoa determined by CASA system using disposable and reusable chambers obtained at 250 frames.

Chambers	Disposable			Reusable	
	D4C	D4C	D4CL	Spk	Spk
Depth	**10 µm**	**20 µm**	**20 µm**	**10 µm**	**20 µm**
VCL	227.91 ± 1.19 ^{d}	238.16 ± 0.83 ^{c}	223.77 ± 0.77 ^{d}	260.47 ± 0.78 ^{b}	268.07 ± 0.54 ^{a}
VSL	70.19 ± 0.67 ^{c,d}	71.16 ± 0.45 ^{c}	68.93 ± 0.42 ^{d}	83.57 ± 0.45 ^{a}	80.46 ± 0.3 ^{b}
VAP	181.57 ± 0.8 ^{a}	177.18 ± 0.52 ^{b}	168.15 ± 0.5 ^{c}	183.8 ± 0.48 ^{a}	181.66 ± 0.33 ^{a}
LIN	29.82 ± 0.27 ^{a,b}	28.3 ± 0.18 ^{c}	29.32 ± 0.19 ^{b}	30.23 ± 0.18 ^{a}	28.46 ± 0.12 ^{a,b}
STR	37.67 ± 0.38 ^{d}	38.09 ± 0.25 ^{d}	39.06 ± 0.24 ^{c}	42.61 ± 0.23 ^{a}	41.47 ± 0.16 ^{b}
WOB	79.1 ± 0.19 ^{a}	73.72 ± 0.13 ^{c}	74.39 ± 0.15 ^{b}	70.19 ± 0.13 ^{d}	67.5 ± 0.09 ^{e}
ALH	1.17 ± 0.01 ^{e}	1.26 ± 0.01 ^{c}	1.22 ± 0.01 ^{d}	1.39 ± 0.01 ^{b}	1.44 ± 0.0049 ^{a}
BCF	42.58 ± 0.2 ^{a}	40.35 ± 0.14 ^{c}	38 ± 0.14 ^{d}	40.79 ± 0.12 ^{b}	39.96 ± 0.08 ^{c}

SEM: Standard Error of the Mean; VCL (µm/s) curvilinear velocity; VSL (µm/s) straight line velocity; VAP (µm/s) average path velocity; LIN (%) linearity; STR (%) straightness; WOB (%) wobble; ALH (µm) amplitude of lateral head displacement; BCF (Hz) beat-cross frequency. Disposable chambers D4C 10 µm and 20 µm depth. Reusable chambers. Spk 10 µm and 20 µm depth. ^{a,b,c,d} Within columns, rates with different superscripts differed ($p < 0.05$).

3.3. Effect of the Capture Field Inside the Counting Chamber

In D4C10, there was no clear tendency observed in the kinematic parameters as the spermatozoids travel from the point of deposition to the last field. However, the velocities (VCL, VSL, VAP) and BCF were higher in the last field while LIN increased along the counting way (form field 1 to 7) (Table 3).

The same results were found in the D4C20 chamber; we can observe an oscillatory change along the counting way. However, we observed a reduction in the velocity value (VCL, VSL, VAP) and the linearity in the last field (Table 3).

Regarding the D4C20L chamber, the highest values for all kinematic parameters (exception from the WOB) were observed closer to the point of deposition. However, the sperm had the lowest kinematic values in the middle of the chamber. The only value that showed no changes in the three counting zones was the BCF (Table 4).

Finally, in the reusable chambers, Spk spermatozoa showed similar behavior for both depths. In both Spk10 and 20 the sperm velocities (VCL, VSL, VAP) and ALH, values decreased significantly from the centre to the edges, while WOB and BCF increased ($p < 0.05$). Yet, VAP and LIN showed the highest value in the second counting ring in Skp20 (Table 5).

Table 3. CASA motility parameters (mean ± SEM) in different fields of disposable chamber ISAS®D4C10 and 20 µm depth.

Chamber	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
D4C10								
1	237.0 ± 3.3 ^{a}	71.96 ± 1.69 ^{b}	186.6 ± 2.71 ^{a}	28.94 ± 0.56 ^{b,c}	37.37 ± 0.71 ^{b,c}	77.73 ± 0.39 ^{b}	1.20 ± 0.01 ^{a}	41.26 ± 0.67 ^{a}
2	226.9 ± 3.1 ^{a,b,c}	66.70 ± 1.59 ^{b}	180.39 ± 2.56 ^{a,b}	28.03 ± 0.53 ^{c}	35.51 ± 0.67 ^{c}	78.79 ± 0.37 ^{a}	1.18 ± 0.01 ^{a}	41.62 ± 0.63 ^{a}
3	218.56 ± 4.22 ^{c,d}	66.62 ± 2.16 ^{b}	177.59 ± 3.47 ^{a,b,c}	29.63 ± 0.72 ^{b,c}	36.63 ± 0.91 ^{b,c}	80.53 ± 0.5 ^{a}	1.14 ± 0.01 ^{a}	42.02 ± 0.86 ^{a}
4	233.54 ± 3.11 ^{a,b}	71.18 ± 1.6b	183.96 ± 2.56 ^{a,b}	29.66 ± 0.53 ^{b}	37.63 ± 0.67 ^{b}	78.58 ± 0.37 ^{a,b}	1.2 ± 0.01 ^{a}	41.99 ± 0.63 ^{a}
5	218.26 ± 4.22 ^{c,d}	67.45 ± 2.16 ^{b}	177.22 ± 3.47 ^{a,b,c}	30.46 ± 0.72 ^{a,b}	37.76 ± 0.91 ^{b}	80.63 ± 0.5 ^{a}	1.14 ± 0.01 ^{a}	42.34 ± 0.86 ^{a}
6	212.65 ± 4.09 ^{d}	69.66 ± 2.1 ^{b}	170.19 ± 3.37 ^{c}	31.97 ± 0.7 ^{a}	40.27 ± 0.88 ^{a}	79.41 ± 0.49 ^{a}	1.12 ± 0.01 ^{a}	40.91 ± 0.83 ^{a}
7	252.96 ± 16.8 ^{a}	93.72 ± 8.61 ^{a}	198.38 ± 13.83 ^{a}	35.64 ± 2.87 ^{a}	45.5 ± 3.61 ^{a}	78.06 ± 1.99 ^{a,b}	1.23 ± 0.06 ^{a}	43.78 ± 3.42 ^{a}
D4C20								
1	238.94 ± 2.64 ^{b}	68.96 ± 1.32 ^{c}	171.01 ± 2.04 ^{b}	26.99 ± 0.4 ^{c}	37.94 ± 0.51 ^{b}	70.54 ± 0.27 ^{c}	1.29 ± 0.01 ^{b}	38.41 ± 0.48 ^{c}
2	215.94 ± 2.58 ^{c}	65.06 ± 1.29 ^{d}	162.94 ± 1.99 ^{c}	28.2 ± 0.39 ^{b}	37.66 ± 0.5 ^{b}	74.28 ± 0.26 ^{a}	1.17 ± 0.01 ^{d}	36.79 ± 0.46 ^{d}
3	241.81 ± 2.51 ^{b}	70.26 ± 1.26 ^{b,c}	180.81±1.94 ^{a}	27.7 ± 0.38 ^{b,c}	37 ± 0.48 ^{b}	74.31 ± 0.26 ^{a}	1.27 ± 0.01 ^{b}	40.52 ± 0.45 ^{b}
4	243.05 ± 2.38 ^{b}	72.97 ± 1.19 ^{b}	182.02±1.84 ^{a}	28.35 ± 0.36 ^{b}	37.88 ± 0.46 ^{b}	74.47 ± 0.24 ^{a}	1.27 ± 0.01 ^{b}	40.88 ± 0.43 ^{b}
5	244.19 ± 2.71 ^{b}	73.77 ± 1.36 ^{b}	182.1±2.09 ^{a}	28.7 ± 0.4 ^{b}	38.39± 0.52 ^{b}	73.99 ± 0.28 ^{a}	1.28 ± 0.01 ^{b}	39.7 ± 0.49 ^{b,c}
6	253.55 ± 3.51 ^{a}	78.47 ± 1.76 ^{a}	184.84±2.72 ^{a}	30.13 ± 0.53 ^{a}	41.1 ± 0.68 ^{a}	72.75 ± 0.36 ^{b}	1.33 ± 0.01 ^{a}	41.51 ± 0.63 ^{a}
7	222.69 ± 3.91 ^{c}	63.82 ± 1.96 ^{d}	169.34 ± 3.02 ^{b,c}	26.61 ± 0.59 ^{c}	34.95 ± 0.75 ^{c}	74.77 ± 0.4 ^{a}	1.22 ± 0.01 ^{c}	38.54 ± 0.71 ^{c}

SEM: Standard error of the mean; VCL (µm/s) curvilinear velocity; VSL (µm/s) straight line velocity; VAP (µm/s) average path velocity; LIN (%) linearity; STR (%) straightness; WOB (%) wobble; ALH (µm) amplitude of lateral head displacement; BCF (Hz) beat-cross frequency. Disposable chambers D4C 10 µm and 20 µm depth. Reusable chambers. Different letters of the alphabet within columns indicate significant differences between different fields ($p < 0.05$).

Table 4. CASA motility parameters (mean ± SEM) in different fields of disposable chamber ISAS®D4C20L.

Capture Fields	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
A	227.53 ± 1.89 ^{a}	70.55 ± 0.95 ^{a}	169.65 ± 1.49 ^{a}	29.54 ± 0.3 ^{a}	39.71 ± 0.38 ^{a}	74.03 ± 0.19 ^{b}	1.24 ± 0.01 ^{a}	38.6 ± 0.37 ^{a}
B	219.63 ± 1.65 ^{b}	65.43 ± 0.83 ^{b}	164.74 ± 1.3 ^{b}	28.28 ± 0.26 ^{b}	37.83 ± 0.33 ^{b}	74.12 ± 0.17 ^{b}	1.21 ± 0.01 ^{b}	37.68 ± 0.32 ^{a}
C	222.51 ± 1.5 ^{b}	69.13 ± 0.76 ^{a}	167.8 ± 1.19 ^{a,b}	29.52 ± 0.24 ^{a}	39.1 ± 0.3 ^{a}	74.58 ± 0.16 ^{a}	1.21 ± 0.01 ^{b}	37.91 ± 0.29 ^{a}

SEM: Standard error of the mean; VCL (µm/s) curvilinear velocity; VSL (µm/s) straight line velocity; VAP (µm/s) average path velocity; LIN (%) linearity; STR (%) straightness; WOB (%) wobble; ALH (µm) amplitude of lateral head displacement; BCF (Hz) beat-cross frequency. Different letters of the alphabet within columns indicate significant differences between different fields ($p < 0.05$). Capture fields for semen analysis proximal (A), medium (B), distal (C) as described in Figure 1.

Table 5. Mean (±SEM) of sperm kinetic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) in donkey semen in reusable chamber Spermtrack® (SpK) 10 and 20 µm depth.

Chamber and Capture Field	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
SpK10								
A	274.11 ± 2.31 ^{a}	88.37 ± 1.28 ^{a}	188.94 ± 1.69 ^{a}	30.33 ± 0.36 ^{a,b}	43.8 ± 0.48 ^{a}	68.53 ± 0.22 ^{b}	1.45 ± 0.01 ^{a}	41.35 ± 0.39 ^{a}
B	257.82 ± 1.24 ^{b}	81.41 ± 0.69 ^{b}	182.23 ± 0.91 ^{b}	29.6 ± 0.19 ^{b}	41.61 ± 0.26 ^{b}	70.3 ± 0.12 ^{a}	1.38 ± 0.0045 ^{b}	39.84 ± 0.21 ^{b}
C	257.6 ± 1.63 ^{b}	83.48 ± 0.91 ^{b}	182.8 ± 1.2 ^{b}	30.69 ± 0.25 ^{a}	43.07 ± 0.34 ^{a}	70.53 ± 0.15 ^{a}	1.37 ± 0.01 ^{b}	40.3 ± 0.28 ^{b}
SpK20								
A	271.45 ± 1.76 ^{a}	82.66 ± 0.93 ^{a}	177.73 ± 1.25 ^{b}	28.08 ± 0.26 ^{b}	42.47 ± 0.34 ^{a}	64.84 ± 0.15 ^{c}	1.47 ± 0.01 ^{a}	38.54 ± 0.29 ^{b}
B	271.84 ± 1.11 ^{a}	81.69 ± 0.59 ^{a}	183.81 ± 0.79 ^{a}	28.71 ± 0.16 ^{a}	41.89 ± 0.22 ^{a}	67.51 ± 0.1 ^{b}	1.45 ± 0.0041 ^{b}	39.42 ± 0.18 ^{a}
C	263.56 ± 1.05 ^{b}	77.72 ± 0.56 ^{b}	180.56 ± 0.74 ^{b}	27.94 ± 0.15 ^{b}	40.27 ± 0.21 ^{b}	68.18 ± 0.09 ^{a}	1.42 ± 0.0038 ^{c}	39.54 ± 0.17 ^{a}

SEM: Standard error of the mean; VCL (µm/s) curvilinear velocity; VSL (µm/s) straight line velocity; VAP (µm/s) average path velocity; LIN (%) linearity; STR (%) straightness; WOB (%) wobble; ALH (µm) amplitude of lateral head displacement; BCF (Hz) beat-cross frequency. Different letters of the alphabet within columns indicate significant differences between different fields ($p < 0.05$). Capture fields for semen analysis central field (A), medium field (B), border field (C) as described in Figure 1.

4. Discussion

In the present study, we attempted to standardize the technique used for the motility assessment of donkey sperm using high-resolution cameras and specific chambers.

The first step was to define the OFR for sperm kinematic analysis. Our work evidenced a direct positive relationship between capture frequency and VCL. Thus, capture frequency is the number of captures that permit the tracking of the sperm from one dot to another, hence we can reconstruct the sperm trajectory while VCL is the speed of the sperm (sperm trajectory/time) and dependent on sperm trajectory. As demonstrated in this study using non-linear regression, we could calculate the maximum frames needed, defined as OFR representing the threshold (α) where more captures will lead to the same sperm path. The OFR for the donkey sperm was different between chambers with a higher value in Spermtrack® (278 fps) having a VCL of 248.36 µm/s. The lower frames were needed in the D4C20L chamber (225.7 fps). It has been noted in other studies in horses that the calculated OFR (309 fps) with a VCL is remarkably superior compared to the donkey (not published). Contrarily to what was found with lower frames (25 fps), the VCL was higher in donkeys compared to the horse [22]. This can be explained by the loss of information with a lower frame. It is believed that donkey sperm is faster than a stallion, but the current study showed less VCL with higher VSL and a straighter trajectory in donkey than the horse. Thus, this explains the lower OFR found in the horse compared to the donkey. Additionally, in recent studies, while using a high-performance camera and the same study design, depending on each species that the sperm shows non-linear trajectory will need more captures to define the correct track. This explains the differences obtained in the calculation of the OFR in the bull (256 fps) [24], the boar (212 fps) [25], and the salmon (250 fps) [26].

It was also found that the OFR is dependent on the chamber type and depth. In fact, the sperm motion or the flagellar beat is affected by the space where it is placed. In a similar study designed for bulls [24], this was also found in a different frame for different chambers.

The second step was to analyse the sperm behavior in different chamber types. As we can observe, the kinematic parameters were high in the reusable chamber compared to disposable chambers, except that the BCF and WOB values were higher in the disposable chamber. In fact, the same results were observed for VC, VSL and VAP on different species like horses, bulls, boars and bucks. Yet, the other parameters were variable depending on the species [24,27–32]. Those differences could be explained by the loading technique that could generate physical forces on the spermatozoa affecting its motility. As well, it was showed the existence of a certain interaction between sperm and ions of the glass mounted on the chamber, which would be toxic in certain species [14]. Furthermore, the chamber's designs can exercise a certain force and affect sperm movement as seen in the D4C20L chamber where donkey sperm showed the lowest values for all kinematic parameters compared to other chambers which could be due to turbulence created inside this chamber [14]. The same results were found for the bull [33]. All these effects, as we can see, are specific to each species. So, it is essential to take these parameters into account for each species when choosing the chamber for sperm motility analysis.

The results of our study additionally demonstrated that the depth of the counting chamber influenced kinematic values. In fact, comparing the chambers of the same type for a different depth, we can observe that donkey sperm had greater VAP, VSL, LIN, STR, WOB and BCF in a narrow chamber of 10 µm rather than 20 µm chamber while VCL and ALH were greater in 20um chamber. The same result was found in other species such as the goat [27]. In the Belgian Blue bull and the Limousine bull breed [24], D4C10, 20 µm depths were used. This kind of linear motility movement is defined by low lateral amplitude and a high straight-line velocity. In actuality, it was found that this movement was essential for the sperm migration from the cervix to the uterus and then to the oviduct [34,35]. Moreover, this movement was also observed in the seminal plasma and the uterine fluids, with a low concentration of glucose. However, a study on the boar [36], the Holstein bull breed [24] and the stallion [29] determined that the sperm was faster (high VCL, VSL and VAP) with higher index values in the deepest chamber 20 µm. Yet, it has also been suggested that sperm kinematics are unaffected by the chamber depth [13]. Looking at the studies referred to above, it was observed that the frame

rate setting and the chambers used to achieve different depths were not identical in all other respects. Therefore, differences in sperm kinematics could not be attributed solely to a chamber depth. A study conducted by [36], using lensless microscopy, showed that the kinetic parameters of boar semen in the deepest chambers of 100 µm increase significantly. Considering all these parameters discussed previously, it was demonstrated that the sperm behave differently depending on the chamber's depth and the species. It is important to contemplate that there is a possibility that in the OFR with a higher chamber depth (more than the size of the sperm), the sperm will show different behavior.

To the best of our knowledge, this is the first work that studies the effect of the chamber field in the analysis of donkey sperm. The semen analysis demonstrated that the zone analysis in the chamber had a significant effect on the kinematic outcomes. The analysis in D4C10 rectangular chamber showed that the sperm increased in velocity as the drop moved to the last field. However, it was observed that the sperm lost its linearity. On the contrary, it was demonstrated that in the D4C20 chamber, the sperm showed opposite comportment for the velocity parameters. The same outcome was found in the fox samples [37] using the same chambers. It was suggested that the Segre–Silderberg effect altered the sperm movement as a consequence of the hydrodynamic drive of the fluid within the capillary-loaded chambers [38]. In this case, it affected the sperm tail [30,32] and the vitality [33]. In fact, we can observe that the results were emphasised in a narrow chamber when observing the sperm of the donkey. In the circle form chamber, Spermtrack®, the drop is moved by the force of the coverslip. In our study, it was found that when the drop moves from the centre to the edges, the motility decreased in both chambers' depth, but the linearity increased. This result could be explained by the force of the fluid in the centre, which is generated by the cover that moves the sperm forward increasing the linearity but affecting the velocity. A similar result was found in the ram using a slide-coverslip [27,32]. However, [39] when using a Makler® for the bull sperm, no differences between the centre and the edges could be determined [39]. As we can see, this could implicate the biology or metabolism of each species which behave differently [40].

Finally, it is important to also consider the dilution rate which had a direct effect on sperm motility parameters as observed in our study. The sperm velocity (VAP and VCL) and the progressiveness (BCF, STR and LIN) values were higher in the high-density sample (80×10^6 sperm/mL) compared to a low density (30×10^6 sperm/mL) sample for all the chambers. As previously reported in many studies, the semen concentration could affect sperm motility parameter values recorded by CASA [41]. The same results were found for different studies in dogs [42] and bulls [43] with a higher initial sperm concentration resulted in an increase in sperm velocity (VAP, VSL and VCL) and progressiveness. This difference can be related to the "dilution effect" referring to the detrimental effect on sperm quality, motility and resistance to a cold shock when adding a high volume of diluent of raw semen. The dilution effect was observed in a low sperm concentration (i.e., $<20 \times 10^6$ sperm/mL) and demonstrated in various species [44–46]. Nevertheless, a study on horses [45,47,48] and humans [49] using a lower sperm density (2.5×10^6 sperm/mL) revealed higher motility parameters due to the minor effect that the "dilution effect" had on the sperm motility comparatively to other species. We propose a concentration of 80×10^6 sperm/mL for further experiments in the study of the donkey when using a narrow chamber of 10 µm and especially in a disposable chamber. Usually, at high concentrations, the sperm aggregates affecting the CASA system results. This was reported for a concentration higher than 100×10^6 sperm/mL [32]. Yet, in the present study, with a concentration of 80×10^6 sperm/mL, the videos were clear, and the trajectories were defined correctly. On the other hand, in reusable chambers, we recommend using a lower concentration since the concentration of 80×10^6 sperm/mL was too dense. Nonetheless, when using the playback facility for sperm tracking, the result showed a wrong trajectory reconstruction due to erroneous head detection in following frames, collision, and cross-tracks. Essentially, a different sperm concentration was proposed for CASA evaluation for different species [43,50,51] such as the horse where the concentration used is between (25×10^6 and 50×10^6 sperm/mL) [46,52]. The differences in the sperm concentration suggested for the analysis,

in different studies, that it is likely due to the different usage of CASA devices and chambers and it is directly related to the species-specificities.

In the present study, a classical skim-milk extender (Kenney extender) was used. Rota et al. [53], by means of a previous CASA system, observed an effect of extender on sperm motility patterns of Amiata donkey spermatozoa. Further studies are needed to better understand the changes in motility patterns of donkey spermatozoa caused by semen extenders, with different composition and fluidity and using new CASA devices and chambers.

5. Conclusions

Each species, including horse and donkey, has its own sperm motility patterns; as a result, each species needs its own CASA system analysis conditions. These conditions are changing according to the constant improvement of related technologies. Current and new high-resolution video-cameras, informatic software and hardware-increased capacities (but limited) or new specific chambers to analyse sperm motility are changing previously defined sperm motility patterns. Then, to define the OFR, the kinds and depths of analysis chambers or the sample dilution are very important to more accurately describe the species-specific sperm motility. Thus, when examining the motility of donkey sperm, the 250 fps in Spk10 chamber and analyzing a minimum of nine fields considering all the capture area (centre and edges) and means determined at a concentration 30×10^6 sperm/mL represents an excellent choice.

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Conflicts of Interest: The authors declare no conflict of interest. Some of the authors (C.S. and D.B.) are employees for the company that manufacture the CASA-Mot system and the counting chambers used or collaborate through a collaborative agreement between the Valèncіa University and the company that manufacture some of the components used here. In any case, it does not imply any conflict of interest, because no publicity (direct or indirect) is done in the manuscript. These components were just used in the same way we could use from other brands and the elements studied are not related with the brand but with physic and biological topics.

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Manuscript 4

A new approach of sperm motility subpopulation structure in stallion and donkey

A new approach of sperm motility subpopulation structure in horse and donkey

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Abstract

This study aimed to characterize the sperm kinematic values with high frames per second, in order to define the subpopulation structure of horse and donkey and compare them. A total of 51 fresh semen ejaculates (26 Spanish and 16 Arabian horse breeds, and 12 donkeys) were collected, and subsequently analyzed for kinematic parameters by using the computer-assisted semen analysis (CASA-Mot) ISAS®v1.2 system and using a Spermtrack® 10µm depth counting chamber. Sequences were recorded at 250 frames per second and eight kinematic parameters were automatically evaluated. All kinematic parameters showed significant differences between donkey and horse, and between horse breeds. All ejaculates evaluated showed excellent semen motility characteristics; with significantly higher values for all kinematic parameters for donkeys compared to horses except for BCF. Donkey sperm was faster and linear than the horse. Regarding horse breeds differences, the Spanish horse had higher VAP, VCL and BCF compared to the Arabian horse. Spanish horse sperm was rapid but Arab horse was more linear. The principal component analysis showed three sperm subpopulations in the ejaculate of donkeys and horses with a significantly different motility characteristic between them. The dominant subpopulation for both donkey and horse was for rapid, straight and linear with a high beat sperm (38.2% and 41.7% respectively) while the lowest subpopulation was for the slowest and non-linear sperms. This, plus

slight differences in the distribution of these subpopulations between Arabian and Spanish horse were found. In conclusion, higher frames permitted to have a new interpretation of motile subpopulations with specie and breed differences. More so, future works on donkey and horse breeds spermatozoa should take into account differences between breeds which may interfere and alter the real analysis performed.

1. Introduction:

All the present living species of equids are assigned to the genus *Equus*, sharing a common ancestor million years ago (1), being distributed worldwide in the most diverse environments. The *Equus* genus comprises two lineages; the caballine which includes domesticated horses (*Equus ferus caballus*) and the wild endangered Przewalski's horse (*Equus ferus przewalskii*) of Mangolia, and non-caballine grouping which comprises the asses and zebras (2). The domestication process for horses was initiated about 450 generations ago, assuming an average generation time of 12 years for wild horses, while that for donkeys was around 6000 years ago (3). Humans have shaped livestock species according to their intentions and contemporary needs producing an artificial selection pressure that has increased particularly since the establishment of studbooks and the development of clear breading objectives affecting the fertility potential (4). Consequently, all the domestic species are now quite far from the original in different aspects involving reproductive characteristics.

The sperm analysis is a prerequisite for breeding soundness examination, and the use of semen in artificial insemination (AI) in horses is like as in all farm animals. The introduction of computer-assisted semen analysis (CASA) technology allowed for an unprecedented degree of sophistication in the study of sperm characteristics, and particularly, in reference to kinematics patterns (5,6). CASA-Mot systems capture sequences from microscopic fields and automatically analyze all sperm trajectories (7,8), offering a battery of kinematic quantitative parameters (9). Such parameters will accurately reflect sperm physiological state and thus be a good fertility predictor potential of the ejaculate. Recently new high-resolution cameras, counting chambers and computer possibilities allowed to define optimum frame rate for kinematic analysis and showed significant differences in the sperm motility descriptors in stallion and donkey (10,11).

Furthermore, it was shown that spermatozoa can be grouped into coherent mathematical subpopulations in a big variety of animals (12–14). These sub-populational structures were also observed for different morphological, biochemical and physiological traits like morphometry (15), DNA fragmentation (16), mitochondrial activity (17), osmotic properties (18), and functional status (19). What is clear nowadays is that the ejaculate is not composed of a homogeneous population of equivalent cells but a different subpopulation regarding a variety of cellular properties. Its origin is related both to the testicular formation of the spermatozoa (20) and with their maturational process along the epididymis (21). Interestingly, it can be conditioned by external social interactions, almost in horses (22).

Our study aims to compare the distribution of these kinematic subpopulations between two close equid species horses and donkeys and two horse breeds Spanish and Arabian and that by using higher frames rate for sperm kinematic calculation.

2. Material and methods

2.1.Animals

The study was conducted at the University Autonoma of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). A total of 51 fresh semen ejaculates (26 of Spanish breeds, 16 Arabian breeds, and 12 donkeys) were collected through the study. Two ejaculates were collected from 13 Spanish pure breeds (PRE) and 8 pure Arabian (PRA) horses, while three ejaculates were obtained from 6 Catalonian donkeys each. Semen was collected from all animals three times a week throughout the year. All animals ranged from 3 to 15 years of age and fed three times a day with a standard diet (mixed hay and basic concentrate), water was also freely available. All the animals were housed in single boxes in the Equine Reproduction Service of the University. The housing facility is an EU-approved semen collection center (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. All animals were semen donors and undergoes regular semen collection, under CEE health conditions (free of equine arteritis, infectious anemia, and contagious metritis). Since this Service already runs under the approval of the Regional Government of Catalonia (Spain) and because no manipulation of the animals other than semen collection was carried out, the Ethics committee of our institution indicated that no further ethical approval was required.

2.2.*Semen collection*

Ejaculates were collected through a prewarmed artificial vagina model Hannover (Minitüb GmbH, Tiefenbach, Germany) with animals excited by an ovariectomized mare or jenny. An in-line nylon mesh filter was used to separate the gel fraction from the semen. Upon collection, gel-free semen was diluted immediately 1:5 (v:v) in skimmed milk (4.9% glucose, 2.4% skim milk, 100 mL double distilled water), previously preheated to 37°C.

Sperm analysis (morphology, concentration) was evaluated upon arrival of semen samples to the laboratory. Sperm concentration was determined using a hemocytometer (Neubauer chamber; Paul Marienfeld, Germany). To this end, samples were previously diluted with a 4% formalin buffered solution, and the sperm count was adjusted for the dilution factor. Sperm morphology was evaluated by the eosin-nigrosin staining technique.

2.3.Semen preparation and CASA analysis

The remaining sample was diluted to a final concentration of 40×10^6 spermatozoa/ml, then a volume of 2 µl was mounted on standardized 10 µm depth counting chambers Spermtrack® (Proiser R+D S.L., Paterna, Spain). All chambers were pre-warmed and maintained at 37ºC on a UB203 (Proiser R+D) heated microscope stage throughout the analysis.

Sperm kinematic parameters were automatically assessed using the motility module of CASA system ISAS®v1 (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain). The device is a combination of a Proiser HS640m digital camera mounted on the referred microscope. For each analysis, up to 10 non-consecutive fields were recorded for 3 seconds at 250 fps in each analysis, permitting the identification of a minimum of 500 spermatozoa per ejaculate.

The settings of the CASA system were those recommended by the manufacturer: particle area > 4 and < 75 μm^2; connectivity: 6; Cut-off values were VAP $\geq$ 10 µm/s for a sperm cell to be considered as motile. The following sperm motility parameters were determined, sperm velocity:

the curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP); and sperm movement trajectory: the frequency with which the actual track crossed the smoothed track in either direction (beat-cross frequency BCF, Hz), and the maximum of the measured width of the head oscillation as the sperm cells swim (the amplitude of lateral head displacement, ALH). Also, three progression proportions were calculated from the velocity measurements: (the linearity, LIN = VSL/ VCL), the departure of actual sperm track from linearity (wobble WOB = VAP/VCL), and linearity of the average path (straightness, STR = VSL/VAP).

2.4.Statistical analysis:

The data obtained from the analysis of all sperm variables 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. Multivariate procedures were performed to identify sperm subpopulations from the set of sperm motility data. All the values for kinematic variables were standardized to avoid any scaling effect.

2.4.1. Multivariate procedures análisis

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. Furthermore, Bartlett's sphericity test and the KMO (Kaiser-Meyer-Olkin) were performed. As a rotation method, the varimax method with Kaiser normalization was used. The second process was to perform a clustering procedure. A two-step cluster procedure was performed, a hierarchical and a non-hierarchical analysis model, with the sperm-derived indices obtained after the PCA, 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. In the first step, to determine the optimal number of clusters, the final centroids were clustered hierarchically using the Ward method (23). All sperm cells within different breeds and species were clustered by using the multivariate k-means clustering procedure was made to classify the spermatozoa into a reduced number of subpopulations (clusters) according to their kinematic variables. The clustering procedure enables the identification of sperm subpopulations because each cluster contributed to a final cluster formed by the spermatozoa linked to the centroids. The ANOVA and χ^2-test procedures were applied to evaluate statistical differences in the distributions of observations (individual spermatozoa) within subpopulations and then a generalized linear model (GLM) procedure was used to determine the effects of the breed and extender type on the mean kinematic variable values defining the different sperm subpopulations (i.e. the cluster centers). Differences between means were analyzed 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).

3. Results:

All the kinematic parameters showed significant differences between both species horse and donkey, being higher for donkey unless BCF that was higher for horse. This means that donkey sperm motility was more linear than horse (Table 1).

When comparing Arabian and Spanish horses VAP, VCL, ALH and BCF were higher in Spanish horse than in Arabian, being the contrary for LIN and WOB. VSL, STR and ALH showed no differences among breeds. Following this results Spanish horse sperm was faster but les linear than Arabian horse (Table 1).

The PCA rendered three PCs for both species, explaining 90.9% for stallion and 89.8% for donkey of the total variance (Table 2). The three PCs were equivalent for donkey and stallion, being PC1, named velocity, positively correlated to the velocity parameters (VCL, VAP) and sperm head oscillation ALH for both species, only BCF was also included for stallion. PC2, named linearity, was positively correlated to progressivity parameters (LIN, STR) and to VSL. Finally, PC3, named oscillation, was positively correlated to WOB in both species and negatively correlated also to BCF for donkey (Table 2).

These PCs were used to identify three well-defined subpopulations (SP1, SP2 and SP3) in both stallion and donkey, showing differences for all the kinematic parameters among them (Table 3, Figure 1). SP1 had the lowest value of all kinematic parameters, being named as the slow and non-linear subpopulation. SP2 included spermatozoa characterized by the highest linear trajectories (LIN and STR) and high speed (VCL, VSL and VAP), ALH and BCF. This subpopulation included the fast, straight and lineal with a high tail beat spermatozoa subpopulation. SP3 was characterized by the highest VCL, VAP, ALH and BCF but low linear trajectories (LIN, STR), being defined as the fast with a high beat and non-linear subpopulation (Table 3).

The percentage of each subpopulation in reference to the total number of spermatozoa varied slightly between donkey and stallion. The stallion and donkey semen have the highest proportion of the fast, straight and lineal with a high beat subpopulation (SP2: 38.2% and 41.7% respectively). SP3 was in the middle with 33.6% for stallion and 31.4% for donkey. While the lowest proportion were for the slow and non-linear subpopulation SP1 (28.2% for stallion and 26.9% for donkey, Table 3).

The proportion of the three stallion subpopulations for Arabian and Spanish horse breeds was almost the same (Table 4).

4. Discussion

Sperm motility is an important determinant for fertilization and so for male fertility and reproductive success since it's related to sperm competition leading to a strong natural selection (24). Sperm competition occurs between sperm from two or more rival males when they make an attempt to fertilize a female within a sufficiently short period of time (25). This fact has been evidenced not only in mammalian but in bird (26), fish (27), reptile (28) and insect (29) species. This relevance affects the sperm morphology leading to a production of longer sperms with larger

mid-piece, where mitochondria is found to produce more energy (30). Also, differences in head morphometry among close related camelids species was observed (31).

Reproductive isolating is one of the most important speciation processes and it is frequently related with sperm characteristics variation (32). In fact, equid species remain very close genetically, being able to produce hybrids viable but not fertile. So, a hinny is a domestic equine hybrid that is the offspring of a male horse (a stallion) and a female donkey (a jenny). It is the reciprocal cross to the more common mule, which is the product of a male donkey (a jack) and a female horse (a mare). Even more, both species can have hybrid offspring with zebras, indicating that evolutive divergency among this species is not enough to avoid reproduction among them. The action of humans along domestication practice implied that natural selection has been replaced by strong artificial selection (33,34). Domestication of equids took place in the Bronze Age for both horses (35) and donkeys (36). Artificial insemination practice meant a considerable advance in reproduction, and consequently in artificial selection, in all farm animals (37,38) and recently started in donkey (39). The results presented here showed how close stallion and donkey species are, almost regarding sperm kinematics. In fact, there are more differences between the two considered stallion breeds than between one of them and the donkey. In some mammalian species, like camelids (31), offspring obtained by crossing different species remains fertile, but as aforementioned this is not the case in equids being expected to find higher differences in sperm kinematics.

From the methodological point of view, improvement in CASA technology and especially the development of new software solutions and new high performing cameras permitted to analyze better the sperm trajectory (8,40). Recent studies have suggested specific optimum video capture frame rates for a mathematically well track definition in a variety of species (40,41). All the previous work using CASA-Mot systems was obtained using suboptimal kinematic data which resulted in a low significance kinematic parameter. Those, being one of the most important limitation in the general use of CASA technology in the past (5). The results showed in the present work were obtained using the optimal frame rate for both species and stallion breeds (10,11). Therefore, the subsequent subpopulation analyzed can be considered of high confidence.

Horse and donkey spermatozoa have a different way to move since the donkey sperm is faster with a more linear trajectory compared to horse. These differences were also appreciable at frame rate 25 fps (42).

To the best of our knowledge only few studies compared different horse breeds motility parameters and semen quality (43). Unfortunately, most of those works didn't took in consideration kinematic variation between breeds. In the present study we observed that Spanish breed present higher sperm velocities (VCL and VAP) than Arab breed, who showed higher linearity and sperm oscillation.

To complete the classical studies based on the comparation of the median values of each parameter as independent variables, the multivariate statistical procedures including a reduction of dimensionality by PCA followed by clustering analysis was developed to define sperm subpopulations (44,45). During the last years a number of studies have showed the universal presence of defined subpopulation structure inside the whole sperm population in the ejaculate

(18,46–48). This fact has changed the previously established paradigm that considered the ejaculate composed for “equivalent” cells competing for reaching the oocyte fertilization. Effectively, some kind of synergies must be present among sperm subpopulations for achieving the final goal of successful fertilization (6,49).

In the present study, the whole collection of kinematic data was grouped in three PCs in donkey ejaculate, named velocity, progressiveness and cell oscillation. Using this two steps approach three subpopulations were obtained showing that the most frequent SP was for the fastest with high linearity (46% of the total). In a previous study conducted on Andalusian donkey four subpopulations were observed, with the main subpopulation (36%) corresponding to low velocity and high progressive spermatozoa and only the 30% corresponded to progressive with high velocity subpopulation (50). But it is important to consider that the authors used a CASA-Mot system with only 25 fps and the statistical procedure was using a simple step for clustering analysis.

Regarding the horse, the patter bot both PCs and subpopulations was very similar to that observed in donkey, even if there were significant differences in the kinematic parameters between both species. Again, a previous work showed four subpopulations but using a frame rate of just 16 fps (48). Even more, up to six subpopulations were found in other work using a frame rate of 25 fps and following a one-step statistical analysis (46).

All these differences can show the frame rate importance in a correct interpretation of sperm trajectories and the errors that can occur with less frames. Those, changing almost the real distribution of subpopulation of an ejaculate resulting of a misunderstanding of real role of each subpopulation in reference to the capacity to arrive to the oocyte and fertilize it.

Finally, in reference to the horse breeds considered here, there were no important differences in the sperm subpopulation structure being little differences in the presence of some of the subpopulations. Instead of this, VCL and VAP for all subpopulations showed significant differences with higher velocities for the Spanish breed compared to Arabian. This could be explained by the history of the domestication of horses who spread out of western central Eurasia, place of origin, that started combined with the continued high genetic input from local wild populations, this hybridization increased genetic differentiation in population which were accentuated by the human force that shaped their need for creating highly competing horses (1). This hybridization has affected the shape and the performance of the spermatozoa in different degree depending on the breed, resulting in a decrease in per-cycle conception rates, at around 60% than those observed for other domestic livestock species (51). Arabian horse, considered one of the most ancestral with pure pedigree even if it was shown with the recent study of mitochondrial DNA sequences that there is heterogenicity and great diversity among this breed (52), while pure Spanish horse is considered the first European "warmblood", a mixture of heavy European and lighter Oriental horses, taking its origin from the Andalusia Spanish region that is recognized as a distinct breed since the 15th century (53).

Similar differences have been observed on other species like bull (54), boar (55) and dog (56), showing how much artificial selection procedures conduces to processes close to speciation process in natural selection (31).
Future work is needed to define the relationship of the observed sperm subpopulation structures and the fertility of the samples, considering the effect of different breeds.

5. Conclusion

New performing camera permitted to acquire higher frames for better sperm motility analysis and therefore get more reliable result approachable to real sperm move. The sperm had significantly higher values for all kinematic parameters for the donkey than the horse. donkey sperm was more rapid and linear than the horse. Regarding horse breeds differences, Spanish horse sperm is rapid but Arab horse is more linear. The cluster analysis showed 3 sperm subpopulations, the predominant motile subpopulation in freshly ejaculated horse and donkey sperm had very fast velocity characteristics and a linear trajectory with high beat frequency.
Finally, the identification and differentiation of the structure of functional sperm subpopulations seem to be an advantageous key element as an alternative valuable tool to successfully detect and improve critical handling of further treatment where the effect of the breed would be considered to avoid alterations in the interpretations of the analysis.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization, C.S. and J.M.; methodology, S.G., J.C. and J.M..; software, C.S.; validation, C.S. and J.M.; formal analysis, A.V.; investigation, S.G., J.C. and J.M..; data curation, S.G., C.S.; writing—original draft preparation, S.G., A.V; writing—review and editing, S.G., C.S., J.M.; visualization, C.S. and J.M.; supervision, C.S. and J.M.. All authors have read and agreed to the published version of the manuscript.

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Data availability

All datasets generated for this study are included in the manuscript and/or the supplementary files.

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Table 1. Sperm kinematic variables (mean ± SEM) in stallions and donkey.

Variable/Breed	Arabian	Spanish	Total stallion	Donkey
VCL	214.52±79.28[a]	232.45±89.55[b]	224.96±85.87[x]	230.08±91.45[y]
VSL	60.14±37.08[a]	59.21±34.83[a]	59.60±35.79[x]	78.07±48.01[y]
VAP	161.59±66.0[a]	167.03±68.89[b]	164.76±67.75[x]	172.63.73±63.08[y]
LIN	26.99±11.95[b]	25.19±11.23[a]	25.94±11.57[x]	33.81±19.26[y]
STR	36.36±15.50[a]	35.49±15.43[a]	35.85±15.46[x]	42.75±19.69[y]
WOB	74.22±9.91[b]	71.21±9.13[a]	72.50±9.58[x]	74.89±14.08[y]
ALH	1.18±0.26[a]	1.30±0.31[b]	1.25±0.30 [x]	1.41±0.34[y]
BCF	37.03±15.69[a]	39.25±14.92[b]	38.32±15.29[y]	33.99±18.78[x]

VCL (µm/s), curvilinear velocity; VSL(µm/s), straight line velocity; VAP(µm/s), average path velocity; LIN(%), linearity; STR(%), straightness; WOB(%), wobble; ALH(µm), amplitude of lateral head displacement; BCF(Hz),beat-cross frequency. SEM = standard error of the mean. [a-b] Different superscripts mean significant statistical differences among stallion breeds. different letters (x, y) indicates differences among stallion and donkey species $P < 0.05$.

Table 2. Eigenvectors of the three principal components obtained in the study of sperm kinematics for stallion and donkey.

	Stallion			Donkey		
Variables	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**
VCL	0.96			0.86		
VSL		0.79			0.82	
VAP	0.90			0.93		
LIN		0.97			0.89	
STR		0.98			0.98	
WOB			0.97			0.90
ALH	0.90			0.84		
BCF	0.73					-0.67
Explained variation (%)	42.47	32.48	15.96	36.92	32.51	20.40

PC1, principal component designated “velocity”; PC2, principal component designated” linearity”; PC3, principal component designated” Oscillation”. VCL (µm/s), curvilinear velocity; VSL(µm/s), straight line velocity; VAP(µm/s), average path velocity; LIN(%), linearity; STR(%), straightness; WOB(%), wobble; ALH(µm), amplitude of lateral head displacement; BCF(Hz),beat-cross frequency. Only eigenvectors > 0.6 are presented for each principal component.

Table 3. Descriptive statistics for the CASA-Mot variables (mean± SD) for each sperm subpopulation species, stallion and donkey samples.

	Stallion			Donkey		
	SP1	**SP 2**	**SP 3**	**SP 1**	**SP 2**	**SP 3**
n	5606	7626	6714	4851	7549	5678
% sperms	28.1	38.2	33.6	26.8	41.7	31.4
VCL	134.6 ± 48.17[a]	233.45 ±59.08[b]	290.77 ± 69.08[c]	142.33 ± 47.41[a]	245.8 ± 75.51[b]	286.73 ± 83.72[c]
VSL	26.29 ± 15.67[a]	85.59 ± 30.66[c]	57.89 ± 28.88[b]	26.18 ± 17.15[a]	116.42 ± 32.80[c]	71.41 ± 36.83[b]
VAP	86.18 ± 30.08[a]	176.51 ± 46.0[b]	217.01 ± 49.33[c]	92.65 ± 29.88[a]	191.89 ± 39.25[b]	215.36 ± 45.55[c]
LIN	18.91 ± 8.14[a]	36.6 ± 7.97[c]	19.7 ± 7.80[b]	18.35 ± 10.73[a]	50.29 ± 15.50[c]	25.1 ± 11.01[b]
STR	29.7 ± 13.48[b]	48.63 ± 10.86[c]	26.48 ± 10.94[a]	27.19 ± 14.18[a]	60.67 ± 10.58[c]	32.21 ± 12.96[b]
WOB	64.67 ± 10.08[a]	75.87 ± 7.47[c]	75.21 ± 7.16[b]	66.04 ± 14.83[a]	79.21 ± 14.11[c]	76.69 ± 13.26[b]
ALH	1.03 ± 0.23[a]	1.23 ± 0.22[b]	1.46 ± 0.27[c]	1.08 ± 0.21[a]	1.46 ± 0.25[b]	1.63 ± 0.29[c]
BCF	22.64 ± 10.89[a]	41.85 ± 13.11[b]	47.41 ± 9.88[c]	22.07 ± 11.66[a]	36.8 ± 19.98[b]	40.43 ± 17.39[c]

SP1, fast and linear subpopulation; SP2, fast and non-linear subpopulation; SP3, slow and non-linear subpopulation. VCL (µm/s), curvilinear velocity; VSL (µm/s), straight line velocity; VAP (µm/s), average path velocity; LIN (%), linearity; STR (%), straightness; WOB (%), wobble; ALH (µm), amplitude of lateral head displacement; BCF(Hz), beat-cross frequency. SD = standard deviation. [a-c] indicates differences among different subpopulation of the same species $P < 0.05$.

Table 4. Descriptive statistics for the CASA-Mot variables (mean ± SEM) for each sperm subpopulation in two stallion breeds Arabian and Spanish.

	Arabian			Spanish		
	SP 1	**SP 2**	**SP 3**	**SP 1**	**SP 2**	**SP 3**
n	2831	3366	2128	3942	4917	2762
%	34%	40%	26%	34%	42%	24%
VCL	136.10±47.84[a]	263.81±59.32[c]	240.88±57.11[b]	142.41±45.87[a]	295.39±69.05[c]	248.89±57.97[b]
VSL	30.05±17.47[a]	61.34±26.72[b]	98.25±34.41[c]	31.51±17.57[a]	61.69±29.51[b]	94.35±27.80[c]
VAP	90.61±32.52[a]	206.71±44.26[c]	184.63±45.64[b]	93.47±31.18[a]	219.44±48.70[c]	178.69±40.09[b]
LIN	21.30±9.45[a]	23.04±8.10[b]	40.79±8.82[c]	21.28±8.91[a]	20.99±8.66[a]	38.25±7.76[b]
STR	32.11±14.28[b]	29.25±10.12[a]	53.24±10.85[c]	32.38±13.34[b]	28.14±11.53[a]	53.02±9.68[c]
WOB	66.86±10.12[a]	78.90±6.82[c]	76.89±7.77[b]	65.86±10.01[a]	74.82±6.65[c]	72.42±7.95[b]
ALH	1.02±0.22[a]	1.31±0.23[c]	1.19±0.23[b]	1.05±0.21[b]	1.47±0.28[c]	0.34±0.25[a]
BCF	23.84±11.91[a]	45.33±11.11[c]	41.46±14.81[b]	25.77±11.94[a]	47.39±9.78[c]	44.00±12.79[b]

VCL (µm/s), curvilinear velocity; VSL (µm/s), straight line velocity; VAP (µm/s), average path velocity; LIN (%), linearity; STR (%), straightness; WOB (%), wobble; ALH (µm), amplitude of lateral head displacement; BCF(Hz), beat-cross frequency. SEM = standard error of the mean. [a-c] values with different superscript letters differ significantly between sperm subpopulations of the same breed. $P < 0.05$.

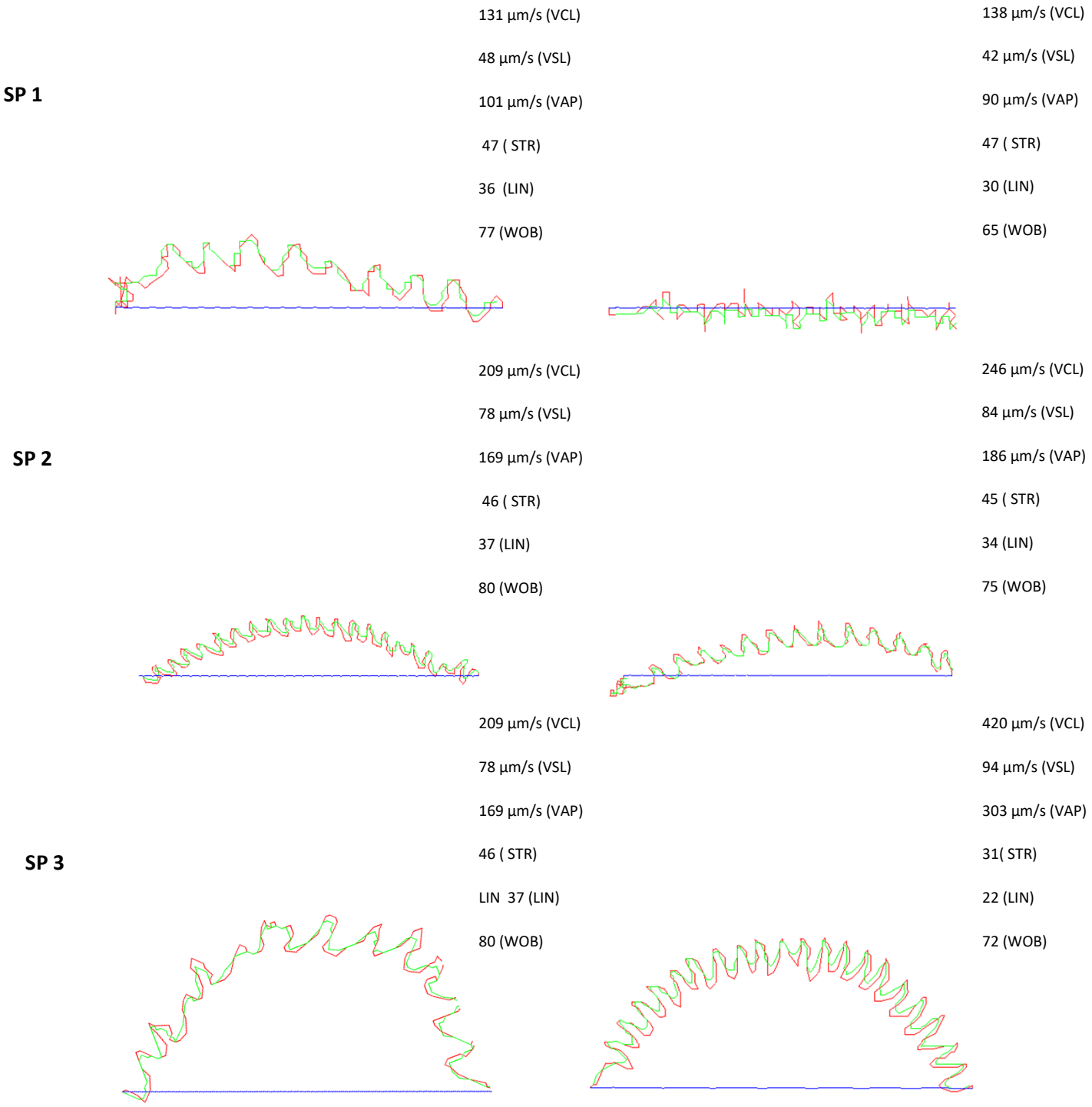

Figure.1 sperm trajectory of the three subpopulations encountered in stallion and donkey semen SP; sperm subpopulation, Blue line: Straight line velocity (VSL), red line: Curvilinear velocity (VCL); green line: Average path velocity (VAP); straightness (STR); Linearity (LIN); Wobble (WOB); Beat cross frequency (BCF).

Manuscript 5

Equine sperm morphology analysis: alive vs dead stained sperms technique

New sperm morphology analysis in equids: Trumorph® vs eosin-nigrosin stain

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Keywords: horse, donkey, sperm morphology, eosin-nigrosin stain, Trumorph®

Authors' declaration of interests

The authors declare no conflict of interest. Some of the authors are working for the company that manufacture the Trumorph® system but the results are not related to the possible interest and no publicity is contained in the manuscript.

Ethical animal research

The animal studies were performed in the Equine Reproduction Service of the Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). This is an EU-approved semen collection center (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. Because of no manipulation of the animals other than semen collection was carried out, the ethical committee of our institution indicated that no further ethical approval was required.

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Authorship

S. Gacem contributed to the study design, study execution, data analysis, interpretation and preparation of the manuscript. J. Catalan contributed to the study execution. E. Yanez-Ortiz contributed to statistical analysis. J. Miro & C. Soler contributed to data curation, funding acquisition, project administration, supervision and writing/review & editing.

All authors gave their final approval of the manuscript.

Data accessibility: The data that support the findings of this study are available from the corresponding author J. Miro, upon reasonable request.

Summary

Reasons for performing study: The evaluation of the male fertility potential is based on the analysis of the basic spermatic characteristics, concentration, motility and morphology. Thus, the study of sperm morphology is a fundamental element in the seminal analysis, but its real meaning has been biased by the techniques used for its evaluation. These techniques involve dehydration phases and subsequent staining, which involves the production of artifacts.

Objective: comparing two methods for equid semen morphology evaluation. The first one is a new technique developed by PROISER R + D (Trumorph®) using alive sperms without using stain. While the second technique is by using the eosin-nigrosine stain.

Study design: experimental study design.

Methods: A total of 49 ejaculates from stallions and donkey were used. After semen collection and dilution, an aliquot was placed on the slide and introduced in the Trumorph® device. Then observation was made with a 40x objective and Negative phase-contrast microscope. Although another aliquot was stained using eosin -nigrosine stain and viewed using 100× magnification.

Results: Well-formed sperms were observed, and different abnormalities were identified using Trumorph®. No significant difference when comparing both techniques for sperm morpho abnormalities count.

Conclusions: The use of eosin-nigrosin staining method and Trumorph® led to the same results and both techniques can be used for stallion and donkey sperm morphological analysis. However, considering the fact that Trumorph® uses living sperms helps prevent sperm cell alteration during sample preparation. Therefore, Trumorph® can be a good alternative to the conventional staining method, which provides a quick test on live sperms.

Introduction

The common use of spermatozoa in assisted reproduction is expanding especially for freezing or FIV, leading to a steady strengthened interest intrigued with the distinguishing sub-fertile or infertile males [1–

5]. The evaluation of the male fertility potential is based on the analysis of basic sperm characteristics motility and morphology. Morphology data are for high predictive value for male fertility potential [6–11] and are critical for selection using the assisted reproductive techniques treatment. Moreover, despite reports on the only percentage of normal and abnormal sperm, an in-depth evaluation is necessary on the types of abnormalities present which will be important in selecting the type of clinical procedure to be adopted for intrauterine insemination (IUI), in vitro fertilization (IVF), or intracytoplasmic injection (ICSI) [12]. Therefore, different stains and herbal dyes have been used for morphology analysis unfortunately, resulting in ambiguous outcomes [14,15, 16]. In this regard, eosin-nigrosin staining has remained the most commonly used technique in equids thus allowing detection of morphological abnormalities and determination of viability [16]. However, it's important to mention that the staining technique passes through 3 steps (staining, smearing, drying), and each one being critical affecting sperm morphology analysis. Today, with advancing technology, a new technique has been introduced for sperm morphology analysis (Trumorph) in humans and other species such as crocodile, mouse...etc. [17]. Thus, Trumorph® focuses on two physiobiological points, one is the immobilization of the sperm by temperature [17], and the second is the pressure applied on the sample that leaves the sperms flattened to the parallel of the surface. Also, it does not affect the viability or the morphology of the spermatozoa [14,17,18].

The need for a standard sperm morphological analysis is important towards establishing a technique that would enable a precise and unambiguous analysis in equid spermatozoa. Therefore, the present study was designed to compare two different methods (eosin-nigrosin staining vs wet preparation with Trumorph® machine) for evaluating sperm morphology of stallion and donkey. The aim is to determine the correlation among the methods and their application to this field.

Materials and methods:

Animals

The study used two ejaculates of 14 stallions and three of 7 donkeys, all collected on separate days. Animals were chosen randomly not taking into consideration their fertility condition. The equids were housed in the Equine Reproduction Service of the Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). This is an EU-approved semen collection centre (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. All animals were

semen donors and were collected under CEE health conditions (free of equine arteritis, infectious anemia and contagious metritis). Since this Service already runs under the approval of the Regional Government of Catalonia (Spain) and because no manipulation of the animals other than semen collection was carried out, the Ethics committee of our institution indicated that no further ethical approval was required.

Semen collection

Ejaculates were collected through a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany) and an in-line nylon mesh filter was used to separate the gel fraction from the semen. Upon collection, gel-free semen was diluted 1:5 (v:v) with Kenney extender, previously preheated to 37°C. The samples were incubated at 20°C until needed for slide preparation. Once in the laboratory, various parameters of importance for seminal fertility were analyzed, such as the concentration using a haemocytometer (Neubauer chamber; Paul Marienfeld) and motility by mean of CASA-Mot system (Proiser R + D, SL, Paterna, Spain).

Sperm morphology was evaluated upon arrival of semen samples at the laboratory, each ejaculate was analyzed with the two methods, Trumorph® (Proiser R + D, Paterna, Spain) system and eosin-nigrosine stain.

Morphological examination of spermatozoa

Trumorph®

For slide preparation, 2 and 3 µl drops of diluted semen of donkey and stallion were used, respectively. The drops were placed on the middle of the slide and covered with a 24/24 mm coverslip. Afterward, the preparation was introduced 2 times into the Trumorph® system (Proiser R+D, S.L., Paterna, Spain, based) to have a total immobilization of all sperms (fig. 1). The machine contains a plate with a preheated stage predefined at a temperature of 60 C·, controlled by a dual-laser infrared thermometer (DT-8861; ATP, Leicestershire, United Kingdom), that allows the maintenance of the sample temperature at 45 ° C, which is ideal for sperm immobilization. The slide is also submitted to a pressure of 6 KP, allowing the sample to extend and the sperms to get flatten in an adequate volume and space.

Sperms were looked at with an optical microscope specifically designed and equipped with a 40x objective of negative phase contrast (UB203 / Proiser R + D, SL, Paterna, Spain), equipped with a CCD video

camera color (Proiser C13-ON, Proiser R+D) of a resolution of 768x576, 8/12 bits per pixel and with room temperature.

An ISAS® v1 (Integrated Semen Analysis System) (Proiser R+D, S.L., Paterna, Spain) was used with the software corresponding to the morphology module to capture the microscopic fields. From each sample, a minimum number of 100 fields was captured randomly comprising around ten cells per field. For each ejaculate it was counted a minimum of 200 cells.

Eosin-nigrosine staining

The stain preparations were made according to the following procedure: a drop of semen (5µl) was placed on a slide preheated to 40°C and mixed with the same volume of the dye mixture (one part 5% bluish eosin solution (Carl Roth Gmbh+Co. KG, Germany) to four parts of 10% nigrosin aqueous solution (Sigma-Aldrich, USA) using a glass rod to produce a smear on the slide. The samples were air-dried at room temperature. Samples were evaluated under a phase-contrast microscope (BX41; Olympus, Hamburg, Germany) at ×1000 magnification (20×0.40 PLAN objective lens, positive phase contrast field; Olympus) using immersion objective lenses. Three replicates were made for each ejaculate and the mean was calculated. All slides were scored blind with a minimum of 200 sperms/sample counted and classified for morpho-abnormalities (fig 1).

Sperm abnormalities classification

The morphological abnormalities were counted as a percentage of the total number of counted spermatozoa. Morphological categories used in this study were: 1. Abnormal heads (including pear-shaped, narrow at the base, abnormal contour, undeveloped, lose abnormal head, narrow, big, little-normal, short-broad), 2. Loose heads (including both those with normal and abnormal head morphology), 3. Abnormal midpieces, 4. Proximal cytoplasmic droplets, 5. Distal cytoplasmic droplets, 6. Bent tail, 7. coiled tails.

Statistics

A statistical analysis of the sperm abnormalities obtained with the two seminal evaluation techniques (staining and Trumorph®) was performed in each animal species (horse and donkey). The difference in the

means of each study variable was compared with the student T-test. Significance was set at $P<0.05$. All calculations were performed using the Statistical Analysis System SAS 9.4.

Results

The different sperm abnormalities were well observed using the eosin-nigrosine staining technique (fig. 1a) as well when using the Trumorph® technique (fig. 1b)

The morpho abnormalities count is summarized in table 1. No significant difference was observed for various abnormalities and total abnormalities using the stained and unstained technique for stallion and donkey sperms (Table. 1).

The percentage of total abnormalities was higher in the stallion than a donkey.

Discussion

The percentage of morphologically normal and abnormal sperm are widely regarded as the most accurate and precise diagnosis of fertility, and also, can be correlated with diseases and exposure to reproductive toxins and hazards [6,14,19,20].

This study represents the first report comparing sperm abnormalities using a Trumorph® technique and the commonly used eosin-nigrosine stain in stallion and donkey. Our results herein showed that the Trumorph® technique kept the sperm alive under observation, unlike eosin-nigrosin method where such observation is done on dead spermatozoa. In this study, both methods were found to be acceptable for morphological evaluation of fresh stallion and donkey spermatozoa. Additionally, no significant difference between the two techniques was observed irrespective of species. It has been demonstrated that the use of staining for sperm morphology analysis might induce morphologic and morphometric alteration of the stallion spermatozoa [21–24]. Those alterations were also observed in other species such as goat, human, monkey [25–27]. Moreover, a study on staining semen smears of purebred Arabian horses demonstrated that the applied staining method of eosin-nigrosine had little influence on the frequency of the observed morphological abnormalities [28]. Another study by [22] found high stallion semen abnormalities using eosin-nigrosine than Papanicolaou stain. However, the presence of a high sperm defect may not always mean artifact because this can be interpreted through a more concise methodology thus revealing sperm

abnormalities. The stain is a hypotonic medium comparatively to semen and this may affect the osmolarity of the mixture, producing shrinking of the sperm head due to the drying process [29]. Also, it has been reported that it can produce an osmotic shock by increasing the coiled and bent tails. As well, the smearing process may detach the head from the midpiece leading to an increase in the percentage of detached heads [22]. However, it is important to note that when studying sperm morphometry, significant differences between staining techniques in head and mid-piece sizes may be seen [23].

According to guidelines by the Society for Theriogenology (SFT), the evaluation of stallion sperm morphology should be performed on fresh unstained samples using a microscope with phase contrast [7]. However, there are very few technicians skilled in this technique in most andrology and veterinary laboratories as well as the scarce resources due to the financial implication. Nevertheless, [22] observed higher abnormalities and defects of rough/swollen midpiece and cytoplasmic droplets in wet-mount preparations when compared to eosin/nigrosin-stained due to the artifact introduced during slide preparation.

The introduction of the new technique Trumorph® was previously validated and tested [18]. It showed that the sperm remained alive and immobile, without implying the use of fixing agents that damage them. It should be noted that in previous work carried out in sperm of various species of mammals, the maintenance of cell life could not be assured. The restriction of cell mobility is a consequence of the 45 ° C thermal shock provided by the system stage. Recently, various works have been carried out with semen of carp, eel in which has proven that the use of Trumorph® does not damage cell life. In the case of carp, the application of pressure by the system led to the activation of sperm motility without the need to suspend the cells in the activation medium [30]. It has been proposed that this is a consequence of the cells being subjected to a natural pressure in spawn conditions at a certain depth. Regarding the eel, it was observed that for at least eight minutes after the application of the pressure, the cells varied their area (and therefore their volume) with oscillating values, which indicates a response by a full and fully functional membrane [30].

In the present study, the comparison between the efficiency of the two methods (live vs dead sperm), it was found that despite the sperm being alive or dead, the incidence of sperm morphology defect remains the same. An explanation to our result could be as a result of a well-articulated and experienced technician in the preparation of eosin-nigrosin stain as this may alter from one individual to another. In practice, however,

both techniques offer different possibilities. The eosin-nigrosin is a negative differential dye which identifies intact live and dead sperm. But this technique needs an experienced technician for smears and sperm identifications. Using Trumorph® all the sperms have a white color (living and dead sperms). Nevertheless, the method is simple, faster, and can be performed by everyone. the observations are made at 400× magnification by taking images that are observed by a computer which can be easily registered. In this regard, the last method diminishes the abnormalities that may be caused by the staining and preparation processes as observed when eosin-nigrosin is used.

Previous studies have demonstrated the effect of cooling on membrane permeability. Consequently, the percentage of abnormalities increases when using different stains on cooled semen due to the effect of different staining techniques. Effectively cooling significantly alter sperm lipid fraction, increase membrane permeability, reduce enzyme activities, change membrane proteins [31] and causes cholesterol loss [32] with concomitant reduction of the stability of sperm cell membrane [33]. However, the lack of differences observed between both methods in our study may be due to the fact that fresh semen was used, therefore further studies are needed on cooled or frozen-thawed semen using Trumorph® for a clearer understanding.

Table 1. Morphological abnormalities of sperm in relation to the technique used (means ± SD)

	Stallion			**Donkey**		
Variables (%)	**Stain**	**Trumorph®**	**P-value**	**Stain**	**Trumorph®**	**P-value**
Total abnormalities	27.72±12.74	29.78±10.13	0.29	15.40±11.37	15.11±10.56	0.12
Bent tail	7.48±7.87	9.69±7.16	0.30	4.35±6.44	8.90±9.04	0.09
Coiled tail	3.66±3.09	2.70±3.47	0.69	6.85±11.38	1.42±3.33	0.92
Abnormal head	2.55±2.35	2.79±1.80	0.78	1.28±1.29	1.33±1.08	0.50
Cabeza suelta	2.94±3.60	2.10±2.79	0.24	1.00±1.17	1.32±1.41	0.10
middle piece	2.83±2.67	3.05±3.03	0.48	0.63±0.60	0.32±0.35	0.85
Proximal droplet	5.56±6.80	7.55±5.00	0.94	0.96±1.33	0.88±0.98	0.06
Distal droplet	2.70±4.17	1.90±3.90	0.52	0.33±0.59	0.94±1.05	0.95

List of figure legends:

Fig1: sperm morphology, A) Stallion sperms stained using eosin-nigrosin stain; B) immobilized stallion sperms using Trumorph technique

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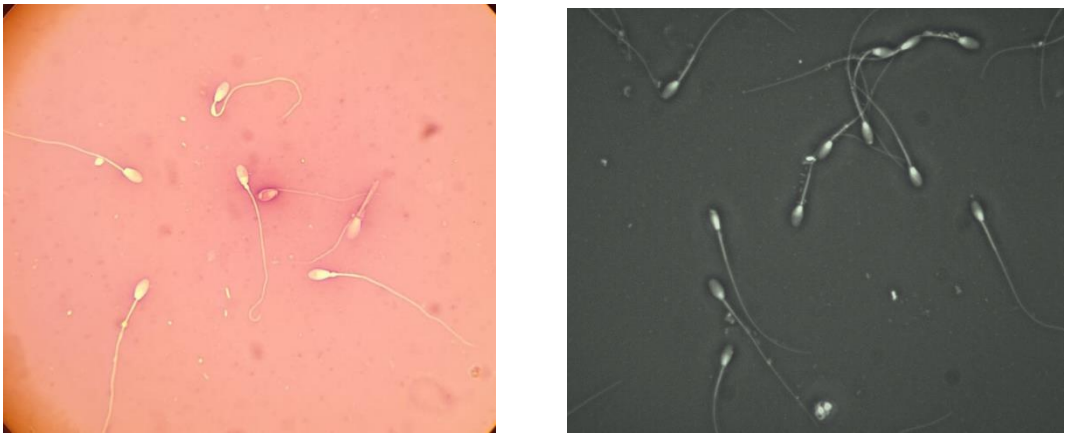

(A) **(B)**

Fig1: sperm morphology

A) Stallion sperms stained using eosin-nigrosin stain; B) immobilized stallion sperms using Trumorph technique

Manuscript 6

Atlas of sperm morpho-abnormalities using Trumorph® in equids

Atlas of sperm morpho-abnormalities using Trumorph® in equids

1. Introduction:

Equidae is the taxonomic family of horses and related animals including extant horses, donkeys and zebra. An estimated 50 million donkeys and 57.7 million horses and 8.5 million mules live in the world, constituting over 85% of the world's equids (FAOSTAT 2020). Today equine reproduction is a rapidly growing field in both donkey and horses especially with the use of different assisted reproductive technologies (ARTs) such as artificial insemination (AI) with cooled or frozen semen who reached about 90 of percentage foaling in stallion and variable in donkey between fresh and freezed semen who remain low (40% pregnancy rate)(Miró et al., 2013; Oliveira et al., 2016), also the use of embryo transfer (ET) and more recently intracytoplasmic-sperm injection (ICSI), are now widely used by veterinarians especially in stallion (Aurich and Aurich, 2006; Hinrichs et al., 2018; Kowalczyk et al., 2019) but didn't reach acceptable fertility rates in donkey and need more deep research (Panzani et al., 2012; Galli et al., 2013; Goudet et al., 2016; Miragaya et al., 2017). A precise diagnosis of ejaculates is necessary to predict fertility and to optimize the male reproductive ability for ART (Varner, 2008; Srivastava and Pande, 2017; Bucci et al., 2018). In bull, boar there is high-quality selection criteria for semen quality, while we no encounter a standard for semen evaluation in equids, those leading to an important proportion of studs with marginal semen quality with possible impaired fertility in males (Aurich and Aurich, 2006; Varner et al., 2015; Gottschalk et al., 2017). There is a manual for clinical assessment of stallion fertility published in 1986 by the Society for Theriogenology (SFT) where they consider a minimum of 500×10^6 progressively motile and morphologically (normal?) as a recommended insemination dosage (Turner, 2005) usually used also for a donkey. However, evidence-based studies to support this figure are lacking. Furthermore, there is evidence that much lower numbers of spermatozoa are enough to ensure good fertility, and the percentage of sperm morphological abnormalities were not predictive of fertility (Whitesell et al., 2019). These two semen quality tests were adapted from the SFT bull breeding soundness examination guidelines for its easy methodology (Ball et al., 1983; Hopkins and Spitzer, 1997). However semen generally is accepted with a value of 60% PMOT in fresh semen, and 30% PMOT for cooled or frozen/thawed semen and have been considered as cut-off values for fertile stallions (Hurtgen, 1992; Loomis and Graham, 2008), without any standard for morphology evaluation, making in consideration also that those values can change within the center depending on the methods and instruments used. Therefore, purchasers can buy a dose at a high price but do not always know the exact quality of

the semen, just that it has passed minimum standards for release and that could affect more when inseminating endangered breed specially in donkeys where limited doses are existing.

The increase in the demand inside this semen market, for a stallion with a highly competitive performance or donkey for mule production, shows the importance of a more serious fertility evaluation. Failure to eliminate poor quality ejaculates may result in poor semen quality, Additionally, accurate estimation of the damage suffered by the sperm cell after cooling or freezing procedures is necessary for the development of newer procedures to maintain sperm integrity and function, and this is the most important issue in the evaluation of a stallion's or donkey's reproductive performance.

The evaluation of sperm morphology is fundamental in the determination of sperm quality for breeding soundness examination. The WHO recommends for human the use of three staining methods: Pap, Shorr or DiffQuik, (WHO, 2010), while in equids the use of different stains was mentioned (India ink, William's, Silver nitrate, Karras, Spermac, Diff-quick, Eosin Aniline blue ……etc.) but the most spread is eosin-nigrosin.

The staining and drying of the samples produce what is known as artifacts, these produce morphological and morphometric changes in the cells as the enlargement of the head (Katz et al., 1986), or increase of coiled and bent tails, or detached sperm heads (Brito et al, 2011).

On the other hand, immature sperm can undergo a great enlargement (swollen) during the staining process (Banaszewska et al., 2015; Whitesell et al, 2019) and is was showed also that immature sperm extracted from the initial regions of the epididymis were highly sensitive to staining than ejaculated sperm, leading to major artifact abnormalities after manipulation. Likewise, the loss or elimination of cytoplasmic drops can be caused, as they are osmotically sensitive (Abraham-Peskir., 2002). It should be noted also that not every morphological artifact is subjectively appreciable, while in parallel, there can be a morphometric modification, only valuable by computer systems. To this we must add the clinician subjectivity, it has been shown that even cells classified as normal from different individuals may have different sizes (Bellastella et al., 2010) and that the percentage of normalities differ also between technician (Brito et al., 2011). Thus, it can be considered that there is a transformation of living cells during the staining process, which impedes the correct assessment of their real morphology.

To choose a staining technique, it must be selected because it causes the least possible change to the cells and to understand the origins of the artifacts, and those by considering the different steps of staining: placing a drop of sample on a slide, fixation, smear, air drying, staining and the length of each step (Soler et al., 2015a). The use of alcohols (such as ethanol or methanol) can cause agglutination and dehydration of the cells with a decrease in size; however, in wet preparations, evaporation of the medium during drying can lead to extracellular saline deposition, which would cause a hypertonic environment that would lead to the exit of water from the cell, causing its

dehydration and shrinking. On the contrary, the sensitivity of the membrane could also undergo the opposite change and swell when rehydrated; nevertheless, the acrosome provides localized " protection " that prevents the expansion of the acrosomal region, but not the post-acrosomal region membrane who is the less stable part (Gago et al., 1999).

We can exemplify the consequence of the artifacts by extrapolating it to a grape: the dehydration of a grape causes a raisin, and the rehydration of the raisin causes a rehydrated raisin, but it is no longer a grape again; It also happens with sperm, after manipulation, they no longer retain their characteristics fresh and what we see and study are not sperm, but artifacts.

When we subjectively assess sperm morphology, its clinical significance is reduced, only when the anomaly is more than evident, and we have a fairly clear diagnostic criterion. Spermatozoa are considered normal if they fall within the classification for a given species (WHO, 2010). Subjective estimates suffer from lack of precision, repeatability, and accuracy, in fact, a study of Bristol et al, (2002) in stallion samples have demonstrated a significant difference of clinician effects ($P< 0.05$) on the proportion of all sperm morphology categories with differences in the proportions of normal sperm (10%), and sperm head (17%) and midpiece defects (11%). For the human, a difference between 10 y el 80% also was encountered between observers (Cooper et al., 2002)

The present study proposes, first of all, to show through a collection of images the result of a sperm analysis of living cells, this implies the non-formation of artifacts caused by their staining. Secondly, the creation of an atlas of stallion and donkey sperm morpho-anomalies. For this, we have used Trumorph® (Proiser R + D, Paterna, Spain), an innovative method for the analysis of morphology based on the examination of wet preparations of immobilized sperm (Soler et al., 2016a).

2. Material and methods

The collection of the biological samples required for our study came from semen of two breed stallions (7 Arabian breeds, and 16 Spanish pure breeds) and 8 Catalan donkeys aged between 2 and 10 years. This study utilized 2 ejaculates of each stallion and 3 ejaculates of donkey. A total of 62 ejaculates were analyzed. The sample repertoire was heterogeneous, including equids with fertility, sub-fertile and fertile problems.

The semen was collected manually by means of an artificial Hannover vagina (Minitüb GmbH, Tiefenbach, Germany). Once semen was collected it was filtered and the volume recorded, then was diluted immediately 1:5(v/v) in a Kenny medium (24 mg/mL skimmed milk and 49 mg/mL glucose) (Kenny, 1983). The samples were incubated at 20°C until needed for slide preparation.

Between the collection and analysis of the semen, no more than 30 minutes passed. Once in the laboratory, various parameters of importance for seminal fertility were analysed, such as the concentration using a NeuBauer® chamber and motility by mean of CASA system (Proiser R + D, SL, Paterna, Spain).

Subsequently, the sample was homogenized manually and distributed in aliquots prior to the analysis of each of the characteristics for each ejaculate. For stallion we followed the technique used as described by Soler (2014), by using an aliquot of 3 µl, placed on the slide, then it was covered with a 22 mm x 22 mm coverslip and introduced into the Trumorph® system (Proiser R + D, SL, Paterna, Spain).

For donkey semen were used 2 µl drop of diluted semen, because more volume didn't immobilize the sperm, then the preparation was introduced 2 times into the Trumorph® system to have a total immobilization of all sperms.

The sample once introduced into the machine it was subject to two changes, temperature and pressure. An increase in the temperature up to 60 ° C, controlled by a dual-laser infrared thermometer (DT-8861; ATP, Leicestershire, United Kingdom), permitted the maintenance of the sample at 45° ideal for sperm immobilization without killing the sperm (Soler et al., 2016a). A pressure of 6 kp is applied, allowing the sample to extend and the sperms to get flatten in an adequate volume and space.

The observation of the cells was carried out at room temperature with an optical microscope specifically designed and equipped with a 40x objective of negative phase contrast (UOP / Proiser R + D, SL, Paterna, Spain), equipped with a CCD video camera colour with a resolution of 768x576, 8/12 bits per pixel.

To capture the microscopic fields, ISAS® v1 (Integrated Semen Analysis System) was used using the software corresponding to the morphology module. The fields were captured randomly but without considering those in which have "dirt" in background (particles, bacteria or cell debris) present in the seminal fluid. This would have made it difficult to obtain images in which 'clean' sperm were seen clearly. The complete process, from the taking of the aliquot to the image captures, was less than a minute.

A minimum number of 100 fields were captured from each sample, comprising around ten cells per field. In each field, all sperms were considered where their parts were well differentiated. All these cells were individualized through the use of the "Cuttings" program, producing a total of about 35.000 individualized cells.

Once the repertoire was compiled, the next step was the classification of each of the images, based on the different anomalies encountered in the head, mid-piece and tail. Finally, prior to the

presentation of the images, they were edited (without modification of the original cell) in order to homogenize their sizes and eliminate background particles. In this phase, it was necessary to use a second program called “GIMP”, as graphic editor.

3. Results:

The most representative images of each type of anomaly were classified according to head, midpiece or tail defects. Since each sperm has these three components, in each image can occur a combination of various morpho-abnormalities. For this reason, the images are accompanied by the corresponding explanatory tables, where the morpho-abnormalities are indicated.

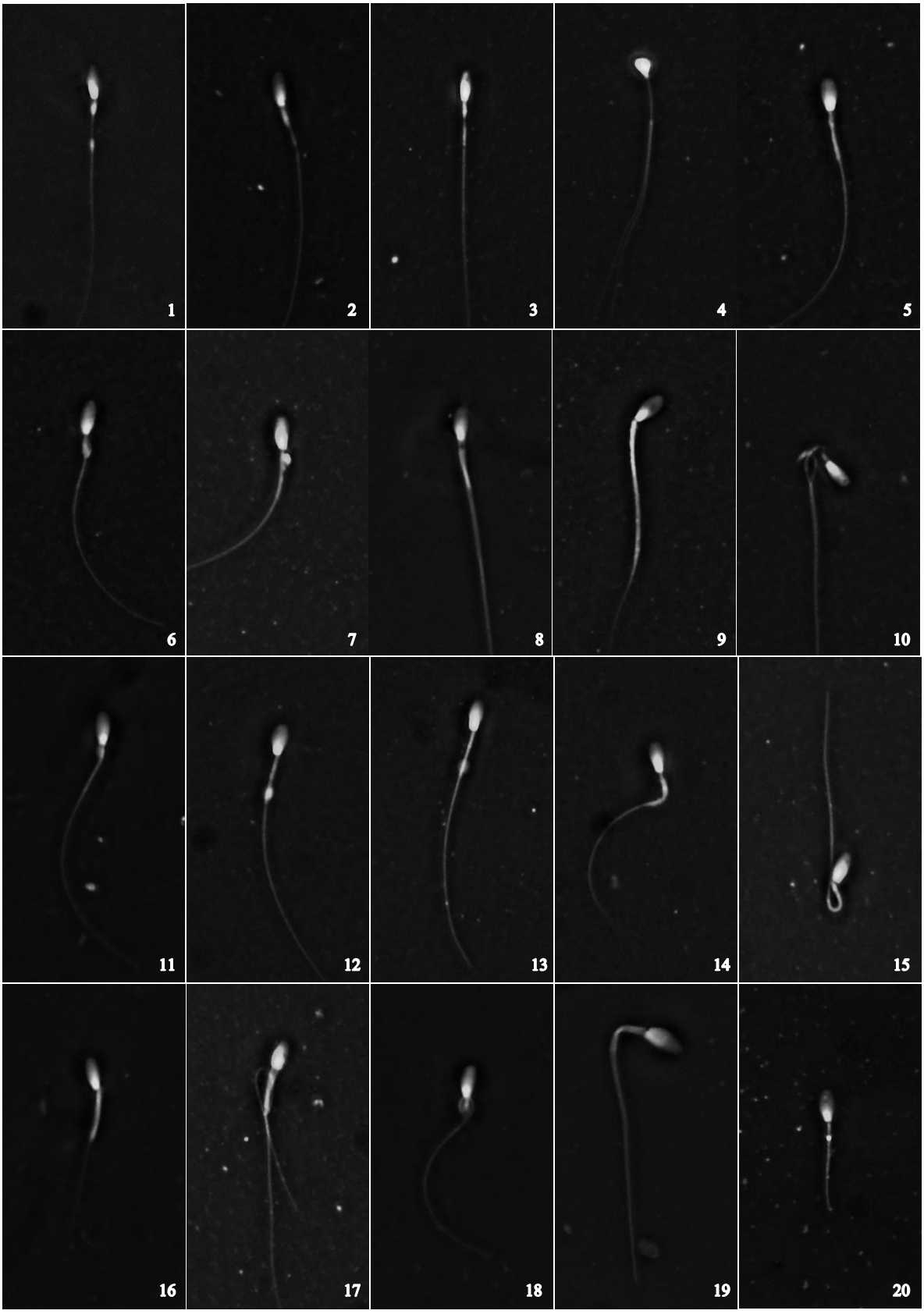
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Figure1: Sperm mid-piece defects

	Head	Mid-piece	Tale
1		Segmental aplasia of the mitochondrial sheet in the middle with proximal droplet	
2		Segmental aplasia of the mitochondrial sheet at the end of mid-piece	
3		A partial segmentation of mitochondrial sheet in the middle of the mid-piece	
4		Total aplasia of mid-piece	
5		Roughed mid-pieces	
6		Swollen mid-piece in the proximal part	
7		Microtubular mass defect	
8		Swollen mid-piece with mal insertion to the head	
9		Abnormal elongation of mid-piece	
10		Protrusion of axonemal fiber	
11		Proximal cytoplasmic droplet	
12		Distal cytoplasmic droplet with rough mid-piece	
13		Distal cytoplasmic droplet	
14		Point of fracture in the in the middle	
15		fracture at the point of insertion of the mid-piece	
16			Tadpole tail
17		Swollen mid-piece enrolled with a tale	Double tail
18		Incomplete mid-piece with retention of cytoplasmic materiel	Coiled tail
19	macrocephaly	Swollen mid-piece don't sure if fractured or bent	
20		Droplet	Short tail

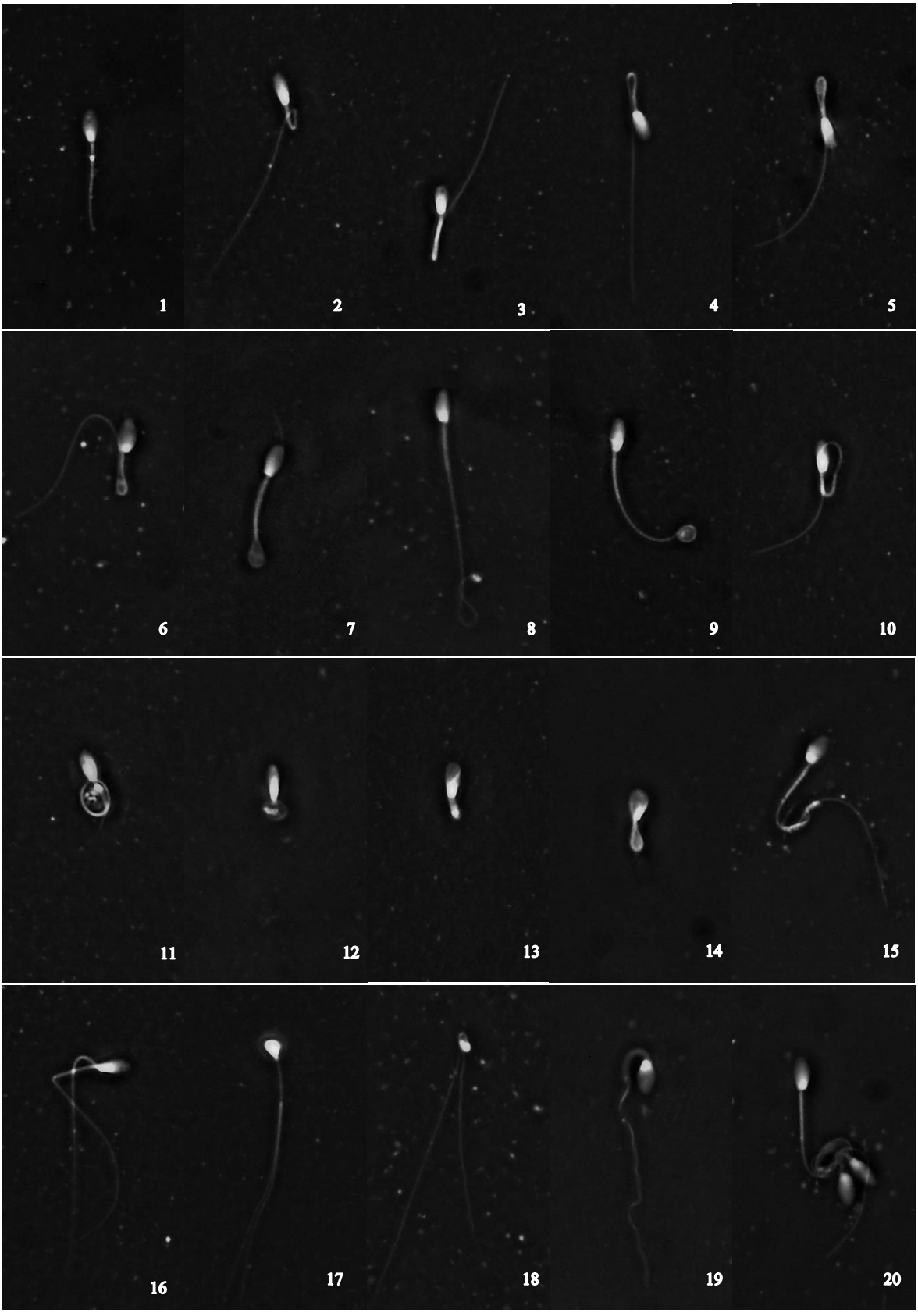
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Figure2: Sperm tale defects

	Head	Mid-piece	Tale
1			Aplasia of the tail
2		Reflex in the middle of mid-piece	Double bend tail
3	Vacuole in the acrosome	Distal mid-piece reflex	Bend tail
4			Bend tail
5			Bend tail with entrapped cytoplasmic droplet
6			Bend tail with entrapped cytoplasm plus a second bend
7			Simple bend tail in the middle of the tail
8			Distal bend tail
9			Bend tail with retained cytoplasm
10			Double bend tail
11			Coiled tail
12	Elongated head		Coiled tail
13	Elongated head		Absence of tail
14	Pyriform		Enrolled tail
15			Thinker tail
16	Pyriform head with vacuole	Absence of mid-piece	Double tail, Bend tail
17	Pyriform and flattened head without acrosome	Aplasia of mid-piece	Double tail
18	Small and pyriform head	Absence of mid-piece	Double tail
19			Abnormal development of the tail
20			Three tails of different sperms enrolled together

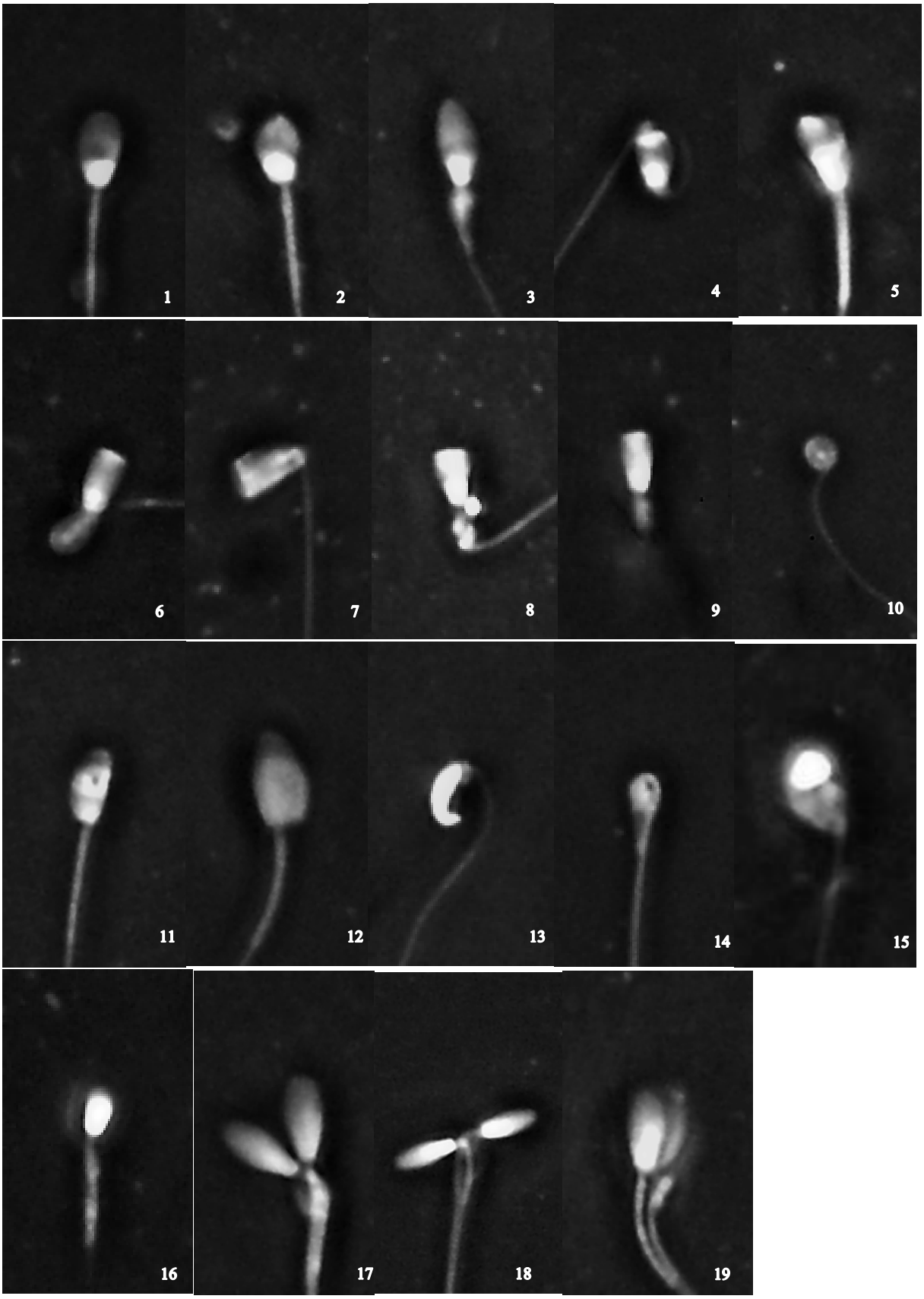
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Figure3: Sperm head defects

	Head	Mid-piece	Tale
1	Normal head shape		
2	Round normal head		
3	Elongated normal head	Microtubular mass defect	
4	Flattened head	Aplasia of mid-piece	
5	Knobbed acrosome with thinking of the apex	Swollen mid-piece	
6	Knobbed acrosome with flattened apex		
7	Knobbed acrosome with edentate apex		
8	Flattened apex	Microtubular mass defect	
9	Knobbed acrosome with flattened head	Entrapped cytoplasm	Bend tail
10	Circle head	Absence of mid-piece	
11	Nuclear vacuole		
12	macrocephaly		
13	Head with cytoplasmic invagination	Aplasia of mid-piece	
14	Pyriform head with vacuole		
15	Extremely abnormal head formation with no acrosome		
16	Extremely abnormal shape with no acrosome	Swollen mid-piece	Tadpole tail
17	Double head	Swollen mid-piece	
18	Double head	Protrusion of axonemal fiber	
19	Triple head with separate mid-piece	Double mid-piece for three head	

4. Discussion

Even admitting that most of the researchers agreed on what the general appearance of a 'normal' sperm looks like, the consensus at the time of their analysis is highly different. To this is added the fact that to study them a large number of different techniques have been used that show in each case advantages and disadvantages that are not always suitable for optimal analysis of the different sperm components (Soler et al., 2015b).

The technique used here, Trumorph®, was previously validated and showed that the sperm remain alive, although they are immobile, without implying the use of fixing agents that damage them. The restriction of cell mobility is a consequence of the 45 ° C thermal shock provided by the system stage. On the other hand, the uniform pressure exerted on the cover ensures the displacement of the surrounding fluid of the cells, which allowed equal dispersion on all surfaces.

Both factors simultaneously cause the alignment of the position of the sperm allowing a uniform observation.

It should be noted that in previous work, carried out with sperm of various species of mammals, the maintenance of cell life could not be assured. Recently, various works have been carried out with semen of carp, eel in which it is proven that the use of Trumorph® does not damage cell life. In the case of carp, the application of pressure by the system led to the activation of sperm motility without the need to suspend the cells in the activation medium (Caldeira et al., 2017). It has been proposed that this is a consequence of the cells being subjected to a natural pressure in spawn conditions at a certain depth. Regarding the eel, it was observed that, for at least eight minutes after the application of the pressure, the cells area change (and therefore their volume) with oscillating values, which indicates a response by a full and fully functional membrane (Caldeira, et al., 2017).

The morphological classifications that have been proposed so far (including that of WHO) are based on subjective observation. These classifications have served as the basis for quantitative approximations using CASA-Morph systems since the 80s of the last centuries (Soler et al., 2016b) following an a priori approach. A priori classifications imply a previous definition based on experience, which serves to define canonical elements that provide the basis for a statistical study of discriminant analysis. It is worth asking if this approach is the most appropriate or if it is better to go to the posterior ranking, based on the analysis of main components and cluster. Since, for the moment, there is no CASA-Morph system capable of analysing the images from the Trumorph®, it is not possible to carry out the statistical work leading to a classification based on quantitative data.

From all the above, it can be concluded that the method used here, based on the Trumorph® system, can be considered validated for the morphological analysis of equids spermatozoa, avoiding the appearance of artifacts as a result of the sample preparation process. As a result, a wide variety of sperm morphologies were obtained, many of them similar to those found in stained preparations. The fact of introducing a new system of analysis implies the reconsideration of everything previously studied. Behaving a redefinition of the concept of ``normal sperm'' that has been used to date, since it shows that the morphological observation has been carried out based on artefactual structures. As we have already indicated, the present work lays the morphological bases for the subsequent morphometric study that derives in a matrix of classification.

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Discussion

Results yielded in this Ph.D. thesis have several and important implications in the development of the utilization of ultrasonography and standardization of CASA motility and morphology analysis. Considered as an important tool of reproductive analysis in stallion and donkey.

Ultrasonography provides an easy, non-invasive means of assessing the ASG and detecting different pathologies (de Souza et al., 2015). However, to detect pathologies, normal reference ultrasound data must be available. Our first manuscript is the first reference on jackass ASG (accessory sex gland) ultrasonographic imaging. Prostate (PROS), bulbourethral gland (BUG) and ducts deferent ampullae's (AMP) were significantly large in donkey if compared with similar-sized stallions (Pozor and McDonnell, 2002), but the vesicular gland (VG) were thinner with no visible lumen. On the other hand, jackass PROS showed anechogenic spaces without sexual stimulation. The jackass AMP had a glandular appearance with anechogenic spaces, whereas in the stallion they are echogenically homogenous and thinner (Pozor and McDonnell, 2002). The AMP of the jackass may contribute significantly to seminal plasma production, resulting in jackasses ejaculating a larger volume of semen than stallions. The inverse correlation between the size of the BUG/AMP and the CASA variables might be explained in that seminal plasma to produce which the jackass has large glands negatively affects sperm preservation and motility (Miró et al., 2009).

Regarding pulsed-Doppler ultrasonography of the testicular artery, a progressive reduction was seen in the values of the blood flow variables towards the testis. This organ receives its arterial blood supply mainly via the testicular artery, with contributions from the vasal and cremasteric arteries. These arteries coil in the pampiniform plexus (PPP) before entering the testes, reducing the amount of blood that enters the organ, thus helping to reduce its temperature some 4-5°C below normal body temperature (Lloyd-Jones et al., 2015) and to reduce the tissue oxygen pressure. As a result, the testis survives on the edge of hypoxia (Bergh et al., 2001) perhaps to protect sperm DNA from the damage caused by oxygen. radicals. Any disturbance in the blood flow could negatively affect spermatogenesis and therefore fertility (Ortiz-Rodriguez et al., 2017). The RI and PI in the stallion are similar to those recorded here. However, the present PSV, EDV and TAMV values were twice those found in stallions and camels, perhaps because of the relatively larger volume of donkey testes and therefore their spermatogenetic requirements (Canisso et al., 2019). The strongest correlation with semen quality was recorded at the level of the capsular artery. The

RI at the level of the capsular artery showed a notable inverse correlation with testis volume. Donkey testes are big, and the diameter of the testicular artery at the level of the CA is large, and there are branches running in the longitudinal plane towards the caudal pole of the testis. However, no correlation was seen between any testicular artery blood flow variable and sperm abnormalities. In the present study, no correlation was made between pulsed-Doppler indices at the pampiniform plexus and semen quality (motility and morphology). The CASA used in the present study provides image captures that didn't exceed 25fps. Today new cameras are implemented and better software to analyse the results. That brings significant changes in the value of sperm kinetic parameters, which must now be considered.

In the second and third articles we attempted to standardize some parameters of CASA used for the motility assessment of donkey and stallion sperm using high-resolution cameras and specific chambers. The first step was to define the optimum frame rate (OFR) for sperm kinematic analysis. The OFR for the donkey and stallion sperm was different between chambers with a higher value in Spermtrack® 20 (278 fps, 309fps). We can note in horses that the calculated OFR (309 fps) with a VCL is remarkably superior compared to the donkey. Contrary to what was found with lower frames (25 fps), the VCL was higher in donkeys compared to the horse (Miró et al, 2005). This can be explained by the loss of information with a lower frame rate. It is believed that donkey sperm run faster than a stallion, but the current study showed less VCL with higher VSL and a straighter trajectory in donkey than in horse spermatozoa. Thus, explain the lower OFR found in the donkey compared to the horse. Additionally, in recent studies, while using a high-performance camera and the same study design, depending on each species that the sperm shows non-linear trajectory will need more captures to define the correct track. This explains the differences obtained in the calculation of the OFR in the bull (256 fps) (Bompart et al., 2019), the boar (212 fps) (Valverde et al., 2019), and the salmon (250 fps) (Valverde et al., 2019).

It can be observed also when comparing the mean value of kinematic parameters that horse sperm is faster and more curvilinear than donkey sperm. Donkey sperm is linear with significantly higher VAP. These differences were inappreciable at frame 25 (Miró et al., 2005). This could be related to sperm morphometric difference between these two species, since donkey sperm has smaller head (11.5±1.3 µm2) with larger midpiece (3.2±0.9 µm2), and an angle between head and midpiece (AHMP) (8.8 ± 17.46 degrees), than a horse (head: 13.42 ± 1.72 µm2, mid-piece: 1.63 ± 0.56 µm2, angle: AHMP: 6.28 ± 6.34 degrees) (Hidalgo et al., 2005). Both studies used the same

staining technique Harris' Haematoxylin. Moreover, the angle between head and midpiece may also affect the curvilinearity of sperm trajectory.

It was also found that the OFR is dependent on the chamber type and depth. In fact, the sperm motion or the flagellar beat is affected by the space where it is placed. As we can observe, the kinematic parameters were high in the reusable chamber compared to disposable chambers in both donkey and stallion. In fact, the same results were observed for VC, VSL and VAP on different species like bulls, boars and bucks. Yet, the other parameters were variable depending on the species (Del Gallego et al., 2017; Bompart et al., 2019; Valverde et al., 2019). Those differences could be explained by the loading technique that could generate physical forces on the spermatozoa affecting its motility. As well, it was showed the existence of a certain interaction between sperm and ions of the glass mounted on the chamber, which would be toxic in certain species (Bompart et al., 2018). The results of our study additionally demonstrated that the depth of the counting chamber influenced kinematic values. In fact, comparing the chambers of the same type for a different depth, we can observe that donkey sperm had greater VAP, VSL, LIN, STR, WOB and BCF in a narrow chamber of 10 µm rather than 20 µm chamber while VCL and ALH were greater in 20um chamber. The same result was found in other species such as the goat (Del Gallego et al., 2017). In the Belgian Blue bull and the Limousine bull breed (Bompart et al., 2019), D4C10, 20 µm depths were used. This kind of linear motility movement is defined by low lateral amplitude and a high straight-line velocity. Actually, it was found that this movement was essential for the sperm migration from the cervix to the uterus and then to the oviduct (Shalgi et al., 1992; Suarez, 2016). Moreover, this movement was also observed in the seminal plasma and the uterine fluids, with a low concentration of glucose. However, in the experiment of stallion the sperm demonstrate the inverse behaviour, the sperm was faster (high VCL, VSL and VAP) with higher index values in the deepest chamber 20 µm. same finding was for the boar (Soler et al., 2018), the Holstein bull breed (Bompart et al., 2019) and the stallion (Hoogewijs et al., 2012). Yet, the kinematics of eel sperm seem to be no different in chambers of either loading type (Gallego et al., 2013). Together, these results indicate that the chamber loading type affects the final motility of the cells differently in different species. However, a study conducted by (Soler et al., 2018), using lensless microscopy, showed that the kinetic parameters of boar semen in the deepest chambers of 100 µm increase significantly. Considering all these parameters discussed previously, it was demonstrated that the sperm behave differently depending on the chamber's depth and the species.

It is important to contemplate that there is a possibility that in the OFR with a higher chamber depth (more than the size of the sperm), the sperm will show different behaviour.

Also, the chamber field was taken into account in the analysis of donkey and stallion sperm. The donkey and stallion sperm had different behaviour for different chambers. In the donkey, the sperm analysed in D4C10 rectangular chamber showed that the sperm increased in velocity and lost its linearity, while the stallion sperm decrease in velocity as the drop moved to the last field. On the other hand, in the D4C20 chamber, an increase was seen in the same direction for VSL, VAP and BCF in both donkey and stallion. The same outcome was found in the fox samples (Soler et al., 2017) using the same chambers. It was suggested that the Segre–Silderberg effect altered the sperm movement as a consequence of the hydrodynamic drive of the fluid within the capillary-loaded chambers (Douglas-Hamilton et al., 2005). In this case, it affected the sperm tail (Lenz et al., 2011; Palacín et al., 2013) and the vitality (Gloria et al., 2013). In fact, we can observe that the results were accentuated in a narrow chamber when observing the sperm of the donkey. In the circle form chamber, Spermtrack®, when the drop moves from the centre to the edges, the motility decreased, but the linearity increased. Only for stallion when using SPK20 no differences were found between fields. Those results could be explained by the force of the fluid in the centre, which is generated by the cover that moves the sperm forward increasing the linearity but affecting the velocity. A similar result was found in the ram using a slide-coverslip (Palacin et al., 2013; Del Gallego et al, 2017). However, when using a Makler® for the bull sperm, no differences between the centre and the edges could be determined (Mortimer et al., 1988; Neuwinger et al., 1990; Valverde et al., 2018). As we can see, this could implicate the biology or metabolism of each species which behave differently (Dresdner and Katz, 1981).

Finally, it was considering the dilution rate within donkey sperm. The sperm velocity (VAP and VCL) and the progressiveness (BCF, STR and LIN) values were higher in the high-density sample (80×10^6 sperm/mL) compared to a low density (30×10^6 sperm/mL) sample for all the chambers. This difference can be related to the "dilution effect" referring to the detrimental effect on sperm quality, motility and resistance to a cold shock when adding a high volume of diluent of raw semen. The dilution effect was observed in a low sperm concentration (i.e., $<20 \times 10^6$ sperm/mL) and demonstrated in various species (Makler et al., 1991; Iguer-ouada et al., 2019). Nevertheless, a study on horses (Hayden et al., 2015; Buss et al., 2019) and humans (Makler et al., 1981) using a lower sperm density (2.5×10^6 sperm/mL) revealed higher motility parameters due to the minor effect that the "dilution effect" had on the sperm motility comparatively to other

species. We propose a concentration of 80×10^6 sperm/mL for further experiments in the study of the donkey when using a narrow chamber of 10 µm and especially in a disposable chamber. On the other hand, in reusable chambers, we recommend using a lower concentration since the concentration of 80×10^6 sperm/mL was too dense.

Higher frame rate and chambers types expressed better the sperm trajectory which is closer to reality. Although the role of the different subpopulations of spermatozoa remains unknown, the work should continue by implementing novel technologies and coordinated tools. The combination of clearly acquired image-sequences and sophisticated image processing allows obtaining reliable kinematic sperm parameters, whose outcomes are improved datasets. This enables a better establishment of real and significant subpopulation structures within and between species (Martínez-Pastor et al., 2011). We wanted in this fourth article to reflect the sperm subpopulation existing in both donkey and horse but also between some horse breed. To the best of our knowledge, it's the first work that uses high frames and the frequencies of these subpopulations were calculated. These differences can be associated with individual variations among ejaculates and individuals (Núñez-Martínez et al., 2006), sperm freezability (Martínez-Pastor et al., 2005), or sperm fertility (Quintero-Moreno et al., 2003).

According to the species, different works have indicated that the 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 dissimilar subpopulation structures (Soler et al., 2017). In the present study, 250 frames were chosen as the average optimum frames for stallion and donkey analysis. Three different sperm subpopulations were identified in donkey and stallion ejaculate, described from three principal components that involved the velocity, progressiveness and cell undulatory. These subpopulations as demonstrated previously had the largest group for the fast and the highest linear sperms (46% for donkey and 38.2% stallion). Previous works on donkeys and stallions demonstrated the existence of four subpopulations and with different percentages. Those works used a lower frame and a different statistical approach. This can show the importance of the frame rate in interpreting sperm trajectories and the errors that can occur with fewer frames, changing almost the real distribution of subpopulation of an ejaculate resulting in a misunderstanding of the real role of

each population and the contribution of each population that represent the state of the sperms (damaged, hyperactivated…etc.) and their capacity to arrive at the oocyte and fertilize it.
between horse breeds sperm subpopulations were slight only 2% between SP2 and SP3, but the kinetic values between a subpopulations of Arabian and Spanish horse breeds showed a significant difference, with high-velocity VCL and VAP for all subpopulations of the Spanish horse compared to the Arabian horse. This can show the effect of the breeding on sperm kinetic parameters resulted. It's indispensable to differentiate among breeds to obtain subsidiary results when analysing physiological accentuates or the effect of cryopreservation on stallion spermatozoa, otherwise the breed variance can cover any variance associated with analysed process.

Only one previous work on foxes' semen was found that 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 when combining both databases (Soler et al., 2017b). This kind of integrative work must be applied in the future, including the assessment of other parameters (DNA fragmentation, viability and membrane stability) to obtain a better comprehension of the ejaculate.

Morphology is a major parameter during the analysis of any mammalian sperm and is critical in selecting the assisted reproductive techniques treatment. The fifth article represents the first report comparing sperm abnormalities using the Trumorph® technique compared to the usual eosin-nigrosin stain in stallion and donkey. Eosin-nigrosin staining techniques were obtained using raw semen and smears were observed in a light microscope. Morphological abnormalities were also studied in unstained wet preparations using the Trumorph® technique. In the current study, testing both methods reported an acceptable estimation of fresh stallion and donkey spermatozoa. Moreover, it was observed that there is no significant difference between the two techniques. Eosin-nigrosin is the most used and spread technique for the study of equid sperm morphology. It has been demonstrated that the use of staining for sperm morphology analysis might induce morphologic alteration of the spermatozoa (Banaszewska et al., 2015; Łacka et al., 2016). Some staining methods cause sperm cell heads to swell (Katz et al., 1986)., others cause them to shrink (Abraham-Peskir., 2002). However, a study on staining semen smears of purebred Arabian horses demonstrated that the applied staining method either eosin-nigrosin or eosin-gentian dye had little influence on the frequency of the observed morphological abnormalities (Kondracki et al., 2017). Other studies demonstrated that staining with eosin-nigrosin yields similar results in stallion sperm morphology analysis as Papanicolaou (Cecere, 2014; Banaszewska et al., 2015; Łacka et al., 2016).

It’s important to note that the most evident changes observed in the different studies were for morphometry of the sperm when comparing different staining techniques (Hidalgo et al., 2005). Regarding the second technique, Trumorph® technique was previously validated and showed that the sperm remain alive, although they are immobile, without implying the use of fixing agents that damage them. It should be noted that in previous work, carried out with sperm of various species of mammals, the maintenance of cell life could not be assured. The restriction of cell mobility is a consequence of the 45°C thermal shock provided by the system stage. On the other hand, the uniform pressure exerted on the cover ensures the displacement of the surrounding fluid of the cells, which allowed equal dispersion in all the surfaces. Both factors simultaneously cause the alignment of the position of the sperm allowing a uniform observation.

Recently, various works have been carried out with semen of carp, eel in which it is proven that the use of Trumorph® technique does not damage cell life. In the case of carp, the application of pressure by the system led to the activation of sperm motility without the need to suspend the cells in the activation medium (Caldeira et al., 2017). It has been proposed that this is a consequence of the cells being subjected to a natural pressure in spawn conditions at a certain depth. Regarding the eel, it was observed that, for at least eight minutes after the application of the pressure, the cells varied their area (and therefore their volume) with oscillating values, which indicates a response by a full and fully functional membrane (Caldeira, et al., 2017). In the present study, it was compared the efficiency of two methods (live vs dead sperm) for sperm morphology analysis and it was found that even if sperm is alive or dead the incidence of sperm morphology defect remains the same. In practice, however, both techniques offer different possibilities. The eosin-nigrosin is a negative differential dye, which permits to identify of intact live and dead sperm. However, this technique needs an experimented technician for the smears and sperm identifications. Using Trumorph® all the sperms have a white colour (living and dead sperms). Nevertheless, the method is simple, faster, and can be performed by everyone. the observations are made at 400× magnification by taking images that are observed by a computer. Also, the last method diminishes the artifacts that can be caused by the staining used and allow the observation of living sperms. Since Trumorph® technique uses living sperms, and it is avoiding the appearance of artifacts as a result of the sample preparation. It can be a good alternative compared to the conventional staining method, which provides a quick test on live sperms.

Conclusions

The present Ph.D. thesis analysed the application of reproductive ultrasonography (B mode and colour pulsed doppler) as a first step. Then focused on the use of CASA technology and multivariate analysis to optimize the semen evaluation of sperm motility and morphology in stallion and donkey. The following section summarizes the main findings and conclusions:

1. Accessory sex gland in Jackass have different size and shape than stallion, affecting seminal plasma volume, and so the sperm quality in both species differently.

2. The reduction of colour pulsed wave doppler indices from the PPA to CA help in maintaining low testis temperature and tissue oxygen pressure, both necessary for spermatogenesis.

3. Pulsed-Doppler ultrasonography indices of CA have a direct correlation with semen parameters (concentration, CASA kinematics, and sperm viability), therefore can be a good predictor of semen quality and quantity.

4. For the optimal use of CASA-Mot technology in the evaluation of equids sperm motility we recommend the use of a FR of 300 fps for stallion and 250 fps for donkey, evaluated on a reusable counting chamber of 20µm depth on almost 9 microscopic fields distributed along the counting area. Using these conditions three sperm subpopulations can be clearly defined in both species.

5. It is important to take into consideration the sperm motility differences between stallion breed when carrying out reproductive studies in these breeds.

6. Trumorph® technique permit the analysis of sperm morphology of alive, unstained sperms and is a good alternative to classical staining approaches in both donkey and stallion.

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