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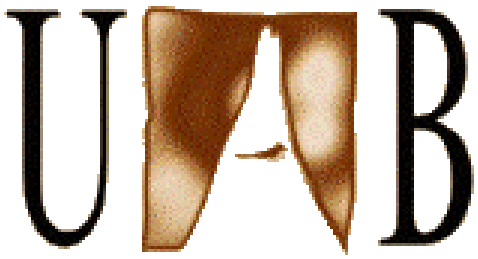

ESTUDIO SOBRE LA DINAMICA DE POBLACIONES ESPERMÁTICAS EN SEMEN DE CABALLO, CERDO Y CONEJO

Armando Quintero Moreno

Bellaterra, Octubre del 2003

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Tesis doctoral presentada como compendio de publicaciones por Armando Arturo Quintero Moreno

Bellaterra, Octubre del 2003

Joan Enric Rodríguez Gil, Profesor Titular del Departamento de Medicina y Cirugía Animales de la Facultad de Veterinaria de la Universidad Autónoma de Barcelona,

CERTIFICA:

Que la tesis titulada **"Estudio sobre la dinámica de poblaciones espermáticas en semen de caballo, cerdo y conejo"** presentada por **Armando Arturo Quintero Moreno** para optar al grado de Doctor en Veterinaria, ha sido realizada bajo su dirección y, considerándola acabada, autoriza su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos que corresponda, firmó la presente en Bellaterra el 23 de Julio del 2003.

Joan Enric Rodríguez Gil

A mi esposa Westalia
A mi hijo Jordi

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ABSTRACT

Quintero-Moreno, Armando Arturo. 2003. Study on dynamic of sperm population in semen of stallion, boar and rabbit. 156 pp.

Text in English

The first aim of this study was to test the presence of separate sperm subpopulations with specific motility characteristics in stallion, boar and rabbit ejaculates by using a computer-assisted semen analysis system (CASA). Sperm motility descriptors were analyzed thorough the clustering of variables based on a covariance matrix. This matrix selects the descriptors of sperm motility that better explain the spermatozoon kinetics. Sperm subpopulations were obtained by disjointing cluster analysis where the observations are divided into clusters by which each observation belongs to one specific cluster. This test showed that three separate sperm subpopulations with different motility characteristics in boar, and four in stallion and rabbit, coexist in the ejaculates. There were significant ($P<0.001$) differences in the distribution of these subpopulations among individuals in all of the studied species, but no clear relationship between motile subpopulation structure and fertility was obtained. A second aim of the study was to test the possibility for a precise estimation of the fertilizing ability of mammalian ejaculate based upon the results of semen analysis. For this purpose, we tested the mathematical combination of several parameters of the boar and rabbit semen quality analysis as predictive "in vivo" fertility tools The main mathematical relations utilized among parameters were logistic and linear regressions. In boar, two mathematical models obtained by logistic regression involving osmotic resistance test, hyperosmotic resistance test and viability of fresh samples, showed a significant ($P<0.05$) relationships between semen characteristics and conception rate. However, none of the obtained models produced a significant relation between semen characteristics and litter size. In rabbits, logistic and linear regression analysis rendered two mathematic, significant ($P<0.05$) models, with related some semen characteristic (sperm viability and abnormalities) with "in vivo" fertility and litter size. In stallion, the study of subpopulations in ejaculates which showed confirmed fertilizing capacity showed that these ejaculates had the majority of their motile spermatozoa included in a subpopulation with high progressiveness and low linear velocity. Moreover, all the ejaculates with proven fertility which have a total sperm count $\geq 20 \times 10^9$ spermatozoa/ ejaculate showed all of their motile sperm included in this subpopulation. Our results support that the use of the values obtained in a standard boar, rabbit and stallion semen quality analysis to predict life fertilizing ability of a single ejaculate can reasonably be achieved by applying logistic and linear regression analyses to the parameters included in this analysis. Thus, our methodology can explain in a systematic manner mammal semen quality, relating it to conception rate and litter size.

Key words: Mammalian semen analysis, Sperm subpopulations, Conception rate, Litter size, Functionality tests.

RESÚMEN

Quintero-Moreno, Armando Arturo. 2003. Estudio sobre la dinámica de poblaciones espermáticas en semen de caballo, cerdo y conejo. 156 pp.

Texto en Castellano

El primer objetivo de este estudio fue determinar la presencia de subpoblaciones espermáticas con pautas específicas de motilidad en semen de caballo, cerdo y conejo. Se utilizó para este fin el análisis computarizado de la motilidad espermática (CASA). La optimización de las variables que mejor explican el movimiento espermático se realizó mediante un análisis de agrupamiento de variables basado en el estudio de su matriz de covarianza. La investigación demostró que tres subpoblaciones espermáticas en semen de cerdos y cuatro en semen de caballo y conejo coexisten en los eyaculados. Se encontraron diferencias significativas ($P<0.01$) en la distribución de estas subpoblaciones en todas las especies, sobretodo en caballos y en conejos. Sin embargo, no existió una relación clara entre las subpoblaciones espermáticas y la fertilidad "in vivo" del semen. Por otra parte, la estimación precisa de la capacidad fecundante del eyaculado de mamíferos sería muy útil para la optimización de las técnicas de reproducción asistida. Con este propósito, se planteó el segundo objetivo de este estudio, que fue determinar la posibilidad de usar la combinación matemática de varios parámetros de calidad seminal en semen de cerdo y conejo, incluyendo el CASA. En cerdo, dos modelos matemáticos obtenidos por regresión logística seleccionaron el test de resistencia osmótica, el test de resistencia hiperosmótica y la viabilidad espermática como los parámetros que mejor predicen la tasa de concepción ($P<0.05$). Sin embargo, ninguno de los modelos hechos por regresión lineal se relacionó con la prolificidad. En conejo, las regresiones logística y lineal proporcionaron dos modelos matemáticos significativos ($P<0.05$) que seleccionaron la viabilidad y las anormalidades espermáticas como los parámetros de mayor predicción de la fertilidad y la prolificidad. En caballos, los eyaculados con al menos una fertilidad confirmada presentaron espermatozoides con gran linealidad y progresividad. Además, la totalidad de las muestras fértiles presentaban un número total de espermatozoides por eyaculado superior a 20 $x10^9$. Nuestras observaciones respaldan la opinión de que la utilización predictiva de los resultados obtenidos en el análisis de semen de estos mamíferos puede conseguirse en forma razonable mediante la aplicación de análisis de regresión que permitan relacionar todos los parámetros de calidad seminal evaluados en cada especie. Así, la metodología empleada explica sistemáticamente la calidad del semen de mamíferos, además de relacionarla con la tasa de concepción obtenida después de la inseminación artificial y la prolificidad en los mamíferos evaluados.

Palabras claves: Análisis seminal en mamíferos, Subpoblaciones espermáticas, Tasa de concepción, Tamaño de la camada, Pruebas funcionales.

RESUM

Quintero-Moreno, Armando Arturo. 2003. Estudi sobre la dinàmica de poblacions espermàtiques en semen de cavall, porc i conill. 156 pp.

Text en Català

El primer objectiu d'aquest estudi va ser determinar la presència de subpoblacions espermàtiques amb pautes específiques de motilitat en semen de cavall, porc i conill. Es va utilitzat per aquest fi l'anàlisi computaritzat de la motilitat espermàtica (CASA). L'optimització de les variables que millor expliquen el moviment espermàtic es va realitzar mitjançant una anàlisi d'agrupament de variables basat en l'estudi de la seva matriu de covariança. La investigació va demostrar que tres subpoblacions espermàtiques en semen de porc i quatre en semen de cavall i conill coexisteixen en els ejaculats. Es varen trobar diferències significatives ($P < 0.01$) en la distribució d'aquestes subpoblacions en totes les espècies, sobretot en cavalls i conills. No obstant, no va existir una relació clara entre les subpoblacions espermàtiques i la fertilitat "in vivo" del semen. P'altra banda, l'estimació precisa sobre la capacitat fecundant de l'ejaculat de mamífers seria molt útil per l'optimització de les tècniques de reproducció assistida. Amb aquest propòsit, es va plantejar el segon objectiu d'aquest estudi, que va ser determinar la possibilitat d'utilitzar la combinació matemàtica de varis paràmetres de qualitat seminal en semen de porc i conill, incloent el CASA. En porc, dos models matemàtics obtinguts per regressió logística varen seleccionar el test de resistència osmòtica, el test de resistència hiperosmòtica i la viabilitat espermàtica com els paràmetres que millor prediuen la taxa de concepció ($P<0.05$). No obstant, cap dels models fets per regressió lineal es va relacionar amb la prolificitat. En conill, les regressions logística i lineal van proporcionar dos models matemàtics significatius ($P<0.05$) que van seleccionar la viabilitat i les anormalitats espermàtiques amb els paràmetres de major predicció de la fertilitat i la prolificitat. En cavalls, els ejaculats amb almenys una fertilitat confirmada van presentar espermatozoides amb gran linealitat i progressivitat. A més, la totalitat de les mostres fèrtils presentaven un número total d'espermatozoides per ejaculat superior a 20×10^9. Les nostres observacions recolzen l'opinió de que la utilització predictiva dels resultats obtinguts en l'anàlisi de semen d'aquests mamífers pot aconseguir-se de forma raonable mitjançant l'aplicació dels anàlisis de regressió que permetin relacionar tots els paràmetres de qualitat avaluats en cada espècie. Així, la metodologia utilitzada explica sistemàticament la qualitat del semen de mamífers, a més de relacionar-la amb la taxa de concepció obtinguda després de la inseminació artificial i la prolificitat en els mamífers avaluats.

Paraules claus: Anàlisi seminal en mamífers, Subpoblacions espermàtiques, Taxa de concepció, Tamany de la camada, Proves funcionals.

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CAPITULO I:
INTRODUCTION Y OBJETIVOS

1. Introducción

Investigaciones recientes han demostrado la existencia de subpoblaciones espermáticas en eyaculados frescos y conservados en algunos mamíferos. Estas subpoblaciones han sido definidas de acuerdo a patrones específicos de movimiento espermático, observándose en especies como el mono tití, la gacela, el cerdo y el perro (Holt, 1996; Abaigar y col, 1999 y 2001; Rigau y col, 2001, Thurston y col, 2001). La existencia de esta estructura en la mayoría de los mamíferos estudiados, a pesar de sus diversos orígenes filogenéticos, sugieren la existencia de alguna relación entre cambios en la estructura subpoblacional de una muestra seminal y su capacidad fecundante. De aquí se infiere que el estudio y caracterización de estas subpoblaciones pueden abrir una nueva vía para mejorar las técnicas del análisis seminal en mamíferos. El cerdo es la especie en la cual se ha profundizado más en esta línea de investigación, habiéndose determinado una estructura dividida en subpoblaciones espermáticas, ya sea por su motilidad o por su morfometría (Abaigar y col, 1999; Thurston y col, 2001; Hirai y col, 2001). En referencia al caballo y al conejo, no se han encontrado referencias bibliográficas previas que soporten esta teoría a pesar del interés comercial y científico de estas especies. Por otro lado, la existencia de subpoblaciones espermáticas con distintos patrones de motilidad no puede apreciarse en el análisis seminal clásico. Este hecho podría explicar, al menos en parte, las limitaciones de la interpretación intrínsecas a dicho análisis en el caso que la estructura de las subpoblaciones espermáticas y la capacidad fertilizante del semen tuvieran alguna relación. De esta manera, los estudios basados en subpoblaciones espermáticas podrían ser importantes para lograr una mejor definición de la calidad seminal en mamíferos .Por lo tanto, estos estudios podrían tener también una repercusión económica muy importante, al ayudar a una mejor comercialización de dosis seminales con mayor garantía de calidad.

La presencia de las subpoblaciones espermáticas con características específicas de motilidad puede ser analizada mediante el análisis computerizado de la motilidad (CASA). El CASA realiza mediciones rápidas y objetivas de los parámetros de motilidad individual. Los detalles técnicos del CASA han sido descritos ampliamente por Boyers y col (1989), mientras que otros estudios mas recientes han explicado aspectos prácticos de operatividad con este sistema (Davis y Katz, 1993; Davis y Siemers, 1995; Irvine, 1995; Krause, 1995; Mortimer, 2000; Verstegen y col, 2002). Los resultados obtenidos en el CASA se procesan posteriormente mediante análisis estadísticos multivariados, que permiten hacer el estudio de las características de las subpoblaciones espermáticas con un alto grado de precisión (Renard y col; 1996; Holt, 1996; Abaigar y col; 1999 y 2001; Thurston y col; 2001). Los análisis multivariados también han permitido estudiar la relación entre las motilidad espermática de muestras criopreservadas de humanos y su capacidad de fecundación "in vitro", con resultados positivos (Davis y col, 1991 y 1995). Estos estudios sugieren por lo tanto, que el uso de los análisis multivariados mejora grandemente la identificación de las subpoblaciones espermáticas con características específicas de motilidad en un eyaculado. A pesar de todo, un inconveniente del CASA es que el software utilizado aporta una gran variedad de descriptores de la motilidad espermática, los cuales en su mayoría están altamente correlacionados. Este hecho dificulta la selección de parámetros que mejor explican el movimiento espermático. Por lo tanto, es necesario buscar el modelo estadístico más adecuado para emplearlo en los análisis, conservando esta manera la mayor cantidad posible de información. La mejor alternativa es hacer análisis estadísticos multivariados basados en el agrupamiento de variables (parámetros) y de observaciones (espermatozoides) en función de sus características comunes. Esto permite separar objetivamente los espermatozoides de la muestra seminal en subgrupos, además de explicar sus patrones cinéticos con las variables que mejor lo describen (Quintero y col, 2001; Rivera y col, 2002).

En referencia al semen equino, no existen citas bibliografícas sobre el estudio de las subpoblaciones espermáticas, a pesar del interés práctico que podría tener este conocimiento en esta especie. Precisamente, las pruebas clásicas suelen tener un valor relativo en la determinación de la calidad seminal equina (Caiza de la Cueva, 1997). Por lo tanto, la sistematización de una prueba basada en el análisis de subpoblaciones puede ser especialmente importante en esta especie. De esta manera, es relevante determinar la distribución de las subpoblaciones en eyaculados fértiles y en semen refrigerado o

descongelado para poder definir de una manera más efectiva la calidad del eyaculado y así distribuir comercialmente dosis seminales con mayor certeza respecto a su calidad.

En referencia al semen porcino, como ya se ha dicho es la especie con más estudios publicados respecto a su estructura espermática subpoblacional. Además, debido a la gran importancia comercial que tiene el cerdo a nivel mundial, existe un gran número de investigaciones donde se discuten aspectos reproductivos, correlacionándose los parámetros de calidad seminal con la fertilidad (Berger y col, 1996; Gadea y col, 1998; Pérez-Llano y col, 2001). No obstante, pocos experimentos han diseñado análisis estadísticos de relación entre análisis seminal y fertilidad en porcino utilizando regresiones lineales o logísticas (Holt y col, 1997; Gadea y col, 1998), así como tampoco aparecen muchos estudios que relacionan la estructura subpoblacional con la fertilidad (Abaigar y col, 2001).

Por último, entre las especies estudiadas, el conejo es mamífero más pequeño al cual se le puede extraer semen fácilmente para evaluar en el laboratorio. El análisis de la calidad espermática de semen del conejo es frecuentemente usado para estudios de toxicidad reproductiva, posteriormente aplicados en medicina humana (Amann, 1982). Sin embargo, los resultados de este análisis presentan grandes limitaciones, ya que todavía se realiza la evaluación de parámetros como la motilidad total de una manera subjetiva, minimizando su importancia como indicador de la calidad seminal. Conjuntamente al interés que tiene el conejo en toxicología humana, la inseminación artificial se ha convertido en un proceso rutinario en explotaciones comerciales intensivas de esta especie, sobretodo en Italia, Francia, Hungría y España (Roca y col, 2000). En este sentido, la optimización del análisis seminal del conejo es necesaria para mejorar la productividad en esta especie, conociéndose muy poco sobre la relación que existe entre los parámetros seminales y la fertilidad o la prolificidad en conejos.

2. Objetivos

El primer objetivo general del presente trabajo es el de explorar la información aportada por el análisis de motilidad espermática (CASA) y determinar las posibles subpoblaciones móviles presentes en los eyaculados de caballo, cerdo y conejo. La finalidad de este estudio es el de conseguir diseñar un procedimiento de aplicación del CASA que permita

mejorar el análisis seminal tradicional en mamíferos. Para ello se planteó la siguiente metodología de trabajo:

1. Optimizar los descriptores de la motilidad espermática aportados por CASA mediante una selección estadística reductora.

2. Determinar la presencia de subpoblaciones espermáticas en muestras seminales diluidas de caballos, cerdos y conejos, además de determinar su relación con la fertilidad.

3. Señalar la asociación existente entre las subpoblaciones encontradas y el semental (caballo/ cerdo) o grupo genético (conejo) evaluado.

4. Precisar la asociación existente entre las subpoblaciones encontradas frente a otros parámetros importantes de calidad seminal.

El segundo objetivo general del estudio fue el de determinar una posible relación entre el análisis seminal en su conjunto de los eyaculados equinos, porcinos y cunícolas y la capacidad fecundante de estos. Para ello se siguió el esquema de:

1. Determinar la relación de los resultados obtenidos del análisis seminal frente a la tasa de concepción en yeguas, cerdas y conejas.
2. Precisar la relación entre los resultados obtenidos del análisis seminal y el tamaño de la camada en cerdas y conejas.

3. Referencias bibliográficas

Abaigar T; Holt W; Harrison R; Del Barrio G. 1999. Sperm subpopulation in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod; 60: 32-41.

Abaigar T; Cano M; Pickard AR; Holt WV. 2001. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (*Gazella dama mhorr*): effects of body weight; electroejaculation technique and short-term semen storage. Reproduction; 122: 265-273.

Amann RP. 1982. Use of animal models for detecting specific alterations in reproduction. Fundam Appl Toxicol; 2: 13-26.

Berger T; Anderson DL; Penedo MCT. 1996. Porcine sperm fertilizing potential in relationship to sperm functional capacities. Anim Reprod Sci; 44: 231-239.

Boyers SP; Davis R; Katz DF. 1989. Automated Semen Analysis. Curr Probl Obstet Gynecol Fertil; 12: 172-200.

Caiza de la Cueva FI; Pujol MR; Rigau T; Bonet S; Miró J; Briz M; Rodríguez-Gil JE. 1997. Resistance to osmotic stress of horse spermatozoa: The role of ionic pumps and their relationship to cryopreservation success. Theriogenology, 48: 947-968.

Davis RO; Overstreet JW; Asch RH; Ord T; Silber; SL. 1991. Movement characteristics of human epididymal sperm used for fertilization of human oocytes in vitro. Fertil Steril; 56: 1128-1135.

Davis RO; Katz DF. 1993. Operational Standards for CASA Instruments. J Androl; 14: 385-394.

Davis RO; Drobnis EZ; Overstreet JW. 1995. Application of multivariate cluster; discriminate function and stepwise regression analyses to variable selection and predictive modelling of sperm cryosurvival. Fertil Steril; 63: 1051-1057.

Davis RO; Siemers RJ. 1995. Derivation and reliability of kinematics measures of sperm motion. Reprod. Fertil Dev; 7: 857-869.

Gadea J; Matás C; Lucas X. 1998. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. Anim Reprod Sci; 56: 95-108.

Hirai M; Boersma A; Hoeflich A; Wolf E; Foll J; Aumuller R; Braun J. 2001. Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): Relation to fertility and seminal plasma growth factors. J Androl; 22: 104-110.

Holt WV. 1996. Can we predict fertility rates? Making sense of sperm motility. Reprod Domest Anim; 31: 17-24.

Holt C; Holt WV; Moore HDM; Red HCB; Curnock RM. 1997. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: Results of two fertility trials. J Androl; 18: 312-323.

Irvine DS. 1995. Computer assisted semen analysis system: sperm motility assessment. Hum Reprod; 10: 53-59.

Krause W. 1995. The significance of computer-assisted semen analysis (CASA) for diagnosis in andrology and fertility prognosis. Hum Reprod; 10: 60-66.

Mortimer ST. 2000. CASA-Practical Aspects. J Androl; 21: 515-524.

Pérez-Llano B; Lorenzo J; Yanes P; Trejo A; García-Casado P. 2001. A short hypoosmotic swelling test for the prediction of boar sperm fertility. Theriogenology; 56: 387-398.

Quintero-Moreno A; Rivera Del Álamo MM; Rigau i Mas T; Rodríguez-Gil; JE. 2001. Optimización de los parámetros de motilidad espermática porcina mediante agrupamiento jerárquico. Revista ITEA; 22: 832-834.

Renard P; Trimeche A; Le Pichon JP; Quero JC; Griveau JF; Chouteau P; Tainturier D; Le Lannou D. 1996. Sperm motility and flagellar motion: a comparison between boar and other mammalian species. Reprod Domest Anim; 31 (1): 249-250.

Rigau T; Farré M; Ballester J; Mogas T; Peña A; Rodríguez-Gil JE. 2001. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology; 56: 801-815.

Rivera del Álamo M; Palomo M; Quintero-Moreno A; Rigau T; Rodríguez-Gil JE. 2002. Sperm subpopulations in boar semen and their relationship with natural photoperiod. 17th

Congress of the International Pig Veterinary Society. June 2-5/ Ames, Iowa; Vol 2, paper 669: 494.

Roca J; Martínez S; Vázquez JM; Lucas X; Parrilla I; Martínez EA. 2000. Variability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15˚C. Anim Reprod Sci; 64: 103-112.

Thurston LM; Watson P; Mileham A; Holt W. 2001. Morphologically distinct sperm subpopulation defined by fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl; 22: 382-394.

Verstegen J; Iguer-Ouada M; Onclin K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology; 57: 149-179.

CAPÍTULO II:
REVISIÓN BIBLIOGRAFICA

Muchos investigadores en el área de reproducción animal están tratando de diseñar el "análisis seminal ideal", que valore adecuadamente y prediga la fertilidad de una muestra seminal. Así, el análisis de semen ideal sería aquel que de forma sencilla y eficaz permitiera conocer de manera predictiva la capacidad fecundante de un eyaculado concreto. Según Graham (1996), las cualidades que deben tener los espermatozoides de un eyaculado fecundante son: motilidad progresiva, morfología normal, metabolismo energético activo, capacidad para desarrollar una motilidad hiperactivada, integridad estructural y funcional de la membrana, integridad de las enzimas asociadas con la fecundación, capacidad de penetración y transferencia óptima del material genético. Sin embargo, este análisis integral es muy difícil de desarrollar, debido a que la enorme complejidad inherente a la función espermática (Amann y Hammerstedt, 1993; Graham, 1996, Caiza de la Cueva, 1998). Esta complejidad eleva a la evidencia el hecho de que el análisis clásico basado en el estudio de la motilidad, la concentración, la viabilidad, las anomalías morfológicas y el estado del acrosoma no permite evaluar todas las característica funcionales espermáticas, sobreestimando o subestimando así el potencial fecundante de una muestra (Butler y Roberts, 1975; Graham y col, 1980; Saacker y col, 1980; Pace y col, 1981).

Los estudios actuales sobre contrastación seminal persiguen como objetivo final identificar algún parámetro cinético, morfológico o bioquímico que indique el estado de la célula espermática en un momento dado y que también pueda ser correlacionado con la fertilidad y calidad del eyaculado. No hay que olvidar que el objetivo del examen cualitativo del semen es asegurar que la fertilidad subsiguiente de dicho semen sea óptimo. Sin embargo, las técnicas de contrastación del semen, tanto para la utilización en investigación como en la práctica deben ser precisas, sencillas, rápidas y económicas. Hasta ahora, las pruebas rutinarias que se realizan en los centros de inseminación artificial para detectar problemas de baja fertilidad e infertilidad cumplen dichas premisas. Ahora

bien, también es cierto que muchas veces dicha pruebas no son totalmente indicativas de la calidad seminal o de la fertilidad de un animal.

Así pues, la evaluación de semen tiene una gran importancia en el diagnóstico de la capacidad fecundante de los espermatozoides de un eyaculado. Ello deriva en un diagnóstico que en caso de ser desfavorable, plantearía la necesidad de un tratamiento clínico o incluso el descarte del animal como reproductor (Caiza de la Cueva, 1998). Actualmente, el análisis seminal clásico ha mejorado mediante la introducción de nuevas técnicas analíticas procedentes de otros campos de la investigación científica. Así, el estudio de la motilidad espermática, la concentración espermática y las anomalías morfológicas que anteriormente se hacían de una manera subjetiva, pueden realizarse hoy en día mediante el uso de métodos computarizados de análisis (Budworth y col, 1988; Samper y col, 1991; Palmer y Magistrini, 1992; Tuli y col, 1992; Varner y col, 1991; Davis y col, 1993; Ball y Mohammed, 1995; Holt, 1996; Vázquez y col, 1997a). La implementación de estos métodos informáticos atenúa en gran parte el factor subjetivo del análisis seminal y garantiza una mejor correlación con la capacidad fecundante del espermatozoide (Jasko y col, 1992).

1. Técnicas de análisis de calidad seminal

El análisis seminal o espermiograma incluye una serie de pruebas que evalúan diversos factores o funciones de la célula espermática. Como resultado del análisis seminal podemos calificar a la muestra como apta o no apta para su uso en inseminación artificial. Entre todas las pruebas disponibles detallaremos las siguientes:

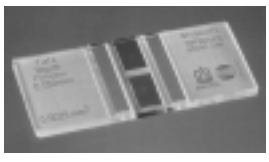
1.1. Concentración espermática

La concentración espermática es una de las pruebas de análisis seminal más importante. Existe una variabilidad muy grande en la concentración de un eyaculado a otro, siendo importante conocer el número de espermatozoides por eyaculado, ya que de este parámetro depende el número de hembras a inseminar. La concentración puede calcularse por varios métodos a partir de una muestra de semen. Entre estos métodos destaca la espectrofotometría, la colorimetría, la citometria de flujo y el uso de cámara de recuento

celular como las de Bürker, Neubauer o Thoma (Woelders, 1990; Hafez, 2000; Noakes y col, 2001).

La espectrofotometría es un método indirecto, capaz de medir la luz monocromática absorbida por las partículas en suspensión o los espermatozoides. La medición de la densidad óptica de la muestra es comparada frente a una curva estándar patrón previamente validada. Esta comparación permite conocer el número de espermatozoides (Woelders, 1990; Noakes y col, 2001). El método de citometria de flujo tampoco es realmente un método directo. Sin embargo, puede determinar el número de partículas por unidad de volumen, aunque no puede asegurar que todas estas partículas son realmente espermatozoides (Woelders, 1990). En eyaculados de conejo este técnica puede inducir a error debido a la gran cantidad de impurezas que presenta el semen (González-Urdiales; 2002).

El método más extendido es la utilización de la cámara de recuento celular. Este análisis se lleva a cabo mediante contaje en un microscopio óptico con el que se examina la muestra de semen previamente diluida con soluciones fijadoras (formaldehído, cloraceno, etc). Los preparados se mezclan cuidadosamente y se cuentan en el microscopio óptico con un aumento de 100X o 400X, empleando por comodidad un contador manual. El contaje se puede realizar de varias maneras; contando todos los espermatozoides que se encuentran dentro de todos los cuadrados de la cámara o contando aquellos que están en los cuadrados externos y centrales (Figura 1). Se cuentan ambos lados del hemocitómetro y finalmente se calcula el promedio. Este método es económico y fácil de llevar a cabo (Hefez, 2000), si bien el número de células contadas puede ser superior al de otros métodos (Woelders, 1990).



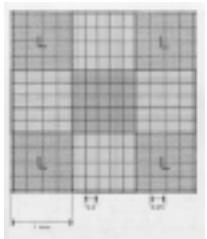


Figura 1. Cámara de Neubauer

Los resultados obtenidos en la cámara de recuento celular presentan mayor coeficiente de variación (12,3%) que en el espectrofotómetro (2,9%) y que la citometria de flujo (2,3%) lo cual ha llevado a afirmar que el espectrofotómetro y la citometria de flujo son las técnicas más precisas para el recuento espermático (Paulenz y Hofmo; 1996).

1.2. La Motilidad total

La motilidad es uno de los parámetros más importantes de la analítica seminal. Existen varias técnicas de estudio de la motilidad, pero la más utilizada y a la vez la más simple es la valoración visual subjetiva del porcentaje de espermatozoides móviles y la calidad de su movimiento. Para la realización de esta valoración todo el material usado debe de estar en condiciones de normocinesis (temperatura de 37˚C).

Los espermatozoides pueden presentar 2 tipos de movimientos:

Movimiento de rotación (alrededor de su eje).

Movimiento progresivo (desplazamiento de la célula), el cual a su vez puede ser: lineal o circular.

Dentro de la motilidad total un caso especial hace referencia a la denominada "motilidad masal" que es únicamente evaluable en eyaculaciones de mamíferos con concentraciones espermáticas muy elevadas, como es el caso de los rumiantes. Se determina depositando una gota de la muestra seminal sin diluir sobre un porta-objetos atemperado en una placa térmica y se visualiza sobre la muestra en un microscopio óptico a 40 aumentos. Se evalúa de forma subjetiva el movimiento de las células espermáticas en su conjunto. Se les da una valoración del 0 al 5 o del 0 al 100% según la preferencia del técnico evaluador. La motilidad individual se obtiene utilizando mediante observación en microscopio óptico a 100-400 aumentos de una gota seminal (generalmente diluida) dispuesta entre portaobjeto y cubreobjeto. En este caso se valoran dos parámetros: Primero, el porcentaje estimado de espermatozoides que muestran algún tipo de movimiento o "motilidad total". Segundo, el porcentaje de espermatozoides motiles que presentan un movimiento progresivo o "motilidad progresiva". Por otro lado, el porcentaje de espermatozoides con movimientos no progresivos nos valorará la motilidad "in situ" de la muestra. La estimación de la motilidad total y la progresiva será una indicación de la calidad de movimiento que presente la muestra seminal.

1.3. Integridad de la membrana espermática

La integridad de la membrana espermática ha sido uno de los parámetros más estudiados, por su papel clave en la función espermática. De hecho, el estado de la membrana espermática marca la integridad morfológica y funcional de la célula (Rodríguez-Martínez y col, 1997). La evaluación morfológica se realiza usando la óptica de contraste diferencial de interferencia o de Nomarski, la óptica de contraste de fase o las tinciones supravitales como el verde rápido/eosina, la eosina/azul de anilina, el azul tripán/Giemsa (figura 2A) o el amarillo de naftol/eritrosina. También el examen a través de la microscopía electrónica de transmisión o de barrido ha sido valiosa para determinar aspectos de integridad espermática (Rodríguez-Martínez y col, 2001). Sin embargo, la mayor parte de estas técnicas aportan solo información estructural parcial, y suelen ser tediosas y costosas. Además, aún cuando algunas técnicas morfológicas informa de los daños de la membrana plasmática, estos resultados no siempre están correlacionados con la fertilidad del semen, a menos que el daño que presenten los espermatozoides sea muy importante (Rodríguez-Martínez y col, 2001). De hecho, posiblemente la tinción más utilizada es la eosina-nigrosina (Figura 2B), la cual es muy económica, asequible y fácil de realizar. Esta técnica tiñe de color rosado aquellos espermatozoides que presentan una membrana alterada, mientras que los espermatozoides vivos se observan de color blanco sobre un fondo púrpura. Uno de los primeros autores que utilizó la eosina como colorante vital fue Murosoff (1930; citado por García-Artiga y col, 1994).

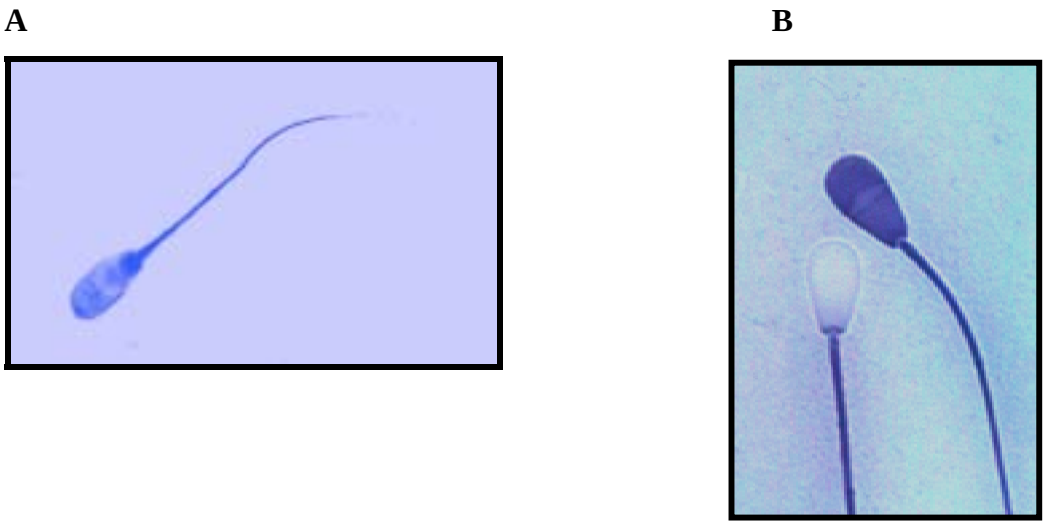


Figura 2.
Arriba: Tinciones espermáticas (A: tinción de azul trypan/Giemsa en espermatozoide de verraco; B: tinción de eosina nigrosina en espermatozoide de verraco.

Posteriormente, son numerosos los trabajos de investigación que determinan las diferencias entre los espermatozoides viables y no viables dentro de un eyaculado utilizando esta tinción, conjuntamente con otras técnicas (Bamba, 1988). Actualmente se están utilizando también diversas tinciones fluorescentes, las cuales presentan una mejor precisión en el estudio de las características de la membrana espermática (Harkema y Boyle, 1992; Magistrini y col, 1997). Así, se ha estado utilizado ampliamente el diacetato de carboxifluoresceina y el ioduro de propidio, Con estas técnicas se visualizan los espermatozoides funcionales de color verde frente a los espermatozoides muertos que se observan de color naranja.

1.4. Estado del acrosoma

El acrosoma juega un papel fundamental en la fecundación y esta importancia hace que convenga realizar una valoración específica del mismo. En un espermatozoide que tenga el acrosoma en perfectas condiciones se pueden distinguir tres regiones claramente diferenciadas en la cabeza: la zona acrosomal, con su borde apical, la zona postacrosomal y el segmento ecuatorial entre ambas. Las muestras seminales con alta proporción de acrosomas alterados o ausentes suelen tener una fertilidad baja. Para determinar el estado del acrosoma se han usado desde hace mucho tiempo diferentes tinciones. Entre éstas tenemos la tinción de Giemsa (Watson, 1975), la eosina–fast green (Wells y Awa, 1970), la eosina/nigrosina (Bamba, 1988), las dobles y triples tinciones basadas en la combinación de azul tripán con otros colorantes (Talbot y Chacon, 1981; Vázquez y col, 1992; Rodríguez–Gil y col, 1994) y tinciones comerciales como el Spermac (Oettlé, 1986). Recientemente, se han utilizado anticuerpos acrosomales específicos marcados con fluorescencia (Blach y col, 1988). Sin embargo, la manera más rápida de determinar el estado del acrosoma en aquellas especies donde el espermatozoide es lo suficientemente grande como para permitirlo es fijar la muestra en una solución de glutaraldehido y visualizas seguidamente de forma directa la estructura acrosomal en un microscopio con contraste de fase. Con este sistema distinguimos un borde apical nítido que se corresponde con el acrosoma, así como las posibles alteraciones del mismo. El estado del acrosoma también puede ser evaluado mediante el empleo de lectinas que, junto con el ioduro de propidio, permiten estudiar el proceso de reacción acrosómica. Las lectinas también se han venido utilizando desde hace más de una década debido a su capacidad de adherirse a las glicoproteínas específicas del acrosoma, permitiendo así un adecuado estudio de su

funcionalidad (Cross y Meizel, 1989). Estas técnicas han sido validadas para semen equino (Farlin y col, 1992; Casey y col, 1993; Meyers y col, 1995; Maxwell y Johnson, 1997) y de verraco (Vázquez y col, 1996).

1.5. Producción de L-lactato:

La producción de L-lactato es un indicador del estatus metabólico de los espermatozoides. La gran capacidad que tiene el espermatozoide para metabolizar azucares como la glucosa o fructosa es la base para desarrollar este tipo de prueba (Rigau y col, 1996). La determinación de la producción de L-lactato a partir de un medio con una concentración conocida de fructosa es una prueba funcional que ha mostrado una correlación aceptable con la fertilidad in vivo en el cerdo (Rigau y col, 1996). La prueba consiste en incubar 100 μL de semen en 900 μL de una solución isoosmótica a pH 7,4 que contiene 2,94% de citrato de sodio y 5,4% de fructosa (presión osmótica de 300 ± 5 mOsm) por 1 hora a 37˚C. Tras la incubación, se centrifuga a 3000X*g* durante 20 minutos. Luego se separa el sedimento obtenido del sobrenadante y se almacenan por separado en un congelador hasta su medición. Se determina la cantidad de L-lactato producida en el sobrenadante según la determinación enzimática descrita por Noll (1984). La cantidad de L-lactato obtenida se ajusta en función de la cantidad de proteína encontrada en el sedimento. Para obtener el valor proteico, el sedimento celular es resuspendido en 100 μL de agua destilada, se homogeniza mediante ultrasonidos y se determina la cantidad de proteína existente usando la técnica de Bradford (1976).

1.6. Técnicas de filtración de espermatozoides

Las nuevas técnicas de laboratorio han permitido determinar más de una propiedad del espermatozoide. Así, la filtración a través de lana de vidrio y Sephadex (Figura 3) ha permitido seleccionar y descartar los espermatozoides con alteraciones morfológicas y de motilidad, así como su grado de reacción acrosomica en el cerdo (Fayemi y col, 1979; Ramio y col, 2003), el toro (Crabo y col, 1992; Anzar y Graham, 1995 y 1996), el bufalo (Ahmad y col, 2003), el caballo (Samper y col, 1991; Samper y Crabo, 1993; Samper y col, 1995; Alghamdi y col. 2002) y el perro (Mogas y col, 1998). Es una técnica que permite seleccionar los espermatozoides de mejor calidad aparente y se basa en el supuesto de que un espermatozoide normal y vigoroso es capaz de atravesar la barrera de filtración

sin modificar sus características funcionales. Así, la utilización de estas resinas mejora la calidad del semen de bovino al atrapar células inmóviles o con motilidades muy bajas (Anzar y Graham, 1995 y 1996) e incrementa la motilidad en semen porcino y equino (Hammit y Martin, 1989; Samper y Crabo, 1993). Sin embargo, en semen de perro el efecto de las resinas depende del tipo de lecho mecánico que sustenta las columnas de Sephadex (Mogas y col, 1998). La filtración con Sephadex G-15 en presencia de lana de vidrio, pero no con una sujeción de polipropileno inerte, atrapa espermatozoides capacitados que han perdido algunas proteínas externas de la membrana, indicando que factores como la clusterina induce una interacción entre los espermatozoides y los filtros en semen de caballo y toro (Samper y col, 1995; Ibrahim y col, 2001).

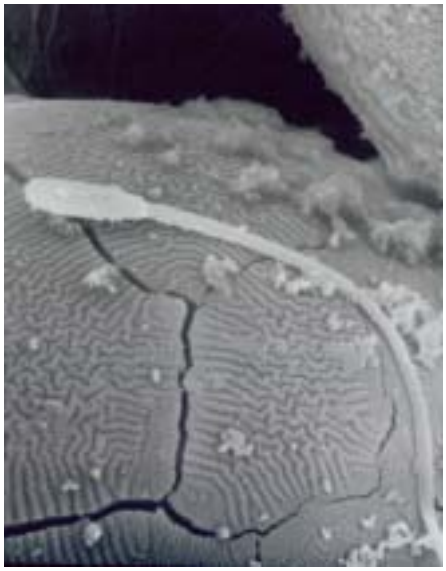


Figura 3. Espermatozoide de perro adherido a una partícula de Sephadex G-15 (Mogas y col, 1998)

1.7. Citometria de flujo

La evaluación del grado de desnaturalización del ADN usando citometría de flujo (SCSA) parece ser un complemento de gran valor para la evaluación espermática (Evenson y col, 1980). Ésta técnica ha permitido analizar entre otras cosas, la concentración espermática, la viabilidad, la función mitocondrial y la integridad del acrosoma (Evenson y col, 1980; Evenson y col, 1982; Evenson y Ballachey, 1986; Auger y col, 1989; Graham y col, 1990; Henry y col, 1993; Wilhelm y col, 1993; Wilhelm y col, 1996; Papaioannou y col, 1997; Evenson, 1999; Evenson y Spano, 2002). El método mide el grado de incremento de la heterogeneidad estructural de la cromatina, la cual se asocia a alteraciones durante la espermatogénesis. Esta heterogeneidad es visible en forma de anormalidades

espermáticas, la que se refleja en problemas de fertilidad (Rodríguez-Martínez y col, 2001). La instauración de esta técnica ha permitido el estudio de la estructura de la cromatina espermática y la medición del grado de desnaturalización del ADN en especies como el cerdo (Evenson y col, 1994), el caballo (Kenney y col, 1995) y el toro (Rodríguez-Martínez, 2000), permitiendo detectar infertilidades idiopáticas, ya que algunos de los parámetros analizados tienen relación significativa con la fertilidad de los reproductores. Cuando se somete la muestra a condiciones acídicas y se incuba con colorantes metacromáticos como la naranja de acridina, el ADN intacto muestra una fluorescencia verde, mientras que el ADN dañado muestra una fluorescencia roja. En verracos, la relación de espermatozoides verdes: rojos se relaciona con la fertilidad (Evenson y Thompson, 1990; Evenson y col, 1994).

1.8. Pruebas de penetración espermática

Las pruebas de penetración espermática permiten una buena determinación de la calidad seminal, al evaluar al mismo tiempo diferentes funciones como la motilidad, la capacidad de sufrir la reacción acrosómica, la penetración del oocito y la descondensación del DNA (Evenson y col, 1980; Didion y col, 1989; Cross y Meizel, 1989; Eaglesome y Miller, 1989). La penetración de oocitos homólogos libres de zonas pelúcida es una técnica utilizada en diferentes especies incluidas la equina y la porcina (Cristanelli y col, 1984; Samper y col, 1989; Zhang y col, 1990; Ivanova y Mollova, 1993; Martínez y col, 1993; Berger y col, 1996; Xu y Foxcroft, 1996; Gadea y col; 1998). En el toro (Graham y Foote, 1987 a y b) también se ha encontrado una alta correlación entre la penetración de oocito de hámster libres de zonas pelúcida y la fertilidad.

1.9. Pruebas de funcionalidad espermática

La membrana espermática es una estructura dinámica que participa en el reconocimiento y transporte de moléculas. Estas funciones permiten que el espermatozoide adapte su metabolismo al medio circundante, proporcionando así un sistema molecular para el reconocimiento del oocito (Hammerstedt y col, 1990). La evaluación de la integridad de membrana constituye una información importante en la evaluación de la fertilidad del macho (Jeyendran y col, 1984). Además, esta integridad no sólo es fundamental para el metabolismo espermático, sino que también lo es para una adecuada

capacitación y reacción acrosomica, y por ende para la fertilidad del macho (Yanagimachi, 1993). Un grupo de pruebas de funcionalidad espermática que ha centrado un gran interés por su simplicidad y su valor predictivo son las de resistencia osmótica. Estas pruebas se basan en los estudios de Drevius y Eriksson (1966), quienes demostraron la capacidad del espermatozoide de toro, conejo y hombre para captar agua en un medio hisposmótico. Estos autores observaron que la hinchazón osmótica esta asociada con el enrollamiento de la cola del espermatozoide, que se desdobla cuando la célula era devuelta a un medio isoosmótico. Estos cambios fueron confirmados por otros autores que relacionaron este fenómeno con la capacidad funcional de la membrana del espermatozoide humano observando una alta correlación entre la capacidad de hinchamiento del espermatozoide humano en un medio hispomótico y su capacidad de penetración en oocito de hámster libre de zona pelúcida (Foote y Bredderman, 1969; Mahi y Yanagimachi, 1973; Jeyendran y col, 1984). Dentro de las pruebas desarrolladas a partir de este fenómeno destacan dos: El test de endósmosis y el test de resistencia osmótica.

1.9.1. Test de endósmosis

El test de endósmosis (Hypoosmotic Swelling test, HOST) consiste en someter a los espermatozoides a un medio de presión osmótica más baja que la fisiológica, lo que causa una entrada de agua en la célula en un intento de equilibrar la presión osmótica interna con la del medio externo. Para que esta respuesta se produzca, la membrana plasmática del espermatozoide debe de estar íntegra y con los mecanismos de intercambio de fluidos funcionando correctamente. La entrada de agua provoca en estas células un hinchamiento y enrollamiento del flagelo. Las células con la membrana física o funcionalmente dañada no experimentarán cambios en la forma del flagelo (Pérez-Llano y col, 1999). Los valores obtenidos en esta prueba se correlacionan con otros parámetros de calidad seminal, como la motilidad, la viabilidad o la morfología. Esta prueba se ha aplicado en el semen del hombre (Zaneveld y Jeyendran, 1990; Jeyendran y col, 1992; Van den Saffele y col, 1992), del toro (Correa y Zavos, 1994; Correa y col, 1997), del perro (Kumi–Diaka, 1993; Kumi–Diaka y Badtram, 1994; Rodríguez–Gil y col, 1994; Sánchez y col, 2002), del caballo (Von Buiten y col, 1989; Caiza de la Cueva, 1997 a y b) y del cerdo (Vásquez y col, 1996; Vázquez y col; 1997 b; Pérez Llano y col, 1998 a y b). Por otra parte, presenta una elevada correlación con el test de penetración en ovocito de hámster en semen humano (Jeyendran y col, 1984). En humanos se ha encontrado una correlación elevada entre los resultados de

HOST y los obtenidos en fecundación “in vitro” (Van der Ven y col, 1986). En cerdos, si la presión osmótica es demasiado baja, la membrana plasmática se rompe y el flagelo aparece de nuevo recto, por lo que se confundiría con un espermatozoide que aún no ha reaccionado (Pérez Llano y col, 1998b). En la misma especie, el HOST detecta una subpoblación de espermatozoides viables que no reacciona al test, siendo así una prueba más sencilla a la hora de evaluar la viabilidad que la tinción de eosina-nigrosina o las tinciones fluorescentes (Pérez-Llano y col, 2003). En la especie humana, el test HOST parece ser un buen método predictivo de la capacidad fecundante “in vitro” e “in vivo” del semen (Check y col, 1989; Zaneveld y Jeyendran, 1990; Verheyen y col. 1997), sin embargo, no todos los autores avalan estos resultados (Rogers y Parker, 1991).

En los espermatozoides funcionalmente alterados no se produce la captación selectiva de agua de forma adecuada alcanzándose así un equilibrio pasivo entre los medios intra y extracelular que no provoca cambios morfológicos apreciables. Por otra parte, el hinchamiento de las células se puede provocar mediante otras vías distintas al descenso de la osmolaridad del medio que rodea a las células. Soluciones isosmóticas de solutos polares como el glicerol puede provocar el hinchamiento celular debido a la capacidad de estas sustancias de arrastrar agua cuando atraviesan la membrana celular, alterando así el equilibrio de las presiones osmóticas internas y extremas (Hammerstedt y col, 1990). De esta manera, las alteraciones celulares atribuídas al glicerol parecen estar más relacionadas con un shock osmótico que con la toxicidad química (Watson, 1979; Critser y col, 1988; Frim y Mazur, 1983; Gao y col, 1992). A pesar de todo ello, todavía existe un gran desconocimiento sobre los mecanismos moleculares específicos que el espermatozoide utiliza para reaccionar frente a un medio hispomótico.

1.9.2. Test de resistencia osmótica

Se ha desarrollado otro test basado en el porcentaje de espermatozoides que presentan una alteración estructural evidente en el acrosoma tras su incubación en un medio hiposmótico (Schilling y col, 1984 y 1986; Tamuli y Watson, 1992, Correa y Zavos, 1994; Gil y col 2000). Esta prueba se denomina test de resistencia osmótica (ORT) y presenta una correlación negativa con la capacidad fecundante del espermatozoide (Schilling y Vengust, 1985; Schilling y col, 1986). Por lo tanto, este test permite analizar el potencial reproductivo de los machos mediante el análisis de la calidad espermática. Se fundamenta

en someter los espermatozoides a un medio hipoosmótico, de forma que aquellos funcionales no mostrarán alteraciones a nivel acrosómico (Pérez-Llano y col, 1998 b,c). La prueba en sí consiste en diluir 100 a 200 microlitros de semen en 1 ml de una solución fisiológica ajustada a 300 mOsm/Kg incubándose durante 30 minutos. La evaluación del estado de los acrosomas tras esta incubación permite comparar la resistencia osmótica de las membranas de distintos eyaculados, así como predecir el comportamiento de los mismos tanto en lo referente a la fertilidad (Schilling y col, 1986) como a la capacidad de conservación (Schilling, 1984, Schilling y Vengust; 1985). Se ha creado una variante de esta prueba que se denominada ORT corto (ORTC) en el que el período de incubación se reduce a 5 minutos, de manera que puede ser usada dentro de la rutina diaria en los centros de inseminación artificial (Pérez-Llano y col, 1998 c).

1.10. Parámetros bioquímicos relacionados con la calidad seminal

Los métodos bioquímicos para la valoración de la calidad seminal se pueden clasificar en métodos de evaluación celular (composición fosfolípidica de la membrana espermática, actividad de enzimas como la aspartato aminotrasferasa o la acrosina y el estado de la cromatina) y de evaluación del plasma seminal (proteínas totales, niveles de zinc, etc.), los cuales se describen a continuación.

1.10.1. Fosfolípidos de la membrana espermática

En los últimos años se han incrementado los estudios referentes a los componentes de membrana espermática, ya que esta juega un papel preponderante en los procesos de conservación de los espermatozoides. En estudios recientes realizados en células espermáticas de verraco, se observa que en eyaculados de alta calidad, la fosfatididilcolina (FC) está presente en mayor cantidad y la esfingomielina en menor proporción, disminuyendo y aumentando respectivamente cuando la calidad es peor (De Alba y col, 1996). Los lípidos que forman la membrana celular están involucrados en la respuesta de los espermatozoides a la conservación del semen (O´Rand, 1979; Quinn y col, 1980). Así, la FC puede tener efecto protector durante el enfriamiento de la célula. Esto explicaría que un mayor contenido de FC en el semen podría mejorar la conservación de dicho eyaculado, ya que la FC colabora a que la motilidad, el estado del acrosoma y el metabolismo celular sean normales (Blesbois y Hemier, 1990). Cuando se refrigera o congela un eyaculado

previamente diluído, la membrana de la célula espermática es el primer lugar donde se manifiestan los efectos del estrés por frío. La sensibilidad al frío está relacionada con la composición y estructura de la membrana de los espermatozoides. Según el modelo de la membrana formulado por Singer y Nicholson (1972), ésta presenta una estructura a modo de mosaico fluido con una doble capa lipídica y proteínas entremezcladas formando una matriz de cristales líquidos, donde tanto los lípidos como proteínas pueden realizar movimientos de traslación dentro de la bicapa (Figura 4). Por lo tanto, la susceptibilidad de la célula al enfriamiento está relacionada con la composición lipídica de la membrana del espermatozoide. Este hecho explica que los espermatozoides de especies como el verraco,

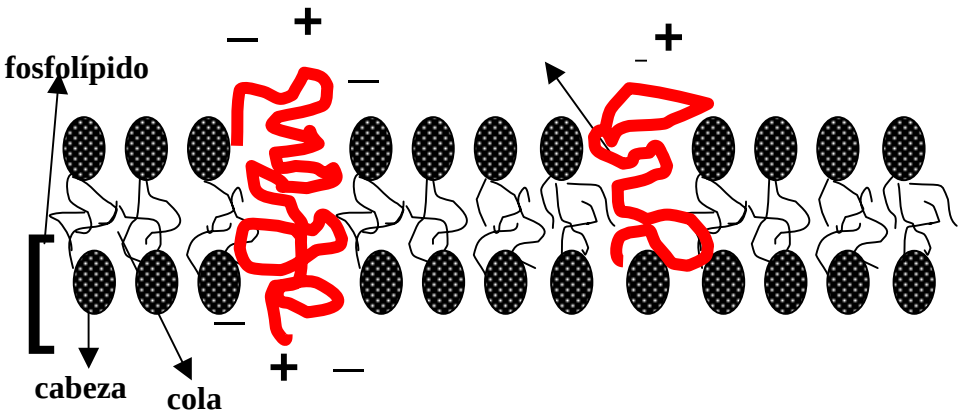


Figura 4. Estructura de membrana en mosaico (Singer y Nicholson, 1972).

el toro o el morueco, en cuya membrana la relación ácidos grasos insaturados/ ácidos grasos saturados es muy alta, sean más sensibles al frío (Koehler, 1985; citado por De Alba y col; 1997). La susceptibilidad al choque térmico se ha relacionado también con el contenido del colesterol y fosfolípidos de la membrana espermática. Así, las especies más sensibles al choque térmico tienen un contenido de colesterol más bajo que el de las especies más resistentes como el hombre y el perro (White y Darin-Bennett, 1976). En estudios realizados por De Alba y col (1996), se observan cambios en la composición fosfolipídica de la membrana espermática en el semen del verraco durante su disolución y posterior conservación a 15º C. Por lo tanto, la disolución produce cambios en la estructura de la membrana, lo que se traduce en la capacidad de conservación de dicho eyaculado. De hecho, la composición fosfolipídica de la membrana espermática del verraco es diferente con respecto a aquellas especies menos sensibles al choque frío (Darin-Bennett y White,

1975; Darin-Bennett, 1977; Watson y Plummer, 1985). Incluso entre eyaculados de distintas razas también existen diferencias en cuanto a la composición fosfolípidica de la membrana (Saiz-Cidoncha y col, 1994). Este hecho podrá estar en la base de las diferencias individuales existentes respecto a la resistencia osmótica en esta especie.

1.10.2. Actividad enzimática de la aspartato aminotransferasa

La aspartato aminotransferasa (AST) es una enzima cuya función consiste en transferir grupos amino del aspartato a moléculas de oxalacetato para formar glutamato. Se encuentra en las células en forma mitocondrial (mAST) y citoplasmática (cAST; Ciereszko y Strzekek, 1989). La actividad de esta enzima en el aparato reproductor de los mamíferos se localiza principalmente en los espermatozoides, en el líquido epididimario y en las secreciones prostáticas. La síntesis de esta enzima esta controlada por los andrógenos (Dubiel y col, 1987). Se ha descrito que todo factor que suponga un aumento de la actividad enzimática en el fluido extracelular dará como resultado un descenso de la fertilidad de ese eyaculado (Strzezek y Swidowick, 1986). Sin embargo, en semen fresco de verraco se ha observado que una actividad de AST elevada es indicativa de una mejor calidad seminal (De Alba y col, 1996). En esta misma línea están los estudios de Ciereszko y col (1990), los cuales describieron la correlación existente entre la motilidad, la concentración espermática y la actividad AST. Según estos autores, la enzima se libera del espermatozoide bajo el efecto de varios factores físicos-químicos, y esta actividad extracelular está correlacionada con la calidad del semen. Del mismo modo, se ha descrito que la actividad AST varía durante la conservación del semen, estando correlacionada con la capacidad de conservación del eyaculado (Strzezel y col, 1987; De Alba y col, 1996). La actividad AST extracelular es 5 veces superior en muestras con espermatozoides móviles que en las que contienen células inmóviles, observándose una correlación significativa entre la concentración espermática y la actividad de la enzima (Cierezko y col, 1990; Saiz-Cidoncha y col, 1994). Esto significa que la AST está implicada en la supervivencia de los espermatozoides, dado que, seguramente el espermatozoide muere inmediatamente después de perder su motilidad

La cuantificación de la AST es relativamente sencilla. El método esta basado en la acción de la enzima sobre un substrato formado por ácido 2-oxoglutotárico y ácido DL-aspártico, en presencia de piridoxal fosfato, añadiendo EDTA para la inhibición de la

acción de las fosfatasas. El color desarrollado en la reacción se mide por espectrofotometría a 505nm (Strzezek, 1984; citado por De Alba, 1997).

1.10.3. Determinación de la acrosina

La acrosina deriva de una proenzima inactiva denominada proacrosina que se ha identificado y cuantificado en el conejo y el verraco (Huang-Yang y Meizel, 1975; Polakoski y Zaneveld, 1984). La acrosina es una enzima con características similares a la tripsina y se encuentra en la pared acrosomal interna del acrosoma (Baccetti, 1979). Esta enzima juega un papel esencial en la penetración del espermatozoide en el óvulo (Brown y Harrison; 1978; Bedford y Cross, 1978). La liberación de acrosina después de un choque frío, al igual que ocurre en el caso de la AST, indica el estado de permeabilidad de la membrana espermática, y consecuentemente el grado de deterioro de la misma.

La cuantificación de la acrosina requiere una diálisis previa para la eliminación de inhibidores del paso de la proacrosina a la acrosina En un trabajo realizado por Marigorta y col (1995) en semen congelado de verraco, se demuestra que la liberación de acrosina y de AST de la célula espermática durante el proceso de congelación son paralelas y están altamente correlacionadas.

1.10.4. Estado de condensación de la cromatina

La determinación del estado de condensación cromática es un parámetro importante para la determinación de la calidad seminal en semen conservado, ya que las lesiones a nivel cromátinico pueden estar induciendo un incremento de la mortalidad embrionaria. De esta manera se complementan los estudios sobre integridad de acrosoma y membrana celular para establecer una mejor correlación con la fertilidad.

El estado de la cromatina en los espermatozoides puede determinarse “in vitro” en base a su susceptibilidad a la descondesación. Ésta se produce mediante la ruptura de los puentes disulfuro, formando complejos con iones metálicos divalentes, especialmente con el zinc, que estabiliza la cromatina espermática. La heparina es uno de los compuestos más importantes que da lugar a la descondensación de la cromatina “in vitro” (Bjomdahl, 1986). Los espermatozoides con un desarrollo normal mantienen una estructura de

cromatina resistente a la desnaturalización del DNA. Evenson y Thompson (1990) admiten la posibilidad de que el DNA en espermatozoides con una estructura cromática anormal sea susceptible de desnaturalizarse “in situ”. En este sentido, Strzezek y Smigielske (1978) observaron cómo se producía la descondesación cromática en semen conservado a -151ºC, mientras que Evenson y Thomson (1990) determinaron una buena correlación del estado de condensación cromática de semen congelado de verracos con la fertilidad. Estos resultados remarcan la importancia de este parámetro como marcador de calidad seminal.

1.10.5. Proteínas totales en el plasma seminal.

El contenido en proteínas totales en el plasma seminal también afecta a la calidad del semen en algunas especies como el verraco (Boursnell y col, 1968; Strzezek, 1976). En experimentos con verracos vesiculectomizados se ha demostrado que la susceptibilidad al choque térmico aumenta en ausencia de secreciones de las vesículas seminales (Davies y col, 1975; Moore y col, 1976). En dicho trabajo se afirma que las proteínas de las secreciones seminales cubren la superficie del espermatozoide, disminuyendo así la permeabilidad de la membrana. Una modificación en el contenido de proteínas totales puede ayudar así a explicar el aumento de susceptibilidad al choque térmico en estos espermatozoides (Moore y Hibbitt, 1977). En concordancia, De Alba y col (1995) encontraron en el eyaculado de calidad seminal alta en contenido un proteínas totales mayor con respecto a los de calidad seminal inferior.

1.10.6. Niveles de zinc en el plasma seminal

El plasma seminal tiene un alto contenido en zinc, encontrándose los iones en forma libre o combinados con proteínas. Según Luberda y Strzezek (1988) la cantidad de zinc en plasma seminal influye sobre la calidad del semen de verraco. Su papel fisiológico y bioquímico no está claro, aunque se piensa que es importante para la estabilidad de la membrana y de la cromatina, así como en el mantenimiento de la motilidad (Blom, 1976). La eliminación del zinc en semen de verraco da lugar a un elevado consumo de oxígeno y a la formación de peróxidos lipídicos (Mac Dermott y Fraser, 1990). Estos datos fueron confirmados posteriormente por De Alba y col (1996), observando que el contenido en zinc disminuye a medida que lo hace la calidad seminal.

2. Sistemas de medición de la motilidad espermática

Los espermatozoides de mamíferos adquieren la capacidad de movimiento durante el trasporte por el epidídimo. La motilidad flagelar es estimulada tras la eyaculación, modulándose durante el tránsito a través del tracto reproductivo de la hembra (Davis y Siemers, 1995). El tránsito de los espermatozoides a través del tracto genital femenino implica cambios secuenciales importantes en los patrones de motilidad espermática. Estos cambios reflejan modificaciones en la actividad metabólica de los espermatozoides, puesto que la motilidad es la principal causa del consumo energético espermático (Roldan, 1998). Por lo tanto, en un eyaculado fértil deberá existir un porcentaje significativo de espermatozoides capaces de llevar a cabo estos cambios de motilidad. Así, la profundización en los estudios sobre el control de la motilidad es importante para llevar a cabo un buen diagnóstico de la capacidad fecundante de un eyaculado (Katz y Overstreet, 1980).

Hasta hace pocos años, el estudio de la motilidad espermática se hacia exclusivamente mediante métodos semicuantitativos. Estos métodos evalúan el porcentaje de espermatozoides móviles, así como el tipo de movimiento que presentaba la media de una población espermática (Vázquez y col, 1997a). Estas medidas ofrecen una descripción general de la motilidad espermática, pero la exactitud y precisión están limitadas por las condiciones del sistema de medida y por la destreza del observador (Deibel y col, 1976). A pesar de ello, la valoración subjetiva de la motilidad por personas experimentadas es de gran valor, debido a que la información se presenta de forma inmediata al tiempo que es un método económico y fácil en su ejecución (Davis y Siemers, 1995). Por este motivo, el análisis de motilidad "*de visu*" en granjas, es el método más usado hoy en día para la evaluación de la motilidad. No obstante, hay que ser conciente que la subjetividad del método puede llevar a la obtención de resultados dispares para una misma muestra (Vázquez y col, 1997a).

Los primeros intentos de objetivizar el movimiento espermático se basaron en exposiciones fotográficas múltiples o en video-micrografías. Estos métodos son tediosos, largos y costosos, por lo que hoy en día no son de elección (Boyers y col, 1989). Sin embargo, la aparición de los sistemas informatizados de digitalización de imágenes abrió un nuevo campo en el estudio de la motilidad de los espermatozoides. Estos sistemas,

denominados genéricamente CASA (Computer Assited Motility Analysis), han automatizado y simplificado el proceso. El análisis computarizado de la motilidad fue propuesto por primera vez hace 24 años (Dott y Foster, 1979) y es usado actualmente en centros de investigaciones en andrología y en centros de reproducción asistida. Las imágenes obtenidas permiten evaluar varios parámetros, incluyendo la concentración, morfología y movimiento espermático. El CASA establece de una manera efectiva medidas cuantitativas del movimiento individual de los espermatozoides (Mortimer, 2000). Con este tipo de análisis se espera obtener medidas correctas de la motilidad espermática que proporcionen información precisa acerca del estado funcional del axonema y de las membranas espermáticas. Cuando el análisis se hace en condiciones óptimas, las medidas detalladas de la motilidad proporcionan información predictiva sobre la función espermática (Davis y Siemers, 1995). Sin embargo, hay que tener en cuenta que la exactitud de los valores obtenidos por técnicas cinemáticas está limitada por factores técnicos y biológicos, que posteriormente se mencionarán. De este modo, la interpretación de los datos es dependiente del conocimiento que tengamos de las condiciones del análisis (Mortimer, 2000 y 2002).

2.1. El sistema de análisis computarizado de la motilidad y los parámetros que mide

Los sistemas automáticos de medición de imágenes se basan en la captura sucesiva de espermatozoides en movimiento provenientes de un microscopio. Estas imágenes se digitalizan identificando las células espermáticas que contienen la primera imagen. Luego se procede al seguimiento de estas células en imágenes sucesivas y al establecimiento de las trayectorias definitivas. Las trayectorias se procesan matemáticamente, obteniendo así unos resultados numéricos precisos (Krause, 1995). Los parámetros determinados para cada espermatozoide son la velocidad del movimiento en base a varios descriptores, la trayectorias que realiza la cabeza del espermatozoide y la frecuencia de los cambios de dirección que esta realiza (Serres, 1984). Los descriptores del movimiento espermático obtenidos en el CASA han sido descritos por Boyers y col (1989). Actualmente, también existen en el mercado varios tipos de CASA que capturan el movimiento espermático y lo analizan tanto a tiempo real como de manera diferida, aportando un gran volumen de información. De una manera global, este último sistema consta de un microscopio con contraste de fase conectado a una platina atemperada que permite mantener las muestras a

37°C, una cámara de vídeo de alta resolución conectada a una pantalla de televisión y un software de análisis de imágenes por ordenador (Figura 5).

Unidad de control

- Ordenador Pentium IV
- 256 Mb RAM
- Tarjeta gráfica de 32 Mb
- Disco duro de 40 Gb
- Regrabadora 24x/10x/40x
- Tarjeta de red 10/100
- Monitor de 17'' (TFT opcional)
- Teclado, ratón y disquetera
- S.O Windows XP / W2000
- Tarjeta Meteor2/4 (Standard)
-

Microscopio triocular

- Nikon Eclipse E400 o similar
- Objetivos Ph 10x, Ph 20x - movilidad
- Objetivos 40x, 100x - morfología
-

Cámara de adquisición

- Sony XC-ES50/CE
- Cable video-alimentación
-

Complementos

- Platina atemperada
- Cámara Makler o similar
- Micrómetro de calibración
- Impresora de datos

Figura 5. Componentes del un sistema CASA (Microptic/ Barcelona, Versión 2002).

El análisis de la motilidad mediante CASA se hace al capturar las imágenes con espermatozoides en movimiento previamente diluídos en un medio adecuado y en el microscopio a 100-200 aumentos. Tras la captura, la información es guardada hasta su análisis. Una vez realizado el análisis, la información obtenida es tranferida a un procesador matemático que fragmenta la motilidad espermática en diversos descriptores de la motilidad individual que caracterizan la linealidad, la angularidad del movimiento espermático y el desplazamiento de la cabeza del espermatozoide (Tabla 1/ Figura 6). Por describir un ejemplo, el sistema CASA diseñado por Microptic (Barcelona) utilizado en

Tabla 1. Parámetros de motilidad que se evalúan mediante CASA.

PARÁMETRO	Unidad	DEFINICIÓN
Velocidad curvilínea (VCL)	µm/sg	Distancia recorrida por el espermatozoide a lo largo de su trayectoria real en función del tiempo.
Velocidad rectilínea (VSL)	µm/sg	Distancia recorrida por el espermatozoide en el primer punto y el último de su trayectoria.
Velocidad media (VAP)	µm/sg	Distancia recorrida por el espermatozoide a lo largo de su trayectoria media.
Índice de linealidad (LIN)	%	Relación porcentual entre la VSL y VCL.
Índice de rectitud (STR)	%	Relación porcentual entre la VSL y VAP.
Índice de oscilación (WOB)	%	Relación porcentual entre la VAP y VCL.
Amplitud media del desplazamiento lateral de la cabeza del espermatozoide (ALHMed)	µm	Desplazamiento medio efectuado por la cabeza del espermatozoide en su trayectoria curvilínea de un lado a otro de la trayectoria media o lineal.
Amplitud máxima del desplazamiento lateral de la cabeza del espermatozoide (ALHMax)	µm	Máximo desplazamiento efectuado por la cabeza del espermatozoide en su trayectoria curvilínea de un lado a otro de la trayectoria media o lineal.
Dance (DNC)	µm	Producto de la VCL por la ALHMed.
Dance medio (DNM)	µm	Cociente entre ALHMed y LIN.
Índice de angularidad (AI)	%	Valor porcentual del ángulo que forma un segmento de la trayectoria con la siguiente.
Velocidad angular media (AV)	(µm/sg)	Es el resultado de (VCL x AI)/100.
Desplazamiento angular medio absoluto (MADAbs)	Grados angulares	Ángulo que toma la dirección de un segmento de la trayectoria y el siguiente en valor absoluto.
Desplazamiento angular medio algebraico (MADAlg)	Grados angulares	Ángulo que toma la dirección de un segmento de la trayectoria y el siguiente, teniendo en cuenta su signo, siendo positivo al sentido contrario de las agujas del reloj.
Frecuencia de batida de la cabeza (BCF)	Hz	Frecuencia con la cual la trayectoria curvilínea atraviesa la lineal en función del tiempo.
Menor oscilación armónica de la cabeza del espermatozoide (HLO)	µm	Menor valor de la amplitud de la trayectoria curvilínea respecto a la trayectoria lineal o media.
Mayor oscilación armónica de la cabeza del espermatozoide (HHI)	µm	Mayor valor de la amplitud de la trayectoria curvilínea respecto a la trayectoria lineal o media.
Oscilación media de la cabeza del espermatozoide (HME)	µm	Valor medio de la amplitud de la trayectoria curvilínea respecto a la trayectoria lineal o media.
Máxima amplitud de la oscilación de la cabeza espermática (HMX)	µm	Distancia máxima en función del tiempo empleado entre 2 cruces sucesivos.
Armónico básico de la oscilación de la cabeza espermática (HBS)	µm	Distancia media en función del tiempo empleado entre 2 cruces sucesivos.
Amplitud del armónico (H_Y)	µm	Distancia mínima en función del tiempo empleado entre 2 cruces sucesivos.

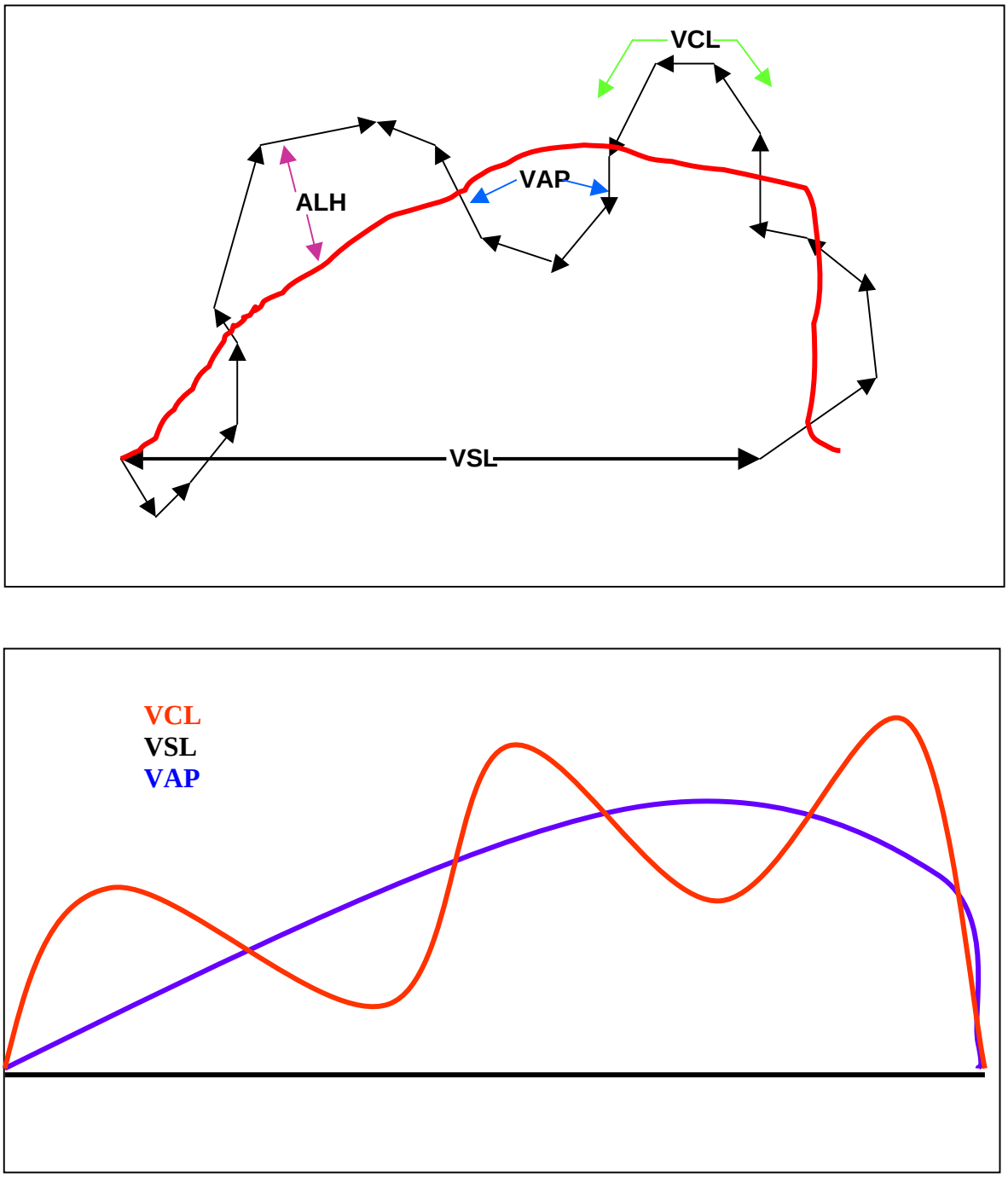


Figura 6. Terminología estándar de algunos parámetros de motilidad (Tabla 1) obtenidos por el sistema CASA.

esta tesis describe hasta 21 parámetros de motilidad, y la captura de las imágenes se basa en el análisis de hasta 100 imágenes fotografiadas de alta resolución (768 x 576), digitalizadas y tomadas en un tiempo de procesamiento de 2 segundos por muestra. Se han comparado datos utilizando diferentes sistemas disponibles en el mercado y se ha demostrado que no hay muchas diferencias en los resultados (Verstegen y col, 2002). En cambio, se han encontrado grandes diferencias entre los operadores y la metodología de preparación de la muestra a analizar (Verstegen y col, 2002).

2.2. Factores que afectan a la motilidad espermática medidas por CASA

Como se ha descrito anteriormente, existen una serie de factores técnicos y biológicos que afectan a los resultados presentados por el CASA. Así, es necesario conocer en todo momento las condiciones del análisis. Como ya se ha dicho, la interpretación de los datos cinéticos es dependiente del conocimiento que tengamos de lo que rodea a las condiciones del análisis (Mortimer y col, 1995). Según Verstegen y col (2002), entre los principales factores de los que depende el resultado final están la temperatura del semen, el volumen analizado, el tipo de cámara utilizada, el tiempo que oscila entre la recogida y el análisis, la concentración espermática de la muestra, el diluyente utilizado, posibles patologías existentes, el tipo de objetivo utilizado y la iluminación del microscopio, los valores del "setup" asociado al CASA y el área de las partículas analizadas.

2.3. Aspectos fisiológicos de morfología y motilidad del espermatozoide de los mamíferos.

Los espermatozoides de mamíferos están formados básicamente por 2 zonas con distintas funciones: la cabeza y la cola. La cola a su vez se divide en 3 segmentos que, por orden cráneo-caudal, se denominan parte intermedia, parte principal y parte terminal. En la figura 7 se describe de una manera general las partes que conforman el espermatozoide de mamíferos (Hafez, 2000). El núcleo de la cola esta formado por un axonema eucariota típico, con una estructura radial de pares de microtúbulos dispuestos en el típico esquema 9+2 (Mortimer, 2000). Alrededor de este axonema se sitúan diversas estructuras que varían respecto al segmento de cola de que se trate. Así, tanto la parte intermedia como la principal contienen una serie de elementos semirígidos (vaina fibrosa, columnas longitudinales) cuya función es la de otorgar direccionalidad y flexibilidad al movimiento

flagelar (Gagnon, 1995). La parte intermedia también contiene las mitocondrias estructuradas en una vaina mitocondrial, cuya función es la de generar la energía necesaria para el mantenimiento del movimiento del axonema (Gagnon, 1995). Todo este sistema genera que la cola presente un patrón de movimiento rotacional y elíptico, el cual se transmite a la cabeza del espermatozoide a través del cuello. Este movimiento rotacional subsidiario de la cabeza, es lo que otorga en última instancia progresividad al movimiento espermático (Mortimer, 2000). Esto explica que dentro de los parámetros de motilidad originados por el CASA, algunos de los más importantes estén relacionados con el desplazamiento de la cabeza. El patrón de movimiento espermático está modulado por numerosos factores y a su vez está íntimamente relacionado con la gestión del metabolismo energético espermático (Rigau y col, 2001). Esto explica los rápidos movimientos que se observan en los patrones de motilidad de los espermatozoides sometidos a incubaciones con cafeína o a una capacitación "*in vitro*" (Abaigar y col, 1999). Estos últimos hallazgos confirman que el patrón de movimiento del espermatozoide está influenciado por el medio ambiente externo (Serres y col, 1984; Mortimer, 2000).

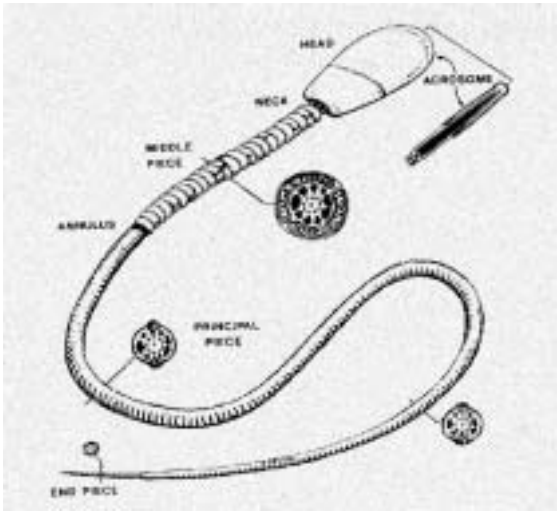


Figura 7. Partes de un espermatozoide (Hafez, 2000)

2.4. El papel de las subpoblaciones espermáticas en el eyaculado: Aplicación del CASA.

La existencia de espermatozoides con características funcionales y de motilidad diferentes dentro de un eyaculado es un fenómeno bien conocido (Chang y Hunter, 1975; Bedford, 1983; Renard y col, 1996; Holt, 1996; Abaigar y col, 1999 y 2001; Rigau y col, 2001). Sin embargo, el papel que puedan tener los grupos de espermatozoides con

características de motilidad similares en la calidad seminal global de un eyaculado no ha sido bien estudiado hasta el momento. Este hecho no deja de sorprender, puesto que parece evidente que cambios entre las proporciones de diversas subpoblaciones dentro de un mismo eyaculado ha de tener consecuencias en la calidad de éste. Sin embargo, el principal problema que existe para el estudio de estas subpoblaciones es su caracterización, puesto que hasta el momento no existían herramientas suficientemente eficaces para ello. Esta situación ha cambiado con la aparición de los sistemas CASA, los cuales permiten diferenciar a los espermatozoides de una manera individual por sus características de motilidad, siempre y cuando asumamos que la motilidad individual es una de las características importantes que definen la funcionalidad de un espermatozoide (Rodríguez-Gil, 2000). De hecho, la importancia real de estudiar los espermatozoides en un eyaculado no como un conjunto homogéneo, si no como el resultado de la combinación de diversas subpoblaciones heterogéneas no es evidente a simple vista. Continúa siendo mucho más simple trabajar con resultados medios de los valores obtenidos en la población entera. Sin embargo, existe una razón muy importante que explica la necesidad de estos estudios. En los últimos siete años se ha demostrado la existencia de estas subpoblaciones espermáticas, caracterizadas por unos patrones propios en su motilidad, en eyaculados de especies tan diferentes como el cerdo, el mono tití, el perro o la gacela (Holt, 1996; Abaigar y col, 1999, Thurston y col, 2001, Rivero y col, 2002; Quintero y col, 2003, ver Tabla 2).

Tabla 2. Publicaciones realizadas en Subpoblaciones Espermáticas en función de la motilidad espermática

Especie	Condición del semen	Subpoblaciones espermáticas (%)				Autor/Año
		1	2	3	4	
Mono Tití	Congelado	50	30	20	-	Holt, 1996
Cerdo	24h/diluido/24˚C	73,4	19,3	7,3	-	Abaigar y col, 1999
	Diluido/refrigerado	48,4	32,5	19,1	-	Thurston col, 2001
	Diluido/refrigerado	45,9	30,7	16,4	7,0	Rivera y col, 2002
		37,0	33,6	19,4	10,0	
Gacela	Congelado	47,4	36,1	10,0	6,5	Abaigar y col, 1999
Gacela	Fresco/diluido	55,0	26,7	10,3	8,0	Abaigar y col, 2001
Perro	Fresco	34,4	24,7	21,5	19,4	Quintero y col, 2003

Además, estos mismos autores también han mostrado que el porcentaje de espermatozoides incluidos en los diferentes subgrupos cambia siguiendo unos patrones fijos cuando los eyaculados son sometidos a procesos como la congelación/ descongelación, la capacitación "*in vitro*" o la incubación con sustancias como la cafeína o el bicarbonato (Holt, 1996; Abaigar y col, 1999).

Nuestro laboratorio también han obtenido resultados parecidos en perro (Mogas y col, 1999; Rigau y col, 2001) y cerdo (Rivera y col, 2002). De hecho, estos resultados nos indican que en un número significativo de especies de mamíferos la capacidad de reacción que tienen los espermatozoides de un eyaculado no es uniforme para todas las células, ya que cada subpoblación muestra una capacidad diferente de reacción frente a situaciones como la congelación/descongelación o la capacitación "*in vitro*". Dicho en otras palabras, dentro de un eyaculado sólo aquellos espermatozoides que integren algún subgrupo con características determinadas serán capaces de mantener una funcionalidad adecuada para proceder a resistir un proceso de congelación/ descongelación o capacitación (Rodríguez-Gil, 2000). La consecuencia de ello es que la aplicación de unos tratamientos estadísticos basados en la asunción de que las poblaciones espermáticas son homogéneas, tal y como se hace rutinariamente, implicará una pérdida considerable de información revelante, ya que quizás sólo una parte de la población espermática será realmente fecundante. Por lo tanto, si ésta subpoblación predominante no es demasiado grande, sus características se verán totalmente enmascaradas por el resto de la población si los resultados son tratados de una manera global dentro del eyaculado. Este hecho quizás explique fenómenos como la poca validez que tiene el estudio de la motilidad global de un eyaculado en algunas especies, como el cerdo. En esta especie se ha observado que la motilidad total y la progresiva, entendidas globalmente, tienen muy poca fuerza estadística como parámetros a considerar en un análisis seminal clásico (Rigau y col, 1996). El problema en esta especie quizás resida en el hecho de que no seamos capaces de observar por separado a las poblaciones espermáticas verdaderamente importantes para entender la calidad seminal. Técnicas como el CASA nos pueden permitir el reconocimiento de estas subpoblaciones marcadoras de la calidad seminal, y de esta manera el estudio concreto de las células seguramente mejorará la capacidad de diagnósticos del análisis seminal (Rodríguez-Gil, 2000).

3. Referencias bibliográficas

Abaigar T; Holt W; Harrison R; Del Barrio G. 1999. Sperm subpopulation in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod; 60:32-41.

Abaigar T; Cano M; Pickard AR; Holt, WV. 2001. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (*Gazella dama mhorr*): effects of body weight, electroejaculation technique and short-term semen storage. Reproduction; 122: 265-273.

Alghamdi AS; Troedsson MHT; Xue J; Crabo BG. 2002. Effect of seminal plasma concentration and various extenders on postthaw motility and glass wool-Sephadex filtration of criopreserved stallion semen. AJVR; 63(6): 880-885.

Ahmad Z; Anzar M; Shahab M; Ahmad N; Andrabi SM. 2003. Sephadex and sephadex ion-exchange filtration improves the quality and freezability of low-grade buffalo semen ejaculate. Theriogenology; 59(5-6):1189-1202.

Amann RP; Hammerstedt RH. 1993. In vitro evaluation of sperm quality: an opinion. J Androl; 14: 397-402.

Anzar M; Graham BF. 1995. Effects of filtration on post-thaw quality of bull semen. Theriogenology; 43: 439-449.

Anzar M; Graham EF. 1996. Role of sperm motility and acrosome integrity in the filtration of bovine semen. Theriogenology; 45: 513-520.

Auger J; Ronot X; Dadoune JP. 1989. Human sperm mitochondrial function related to motility: a flow and image cytometric assessment. J Androl; 10: 439-448.

Baccetti B. 1979. The evolution of the acrosomal complex In: Fawcett y Bedford (Eds.). The Spermatozoon; pp. 305-329.

Ball BA; Mohammed HO. 1995. Morphometry of stallion spermatozoa by computer assisted-image analysis. Theriogenology; 44: 367-377.

Bamba K. 1988. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. Theriogenology; 29:1245-1251.

Bedford JM; Cross NL. 1978. Normal penetration of rabbit spermatozoa through a trypsin- and acrosin-resistant zone pellucida. J Reprod Fertil; 54: 385-392.

Bedford JM. 1983. Significance of the need for sperm capacitation before fertilization. Biol. Reprod; 28: 108-120.

Berger T; Anderson DL; Penedo MCT. 1996. Porcine sperm fertilizing potential in relationship to sperm functional capacities. Ani Reprd Sci; 44. 231-239.

Bjorndahl L. 1986. On serum nuclear zinc and chromatin descondensation. Thesis. Kongl. Carolinska Medico Chirusrgiska Instituter Stockholm, p. 1-32.

Blach EL; Amann RP; Bowen RA; Sawyer HR; Hermenet MJ. 1988. Use of monoclonal antibody to evaluate integrity of the plasma membrane of stallion sperm. Gamete Res; 21: 233-241.

Blesbois E; Hemier D. 1990. Effects of high-density lipoproteins on storage at 4˚C of flow spermatozoa. J Reprod Fertil; 90: 473-482.

Blom E. 1976. Zinc as a possible causal factor in the sterilizing sperm defect in Jersey bulls nord. Vet Med; 28: 515-518.

Boyers SP; Davis R; Katz DF. 1989. Automated semen analysis. Curr Probl Obstet Gynecol Fertil; 12:172-200.

Boursnell JC; Briggs PA; Cole DM. 1968. Boar vesicular secretion protein: further comparison with seminal plasma protein. J Reprod Fert; 16: 457-461.

Bradford M. 1976. A rapid and sensitive method for the quantization of micrograms quantities of protein utilizing the principle of protein-dye binding. Anal Biocem; 72: 248-256.

Brown C; Harrison R. 1978. The activation of proacrosin in spermatozoa from ram, bull and boar. Biochymica et Biophysica acta; 526: 202-217.

Budworth PR; Amann RP; Chapmen PL. 1988. Relationship between computerized measurements of motion in frozen-thawed bull spermatozoa and fertility. J Androl; 9:41-54.

Butler WJ; Roberts TK. 1975. Effects of some phosphatidyl compounds on boar spermatozoa following cold shock or slow cooling. J Reprod Fertil; 43: 183-187.

Caiza de la Cueva FI; Pujol MR; Rigau T; Bonet S; Miró J; Briz M; Rodríguez-Gil JE. 1997[a]. Resistance to osmotic stress of horse spermatozoa: the role of ionic pumps and their relationship to cryopreservation success. Theriogenology, 48: 947-968.

Caiza de la Cueva FI; Rigau T.; Bonet S; Miro J; Briz M.; Rodríguez J. 1997[b]. Subjecting horse spermatozoa to hypoosmotic incubation: effects of ouabain. Theriogenology, 47: 765-784.

Caiza de la Cueva FI. 1998. Estudio sobre la resistencia al estrés osmótico de espermatozoides porcinos y equinos. Tesis doctoral, Universidad Autónoma de Barcelona/ España.

Casey PJ; Hilman RB; Robertson KR; Yudin AI; Liu IKM; Drobins EZ. 1993. Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. J Androl; 14: 289-297.

Chang MC; Hunter RHF. 1975. Capacitation of mammalian sperm: biological and experimental aspects. In: Hamilton DW, Greep RO (eds.), Handbook of Physiology. Endocrinology V. Washington: American Physiological Society, 339-351 pp.

Check JH; Epstein R; Nowroozi K; Shanis BS; Wu CH; Bollendorf A. 1989. The HOS test as a useful adjunct to the semen analysis to predict fertility potential. Fertil Steril; 52 (1): 159-161.

Ciereszko A; Strzezek J. 1989. Isolation and characteristics of aspartate aminotransferase from boar spermatozoa. Int J Biochem; 21 (12): 1343-1351.

Ciereszko A; Jablonowska C; Strzezek J. 1990. Aspartate aminotransferase activity in motile and inmotile spermatozoa fractions of frozen-thawed semen. Ani Reprod Sci; 23: 237-244.

Correa JR; Zavos PM. 1994. The hypoosmotic swelling test: its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology; 42: 351-360.

Correa JR; Heersche G; Zavos PM. 1997. Sperm membrane functional integrity and response of frozen-thawed bovine spermatozoa during the hypoosmotic swelling test incubation at varying temperatures. Theriogenology; 47: 715-721.

Crabo BG; Loseth KJ; Weidel L. 1992. Trapping of morphological types of bull spermatozoa by Sephadex/glass whool filters. Proc 12th Int Congr Ani Reprod AI; La Haya, Vol 1: 423-425.

Cristanelli MJ; Squires EL; Amann RP; Pickett BW. 1984. Fertility of stallion processed, frozen and thawed by a new procedure. Theriogenology; 22: 39-45.

Critser JK; Huse-Benda AR; Aaker DV; Arneson BW; Ball GD. 1988. Cryopreservation of human spermatozoa. The effect of holding procedure and seeding on motility, fertilizability and acrosome reaction. Fertil Steril; 50: 314-320.

Cross NL; Meizel S. 1989. Methods for evaluating the acrosomal status of mammalian sperm. Biol Reprod; 41: 653-641.

Darin-Bennett A; White I. 1975. Cholesterol and phospholipids content of mammalian spermatozoa and its relation to membrane structure and cold-shock. J Reprod Fert; 43 (2): 383-384.

Darin-Bennett A. 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold shock. Cryobiology; 14: 466-470.

Davies DC; Hall G; Hibbit HK; Moore HDM. 1975. The removal of the seminal vesicles from the boar and the effects on the semen characteristics. J Reprod Fert; 43: 305-312.

Davis RO; Gravance CG; Casey PJ. 1993. Automated morphometric analysis of stallion spermatozoa. Am J Vet Res; 54: 1801-1811.

Davis RO; Siemers RJ. 1995. Derivation and reliability of kinematic measures of sperm motion. Reprod Fertil Dev; 7: 857-869.

De Alba C. 1995. Variaciones de la composición fosfolipídica de la membrana espermática del verraco durante el equilibrio del semen en procesos de conservación. Tesis doctoral. Universidad de Complutense, Madrid.

De Alba C; Marigorta P; Saiz F; Strzezek J; Lyczynski A; Martin-Rillo S. 1996. Aspartate aminotransferase activity changes during boar semen preservation at 15˚C. Journal of Phisiol Pharmacol; 47 (2), Suppl 1.

De Alba C; Corcuera B; García-Artiga C; Strzezek J. 1997. Determinación de parámetros bioquímicos relacionados con la calidad seminal. En: IV Simposium Internacional de Reproducción e IA porcina. Madrid, 91-101 pp.

Deibel FC; Smith JE; Crabo BG; Graham EF. 1976. Evaluation of six assays of sperm quality by means of their accuracy, precision and sensitivity in separating known induced levels of damage. Proc. 8th Int Congr Ani Reprod AI, 4: 888-891.

Didion BA; Dobrinsky JR; Giles JR; Graves CN. 1989. Staining procedure to detect viability and the true acrosome reaction in spermatozoa of various species. Gam Res; 22: 51-57.

Dott HM, Foster GC. 1979. The estimation of sperm motility in semen, on a membrane slide, by measuring the area change frequency with an image analyzing computer. J Reprod Fertil; 55: 161-166.

Drevius LO; Eriksson H. 1966. Osmotic swelling of mammalian spermatozoa. Exp Cell Res; 42: 136-156.

Dubiel A; Stanzyk JF; Barcikowski B; Ronowics K; Holszka M. 1987. Testosterone concentrations in the plasma of boars of different ages. Medycina Weterynaryjna; 43 (11): 687-691.

Eaglesone MD; Miller SA. 1989. Prediction of fertility of bovine semen: preliminary studies with the hamster egg penetration test. Theriogenology, 31: 643-651.

Evenson DP; Darzynkiewicz Z, Melamed MR. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. Science, 210: 1131-1133.

Evenson DP; Darzynkiewics Z; Melamed MR. 1982. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membranes potential related to cell motility. J Histochem Cytochem; 30: 279-280.

Evenson DP; Ballachey BE. 1986. Flow cytometric evaluation of bull sperm chromatin structure, mitochondrial activity, viability and concentration. Proc 11th NAAB Tech Conf AI Reprod; p. 109.

Evenson DP, Thompson L. 1990. Flow cytometric analysis of boar sperm chromatin structure as related to cryopreservation and fertility. Reprod Dom Anim; Suppl 1: 165-183.

Evenson DP, Thompson L, Jost L. 1994. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology; 41: 637-651.

Evenson DP. 1999. Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. Reprod Fertil Dev; 11: 1-15.

Evenson DP, Spano M. 2002. Flow cytometry and human sperm analysis. In: 9th International Symposium on Spermatology. October 6-11, Cape Town/South Africa, p.80.

Fayemi EO; Crabo BG; Graham EF. 1979. Assay of frozem boar semen with Sephadex filtration. Theriogenology; 12: 13-17.

Farlin ME; Jasko DJ; Graham JK; Squires EL. 1992. Assesment of *Pisum sativum* agglutinin in identifying acrosomal damage in stallion spermatozoa. Mol Reprod Dev; 32: 223-277.

Foote RH; Bredderman PJ. 1969. Sizing of aging bull spermatozoa with an electronic counter. J Dairy Sci; 52: 117-120.

Frim J; Mazur P. 1983. Interactions of cooling rate, glycerol concentration, and dilution procedure on the viability of frozen-thawed human granulocites. Cryobiology; 20: 657-676.

Gadea J, Matás C, Lucas X. 1998. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. Anim Reprod Sci; 56: 95-108.

Gagnon C. 1995. Regulation of sperm motility at the axonemal level. Reprod Fertil Dev; 7: 847-855

Gao DY; Mazur P; Kleinhans FW; Watson PF; Noiles EE; Critser JK. 1992. Glycerol permeability of human spermatozoa and its activation energy. Cryobiology; 29: 657-667.

García-Artiga C; Fontanillas JC; Pérez J; García-Cuenca I; Martín-Rillo S; Pérez-García T. 1994. Técnicas de Tinción Espermática. Porci; 21: 11-18.

González-Urdiales R. 2002. Contrastación seminal. Cunicultura; 160: 394-399.

Graham EF; Schmehl MKL; Nelson DS. 1980. Problems with laboratory assays. Proc 8th NAAB Tech Conf AI Reprod, p. 1-8.

Graham JK; Foote RH. 1987[a]. Dilauroylphosphatidylcholine liposome effects on the acrosome reaction and in vitro penetration of zona-free hamster eggs by bull sperm. I. Fertility assay for fresh semen. Gamete Res; 16: 133-145.

Graham JK; Foote RH. 1987[b]. Dilauroylphosphatidylcholine liposome effects on the acrosome reaction and in vitro penetration of zona-free hamster eggs by bull sperm. II. Fertility assay for frozen-thawed semen. Gamete Res; 16: 147-158.

Graham JK; Kunze E; Hammerstedt; RH. 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol Reprod; 43: 55-64.

Graham J. 1996. Cryopreservation in stallion spermatozoa. Vet Clin North Ame; 12: 131-147.

Gil J; Januskaukas A; Håård M Ch; Håård MGM; Johanisson A; Söderquist L; Rodríguez-Martinez H. 2000. Functional sperm parameters and fertility of bull semen extended in Biociphos-plus ® and Triladyl ®. Reprod Dom Anim; 35: 69-77.

Hammit DG; Martin PA. 1989. Correlations among assays of porcine sperm quality following cryopreservation. Theriogenology; 32: 369-384.

Hafez ESE; Hafez B. 2000. Reproduction in farm animals. 7th edition, Baltimore/USA. 509 pp.

Hammerstedt RH; Graham JK; Nolan JP. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J Androl; 11: 73-88.

Harkema W; Boyle MS. 1992. Use of fluorescent stain to assess membrane integrity of equine spermatozoa. Proc 12th Int Congr Anim Reprod AI, La Haya; Vol 2: 1424-1426.

Henry M; Noiles EE; Gao D; Manzur P; Critser JK: 1993. Cryopreservation of human spermatozoa. IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity and mitochondrial integrity. Fertil Steril; 60: 911-918.

Holt WV. 1996. Can we predict fertility rates? Making sense of sperm motility. Reprod Dom Anim, 31:17-24.

Huang-Yang YHJ; Meizel S. 1975. Purification of rabbit testis proacrosina and studies of its active form. Biol Reprod; 12: 232-238.

Ibrahim NM; Foster DN; Crabo BG. 2001. Localization of clustering on freeze-preserved bull spermatozoa before and after glass wool-Sephadex filtration. J Androl; 22: 891-902.

Ivanova M; Mollova M. 1993. Zona–penetration in vitro test for evaluating boar sperm fertility. Theriogenology; 40: 397-410.

Jasko DJ; Little TV; Lein DH; Foote RH. 1992. Comparison of spermatozoa movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). JAVMA; 200: 979-985.

Jeyendran RS; Van der Ven HH; Pérez-Peláez M; Crabo BJ; Zaneveld LJD. 1984. Development of an assay to asses the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil; 70:219-228.

Jeyendran RS; Van der Ven HH; Zaneveld LJ. 1992. The hypoosmotic swilling test: an update. Arch Androl; 39: 1279-1289.

Katz DF; Overstreet JW. 1980. Mammalian sperm movement in vitro secretions of the male and female genital tracts. In: Testicular development, structure and function. Eds: A. Steinberger y E. Steinberger. Raven Press: New York: pp. 481-489.

Kenney RM; Evenson DP; García MC; Love CC. 1995. Relationship between sperm cromatin structure, motility and morphology of ejaculated sperm and seasonal pregnancy rate. Biol Reprod; 1: 647-653.

Krause W. 1995. The significance of computer-assisted semen analysis (CASA) for diagnosis in andrology and fertility prognosis. Hum Reprod; 10: 60-66.

Kumi-Diaka J. 1993. Subjecting the canine spermatozoa to the hypoosmotic swelling test. Theriogenology; 39: 1279-1289.

Kumi-Diaka J; Badtram G. 1994. Effect of storage on sperm membrane integrity and other functional characteristics of canine spermatozoa: in vitro bioassay for canine semen. Theriogenology; 41: 1355-1366.

Luberda A; Strzezek J. 1988. Effect of zinc on boar seminal plasma over DNSA and phosphatase activity. Medycyna Weterynbaryjna; 5 (XLIV):298-301.

Mac Dermott C; Fraser LR. 1990. Calcium-related changes in the mouse sperm capacitation state assessed with chlorotetracycline. J Reprod Fertil; 6 (abstr.): 5,1.

Magistrini M; Guitton E; Levern Y; Nicolle J Cl; Vidament M; Kerboeuf D; Palmer E. 1997. New staining methods for sperm evaluation estimated by microscopy and flow cytometry. Theriogenology; 48: 1229-1235.

Mahi CA; Yanagimachi R. 1973. The effects of temperature, osmolarity and hydrogen ion concentration on the activation and acrosome reaction of golden hamster spermatozoa. J. Reprod Fertil; 35: 55-66.

Marigorta P; Saiz F; De Alba C; Corcuera D; Sagges A; Martin-Rillo S. 1995. Acrosine and AAT activities in boar semen during the freezing process as indicative of the cell status. 46th FEZ, Prague.

Martín-Rillo,S. 1985. Aportación al estudio de la congelación del semen de verraco. Tesis doctoral. Universidad de Zaragoza, Zaragoza/ España. 197 pp.

Martínez E; Vázquez JM; Matás C; Roca J; Coy P; Gadea J. 1993. Evaluation of boar spermatozoa penetrating capacity using pig oocyte at the germinal vesicle stage. Theriogenology; 40: 547-557.

Maxwell WMC; Johnson LA. 1997. Membrane status of boar spermatozoa after cooling or cryopreservation. Theriogenology; 48: 209-219.

Meyers SA; Overstreet JA; Liu IKM; Drobnis EZ. 1995. Capacitation in vitro of stallion spermatozoa: comparison of progesterone-induced acrosome reaction in fertile and subfertile males. J Androl; 16: 47-54.

Mogas T; Rigau T; Piedrafita J; Bonet S; Rodríguez-Gil JE. 1998. Effects of column filtration upon the quality parameters of fresh dog semen. Theriogenology; 50:1171-1189.

Moore HDM; Hall GA; Hibbitt KG. 1976. Seminal plasma protein and the reaction of spermatozoa from intact boars and from boars without seminal vesicles to cooling. J Reprod Fertil; 47: 39-45.

Moore HDM; Hibbitt KG. 1977. Fertility of boar spermatozoa after freezing in the absence of seminal vesicles protein. J Reprod Fertil; 50: 349-352.

Mortimer D; Aitken RJ; Mortimer ST; Pacey AA. 1995. Workshop report: Clinical CASA. The quest for consensus. Reprod. Fertil Dev; 7: 951-959.

Mortimer, S. 2000. CASA - Practical aspects. J Androl; 21: 515-524.

Mortimer ST. 2002. Practical application of computer-aided sperm analysis (CASA). In: 9th International Symposium on Spermatology. October 6-11, Cape Town/South Africa, p.79.

Noakes DE; Parkinson TJ; England GCW. 2001. Arthur´s Veterinary Reproduction and Obstetrics. 8th edition; W Sauders; London, 868 pp.

Noll F. 1984. *L*-Lactate. In: HU Bermeyer (Editor), Methods of Enzymatic Analysis. Vol. VI, Verlag Chemie, Weinheim, pp. 582-588.

Oettlé EE. 1986. Using a new acrosome stain to evaluate sperm morphology. Vet Med; 81: 263-266.

O`Rand MG. 1979. Changes in sperm properties correlated with capacitation. En: Fawcett y Bedford (Eds.); The Spermatozoon; 195-204.

Pace MM; Sullivan JJ; Elliot FI; Graham EF; Coulter GH. 1981. Effects of thawing temperature, number of spermatozoa and spermatozoa quality on fertility of bovine spermatozoa package in 0.5 ml fresh straw. J Ani Sci; 53: 693-701.

Palmer E; Magistrini M. 1992. Automated analysis of stallion semen post-thaw motility. Acta Vet Scand (Suppl.); 88: 137-152.

Papaioannou KZ; Murphy RP; Monks RS; Hynés N; Ryan MP; Boland MP; Roche JF. 1997. Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. Theriogenology; 48: 299-312.

Paulenz H; Hofmo PO. 1996. Routine assessment of sperm concentration at a boar AI station using a coulter counter. Reprod Dom Ani; 31(1): 257-258.

Pérez-Llano B; García-Casado P; Lorenzo JL; Sánchez-Sánchez R. 1998[a]. Response to the boar sperm to the Host test and relationship between HOST and ORT results. 15th IPVS Congress, Birmingham, England, 5-9/Julio, Abstr. 69.

Pérez-Llano B; Sánchez R; Lorenzo JL; García-Casado P. 1998[b]. A short version of the osmotic resistance test for boar semen. 15th IPVS Congress, Birmingham, England, 5-9/Julio, Abstr. 61.

Pérez-Llano B; González JL; García-Casado P. 1998[c]. Nueva técnica de ORT corta para su evaluación. Albéitar; 21:6-7.

Pérez-Llano B, González JL, Clemente MJ, García-Casado P. 1999. El test de endósmosis (HOST) en semen de ganado porcino. Albéitar; 30:16-17.

Pérez-Llano B; Yanes-García P; García-Casado P. 2003. Four subpopulations of boar spermatozoa defined according to their response to the short hypoosmotic swelling test and acrosome status during incubation at 37˚C. Theriogenology; in press.

Polakoski KL; Zaneveld LJD. 1984. Proacrosina. Proteasas de gametos y embriones; 26: 325-329.

Quinn PJ; Chow PYW; White IG. 1980. Evidence that phospholipids protects ram spermatozoa from cold shock at a plasma membrane site. J Reprod Fert; 60: 403-407.

Quintero-Moreno A; Madrigal O; Gallardo F; Ramió L; Peña A; Miró J; Rigau T; Rodríguez-Gil JE. 2003. Subpoblaciones espermáticas en mamíferos. Biologia de la Reproducció; 8: 47-49.

Ramió-Lluch L; Quintero-Moreno A; Rodríguez-Gil JE. 2003. Effect of column filtration upon the quality parameters of diluted boar semen. 5th International Conference on Boar Semen Preservation, Ultrecht/The Netherlands, August 24-27. In press.

Renard P; Trimeche A; Le Pichon JP; Quero JC; Griveau JF; Chouteau P; Tainturier D; Le Lannou D. 1996. Sperm motility and flagellar motion: a comparison between boar and other mammalian species. Reprod Domestic Anim; 31 (1): 249-250.

Rigau T; Piedrafita J; Reverter A; Canal M; Rodríguez-Gil JE. 1996. The rate of L-lactate production: a feasible parameter for the fresh diluted boar semen quality analysis. Ani Reprod Sci; 43: 161-172.

Rigau T ; Farré M ; Ballester J ; Mogas T; Peña A; Rodríguez-Gil JE. 2001. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates; Theriogenology; 56: 801-815.

Rivera del Álamo M; Palomo M; Quintero-Moreno A; Rigau T; Rodríguez-Gil JE. 2002. Sperm subpopulations in boar semen and their relationship with natural photoperiod. 17th Congress of the International Pig Veterinary Society. June 2-5/ Ames, Iowa; Vol 2, paper 669: 494.

Rodríguez-Gil JE; Montserrat A; Rigau T. 1994. Effects of hypoosmotic incubation on acrososme and tail structure on canine spermatozoa. Theriogenology; 42: 815-829.

Rodríguez-Gil, J. E. 2000. Aspectos funcionales de espermatozoides de mamífero. En: Jornadas sobre la fecundación en mamíferos: conceptos actuales. Universidad Autónoma de Barcelona, Facultad de Veterinaria, Unidad de Reproducción Animal, p. 1-6.

Rodríguez-Martínez H; Larsson B; Pertoft H. 1997. Evaluation of sperm damage and techniques for sperm clean-up. Reprod Fertil Dev; 9: 297-308.

Rodríguez-Martínez H. 2000. Evaluación de semen congelado: Métodos Tradicionales y de Actualidad. Internacional Veterinary Infiormation Service (www.ivis.org), Ithaca, New Cork, USA.

Rodríguez-Martínez H; Wallgren M; Selles E; Tienthai P. 2001. Aspectos de función espermática en cerdos. III Congresso Ibérico de Reproduçao Animal. Porto, 6-8/Portugal. Livro de Resumos: 51-60.

Roger BJ; Parker RA. 1991. Relationship between the human sperm hypoosmotic swelling test and sperm penetration assay. J Androl; 12: 152-158.

Roldan ERS; Cassinello J; Abaigar T; Gomendio M. 1998. Inbreeding, fluctuating asymmetry and ejaculate quality in a endangered ungulate. Proc R Soc Lond B, 265: 243-248.

Saacker RG; Vinson WE; O'Connor ML; Chandler JE; Mullins J; Amann RP; Marshall CE; Wallace RA; Vincel WN; Kellgren HC. 1980. The relationship of semen quality and fertility: a heterospermic study. Proc 8th NAAB Tech Conf AI Reprod; 71-78.

Saiz-Cidoncha F; De Alba C; Marigorta P; Corcuera BD; Martin-Rillo S. 1994. Estudio de la calidad del semen de verraco a través de la evaluación de parámetros bioquímicos. Porci; 21: 57-76.

Samper JC; Behnke EJ; Byers AP; Hunter AG; Crabo BG. 1989. In vitro capacitation of stallion spermatozoa in calcium-free tyrode`s medium and penetration of zona-free hamster eggs. Theriogenology; 31: 875-884.

Samper JC; Hellander JC; Crabo BG. 1991. Relationship between the fertility of fresh and frozen stallion semen and semen quality. J Reprod Fert; 44 (suppl.): 107-114.

Samper JC; Crabo BG. 1993. Assay of capacited, freeze-damage and extended stallion spermatozoa by filtration. Theriogenology; 39: 1209-1220.

Samper JC; Hamilton DW; Pryor JL; Loseth KJ; Troedsson MHT; Crabo BG. 1995. Mechanism of Sephadex trapping of capacitated stallion spermatozoa. Biol Reprod; Equine Reproduction VI: 227-235.

Sánchez A; Rubilar J; Gatica MV. 2002. Evaluation of fresh and frozen canine semen by the hypoosmotic swelling test. Arch Med Vet; 34 (1): 123-130.

Schilling E; Vengust M; Smidt D. 1984. ORT: Un nuevo sistema para predecir la congelabilidad y la capacidad de almacenamiento de los espermatozoides de verraco. Proc 8th IPVS Congress, Belgica, pp. 346 (abstr.).

Schilling E; Vengust M. 1985. Determination of osmotic resistance of boar spermatozoa and its relationship with the storage ability of semen samples. Zuchthygiene; 20: 61-78.

Schilling E; Vengust M; Bajt G; Tomcic M: 1986. The osmotic resistance (ORT) of boar spermatozoa and the relation to pregnancy rate and litter size. Proc 9th IPVS Congress, Barcelona, pp. 77 (abstr.).

Serres C; Feneux D; Jouannet P; David G. 1984. Influence of the flagellar wave development and propagation on the human sperm movement in seminal plasma. Gam Res; 9: 183-195

Singer SJ; Nicholson GL. 1972. The fluid mosaic model of the structure of cell membranes. Science; 175: 720-731.

Strzezek J. 1976. The protein of boar seminal plasma and its biochemical function. Symposium of boar semen preservation. Uppsala, Switzerland.

Strzezek J; Smigielska J. 1978. Immunological characteristics of deoxyribonucleoproteins (DNP) insolated from fresh and stored boar spermatozoon. Medycyna Wet; 10: 617-621.

Strzezek J; Swidowicz K. 1986. Cambios crió-bioquímicos del espermatozoide y su capacidad fertilizante. Zuchthygiene; 21: 64-70.

Strzezek J; Cieresko A. 1987. Heterogeneity of aspartate aminotransferasa (AAT) in bull semen. Comp Biochem Physiol; 86 B. 2: 373-375.

Talbot P; Chacón RSA. 1981. A triple-stain technique for evaluating normal acrosome reactions in human sperm. J Exp Zool; 215: 201-208.

Tamuli MK; Watson PF. 1992. Effects of temperature of incubation on the development of resistance to cold stress and hypoosmotic stress in boar spermatozoa incubated for up 24 hours. Proc. 12th ICAR Congress. The Hague, pp. 1484-1486.

Thurston LM; Watson P; Mileham A; Holt W. 2001. Morphologically distinct sperm subpopulation defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl; 22 (3): 382-394.

Tuli RK; Schmidt-Baulain R; Holt W. 1992. Computer assisted motility assessment of spermatozoa from fresh and frozen-thawed semen of bull, boar and goat. Theriogenology; 38: 487-490.

Van den Saffele J; Vermeulen L; Schoojans F; Comhaire FH. 1992. Evaluation of the hypoosmotic swelling test in relation to advanced methods of semen analysis. Andrologia; 24: 213-217.

Van der Ven HH; Jeyendran RS; Al-Hasani S; Pérez-Peláez M; Diedrich K; Zaneveld LJD. 1986. Correlation between human sperm swelling in hypoosmotic médium (Hypoosmotic Swelling Test) and in vitro fertilization. J. Androl; 7: 190-196.

Varmer D; Vaughan S; Johnson L. 1991. Use of computerised system for evaluation of equine spermatozoa motility. Am J Vet Res; 52: 224-230.

Vázquez JM; Martínez EA; Roca J; Coy P; Ruiz S. 1992. Use of triple stain technique for simultaneous assessment of viability and acrosomal status in boar spermatozoa. Theriogenology; 38: 843-852.

Vázquez JM; Martínez EA; Pastor LM; Roca J; .Matas C; Calvo A. 1996. Lectin histochemistry during "in vitro" capacitation and acrosome reaction in boar spermatozoa: new lectins for evaluating acrosomal status of boar spermatozoa. Acta Histochem (Jena); 98: 93-100.

Vázquez JM; Martínez EA; Roca J; Blanco O; Lucas X; Matas C. 1997[a]. Utilización del analizador de imágenes para la evaluación de la motilidad de los espermatozoides de verraco. IV Simposium Internacional de Reproducción e IA porcina. Madrid, 83-90 pp.

Vázquez JM; Martínez EA; Martínez P; García-Antiga C; Roca J. 1997[b]. Hypoosmotic swelling of boar spermatozoa compared to other methods for analyzing the sperm membrane. Theriogenology; 47: 913-922.

Verheyen G; Joris H; Crits K; Nagy Z; Tounare H; Van Steirteghem A. 1997. Comparison of different hypo-osmotic swelling solutions to select viable inmotile spermatozoa for potential use in intracytoplasmatic sperm injection. Human Reprod (Update); 3: 195-203.

Verstegen J; Iguer-Ouada M; Onclin K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology; 57: 149-179.

Von Buiten A; Zhang J; Boule MS. 1989. Integrity of plasma membrane of stallion spermatozoa before and after freezing. J Reprod Fertil; 4: 11-18.

Watson PF. 1975. Use of Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. Vet Rec; 97: 12-15.

Watson PF. 1979. The preservation of semen in mammals. In: Oxford Reviews of Reproducive Biology. Finn, CA (ed.). Oxford, pp. 283-350.

Watson PF; Plummer JM. 1985. The responses of boar semen membrane to cold shock and cooling. In. Deep freezing of boar semen. L.A. Johson and K. larson (eds.), Swed. Univ of Agr Sci, Uppsala, Sweden, pp. 113-127.

Well ME; Awa OA. 1970. New technique for assessing acrosomal characteristics of spermatozoa. J Dairy Sci; 53: 227-232.

White IG; Darin-Bennett A. 1976. The lipids of sperm in relation to cold shock. Proc VIII Int Congr Anim Reprod Artf Insem (Krakow)/4: 951-954.

Wilhelm KM; Hinds K; Squires EL; Graham JK. 1993. Development of new extenders for preserving stallion sperm at 39˚C and -196˚C. Biol Reprod; 48 (suppl.): 165.

Wilhelm KM; Graham JK; Squires EL. 1996. Comparison of the fertility of criopreserved stallion spermatozoa with sperm motion analyses, flow citometric evaluation and zona-free hamster oocyte penetration. Theriogenology; 46: 559-578.

Woelders H. 1990. Overview of in vitro methods for evaluation of semen quality. Reprod Dom Ani; Suppl (1): 145-164.

Xu X; Foxcroft GR. 1996. IVM/IVF technology for assessment of semen quality and boar fertility. Reprod Domest Anim; 31:31-36.

Yanagimachi R. 1993. Mammalian Fertilization. In: Knobil E (Eds) Phisiology of Reproduction. Raven Press, NY; pp. 189-317.

Zaneveld LJD; Jeyendran RS; 1990. Hypoosmotic swelling test. In: Handbook of the laboratory Diagnosis and treatment of infertility. Keel, BA; Webster BW (eds.). CRC Press, Boca Ratón, pp. 91-110.

Zhang J; Boylen MS; Smith CA; Moore HDM. 1990. Acrosome reaction of stallion spermatozoa evaluated with monoclonal antibody and zona-free hamster eggs. Mol Reprod Dev; 27: 152-158.

CAPITULO III

IDENTIFICATION OF SPERM SUBPOPULATIONS WITH SPECIFIC MOTILITY CHARACTERISTICS IN STALLION EJACULATES

IDENTIFICACION DE SUBPOBLACIONES ESPERMÁTICAS CON CARACTERISTICAS ESPECÍFICAS DE MOTILIDAD EN EYACULADOS DE CABALLOS

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ABSTRACT

The aim of this study is to test the presence of separate sperm subpopulations, with specific motility characteristics, in stallion ejaculates by using a computer-assisted semen analysis (CASA) system. Motility data were analyzed with a hierarchical clustering of variables based on a correlation or covariance matrix to select like parameters of sperm motility descriptors that better explain the kinetics of spermatozoa. The statistical analyses clustered the whole motile sperm population in both fresh and 24-h stored ejaculates into four separate groups. There were significant differences in the distribution of the four subpopulations ($P<0.001$) as well as in the total sperm number and the percentage of total motility ($P<0.01$) in fresh semen among the five stallions tested. Our results show that separate subpopulations of spermatozoa with different motility characteristics coexist in stallion ejaculates. These subpopulations were maintained, although with a less-progressive motion pattern, after 24 hour of storage. The study of subpopulations in ejaculates that have confirmed fertilizing capacity showed that the majority of the motile spermatozoa in these ejaculates are included in a subpopulation with high progressive motility and low linearity, and the ejaculates with proven fertility that have a total sperm count $\geq 20 \times 10^9$ spermatozoa/ejaculate show all of their motile sperm included in this subpopulation. Our results show that the use of the CASA system is a relatively simple approach to the study of sperm subpopulation patterns in equine ejaculates.

Key words: Sperm subpopulation, Motility pattern, Stallion spermatozoa.

1. INTRODUCTION

Studies carried out by several researchers demonstrate the existence of sperm subpopulations in mammals such as the golden hamster, marmoset, gazelle, boar or dog (1,10,17,26,29). These subpopulations were defined by separate parameters, such as motion characteristics (1,17,26), the precise behavior in front of a flux cytometry analysis (15) or the specific distribution pattern of membrane glycoconjugates (28,30). These studies indicate the presence of functionally different sperm in a single ejaculate, which acts specifically when the cells are subjected to processes such as “in vitro” capacitation or incubation with a specific sugar (1,15,16,26,32). These results have led researchers to

suggest a strong relationship between changes in the percentages of diverse populations and the fertilization capacity of a specific ejaculate, at least in boar (1,2). Therefore, the study and characterization of these subpopulations open up new ways to improve semen analysis techniques. With regard to equines, very few bibliographical references to the study of identified subpopulations in equines are avaible, in spite of the potential practical interest of having such knowledge for this species. Amann and Hammersteddt (4) have shown a heterogeneous distribution of sperm population using the association of criteria of different physiological functions of the spermatozoa. The existence of these subpopulations is neglected in the classical semen quality analysis, and this can seriously interfere with a correct evaluation of sperm quality. Thus, it would be important to initiate further studies of sperm subpopulations in order to reach a better definition of equine semen quality. This could also have significant economic repercussion, since better semen analysis could lead to the commercialization of equine semen doses which offer greater guarantees of quality. However, although several techniques such as the computer-assisted semen motility analysis (CASA) have been applied to equine semen, the results obtained have been based on average values which were selected without any statistical criteria, thus impeding a serious analysis of the presence of sperm subpopulations (13). This has, to a great extent, limited the practical application of CASA as part of the improvement in the quality analysis of stallion semen.

As described above, the CASA system is one of the simplest and most reliable methods for studying sperm subpopulations (1,2,29). The CASA is based upon the capture of successive microscopic images which are then digitalized. The motile spermatozoa observed in these images are subsequently identified in the successive images, thus allowing for the establishment of their trajectories. Finally, the obtained trajectories are mathematically processed, which allows for the definition of these trajectories in a numerical form (7,20). The results of this processing are reflected in a series of parameters which precisely define the exact movement for each individual spermatozoon. This allows for the study of the individual sperm movement and also of the extraction of the putative sperm subpopulations, in accordance with the different trajectories obtained from the analysis of each ejaculate.

Until now, most studies that use CASA have focused on the identification of the parameters of movement which present the best correlation with respect to the other parameters of semen quality. However, the results have demonstrated great variability, depending upon the studied species (16,21). Additionally, CASA reduces the informative value of its data by assuming a normal distribution of all of the variables. The evaluation of the possible types of distribution of the motion parameters often reveals a high degree of asymmetry in the observations. Thus, it is not surprising that the results have often been disappointing. The analysis of correlation and multiple regression to analyze the parameters of sperm motility provided by CASA has been used for a decade, and few researchers have used clustering procedures to evaluate the analysis of sperm motility descriptors (1,2,11,12,16,26). Another great problem of CASA is the appearance of a large variety of separate motility parameters (21 in our specific system), which in most cases are highly correlated. This makes fact makes it difficult to objectively select parameters which explain the overall sperm movement accurately. As a result, it is necessary to look for a more correct statistical method to employ in the CASA analysis in order to conserve all of the relevant information. One possibility is an analysis based on the clustering of variables and observations, which would make it possible to apply CASA in the study of sperm subpopulations.

The main objective of this study was to determine the presence of separate sperm subpopulations, with specific motion characteristics, in fresh ejaculates. Moreover, the presence of these subpopulations was also tested after 24 h of storage at 4°C, in order to observe the response of these subpopulations after cold storage. A further, preliminary analysis was carried out in order to observe the specific subpopulation structure of equine ejaculates with proven fertilizing ability. To this end, priority consideration was given to the optimization of the parameters of CASA by means of reduced sperm selection and the existing association among these subpopulations with regard to parameters such as the percentage of total motility, the total sperm number and the individual stallion from which the ejaculates were obtained.

2. MATERIALS AND METHODS

2.1. Semen collection

Semen was collected from five healthy, mature stallions, age 3-11 years. They were of three different breeds (two Arabian, two Spanish and one cross-breed). Collections were performed at 2-7 days intervals using an artificial vagina with the aid of an ovariectomized mare that was induced into estrus with estrogens.

2.2. Sperm evaluation

An aliquot of the ejaculate was used to measure sperm concentration and pH, and the semen was immediately diluted in prewarned (37°C) Kenney's diluent (19) to a final concentration of $3x10^7$ spermatozoa/mL. Afterwards, aliquots of the diluted semen were taken as needed to carry out the appropriate analyses. Samples which showed pH values below 6.8 were excluded from this study. Percentages of viability, altered acrosomes and morphological abnormalities were determined by counting 200 spermatozoa (at magnification 1000X) using a vital Trypan Blue/Giemsa double stain as described Caiza de la Cueva (9), to evaluate stallion morphology. Sperm concentration was evaluated using a haemocytometer (24). At the same time, another 1-ml aliquot of the diluted semen was incubated for 5 min at 37°C. This sample was used to perform CASA analysis by using a commercial system (Sperm Class Analyzer, Microptic; Barcelona, Spain) added to an optical, phase-contrast microscope with heatable (37°C) plate. This was done by analyzing three consecutive 5-µL drops for each sample in all of the studied ejaculates in this study (Total number of ejaculates in the study, including those used for storage and "in vivo" fertilization studies was 88). Three fields per drop were taken, and the total number of spermatozoa analyzed in each semen sample (including those not motile) was from 20 to 50. The sperm motility descriptors obtained after CASA are defined in Table 1, based on those described by Boyers et al (7), Davis et al (12), and Rigau et al (26). Our CASA system was based upon the analysis of 16 consecutive, digitalized photographic images obtained from a single field at magnification 200X augmentations on a dark field. These 16 consecutive photographs were taken in a time lapse of 0.64 s, which implied a velocity of image capturing of 1 photograph each 40 ms. For 18 samples, a 20-ml aliquot of the remainder of the diluted semen was stored, in anaerobic conditions, for 24 h at 4°C. After this, this stored semen was analyzed as described above to determine the CASA-derived motility characteristics.

Table 1.

Definition of the motility descriptors

Name	Units	Description
Curvilinear velocity (VCL)	μm/sec	The instantaneously recorded sequential progression along the entire trajectory of the spermatozoon
Linear velocity (VSL)	μm/sec	The straight trajectory of the spermatozoa per unit of time
Mean velocity (VAP)	μm/sec	The mean trajectory of the spermatozoa per unit of time
Linear coefficient (LIN)	%	VSL/VCL
Straightness coefficient (STR)	%	VSL/VAP
Wobble coefficient (WOB)	%	VAP/VCL
Mean lateral head displacement (ALHMED	μm	Mean head displacement along its curvilinear trajectory around the mean trajectory
Maximal lateral head displacement (ALHmax)	μm	Maximal head displacement along its curvilinear trajectory around the mean trajectory
Dance (DNC)	μm^2/sec	VCL x ALHMED
Mean dance (DNM)	μm	ALHMED/LIN
Angularity index (AI)	%	The percent value of the angle formed by two successive trajectory segments
Angular velocity (AV)	μm/sec	(VCL x AI)/100
Absolute angular mean displacement (MADABS)	angular degrees	The absolute value of the advancing angle of the sperm trajectory
Algebraic angular mean displacement (MADALG)	angular degrees	The algebraic value of the advancing angle of the sperm trajectory. Negative values indicate a clockwise displacement
Frequency of head displacement (BCF)	Hz	The number of lateral oscillatory movements of the sperm head around the mean trajectory
Minor harmonic oscillation of the head (HLO)	μm	The minimum value of the distance between the curvilinear trajectory, with respect to the mean trajectory
Mean harmonic oscillation of the head (HME)	μm	The mean value of the distance between the curvilinear trajectory, with respect to the mean trajectory
Major harmonic oscillation of the head (HHI)	μm	The maximal value of the distance between the curvilinear trajectory, with respect to the mean trajectory
Harmonic amplitude (H_Y)	μm	Minimal distance between two successive crosses around the mean trajectory
Basic harmonic oscillation of the head (HBS)	μm	Mean distance between two successive crosses around the mean trajectory
Maximal amplitude of the oscillation of the head (HMX)	μm	Maximal distance between two successive crosses around the mean trajectory

2.3. Studies of the fertilizing ability of equine semen in vivo

Twenty-six ejaculates which were processed as described above, were used to perform artificial insemination (AI). For this, ejaculates were diluted in Kenney's diluent at a concentration of $3x10^7$ spermatozoa/ ml and kept at 4ºC until their use. The A.I. with these doses was carried out from 3 to 24h after obtaining and processing of the ejaculates. The AI was carried out on healthy mature cross-breed mares, age from 3 to 8 years, that were in spontaneous estrus. They were from private owners, who called us to carry out the service. Prior to AI., the mares were examined using transrectal ultrasonography to determine the follicle structure and the presence of a normal, non-pathological uterus structure. The ultrasonography was repeated once daily until the follicular size (≥40 mm of diameter) and shape (slightly irregular) indicated the proximity of ovulation. When this occurred, AI was performed by intrauterine deposition of the prewarmed (35ºC) 20 mL diluted semen. The ovulation was not hormonally induced and we were not able to determine the exact insemination-ovulation interval. The number of mares which were inseminated with a single ejaculate varied from 1 to 3. This procedure does not allow for an accurate evaluation of the overall potential fertility of a single ejaculate. However, since our main interest was to determine how a single sample with a characteristic motility subpopulation pattern might correspond to a concrete proven pregnancy rather than to establish the general potential fertility of an ejaculate, we considered that our experimental design was suitable for this study.

The confirmation of the pregnancy in the inseminated mares was done by transrectal ultrasonography 15 days after AI, and another ultrasonography was performed 20 days after AI. An ejaculate was considered of proven fertility when the presence of a recognizable embryo was determined in either of the ultrasonographic explorations, provided that, following our methodology, the absence of a proven pregnancy did not necessarily indicate that the ejaculates used were non fertile. The total number of mares which were inseminated was 50 (mean number of inseminations/ejaculate: 1.92), and 26 of them had a confirmed pregnancy. These confirmed pregnancies corresponded to 22 separate ejaculates. Thus, the number of ejaculates which were considered of proven fertility was 22, and they were used for the specific studies.

2.4. Statistic analysis of sperm subpopulations

Sperm motility descriptors obtained from CASA were analyzed by the VARCLUS clustering procedure of the SAS statistical package system (27). In this system, clusters are chosen to maximize the variation accounted for by either the principal component or the centroid component of each cluster. Thus, the VARCLUS procedure can be used to reduce the number of sperm motility descriptors.

The VARCLUS procedure allows for the determination of the optimal number of motion parameters which can be used to define sperm motion characteristics. Following this, the next point was to use another statistical procedure which allows us to allocate an individual, motile spermatozoon into a specific subpopulation. Another clustering procedure, the FASTCLUS (27), was used for this purpose. This procedure performs a disjointed cluster analysis on the basis of Euclidean distances computed from one or more quantitative variables (sperm motility descriptors), which permits the study of large sets of observations, from approximately 100 to 100,000. The spermatozoa were divided into clusters such that every observation belonged to one and only one cluster. Spermatozoa that are very close to each other are usually assigned to the same cluster, while spermatozoa which are far apart are in different clusters. The separation of spermatozoa into clusters was performed over the total number of spermatozoa obtained in the fresh semen samples (total analyzed sperm in fresh semen= 2649). A General Lineal Model (PROC GLM) was used to evaluate significant differences ($P<0.05$) among clusters of sperm subpopulations, while the LSMEANS procedure was used to compare the obtained sperm subpopulations among them (27). Finally, a chi-squared test was used to determine the relation between sperm subpopulations in fresh semen with the individual stallion used, as well as with the percentage of total motility (defined as the percentage of spermatozoa with a curvilinear velocity >25 μm/sec) and the sperm concentration in the entire fresh ejaculate. For this latter purpose, the whole ejaculates were arbitrarily categorized after using both total motility and total sperm number in the following form:

Total motility:
Group A whole ejaculates: Total motility ≥90%.

Group B whole ejaculates: total motility <90% ≥80%.

Group C whole ejaculates: total motility <80% ≥70%.

Group D whole ejaculates: total motility <70%.

Total sperm-number per ejaculate:

Group A whole ejaculates: $<10x10^9$ spermatozoa/ejaculate.

Group B whole ejaculates: $\geq 10x10^9$ spermatozoa/ejaculate $<20x10^9$ spermatozoa/ejaculate.

Group C whole ejaculates: $\geq 20x10^9$ spermatozoa/ejaculate.

Thus, the main objective of these arbitrary categories was to have some sort of references that allowed us to observe changes in the distribution of motile subpopulations depending upon either total motility or the total number of spermatozoa in the ejaculates

3. RESULTS

3.1. Mean results for semen quality analysis

The mean values for the studied semen characteristics are shown in Table 2. There were significant differences in the sperm concentration and the total motility in fresh semen among the 5 stallions sampled ($P<0.05$; data not shown), although the percentage of total abnormality did not significantly differ among individuals.

Table 2.

General characteristics of fresh stallion semen samples.

Filtered volume of the ejaculates (mL)	49.2 ± 0.2
Concentration in hematcytometer chamber (x 10^6 /mL)	304.8 ± 1.9
Total motility (curvilinear velocity >25μm/sec, %)	88.9 ± 0.1
pH	7.6 ± 0.01
Viability by Trypan Blue/Giemsa staining (%)	82.1 ± 0.1
Sperm with structurally abnormal acrosomes (%)	1.1 ± 0.01
Head morphological abnormalities (%)	1.0 ± 0.01
Neck and midpiece abnormalities, including cytoplasmic droplets (%)	3.8 ± 0.01
Tail abnormalities (%)	3.2 ± 0.03

Values are means ± S.E.M. (n=88 ejaculates; of these, 7 ejaculates were from stallion 1, 28 from stallion 2, 12 from stallion 3, 8 from stallion 4 and 33 from stallion 5).

3.2. Selection of the parameters for study of stallion sperm motility.

The application of the VARCLUS procedure combined with subsequent analyses of variance from 2649 observations obtained from fresh ejaculates grouped the 21 motion variables obtained in the CASA into 5 clusters which explain 72.98% from the total variation (Table 3). The comparison of the statistical proximity among the separate motion variables gathered in the same clusters led to the choosing of one or two motion parameters in each cluster, which conserved the maximal statistical information contained in each cluster. From this analysis, the chosen variables for each cluster were: mean velocity (VAP), dance (DNC), mean amplitude of head lateral displacement (ALHMED), frequency of head displacement (BCF), lateral head displacement (HME), wobble coefficient (WOB) and linear coefficient (LIN, see Table 1 for the definition of all of these variables).

3.3. Sperm subpopulation analysis in fresh semen

Four sperm subpopulations were defined after the application of the FASTCLUS procedure and subsequent analysis of variance of 2649 individual spermatozoa using the chosen motion variables. Summarized statistics for these subpopulations are shown (Table 4) and qualitative interpretations of these data are as follows:

3.3.1. Subpopulation 1.

This shows the highest degree of progressive motility, as inferred by the greater LIN values. The VAP is relatively low compared with other subpopulations, indicating that many trajectories must be complex and nonlinear. Moreover, the ALHMED, the HME and the DNC values are the lowest of all of the subpopulations, indicating movement with low undulatory characteristics (Table 4). The BCF was similar to other subpopulations. More than 70% of the spermatozoa in the data set were assigned to this subpopulation (Table 4).

3.3.2. Subpopulation 2.

This shows highly active spermatozoa, with high values of VAP. Trajectories were generally less straight than Subpopulation 1, since LIN is significantly lower (Table 4). However, this subpopulation was straighter than those numbered 3 and 4. The WOB values

were similar to that of Subpopulation 1 and were higher than those of Subpopulations 3 and 4 (Table 4). ALHMED, HME and DNC values are lower than Subpopulation 1 but higher than Subpopulation 3 and 4 (Table 4). About 20% of the total motile spermatozoa were included in this subpopulation.

Table 3.

Hierarchical clustering of the sperm motility descriptors

Hierarchical clustering	Sperm motility descriptors	R^2 with own cluster (OC)	R^2 with next closest (NC)	Proportion ($1- R^2_{oc} /1-R^2_{nc}$)
Cluster 1	VCL	0.7438	0.4886	0.5009
	VSL	0.7321	0.5283	0.5678
	VAP*	0.9450	0.2894	0.0774
	AV	0.8912	0.1992	0.1359
	HMX	0.5005	0.2741	0.6882
	HBS	0.7243	0.1088	0.3094
	H_Y	0.5903	0.3029	0.5878
Cluster 2	ALHMED*	0.9220	0.2236	0.1005
	ALHMAX	0.8746	0.2175	0.1602
	DNC*	0.8905	0.2393	0.1440
	DNM	0.5267	0.1361	0.5479
Cluster 3	MADABS	0.7841	0.4760	0.4120
	BCF *	0.7841	0.1226	0.2461
Cluster 4	HLO	0.7450	0.0653	0.2728
	HME *	0.9753	0.1736	0.0298
	HHI	0.7039	0.4631	0.5514
Cluster 5	LIN *	0.8762	0.2150	0.1577
	STR	0.5437	0.2766	0.6308
	WOB *	0.8497	0.3473	0.2302
	AI	0.6832	0.4246	0.5505
	MADALG	0.2608	0.1097	0.8303

n=2649 spermatozoa from 88 ejaculates. Of these, 7 ejaculates were from stallion 1, 28 from stallion 2, 12 from stallion 3, 8 from stallion 4 and 33 from stallion 5. The ejaculates were distributed among the stallions as shown in Table 1. The spermatozoa were distributed among the stallions as follow: 209 motile spermatozoa were from stallion 1, 859 from stallion 2, 387 from stallion 3, 215 from stallion 4 and 979 from stallion 5. For a definition of the motility descriptors, see Table 1. The cluster summary gives the number of sperm motility descriptors in each cluster and the variation explained by the cluster component. Two squared correlations are printed for each cluster; the column labeled "R^2 with own cluster" gives the squared correlation of the sperm motility descriptor with its own cluster component. The larger the squared correlation is, the better. The column labeled "R^2 with next closest" contains the next highest squared correlation of the sperm motility descriptor with a cluster component. This value is low if the clusters are well separated. The column headed "$1- R^2_{oc} /1-R^2_{nc}$" gives the ratio of one minus its own cluster R^2 to one minus the next closest R^2. A small "$1- R^2_{oc} /1-R^2_{nc}$" indicates a good clustering.
* Selected variables.

Table 4.

Sperm subpopulations and motility descriptors in fresh stallion semen

Sperm motility descriptors	Subpopulations			
	1	2	3	4
n	1917	523	174	35
(%)	72.4	19.7	6.6	1.3
VAP (μm/sec)	40.3 ± 0.4^{a}	79.1 ± 0.5^{b}	82.0 ± 0.5^{b}	86.6 ± 0.5^{b}
LIN (%)	56.13 ± 0.4^{a}	47.0 ± 0.4^{b}	31.0 ± 0.3^{c}	24.8 ± 0.3^{d}
WOB (%)	64.7 ± 0.3^{a}	64.5 ± 0.3^{a}	52.4 ± 0.3^{b}	49.1 ± 0.3^{b}
ALHMED (μm/sec)	1.96 ± 0.02^{a}	4.29 ± 0.02^{b}	6.98 ± 0.02^{c}	10.98 ± 0.03^{d}
DNC (μm^2/sec)	107.3 ± 1.2^{a}	496.8 ± 2.0^{b}	1053.8 ± 2.7^{c}	1926.2 ± 5.3^{d}
BCF (Hz)	13.56 ± 0.10^{a}	13.39 ± 0.08^{a}	14.29 ± 0.10^{a}	13.16 ± 0.11^{a}
HME (μm)	1.04 ± 0.02^{a}	2.21 ± 0.02^{b}	2.75 ± 0.03^{c}	4.46 ± 0.07^{d}

Motility descriptors are described in Table 1. Different superscripts within a row indicate significant differences ($P<0.05$). Results are expressed as means ± S.E.M of spermatozoa from 88 ejaculates. Of these, 7 ejaculates were from stallion 1, 28 from stallion 2, 12 from stallion 3, 8 from stallion 4 and 33 from stallion 5. The total number of motile spermatozoa analyzed was 2649. The spermatozoa were distributed among the stallions as follows: 209 motile spermatozoa were from stallion 1, 859 from stallion 2, 387 from stallion 3, 215 from stallion 4 and 979 from stallion 5.

3.3.3. Subpopulations 3 and 4.

These contained spermatozoa with non-linear and circular trajectories. Although they were very active, with high VAP values, their non-linearity and poor progressive motility characteristics were evident by their high values of ALHMED and DNC (Table 4). The percentage of motile spermatozoa which were included in Subpopulation 3 was about 7%, while those included in Subpopulation 4 were about 1% (Table 4). The comparison of the subpopulation distribution and the overall characteristics of the entire ejaculates showed very interesting results. First, there were significant ($P<0.001$) differences in the distribution of the four sperm subpopulations in whole fresh ejaculates depending on stallion donor. Thus, the percentage of spermatozoa classified in Subpopulation 1 ranged from 52.6% to 81.0 % depending upon the individual, while the percentage identified in Subpopulation 2 ranged from 13.4 % to 32.0%. Subpopulation 3 varied from 4.7 % to 12.9 % and Subpopulation 4 from 0.83 % to 2.4 % among the stallions (Table 5).

Table 5.

Relationship among the proportion of the motile sperm subpopulations for fresh ejaculates and stallions

Subpopulation	Stallions				
	1	2	3	4	5
1	52.6	63.4[b]	77.0[c]	78.3[c]	81.0[c]
2	32.1	26.6[b]	16.5[c]	16.5[c]	13.4[c]
3	12.9	8.6[b]	4.4[c]	4.3[c]	4.7[c]
4	2.4	1.4[b]	2.1[a]	0.9[c]	0.8[c]

Sperm subpopulations are described in Section 3. Results express the percentage of all of the spermatozoa evaluated in each stallion which were included in the separate subpopulations. The results were obtained from 88 ejaculates. Of these, 7 ejaculates were from stallion 1, 28 from stallion 2, 12 from stallion 3, 8 from stallion 4 and 33 from stallion 5. The total number of motile spermatozoa analyzed was 2649. The spermatozoa were distributed among the stallions as follows: 209 motile spermatozoa were from stallion 1, 859 from stallion 2, 387 from stallion 3, 215 from stallion 4 and 979 from stallion 5.

Moreover, there were significant ($P<0.001$) differences when the distribution of the four sperm subpopulations was compared to the classification of the ejaculates according to their percentages of total motility. Thus, Subpopulation 1 was increased from about 70% in those ejaculates with a total motility ≥90% (Group A ejaculates, see section 2) to about 85% in ejaculates with a total motility <70% (Group C ejaculates, see section 2, and Table 6). On the contrary, Subpopulations 2, 3 and 4 progressively decreased when total motility also decreased. Thus, Subpopulation 2 ranged from 22% in Group A ejaculates to 13% in Group D ejaculates, Subpopulation 3 varied from below 10% in Group A ejaculates to about 2% in Group D ejaculates and Subpopulation 4 ranged from about 2% in Group A ejaculates to 0% in Group D ejaculates (Table 6). Thus, although the Group A - D classification was arbitrary, such classification allows us to clearly show the presence of a clear relationship between the percent distribution of motile subpopulations and the total motility of the ejaculates.

Finally, the distribution of the sperm subpopulations also varied depending upon the total number of spermatozoa of the whole ejaculates, although in this case the variations were less marked than those observed for percentage of total motility. Thus, as shown in Table 7, the most intense change was observed in Subpopulation 3, which ranged from about 6% in ejaculates with a total sperm count<$10x10^9$ spermatozoa/ejaculate (Group A ejaculates, see section 2) to about 10% in ejaculates with a total sperm number≥$20\ x10^9$ spermatozoa/ ejaculate (Group C ejaculates, see section 2). As in the case of total motility,

although the Group A - C classification was arbitrary, it allows us to show the presence of a relationship between the percent distribution of motile subpopulations and the total number of spermatozoa of the ejaculates.

Table 6.

Relationship between the proportion of the motile sperm subpopulations in fresh ejaculates and the total motility of the whole ejaculates

Subpopulation	Total motility groups			
	≥90%	<90% ≥80%	<80% ≥70%	<70%
1	68.7^{a}	77.5^{b}	74.4^{b}	85.2^{c}
2	22.1^{a}	16.0^{b}	18.9^{b}	13.0^{c}
3	7.4^{a}	5.9^{b}	6.1^{b}	1.8^{c}
4	1.8^{a}	0.6^{b}	0.6^{b}	0.0^{c}

Sperm subpopulations are described in Section 3. Fresh ejaculates were classified in arbitrary groups according to values of total motility, as described in Section 2. Thus, the table expresses the percentages of the motile spermatozoa included in each subpopulation depending upon the total motility values of the whole ejaculates. Different superscripts within a row indicate significant differences ($p<0.05$) following the Chi-square test. The results were obtained from 88 ejaculates. Of these, 7 ejaculates were from stallion 1, 28 from stallion 2, 12 from stallion 3, 8 from stallion 4 and 33 from stallion 5. The total number of motile spermatozoa analyzed was 2649. The spermatozoa were distributed among the stallions as follows: 209 motile spermatozoa were from stallion 1, 859 from stallion 2, 387 from stallion 3, 215 from stallion 4 and 979 from stallion 5.

3.4. Sperm Subpopulation Analysis in semen stored for 24 h

The storage of semen samples for 24 h at 4°C induced a slight, although significant ($P<0.01$), decrease in the percentages of viability and total motility of the samples. Thus, viability fell from 82.1 ± 0.1% in fresh ejaculates to 70.3 ± 0.1% in stored ones; while the percentage of altered acrosomes rose from 1.1 ± 0.1% in fresh samples to 7.9 ± 0.1% in stored samples (data as means±SEM; Table 2 and data not shown). On the other hand, total motility dropped from 88.9 ± 0.1% in fresh semen to 70.3 ± 0.1% in stored samples. (data as means±S.E.M.; Table 2 and data not shown).

Table 7.

Relationship between the proportion of the motile sperm subpopulations in fresh ejaculates and the total spermatozoa number of the whole ejaculates

Subpopulation	Total Spermatozoa-Number Groups		
	$<10x10^9$ sperm/ejaculate	$<20x10^9 \geq 10x10^9$ sperm/ejaculate	$\geq 20x10^9$ sperm/ejaculate
1	72.4^a	76.0^a	67.6^b
2	20.3^a	17.8^b	20.4^a
3	6.1^a	5.2^a	9.9^b
4	1.2^a	0.9^a	2.1^b

Sperm subpopulations are described in Section 3. Fresh ejaculates were classified in arbitrary groups according to values for total spermatozoa number, as described in Section 2. Thus, the table expresses the percentages of the motile spermatozoa included in each subpopulation depending upon the total spermatozoa number values of the whole ejaculates. Different superscripts within a row indicate significant differences ($p<0.05$) following the chi-squared test. The results were obtained from 88 ejaculates. Of these, 7 ejaculates were from stallion 1, 28 from stallion 2, 12 from stallion 3, 8 from stallion 4 and 33 from stallion 5.The total number of motile spermatozoa analyzed was 2649. The spermatozoa were distributed among the stallions in the following manner: 209 motile spermatozoa were from stallion 1, 859 from stallion 2, 387 from stallion 3, 215 from stallion 4 and 979 from stallion 5.

Cold storage also modified the subpopulation distribution of these ejaculates. Thus, the application of the FASTCLUS procedure and the subsequent analysis of variance of the 24-h stored semen samples also showed the presence of four separate, well-defined motile subpopulations, which correspond to those observed in fresh samples (Table 8). However, storage modified some of the motility parameters which define the subpopulations. Thus, there was a decrease in the mean values of VAP and DNC in all of the four subpopulations from stored semen when compared with the homologous subpopulations from fresh samples (Tables 4 and 8). Moreover, Subpopulation 1 from stored samples had significant ($P<0.001$) lower mean values of WOB than Subpopulation 1 from fresh samples. Subpopulations 3 and 4 from stored semen showed significantly ($P<0.001$) lower mean values of ALHMED, BCF and HME than those from fresh samples (Tables 4 and 8). Cold storage also induced changes in the distribution percentages of spermatozoa in each subpopulation. Thus, storage for 24 h at 4°C induced a significant ($P<0.001$) decrease in the percentage of Subpopulation 1 from 72.4% in fresh samples to 63.6% in stored samples, whereas the other subpopulations showed a slight, concomitant increase after 24 h storage when compared to the fresh semen samples (Tables 4 and 8).

Table 8.

Sperm subpopulations and motility descriptors of stallion semen stored for 24 h at 4ºC

Sperm Motility descriptors	Subpopulations			
	1	2	3	4
n	236	91	32	12
(%)	63.6	24.6	8.6	3.2
VAP (μm/sec)	33.0 ± 0.6^{a}	61.1 ± 0.5^{b}	64.8 ± 0.5^{b}	63.0 ± 0.5^{b}
LIN (%)	51.2 ± 0.7^{a}	56.8 ± 0.5^{a}	35.4 ± 0.5^{b}	16.3 ± 0.3^{b}
WOB (%)	45.3 ± 0.7^{a}	65.5 ± 0.4^{b}	53.5 ± 0.3^{ab}	40.6 ± 0.3^{a}
ALHMED (μm/sec)	2.18 ± 0.05^{a}	2.96 ± 0.02^{b}	4.57 ± 0.02^{c}	7.64 ± 0.02^{d}
DNC (μm^2/sec)	55.3 ± 1.0^{a}	269.0 ± 1.6^{b}	544.3 ± 2.1	1165.8 ± 2.8^{d}
BCF (Hz)	13.11 ± 0.20^{a}	14.51 ± 0.10^{ab}	16.60 ± 0.14^{ab}	20.12 ± 0.19^{b}
HME (μm)	1.02 ± 0.02^{a}	1.74 ± 0.02^{b}	2.27 ± 0.02^{b}	2.02 ± 0.02^{b}

Motility descriptors are described in Table 1. Different superscripts within a row indicate significant differences ($P<0.05$). Results are expressed as means ± S.E.M. of spermatozoa from 18 ejaculates. Of these, 7 ejaculates were from stallion 1, 5 from stallion 2 and 6 from stallion 3. The total number of motile spermatozoa analyzed was 371. The spermatozoa were distributed among the stallions as follows: 146 motile spermatozoa were from stallion 1, 80 from stallion 2 and 145 from stallion 3.

3.5. Sperm subpopulation distribution in ejaculates with at least one proved fertilization

Finally, our studies were centred on those ejaculates used for AI in which it was possible to demonstrate the existence of at least one proved fertilization. It is worth noting that the 66.5% of the ejaculates with proven in vivo fertility have a total motility >90%, whereas the other 33.5% of the ejaculates have total motility values between 80% and 90% (data not shown). Additionally, 32.7% of the ejaculates with proven "in vivo" fertility had a total number of spermatozoa below $10x10^9$ spermatozoa/ejaculate, 28.9% have a total sperm count between $10x10^9$ spermatozoa/ejaculate and $20x10^9$ spermatozoa/ejaculate, and 38.4% had a total spermatozoa number above $20\ x10^9$ spermatozoa/ejaculate (data not shown). The ejaculates with proven fertility showed the presence of 3 separate subpopulations of motile spermatozoa in these ejaculates, which were homologous to Subpopulations 1, 2 and 3 described above (Table 9).

Table 9.

Sperm subpopulations and motility descriptors of stallion semen with proven fertility

Sperm motility descriptors	Subpopulations		
	1	2	3
n	298	78	12
(%)	76.8	20.1	3.1
VAP (μm/sec)	40.4 ± 0.7^{a}	113.0 ± 1.1^{b}	95.3 ± 1.3^{b}
LIN (%)	62.7 ± 0.7^{a}	67.5 ± 0.7^{a}	35.5 ± 0.7^{b}
WOB (%)	70.8 ± 0.5^{a}	78.8 ± 0.5^{b}	53.4 ± 0.7^{c}
ALHMED (μm/sec)	1.80 ± 0.02^{a}	4.33 ± 0.03^{b}	7.61 ± 0.05^{c}
DNC (μm^2/sec)	110.7 ± 2.3^{a}	$595.0 \pm .4^{b}$	1314.7 ± 9.0^{c}
BCF (Hz)	$14.02 \pm .14^{a}$	12.80 ± 0.11^{a}	14.67 ± 0.24^{a}
HME (μm)	0.87 ± 0.02^{a}	2.26 ± 0.03^{b}	4.26 ± 0.06^{c}

Motility descriptors are described in Table 1 and ejaculates with proven fertility are defined in Section 2. Different superscripts within a row indicate significant differences ($P<0.05$). Results are expressed as means ± S.E.M. of spermatozoa from 22 ejaculates. From these, 4 ejaculates were from stallion 1, 7 from stallion 2 and 11 from stallion 3. The total number of motile spermatozoa analyzed was 388. The spermatozoa were distributed among the stallions as follows: 67 motile spermatozoa were from stallion 1, 126 from stallion 2 and 195 from stallion 3.

Next, the subpopulation distribution of these ejaculates was compared with the total motility and the total number of spermatozoa in the ejaculates. This comparison showed that there were no changes in the percentages of distribution of Subpopulations 1, 2 and 3 in ejaculates of both Group A and Group B total motility (data not shown). However, the sperm number of the ejaculates with at least one proved fertilization influenced the subpopulation distributions. As shown in Table 10, ejaculates with a total sperm count $<10x10^9$ spermatozoa/ejaculate (Group A ejaculates, see section 2) had about 70% of motile spermatozoa included in Subpopulation 1, about 25% in Subpopulation 2 and about 5% in Subpopulation 3. Nevertheless, ejaculates with a total sperm number between $10x10^9$ spermatozoa/ejaculate and $20x10^9$ spermatozoa/ejaculate (Group B ejaculates, see section 2) showed no spermatozoa in Subpopulation 3, whereas more than 90% were included in Subpopulation 1 (Table 10). The results were more pronounced in ejaculates with a total sperm number ≥ 20 $x10^9$ spermatozoa/ejaculate (Group C ejaculates, see section 2) where all of the motile spermatozoa were included in Subpopulation 1 (Table 10).

Table 10.

Relationship between the proportion of the motile sperm subpopulations in ejaculates with proven fertility and the total spermatozoa number of the whole ejaculates

Subpopulation	Total Spermatozoa-Number Groups		
	$<10x10^9$ sperm/ejaculate	$<20x10^9 \geq 10x10^9$ sperm/ejaculate	$\geq 20x10^9$ sperm/ejaculate
1	69.5^a	92.1^b	100.0^c
2	26.0^a	7.9^b	0.0^c
3	4.5^a	0.0^b	0.0^b

Sperm subpopulations are described in Section 3. Fresh ejaculates were classified in arbitrary groups according to values for total spermatozoa number, as described in Section 2. Thus, the table expresses the percentages of the motile spermatozoa included in each subpopulation depending upon the total spermatozoa number values of the whole ejaculates. Different superscripts within a row indicate significant differences ($p<0.05$) using the Chi-squared test. Data were obtained from 22 ejaculates. Of these, 4 ejaculates were from stallion 1, 7 from stallion 2 and 11 from stallion 3. The total number of motile spermatozoa analyzed was 388. The spermatozoa were distributed among the stallions as follows: 67 motile spermatozoa were from stallion 1, 126 from stallion 2 and 195 from stallion 3.

4. DISCUSSION

Our results have shown the presence of specific, well-defined sperm subpopulations in stallion ejaculates, which can be easily defined by their motility characteristics. The combination of CASA with the statistical VARCLUS procedure generates a very powerful statistical procedure that was instrumental in clearly establishing these subpopulations, in spite of the relatively low number of stallions, ejaculates and spermatozoa used. In fact, the use of a relatively low number of data (animals, ejaculates, etc.), if they are appropriately processed and the results are sufficiently clear, can suggest very interesting proposals, which allow for the opening of new perspectives in the study of equine semen. There are many recent studies involving a relatively low number of observations that can confirm this assertion (5,14,25). Our results suggest that the study of the distribution of motile spermatozoa subpopulations could be a powerful tool in improving the overall semen quality analysis of stallion ejaculates by introducing a new point of view on the classical equine semen quality analysis.

There are relatively few studies involving evaluation of stallion sperm motility using CASA (5,8,23,25,31). Moreover, the majority of these studies have used the selection of parameters based on purely biological criteria without subjecting the data to the many

types of analysis available in statistical packages. This system of selection makes the interpretation of the results obtained with CASA very difficult, since a great deal of the statistical information which is obtained by the analysis is neglected. In fact, the importance of a purely statistical selection approach to the CASA results has been pointed out. Thus, Abaigar et al. (1,2), working on boar and gazelle spermatozoa, demonstrate the presence of well-defined motile subpopulations, which were defined by the VCL, VAP, VSL, ALH, BCF, LIN and STR motion parameters (1). In this case, the investigators selected these concrete motion variables after analyzing the data by the SAHN–UPGMA selection procedure from the PATN statistical package for population analysis through various types of analyses (6).The presence of sperm subpopulations with separate motility characteristics has also been observed in dog spermatozoa, although in this case the statistical approach was different, without allowing a precise identification of these subpopulations (26). It is difficult to determine the most accurate statistical methodology for application to CASA which will allow researchers to offer the best explanation of the different trajectories taken by the sperm. This is due to the fact that, with the exception of BCF and HLO, almost all of the motion parameters obtained after CASA show elevated or average relations to each other, making it difficult to choose the most adequate motility variables. This occurs because the majority of these variables are products, quotients or percentages derived from relationships among them. Furthermore, when comparing our results to those published for other species, especially boar, there were clear differences in the location of each motion variable within each independent group among species. For this reason, the selection and degree of importance of motility variables, and thus the degree of association among the parameters, will also vary among species. Hierarchical clustering is a good method for selecting variables in this type of study and reveals that differences exist between equine and other species, with regard to the selection of motility parameters. This implies that the accurate application of CASA to a specific concrete species will need a previous, accurate statistical analysis, probably using some type of cluster analysis, in order to establish the precise motility variables which will afford the maximal information from the ejaculates of this specific species.

In our study, the presence of four motile sperm subpopulations was observed. The dominant population in all of these ejaculations showed very similar average values in fresh and cooled semen. The other subpopulations of the analysis showed a high VAP. Caiza de la Cueva et al (8), described average values per ejaculation in fresh semen,

without dilution, of: 32.5 μm/sec, 47.5%; 60.8%; 7.27 Hz and 0.32 μm, respectively, for VAP, LIN, WOB, BCF and, HME (8). These values are numerically lower than those obtained in our subpopulations. However, using diluted and cold-stored semen found similar values were found to those obtained in our study (8). Sperm motility is quicker and more efficient in diluted samples, at least partially, of the fall in cross-over among sperm that follow a trajectory. Another important point to stress about each of the subpopulations found is the fact that when the subpopulation presents a numerical increase in the parameters under study (VAP, ALHMED, DNC, HME), the linearity of its trajectory curve (LIN) and the oscillation rate (WOB) also fall, indicating a clear relationship between linearity of movement and the strength developed for the cell to originate its own motility pattern.

Our results show that Subpopulation 1 represented the most progressive spermatozoa in both fresh and stored samples. The others sperm subpopulations had highly nonlinear patterns of motility, but differed in trajectory and vigor. Thus, Subpopulation 2 was characterized by highly vigorous but mean-linear motion. Subpopulations 3 and 4 differed from each other in terms of vigor and shape of trajectory and were characterized by nonlinear motion, possibly representing a hyperactivated or uncoordinated motility. In human spermatozoa analyzed at 60 Hz, the definition for hyperactivated motility is VCL ± 150 μm/s, LIN ≤ 50% and ALHMAX ± 7.0 μm (22). An interpretation of this analysis is that various subpopulations represent spermatozoa in different physiological states (2). Spermatozoa are known to change their behavior in response to environmental changes and storage and, therefore, it is likely that changes in the sperm motility pattern reflect the subtleties of this process (2). Thus, a possible sequence for this progression of spermatozoa through the subpopulation structure can be constructed. As a consequence, the observed differences among the motion of individual cells can be explained, at least partially, by the efficiency of the protein kinase/phosphatase cascades which result in protein phosphorylation on the flagellum and the initiation of movement (3)

Our results show that the specific subpopulation structure of an ejaculate is related to several parameters such as the individual stallion, the total motility or the tool sperm number. The relationship of stallion and sperm subpopulation were expected because other

researches have found a similar relationship between motility and the influence of an individual stallion on mean values and other semen characteristics (18,23,31). Our results confirm these previous observations. Moreover, the comparison between motile subpopulations structure and total motility and total sperm count indicates that sperm trajectories vary according to the sperm number and the total motility. This effect would be considered logical if we assumed that the motility of a single spermatozoon would depend on the different interactions that it establishes with other sperm cells. Thus, these factors would be strongly affect both the specific percentages and the motility characteristics of the motile subpopulations of equine ejaculates.

During storage, the Subpopulation 1 seems to slightly decrease while Subpopulations 2, 3, and 4 concomitantly increase. This result could be a consequence of some spermatozoa included in Subpopulation 1 converting to behaviors classified into Subpopulation 2, 3, and 4, possibly through alteration of their functionality. Following this rationale, the increase in Subpopulations 2, 3 and 4 during cold storage might be a consequence of the functional alterations that equine spermatozoa suffer during this process. Thus, it is possible that the increase in the percentages of Subpopulations 2, 3 and 4 was an early signal of a deteriorating semen quality, that would be further detected by alterations in other parameters of semen quality, such as viability, total motility or altered acrosomes. As such, the study of motile subpopulations could be useful in improving equine semen quality analysis by detecting early and subtle changes of sperm function.

Evidence of the presence of three or four motile sperm subpopulations with very strong separate characteristics were observed in fresh and frozen/thawed semen of the common marmoset (17). Further studies were carried out to determine the population pattern of the semen of boar and gazelle (1,2, 17). These studies reaffirmed the presence of four different sperm subpopulations in the semen of the gazelles and three in that of the boar (1). Therefore, our results are in accordance with those found in other mammalian species. In fact, observing the great phylo-genetic differences among the species studied, it could be assumed that the appearance of three to four subpopulations is a widespread phenomenon in the ejaculation of mammals, thus opening a new insight in their study.

It is worth noting that, in our study, the ejaculates that hade, at least one proven fertilization did not show the Subpopulation 4, whereas the overall characteristics of the other three subpopulations were not greatly different from those defined in the study of the fresh samples. Additionally, there was a clear relationship between the percentage of the subpopulations and the total number of the ejaculates and, thus, the motile spermatozoa of the ejaculates with the greatest sperm number are all included in Subpopulation 1. We have to be very cautious in the interpretation of these results, since we will need more data to completely establish a firm conclusion. However, these results seem to indicate that fertility is associated with those spermatozoa which could be included in Subpopulation 1, since there are confirmed pregnancies using ejaculates that had only spermatozoa from this subpopulation. The inclusion of the spermatozoa with proven fertility potential in a concrete subpopulation, if further investigations confirm this assertion, could have practical consequences in the future development of equine quality analysis techniques, focusing some of these on the detection of those spermatozoa with specific physiological and thus motile characteristics. Thus, the study of motile subpopulations could lead to a considerable improvement of equine semen analysis.

5. REFERENCES

[1] Abaigar T; Holt, W; Harrison, R; Del Barrio, G. Sperm subpopulation in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod. 1999; 60:32-41.

[2] Abaigar T; Cano M; Pickard AR; Holt WV. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor azelles (*Gazella dama mhorr*): effects of body weight, electroejaculation technique and short-term semen storage. Reproduction. 2001; 122: 265-273.

[3] Aitken, J. Possible Redox Regulation of sperm motility activation. J. Andrology. 2000; 21 (4): 491-496.

[4] Amman RP; Hammerstedt, RH. *In vitro* evaluation of sperm quality: an opinion. J. Androl. 1993; 14 (6): 397-406.

[5] Ball BA; Medina V; Gravance CG; Baumber J. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. Theriogenology. 2001; 56: 577- 589.

[6] Belbin, L. Pattern Analysis Package, Software Division of Wildlife and Ecology. Canberra/ Australia: CSIRO. 1993.

[7] Boyers SP; Davis R; Katz DF. Automated Semen Analysis. Curr. Probl. Obstet. Gynecol. Fertil. 1989; 12: 172-200.

[8] Caiza de la Cueva FI; Pujol MR; Rigau T; Bonet S; Miró J; Briz M; Rodríguez-Gil, J. E. Resistance to osmotic stress of horse spermatozoa: The role of ionic pumps and their relationship to cryopreservation success. Theriogenology. 1997; 48: 947-968.

[9] Caiza de la Cueva F; Rigau T; Bonet S; Miro J; Briz M; Rodríguez-Gil, J. E. Subjecting horse spermatozoa to hypoosmotic incubation: Effects of ouabain. Theriogenology. 1997; 47: 765- 784.

[10] Chang MC; Hunter RHF. Capacitation of mammalian sperm: biological and experimental aspects. In: Hamilton D. W, Greep RO (eds.), Handbook of Physiology. Endocrinology V. Washington: American Physiological Society. 1975; 339-351.

[11] Davis RO; Overstreet JW. Asch RH; Ord T; Silber SL. Movement characteristics of human epididymal sperm used for fertilization of human oocytes “in vitro”. Fertil. Steril. 1991; 56: 1128-1135.

[12] Davis RO; Drobnis EZ; Overstreet JW. Application of multivariate cluster, discriminate function and stepwise regression analyses to variable selection and predictive modelling of sperm cryosurvival. Fertil Steril. 1995; 63: 1051-1057.

[13] Davis RO; Siemers RJ. Derivation and reliability of kinematics measures of sperm motion. Reprd Fertil Dev. 1995; 7: 857-869.

[14] Griggers S; Paccamonti DL; Thompson RA; Eilts BE. The effects of pH, osmolarity and urine contamination on equine spermatozoa motility. Theriogenology 2001; 56: 613-622.

[15] Harrison, RAP. Capacitation mechanisms and the role of capacitation as seen in eutherian mammals. Reprod Fertil Dev. 1996; 8: 581-594.

[16] Holt WV; Moore H; Hillier S. Computer-assisted measurement of sperm swimming speed in human semen: correlation with “in vitro” fertilization assays. Fertil Steril. 1985; 44: 112-119.

[17] Holt WV. Can we predict fertility rates? Making sense of sperm motility. Reprod. Domest. Anim. 1996; 31: 17-24.

[18] Jasko DJ; Little TV; Lein DH; Foote RH. Comparison of spermatozoa movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). JAVMA. 1992; 200: 979-985.

[19] Kenney RM; Bergman RV; Cooper WL; Morse GW. Minimal contamination techniques for breeding mares: Technique and preliminary finding. Proc Ame Assoc Equine Practn. 1975; 327-336.

[20] Krause W. The significance of computer-assisted semen analysis (CASA) for diagnosis in andrology and fertility prognosis. Hum Reprod. 1995; 10: 60-66.

[21] MacLeod IC; Irvine DS. The predictive value of computer-assisted semen analysis in the context of a donor insemination programme. Hum Reprod. 1995; 10: 580-586.

[22] Mortimer ST; Swan MA; Mortimer, D. Effects of seminal plasma on capacitation and hyperactivation in human spermatozoa. Hum Reprod. 1998; 13: 2139-2146.

[23] Palmer E; Magistrini M. Automated analysis of stallion semen post-thaw motility. Acta Vet Scand. 1992 (Suppl); 88: 137-152.

[24] Pickett BW; Back DG. Procedures for preparation, collection, evaluation and inseminating of stallion semen. Bull Colo St Univ Agric Exp Stn Anim Reprod Lab Gen Ser. 935; 1973: 3-52.

[25] Rathi R; Colenbrander B; Bevers MM; Gadella BM. Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprod. 2001; 65:462-470.

[26] Rigau T; Farré M; Ballester J; Mogas T; Peña A; Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 2001; 56: 801-815.

[27] SAS. SAS/STAC Software: SAS Inst. Inc; Carry, NC. USA. 1996.

[28] Sukardi S; Curry M; Watson P. Simultaneous detection of the acrosomal status and viability of incubated ram spermatozoa using fluorescent markers. Ani Repro Sci. 1997; 46: 89-96.

[29] Thurston LM; Watson P; Mileham A; Holt W. Morphologically distinct sperm subpopulation defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl. 2001; 22(3): 382-394.

[30] Valcárcel A; De las Heras MA; Pérez L; Moses DF; Baldassarre H. Assessment of the acrosomal status of membrane–intact ram spermatozoa after freezing and thawing, by simultaneous lectin/ Hoechst 33258 staining. Anim Repro Sci. 1997; 45: 299-309.

[31] Varmer D; Vaughan S; Johnson L. Use of computerised system for evaluation of equine spermatozoa motility. Am J Vet Res. 1991; 52: 224-230.

[32] Williams A; Ford C. The role of glucose in supporting motility and capacitation in human spermatozoa. J. Androl. 2001; 22 (4): 680-695.

CAPÍTULO IV

REGRESSION ANALYSES AND MOTILE SPERM SUBPOPULATION STRUCTURE STUDY AS IMPROVING TOOLS IN BOAR SEMEN QUALITY ANALYSIS

USO DEL ANALISIS DE REGRESION PARA ESTUDIAR LA MOTILIDAD DE LAS SUBPOBLACIONES ESPERMÁTICAS COMO HERRAMIENTA PARA MEJORAR EL ANALISIS SEMINAL EN CERDOS

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ABSTRACT

A precise estimation of the fertilizing ability of a boar ejaculate would be very useful to improve pig assisted reproduction results. For this purpose, we tested the mathematical combination of several parameters of the boar semen quality analysis, including the computer-assisted semen motility analysis (CASA), as a predictive fertility tool. The utilized mathematical relations among parameters were logistic and linear regressions. Two mathematical models obtained by logistic regression involving Osmotic Resistance Test, Hyperosmotic Resistance Test and viability of fresh samples, showed a significant ($P<0.05$) correlation between semen characteristics and conception rate. However, none of the obtained models produced a significant correlation model between semen characteristics and prolificacy. The CASA analyses show that three separate subpopulations of spermatozoa with different motility characteristics coexist in boar ejaculates. There were significant ($P<0.001$) differences in the distribution of these subpopulations among boars, but no clear relationship between motile subpopulation structure and fertility was obtained. Our results support the belief that the predictive use of the results obtained in a standard boar semen quality analysis can reasonably be achieved by applying logistic correlation analyses among several function parameters of boar semen quality analysis and in vivo conception rates obtained after artificial insemination.

Key words: Boar semen analysis, Functionality tests, Sperm subpopulations, Conception rate, Litter size.

1. INTRODUCTION

The wide use of artificial insemination (AI) in the pig industry has raised the interest in improving the quantitative analysis of boar semen samples, in order to estimate the fertility potential of males. In a practical sense, the main aim of these analyses would be to use this as a tool to make a concrete decision about the use of a male in a commercial AI farm. Many studies have been carried out to achieve this aim. However, there is not a single test included in the boar sperm semen quality analysis that fulfills this purpose, and only some functional tests, such as the Osmotic Resistance Test (ORT Test; 1), the rhythm of L-lactate production (2) or in vitro penetration tests (3) can be partially used as single, weak

predictor parameters for the in vivo fertility of a specific sample. These results indicate that only a combination of several tests, mainly those which are centered in functional aspects of boar spermatozoa, can be useful for this aim. The manner in which these parameters must be related or combined in order to obtain their maximal information is troublesome, and only relatively complex statistical procedures would allow for the extraction of the maximal potential of these combined parameters. In boar, a significant amount of bibliography has been published dealing with this question by using several correlation analysis systems (3-11). Nevertheless, the majority of these works applied these systems, namely linear or logistic correlations, on few numbers of tests, such as motion characteristics (3,9,12) and in vitro penetration assays (3). As a consequence, the results obtained were, in many cases, partial and, hence, difficult to interpret, thus making the achieving of a global, general statistical system that would allow professionals to direct the boar semen quality analysis towards a predictive status difficult.

One of the most utilized parameters in boar semen quality analysis is the study of motility. Until only a few years ago, the observation of boar sperm motility was troublesome, since the motion characteristics of these cells make an accurate subjective estimation of the samples difficult. In fact, subjective determination of boar sperm motility seems to not be a very useful tool for semen quality analysis (2, 13). The appearance of computer-assisted semen analysis (CASA) systems has greatly aided in overcoming this problem, since it provides an objective and accurate system to determine the motion characteristics of the sample. To make motility determination objective is important in the sense that this can be considered like a functional test, since a significant percentage of the energy that is produced by mammalian sperm is directed towards maintaining motility (14,15). Thus, the exact motion parameter that is shown by a cell is a direct determination of the energy status of this sperm. As a result, the CASA-subjected study of motility can be included in the group of functional tests that can be considered as useful for in vivo predictive evaluation of an ejaculate.

Moreover, CASA analysis has allowed researchers to determine the existence of a structure of separate motile subpopulations in a whole ejaculate. This structure has been described in many separate mammalian species, such as golden hamster (16), gazelle, (17), dog (18), horse (19) and boar (10,17,20,21). From a practical point of view, the existence

of separate motile subpopulations in an ejaculate is important, since this structure has to be taken into account when applying a CASA system in the evaluation of motility as a functional test of boar semen quality. The simpler manner to apply CASA considers that motile spermatozoa are distributed in a boar semen sample in a uniform, normal distribution. However, this simplistic view will result in a substantial loss of information when statistical procedures are applied to the results, since the real distribution of motile sperm is not uniform and normal, but rather structured in separate subpopulations (22). Moreover, the presence of this subpopulation structure raises the question as to whether the fertilizing ability of an ejaculate is (or not) localized in one or another of these subpopulations. In this sense, a strong relationship between changes in the percentages of diverse populations and the fertilization capacity of a specific ejaculate has been suggested, at least in boar (17,20) and horse (19). Thus, the study of the concrete motile subpopulation structure of a boar ejaculate can be of the greatest importance to optimize the predictive ability of boar semen quality analysis.

The main objective of this study is to find a global system which allows a reasonable predictive use of boar semen quality analysis in conception rate and prolificacy results. For this purpose, the main tests of boar semen analysis (excepting those dealing with computerized analyses of sperm morphology and in vitro penetration) were tested and related both among themselves and with the conception rate and prolificacy results by using both linear and logistic correlation techniques. This will allow us to obtain a global, statistical link among the parameters. Moreover, this approach was completed with a study of the motile subpopulation structure of the ejaculates relating to their in vivo fertility abilities. In this way, the sum of both approaches to boar semen quality analysis would allow for the optimization of the predictive usefulness of this analysis when applied to the results of in vivo fertility.

2. MATERIALS AND METHODS

2.1. Semen Collection and Processing

Boar semen was collected from 6 healthy, mature boars from a commercial farm. Boars were 2 Large White, 3 Pietrain x Large White and 1 Pietrain x White Belgium, which were

from 2 to 3 years old. The ejaculates were manually collected and immediately placed in a water-bath at 37°C. The semen was immediately diluted to a concentration of 2 x 10^7 spermatozoa/ml in a commercial extender for refrigerated semen (MR-A Extender; Kubus; Majadahonda, Spain) and distributed in 100-mL commercial AI recipients. Concentrations of these samples were determined at x200 with either a Neubauer or Thomas hemocytometer cell chamber. When necessary, some of these doses were used for AI in the same farm. One of the obtained 100-mL doses was placed in a portable refrigerator at 16°C for approximately 90 min, which was the time required to arrive at the laboratory. Here, a 5-mL sample was placed in a water-bath at 37°C and aliquots were taken immediately to evaluate the analyzed parameters in fresh diluted semen.

2.2. Analytical Procedures

Percentages of viability, altered acrosomes and morphological abnormalities were determined by using the Nigrosin-Eosin stain (23). These percentages were determined after counting 200 to 300 spermatozoa/slide at x1,000. Altered acrosomes were considered to be those which did not show a clear and uniform acrosomal ridge. Morphological abnormalities were classified according to their location in head, neck or midpiece, and tail abnormalities. Proximal and distal cytoplasmic droplets were counted as separate abnormalities.

The ORT Test was carried out as described by Rodriguez-Gil and Rigau (24), whereas L-lactate production in an isoosmotic medium was determined enzymatically, as described in (2,24).

A previous report showed that the resistance of boar sperm to sudden changes of osmolarity could be a feasible parameter to estimate cell function (25). Following this, we have developed a functional test based upon the ability of boar sperm to resist osmolarity changes from a hyperosmotic medium to an isoosmotic medium. This test, which we have named the Hyperosmotic Resistance Test (HRT Test), was performed as follows:

A 100-μL aliquot of semen was added to 900 μL of a hyperosmotic solution containing 0.9 mol/Kg glucose (π = 1169 ± 13 mOsm, mean ± SEM for 8 separate determinations).

The mixture was incubated for 5 min at 37ºC, and after this a 100-µL aliquot was quickly returned to an isoosmotic environment by placing it in an Eppendorf tube containing 1000 µL of a Krebs-Henseleit-Ringer solution at 37ºC (π = 296 ± 7 mOsm, mean ± SEM for 8 separate determinations). Both, the remaining 900 µL of the hyperosmotic medium (undisturbed solution) and the 1100 µL isoosmotic medium with hyperosmotic stressed spermatozoa (disrupted solution), were quickly observed by using the modified dual Trypan Blue-Giemsa stain (26). In these stained samples, the percentages of viability and altered acrosomes were determined, in a manner similar to that previously published (25). Following this, the HRT Test was based upon the attaining of the relationships between the percentages of viability (VHIPER) and altered acrosomes (ACROHIPER) as indicated in the following formulae:

VHIPER = VD/VU,
ACROHIPER = AD/AU,
Where:
VD = Percentage of viability in the disrupted medium;
VU = Percentage of viability in the hyperosmotic, undisturbed medium;
AD = Percentage of altered acrosomes in the disrupted medium;
AU = Percentage of altered acrosomes in the hyperosmotic, undisturbed medium.

2.3. Computer-Assisted Motility Analysis

As previously stated, motion characteristics of the samples were determined by using a CASA system (Sperm Class Analyzer, Microptic; Barcelona, Spain). In this, a 5-mL aliquot of the diluted semen was incubated for 5 min in a water-bath at 37°C. After this, a 5-µL drop of the sample was placed on a warmed (37°C) slide and covered with a 25-mm^2 coverslip. Our CASA system was based upon the analysis of 16 consecutive, digitalized photographic images obtained from a single field at magnification of x200 on a dark field. These 16 consecutive photographs were taken in a time lapse of 0.64 sec, which implied a velocity of image capturing of 1 photograph every 40 milliseconds. From 2 to 3 separate fields were taken for each sample. The obtained sperm motility descriptors were described following Boyers et al. (27), Davis and Siemers (28) and Rigau et al. (18). Total motility

was defined as the percentage of spermatozoa which showed a mean velocity (VAP; see Table 1 for definition) above 10 μm/sec.

2.4. Selection of the Parameters to Perform the Boar Sperm Motility Study

The application of the VARCLUS procedure, combined with subsequent variance analyses, was performed, as described in (19). The analysis of the data obtained from 6,297 spermatozoa, grouped the 21 motion variables obtained in the CASA into 8 clusters, which explain 84.22% of the total variation. The comparison of the statistical proximity among the separate motion variables gathered in the same clusters led to the choosing of one or two motion parameters in each cluster, which conserved the maximal statistical information contained in each cluster. From this analysis, the chosen variables for each cluster were: the mean amplitude of head lateral displacement (mean ALH), linear coefficient (LIN), VAP, frequency of head displacement (BCF), minor harmonic oscillation of the head (HLO), algebraic angular mean displacement (AlgMAD), dance (DNC) and the maximal amplitude of the oscillation of the head (HME). For a better definition of these parameters, see Table 1.

Table 1.

Definition of the choosen motility descriptors

Name	Units	Description
Mean velocity (VAP)	μm/sec	The mean trajectory of the spermatozoa per unit of time
Linear coefficient (LIN)	%	VSL/VCL
Mean lateral head displacement (mean ALH)	μm	Mean head displacement along its curvilinear trajectory around the mean trajectory
Dance (DNC)	μm^2/sec	VCL x ALHMED
Algebraic angular mean displacement (AlgMAD)	angular degrees	The algebraic value of the advancing angle of the sperm trajectory. Negative values indicate a clockwise displacement
Frequency of head displacement (BCF)	Hz	The number of lateral oscillatory movements of the sperm head around the mean trajectory
Minor harmonic oscillation of the head (HLO)	μm	The minimum value of the distance between the curvilinear trajectory with respect to the mean trajectory
Maximal amplitude of the oscillation of the head (HMX)	μm	Maximal distance between two successive crosses around the mean trajectory

2.5. Fertility Trials

The in vivo fertility study was conducted in the same farm, using a total of 133 multiparous (two to six pregnancies) crossbred sows (Large White x Landrace). Estrus was checked daily in the presence of a mature teaser boar. Occurrence of estrus was defined by the standing reflex in front of a boar (back pressure test) and reddening and swelling of the vulva. The sows were inseminated with diluted semen, using disposable AI-catheters. Insemination took place 12 hours after diagnosis of estrus and was repeated 12 hours later. Pregnancy diagnosis was performed 21-30 days after AI by ultrasonography. When several anechoic (dark) areas (which represent the embryonic vesicles) were found in the image, it was considered a positive diagnosis of pregnancy. In the absence of dark areas, the sows were classified as non-pregnant, and it was confirmed by the return to estrus or nonfarrowing at the predicted time. Semen fertility was measured by relating the semen characteristics with the pregnancy diagnosis (0=non-pregnant, 1=pregnant). For each sow that farrowed, the number of dead and live piglets was counted and the sum was defined as the total number of piglets born.

2.6. Statistical Analysis

All data recorded (sperm motility descriptors, morphometry and fertility) were imported into a database (Excel, 2000) and analyzed by SAS/Statistical Analysis System for Windows (29). Sperm motility descriptors obtained from CASA were analyzed by the VARCLUS clustering procedure. In this system, clusters are chosen to maximize the variation accounted for by either the principal component or the centroid component of each cluster. Thus, the VARCLUS procedure can be used to reduce the number of sperm motility descriptors. This procedure allows us to determine the optimal number of motion parameters which can be used to define sperm motion characteristics. The separation of variables into clusters was carried out over 64 evaluations of 32 ejaculates with 6,297 spermatozoa. Following this, the next point was to use another statistical procedure which allows for allocating an individual, motile spermatozoon into a specific subpopulation. Another clustering procedure, the FASTCLUS one, was used for this purpose. This procedure performs a disjointed cluster analysis on the basis of Euclidean distances computed from one or more quantitative variables (sperm motility descriptors), which

allows for the study of large sets of observations, from approximately 100 to 100,000. The spermatozoa were divided into clusters such that every observation belongs to one and only one cluster. Spermatozoa that are very close to each other are usually assigned to the same cluster, while spermatozoa that are far apart are in different clusters. The separation of spermatozoa into clusters was carried out from the total number of spermatozoa obtained in evaluations of 28 ejaculates (n= 5,515). A General Lineal model (PROC GLM) was used to evaluate significant differences ($P<0.05$) among clusters of sperm subpopulations, whereas the LSMEANS procedure was used to list these mean differences. A Chi-square test was used to determine the relationship among sperm subpopulations in diluted semen with the individual boar used, as well as with the total L-lactate in the ejaculates, percentage of spermatozoa viability in ORT Test and the viability between disturbed and undisrupted media (VHIPER) in the whole diluted ejaculates. The results obtained after the Chi-square tests allowed us to categorize the motile spermatozoa in the following form, after application of the FASTCLUS procedure:

Following the rate of L-lactate production:

Group 1 whole ejaculates: L-lactate production 1 μmol/mg protein x 60 min $>x\leq$ 6.5 μmol/mg protein x 60 min.
Group 2 whole ejaculates: L-lactate production 6.5 μmol/mg protein x 60 min $>x\leq$ 11.5 μmol/mg protein x 60 min.
Group 3 whole ejaculates: L-lactate production 11.5 μmol/mg protein x 60 min>x.

Following the results of the ORT Test:

Group 1 whole ejaculates: ORT Test percentage 45% $>x\leq$ 59%
Group 2 whole ejaculates: ORT Test percentage 59% $>x\leq$ 78%.
Group 3 whole ejaculates: ORT Test percentage 78%.>x.

Following the results of the HRT Test:

Group 1 whole ejaculates: VHIPER results 0.01 $>x\leq$0.35.
Group 2 whole ejaculates: VHIPER results 0.35 $>x\leq$ 0.7

Group 3 whole ejaculates: VHIPER results 0.7 >x.

Logistic regression analyses were used to relate the dichotomous conception rate to the sperm parameters. Finally, linear regression analyses (Pearson correlation and multiple regressions) were used to examine the relationship between litter size and measured semen parameters (9,30).

3. RESULTS

3.1. Mean Results for the Semen Quality Analysis of Boar Ejaculates

The mean values for the overall semen quality parameters are shown in Table 2. The presence of relatively great values of SEM in several parameters is noteworthy. This was due to the existence of great, significant (χ^2= 36.58, df= 12, P<0.001) differences in parameter values among boars. Thus, the percentage of proximal cytoplasmic droplets varied from 3.0±0.5% in Boar 206 to 46.0±5.09% in Boar 410 (Table 3). A similar variability can be observed in other parameters, such as the percentages of altered acrosomes, ORT and HRT Tests (Table 3). This indicates the importance of the individual in the precise result of quality seminal analysis.

3.2. Relationship between Seminal Characteristics and In Vivo Fertility Results

No significant correlations were obtained between values of a single semen quality parameter and either conception rate or prolificacy (data not shown). In fact, when we observed the data segregated by boars there were some surprising facts, such as that individuals with a high percentage of proximal cytoplasmic droplets showed similar fertility results to those with much lower percentages of this morphologic abnormality (Table 3). Only those quality parameters involving functional aspects of ejaculates, especially ORT and HRT Tests, showed a tendency to correlate with conception rate, although none of them, individually, showed a significant correlation (data not shown).

3.3. Sperm Subpopulations Analysis in Diluted Semen

Three sperm subpopulations were defined after the application of the FASTCLUS procedure and subsequent variance analysis (Table 4). The qualitative interpretations of the data obtained from each subpopulation, are the following:

Table 2.

General characteristics of the diluted sperm fraction of boar ejaculates

Parámetro	n	Means ± SEM	Confidence intervals (95%)
Percentage of viability by Eosin/ nigrosin staining (%)	32	84.7 ± 2.3	80.0-89.3
Altered acrosomes (%)	33	12.0 ± 1.1	9.8-14.2
Total abnormalities (%)	32	55.6 ± 5.1	45.4-65.8
Proximal cytoplasmic droplet (%)	32	32.7 ± 3.1	26.4-38.9
Distal cytoplasmic droplet (%)	32	11.6 ± 1.7	8.2-15.0
Total Motility (%)	33	81.0 ± 1.6	77.7-84.3
Osmotic resistance test (%)	33	76.4 ± 1.2	74.0-78.9
L-Lactate production (μ mol/mg protein x 60 min)	24	8.1 ± 0.7	6.7-9.5
Percentage of viability in hyperosmotic, undisrupted medium (%)	30	81.7 ± 2.1	77.6-85.9
Percentage of altered acrosomes in hyperosmotic, undisrupted medium (%)	30	22.8 ± 2.1	18.7-26.9
Percentage of viability in disrupted medium (%)	29	50.3 ± 3.7	42.9-57.6
Percentage of altered acrosomes in disturbed medium (%)	29	58.0 ± 2.4	53.2-62.7
Relation of the viability between disturbed and undisrupted media (VHYPER)	28	0.6 ± 0.1	0.5-0.7
Relation of altered acrosomes between disturbed and undisrupted media (ACROHYPER)	28	0.39 ± 0.03	0.33 a 0.46

Total motility is defined as the percentage of spermatozoa with a VAP > 10 μm/sec.

Subpopulation 1.

This contains fast spermatozoa, with high values of VAP. Trajectories show low linearity, although it was higher than Subpopulation 3 (LIN of 38.5±1.1% vs. 30.1±2.6% in Subpopulation 3). Mean ALH and DNC are higher than Subpopulation 2 but lower than Subpopulation 3. About 9 % of the total motile spermatozoa were included in this subpopulation.

Subpopulation 2.

Table 3.

Individual boar results of sperm quality parameters and values of conception rate and litter size after A.I. with the analyzed ejaculates.

Variables	Boar					
	206	210	409	410	411	412
Number of ejaculates	2	2	3	9	12	4
Proximal cytoplasmic droplets (%)	3.0 ± 0.5^{a}	2.0 ± 1.0^{a}	24.2 ± 7.8^{b}	46.0 ± 5.9^{c}	43.2 ± 0.6^{c}	3.66 ± 0.5^{a}
Total Motility (%)	88.8 ± 0.4^{a}	78.8 ± 1.3^{b}	76.2 ± 2.6^{b}	89.7 ± 2.4^{a}	82.4 ± 2.1^{b}	60.2 ± 2.2^{c}
VAP (μm/sec)	46.7 ± 1.0^{a}	42.8 ± 1.8^{b}	38.6 ± 1.0^{c}	47.3 ± 0.6^{a}	36.6 ± 0.3^{cd}	35.1 ± 0.8^{d}
LIN (%)	56.6 ± 1.2^{a}	50.1 ± 1.6^{b}	55.2 ± 1.1^{a}	55.3 ± 0.6^{a}	48.2 ± 0.4^{b}	47.5 ± 1.0^{b}
MeanALH (μm)	2.56 ± 0.05^{a}	2.69 ± 0.08^{a}	3.77 ± 0.10^{b}	2.66 ± 0.03^{a}	2.60 ± 0.02^{a}	2.47 ± 0.05^{a}
DNC (μm^2/sec)	203.7 ± 9.0^{a}	213.3 ± 17.3^{ab}	242.2 ± 22.5^{c}	222.7 ± 5.6^{b}	198.7 ± 4.6^{a}	171.4 ± 8.5^{d}
BCF (Hz)	15.3 ± 0.2^{a}	14.4 ± 0.4^{ab}	13.0 ± 0.2^{c}	14.4 ± 0.1^{ab}	14.6 ± 0.1^{ab}	14.3 ± 0.3^{b}
HLO (μm)	0.11 ± 0.01^{a}	0.10 ± 0.01^{a}	0.18 ± 0.03^{b}	0.14 ± 0.01^{ab}	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}
HMX (μm)	2.38 ± 0.06^{abc}	2.54 ± 0.13^{a}	2.33 ± 0.08^{abc}	2.40 ± 0.03^{ab}	2.20 ± 0.02^{bc}	2.17 ± 0.05^{c}
AlgMAD (angular degrees)	-10.38 ± 1.14^{ab}	-14.38 ± 1.80^{c}	-9.55 ± 1.03^{ab}	-11.70 ± 0.56^{bc}	-15.02 ± 0.46^{cd}	-18.50 ± 0.94^{d}
Inseminated sows in this trial	6	0	17	42	55	13
Conception rate in this trial (%)	50.0	-	94.1	83.3	81.8	92.3
Conception rate in the farm (%)	71.6	-	76.0	80.8	76.3	85.7
Litter size in this trial (means)	12.0	-	11.02	13.0	10.97	10.83
Litter size in the farm (means)	10.84	-	10.07	10.35	10.31	10.44

Data are expressed as means ± SEM. Motility descriptors and general characteristics of diluted semen have been described in Table. Different superscripts in a row indicate significant differences ($P<0.05$).

These spermatozoa showed a relatively low velocity together with high progressiveness, as indicated by their VAP and LIN values. Moreover, mean ALH, DNC, HLO and HMX values are the lowest among the subpopulations, indicating a type of movement with low ondullatory characteristics. The BCF is similar to the other subpopulations. AlgMAD is high and negative (-12.81±0.68 angular degrees), indicating a high angular, clockwise-oriented displacement. About 90% of the spermatozoa in the data set were assigned to this subpopulation.

Table 4.

Motile subpopulation characteristics of diluted boar samples from the CASA data set.

Sperm motility descriptors	Sperm Subpopulations		
	1	2	3
N° spermatozoa	510	4928	77
Percentage from the total	9.2	89.4	1.4
VAP (μm/sec)	65.2 ± 0.9^{a}	39.0 ± 0.5^{b}	69.3 ± 2.2^{a}
LIN (%)	38.5 ± 1.1^{a}	53.4 ± 0.6^{b}	30.1 ± 2.6^{c}
mean ALH (μm)	4.94 ± 0.04^{a}	2.29 ± 0.02^{b}	9.89 ± 0.01^{c}
DNC (μm^2/sec)	570.1 ± 6.2^{a}	144.5 ± 3.2^{b}	1757.3 ± 13.0^{c}
BCF (Hz)	14.9 ± 0.3^{a}	14.2 ± 0.1^{a}	13.7 ± 0.6^{a}
HLO (μm)	0.28 ± 0.02^{a}	0.091 ± 0.01^{b}	0.12 ± 0.05^{ab}
HMX (μm)	3.84 ± 0.07^{a}	2.23 ± 0.03^{b}	6.39 ± 0.16^{c}
AlgMAD (angular degrees)	-9.44 ± 1.21^{a}	$- 12.81 \pm 0.68^{b}$	-7.30 ± 2.79^{a}

Results are expressed as means ± S.D. of 5,515 spermatozoids from 28 separate ejaculates. Motility descriptors have been described in Table 1. Different superscripts in a row indicate significant differences ($P<0.05$).

Subpopulation 3.

These spermatozoa showed non-linear trajectories. Cells were very active, with high values of VAP, although their non-linearity and poor progressiveness characteristics were evident from their high values of mean ALH and DNC. The percentage of motile spermatozoa which were included in Subpopulation 3 was 1.4%.

Since values of several semen quality parameters were dependent on the precise boar from which the semen sample came, it was also considered of interest to study if the precise subpopulation structure of ejaculates varied among individuals. Our results indicate that, whereas the percentage of sperm included in each subpopulation did not greatly vary among boars, there were great variations in the single values of several motility parameters included in each subpopulation. Thus, centering on VAP values, these varied, in

Subpopulation 1 from 55.0±3.6 μm/sec in Boar 409 to 90.4±5.3 μm/sec in Boar 210; in subpopulation 2 from 31.7±0.9 μm/sec in Boar 409 to 46.4±0.9 μm/sec in Boar 206, and in Subpopulation 3 from 37.3±5.4 μm/sec in Boar 409 to 128.7±13.1 μm/sec in Boar 210. Similar variations were observed in all of the other motility parameters (data not shown). This indicates that the overall motile sperm subpopulation structure was similar in all of the boar studied despite the existence of individual differences in the values of motility parameters per each subpopulation.

3.4. Relationship between Motility Parameters and L-Lactate Formation, ORT Test and HRT Test

A common assertion indicates that sperm motility is directly related to the functionality of this sperm. This would imply that there is a more or less direct relationship between the motility characteristics of boar sperm and the results of the seminal quality tests that reflect sperm functionality. In our sperm analysis, these functionality tests are three: The production of L-lactate and the ORT and HRT Tests. Thus, the motility parameters were compared, both in their overall results and under the motile subpopulation structure, with a categorization of the results obtained by the FASTCLUS procedure, as opposed to these tests, in order to determine the existence of such a relationship.

Mean motility parameters varied when spermatozoa were classified according to their production of L-lactate. As shown in Table 5, VAP, LIN and DNC were significantly ($P<0.05$) lower, and mean ALH and HMX were significantly ($P<0.05$) higher in samples with a production of L-lactate greater than 11.5 μmol/mg protein x 60 min as compared to those samples between 1 and 6.5 μmol/mg protein x 60 min. Samples with an L-lactate formation between 6.5 and 11.5 μmol/mg protein x 60 min showed motility parameters, excepting VAP and AlgMAD, similar to those of the group with lower values of L-lactate formation, which is compatible to its intermediate position in the L-lactate formation groups (Table 5).

The segregation of separate motion characteristics associated with a specific rate of L-lactate formation was also shown when samples were studied under a motile subpopulation

structure. Thus, the greater percentage of spermatozoa included in Subpopulation 1 (10.0%, which corresponded to 248 spermatozoa) was observed in samples with a production of L-lactate between 1 and 6.5 μmol/mg protein x 60 min. Similarly, Subpopulation 3 practically disappeared in ejaculates with an L-lactate formation rate above 11.5 μmol/mg protein x 60 m (1.6%, which corresponded to 39 spermatozoa). This indicates that the precise subpopulation structure can be related to the ability of L-lactate formation in these samples. Moreover, subpopulations varied not only in their percentage, but also in their motion characteristics. Thus, samples with an L-lactate formation rate above 11.5 μmol/mg protein x 60 m showed higher, significant ($P<0.05$) values of VAP in Subpopulation 3 (103.0±7.6 μm/sec, mean±S.D. from 6 spermatozoa) than those of Subpopulation 1 (63.6±2.1 μm/sec, mean±S.D. from 79 sperm) and Subpopulation 2 (39.2±0.7 μm/sec, mean±S.D. from 888 spermatozoa). Accordingly, the VAP of Subpopulation 1 was significantly higher in samples with an L-lactate formation between 6.5 and 11.5 μmol/mg protein x 60 m (66.2±2.1 μm/sec vs. 34.2±0.7 μm/sec in Subpopulation 2 and 58.5±3.8 μm/sec in Subpopulation 3; results as means ±S.D. from 85, 1294 and 24 spermatozoa, respectively). Similar results were observed in the other motility parameters (data not shown). Thus, the motion characteristics of each subpopulation seem to also be related to the overall capacity to form L-lactate of the ejaculates.

Similarly to that observed in the rate of L-lactate formation, there were clear variations in many mean values of motility parameters related to the response of an ORT Test. Thus, after determining means±S.D. results of 55,515 spermatozoa from 28 ejaculates, values of LIN were significantly ($P<0.05$) lower in samples with ORT values above 59%, and this decrease was greater as ORT values increased (56.4±1.5 % in the ORT group in the range of 59%-to-58 % and 50.0±1.2 % in the ORT group above 78%). Mean ALH, DNC, BCF, HLO and HMX increased when ORT values also increased and, thus, these motion parameters were significantly ($P<0.05$) greater in samples with ORT values above 59% than in those with ORT scores below 59% (data not shown). This relationship between ORT and mean motility values was translated into specific modifications in the subpopulation structure related to ORT values. In this respect, it is worth noting that Subpopulation 3 was practically absent (0.3% of the total motile sperm population) in samples with ORT values below 59%. Moreover, Subpopulation 1 greatly increased in samples with ORT scores above 78%. In these samples, Subpopulation 1 was 11.5% of the

total motile sperm, which was significantly (P<0.05) greater than that observed in samples with ORT values below 59% (4.6%) and with an ORT score between 59% and 78% (6.9%). On the other hand, the VAP did not significantly differ in subpopulations when classified according to the ORT values of the whole ejaculate, and the high VP value seen in Subpopulation 3 can be explained by the fact that only one spermatozoon was found in this subpopulation in ejaculates with ORT values below 59%, thus invalidating the statistical procedure applied in the samples (data not shown).

Table 5.

Relationship between the mean motion characteristics with respect to the rhythm of *L*-lactate formation in whole samples.

Sperm motility descriptors	L-Lactate Groups		
	>1-≤6.5	>6.5-≤11.5	>11.5
VAP (μm/sec)	44.2 ± 1.3^{a}	40.0 ± 1.7^{b}	33.8 ± 1.5^{c}
LIN (%)	55.0 ± 1.9^{a}	53.0 ± 2.5^{a}	48.8 ± 2.3^{b}
mean ALH (μm)	1.6 ± 3.3^{a}	1.0 ± 4.4^{a}	6.6 ± 3.9^{b}
DNC (μm^2/sec)	193.0 ± 9.7^{a}	172.5 ± 12.8^{a}	134.2 ± 11.5^{b}
BCF (Hz)	14.3 ± 0.4^{a}	14.8 ± 0.5^{a}	14.0 ± 0.4^{a}
HLO (μm)	0.11 ± 0.04^{a}	0.09 ± 0.05^{a}	0.10 ± 0.05^{a}
HMX (μm)	1.91 ± 1.91^{a}	1.52 ± 2.54^{a}	4.65 ± 2.26^{b}
AlgMAD (angular degrees)	-12.5 ± 1.5^{a}	-19.0 ± 2.0^{b}	-17.2 ± 1.8^{a}

Results are shown as means ± S.D. of 4,856 spermatozoa from 25 ejaculates. Motility descriptors have been described in Table 1. Different superscripts in a row indicate significant (P<0.05) differences among groups. L-lactate production is expressed as μmol/mg protein x 60 min.

Comparable results were obtained when samples were classified according to their mean motion parameters, with respect to the changes in the HRT Test. Samples with VHIPER above 0.7 showed values of LIN significantly (P<0.05) lower, and values of mean ALH, DNC, HLO, HMX and AlgMAD significantly (P<0.05) higher than those obtained in samples with VHIPER below 0.35 (data not shown). Samples with VHIPER above 0.35 and below 0.7 showed intermediate, mean motion values between those of the extreme subpopulations (data not shown). According to that observed with the L-lactate formation rate and the ORT Test, the modifications observed in the mean values of motion parameters were related to variations in the specific subpopulation structure of the ejaculates. Thus, again Subpopulation 3 was practically absent (0.8% from the total motile spermatozoa) in samples with VHIPER values below 0.35, and Subpopulation 1 increased

from 7.4% with VHIPER scores between 0.01 and 0.35 to 9.6% in samples with VHIPER values above 0.7. Moreover, VAP values did not change in any subpopulation when compared to the VHIPER values of the whole samples (data not shown).

The next step was to verify whether a mathematical relationship among the seminal quality parameters could render some correlation between boar semen analysis and fertility in vivo. For this purpose, a study of logistic regression between the semen quality parameters and the in vivo conception rate was performed. This study finally rendered five separate logistic regression models. Of these, only two of them were statistically significant ($P<0.05$; Table 6). It is noteworthy that both models only included two (ORT and HRT Tests) or three (ORT Test, HRT Test and viability) semen parameters, with motility characteristics not being considered important for these logistic regressions (Table 6). Thus, in our conditions, the combination of two functional probes was the best model to explain the in vivo conception rate from the utilized ejaculates.

A similar study by using multiple linear regression models between semen quality parameters and litter size was performed. In this, various statistical models were generated that explained 10%-24% of the variation in litter size (data not shown). However, none of these models was statistically significant (as much as $P>0.08$) and they were not capable of explaining the variation in prolificacy (data not shown).

Although the results obtained with the mean values of semen parameters were not promising, we next studied the possibility of a relationship between the precise, motile subpopulation structure of ejaculates and in vivo fertility results. To this end, the sows inseminated with the ejaculates were classified into two categories; those that were positive for pregnancy after AI and those that were not, and then the subpopulation structure of the mean of the ejaculates utilized in both groups was analyzed. Table 7 indicates that the only subpopulation that showed some differences between positive and negative sows was Subpopulation 3. This group presented significantly ($P<0.05$) higher values of mean ALH and DNC, and lower values of VAP in the sows that were negative for pregnancy after AI. No other significant difference was observed in this study (Table 7).

Table 6.

Details of the two significant models obtained after carrying out a logistic regression study of seminal characteristics relating to conception rate data obtained with the same samples.

Statistical Model	Regression Coefficient	SE	Significance of individual parameters (P)	Significance of Statistical model (P)	Percent Concordant
Model 1	-	-	-	0.0318	69.0
ORT	-0.1729	0.0885	0.0507		
VHIPER	-2.0362	1.6549	0.2186		
Constant	17.2977	7.8025	0.0266		
Model 2				0.0487	67.1
Viability	0.0280	0.0280	0.3166		
ORT	-0.1743	0.0883	0.0483		
VHIPER	-1.9014	1.5733	0.2268		
Constant	14.8378	8.0115	0.0640		

The logistic function for Model 1 is $p=1/[1+e^{-(17.297-0.172*ORT-2.036*VHIPER)}]$, whereas the logistic function for Model 2 is $p=1/[1+e^{-(14.837+0.028*viability-0.174*ORT-1.9014*VHIPER)}]$.

Only the most significant effects ($P<0.10$) are shown. The other parameters of seminal characteristics were removed by logistic regressions.

Viability: Percentage of viability in fresh, diluted samples.

ORT: Results from the ORT Test.

VHIPER: Relationship between the percentages of viability between disturbed and hyperosmotic undisrupted

4. DISCUSSION

The analysis of our results shows several interesting conclusions that have to be taken into account when interpreting data from a boar semen quality analysis. First of all, the mathematical combination of the parameters of this semen analysis, especially in the shape of logistic regressions, can render much more valuable information than the individual observation of separate parameters. This is an obvious conclusion, and it has been reported before (9,31,32). However, the importance of our model is found in the fact that the most important parameters for the interpretation of semen quality analysis were those based on sperm functionality, such as the ORT and HRT Tests. Conversely, some classical parameters, such as morphological abnormalities or total motility, seem to not be of great importance in the analysis of fertile, boar semen samples. This is highlighted, in the case of abnormalities like proximal cytoplasmic droplets, when observing that semen samples with very different percentages of these anomalies rendered high and similar results on in vivo fertility (see Table 3). Of course, it must be stressed that this result is only applicable to semen with proven fertility, and the mathematical integration of the quality analysis

Table 7. Motile sperm subpopulation structures of boar semen samples with positive or negative pregnancy results after artificial insemination.

Sperm motility descriptors	Subpopulation 1		Subpopulation 2		Subpopulation 3	
	Positive Pregnancies	Negative Pregnancies	Positive Pregnancies	Negative Pregnancies	Positive Pregnancies	Negative Pregnancies
Percentage (%)	8.8^{a} (322)	10.2^{a} (188)	90.0^{b} (3298)	88.1^{b} (1630)	1.2^{c} (46)	1.7^{c} (31)
VAP (μm/sec)	66.0 ± 1.1^{a}	64.3 ± 1.4^{a}	38.4 ± 0.5^{b}	39.5 ± 0.7^{b}	75.9 ± 2.8^{c}	62.7 ± 3.4^{a}
LIN (%)	38.6 ± 1.3^{a}	38.4 ± 1.7^{a}	53.7 ± 0.6^{b}	53.1 ± 0.8^{b}	29.2 ± 3.3^{a}	31.0 ± 4.0^{a}
Mean ALH (μm)	4.93 ± 0.04^{a}	4.95 ± 0.06^{a}	2.26 ± 0.02^{b}	2.32 ± 0.02^{b}	9.43 ± 0.11^{c}	10.34 ± 0.14^{d}
DNC (μm^2/sec)	570.0 ± 6.5^{a}	570.2 ± 8.6^{a}	140.3 ± 3.1^{b}	148.7 ± 4.0^{b}	1599.0 ± 16.3^{c}	1915.5 ± 20.0^{d}
BCF (Hz)	14.7 ± 0.3^{a}	15.1 ± 0.4^{a}	14.1 ± 0.1^{a}	14.3 ± 0.2^{a}	13.4 ± 0.8^{a}	14.0 ± 1.0^{a}
HLO (μm)	0.31 ± 0.02^{a}	0.24 ± 0.03^{a}	0.08 ± 0.01^{b}	0.09 ± 0.01^{b}	0.16 ± 0.06^{ab}	0.07 ± 0.01^{b}
HMX (μm)	3.86 ± 0.07^{a}	3.82 ± 0.10^{a}	2.21 ± 0.03^{b}	2.25 ± 0.03^{b}	6.40 ± 0.20^{c}	6.38 ± 0.24^{c}
algMAD (angular degrees)	-9.6 ± 1.4^{a}	-9.2 ± 1.8^{a}	-13.0 ± 0.7^{b}	-12.7 ± 0.9^{b}	-12.1 ± 3.5^{b}	-12.5 ± 4.3^{b}

The results were obtained from 5,515 spermatozoa included in 28 separate ejaculates. These were utilized to inseminate 133 sows. Nineteen of these ejaculates showed positive pregnancies, involving 111 sows, whereas 7 ejaculates and 22 sows inseminated with them, showed negative pregnancies. Different superscripts in a row indicate significant ($P<0.05$) differences among groups. Results are expressed as means ± S.E., excepting those of the percentage of motile spermatozoa included in each subpopulation. In the lane corresponding to the percentage of each subpopulation with respect to the whole sample, the number of spermatozoa analyzed in each point is shown in parentheses. Motility descriptors have been defined in Table 1.

parameters from sub-, or infertile samples might render other results. Nevertheless, in our conditions of a commercial farm, which uses semen of good average quality for AI, the most classical parameters of seminal quality add very little information about the fertilizing ability of a concrete sample.

The importance of functional tests on boar semen quality analysis has been previously shown by separate working groups. Thus, from its appearance, the ORT Test has shown itself to be one of the most potent quality parameters (1,3,33). The HRT Test that has been developed by us from former works (25,26) seems to also be a potent functional parameter. This is not difficult to understand, since the HRT Test is also a test that determines the ability of boar spermatozoa to react against osmotic changes of the environment. The main difference between both parameters is that, whereas the ORT Test determines the resistance of sperm to a single osmotic condition, the HRT Test evaluates the ability of these cells to resist sudden changes of osmolarity, thus observing the behavior of sperm in two consecutive osmolar conditions. Other functional tests, such as those based on the in vitro penetration ability of boar sperm, have also shown themselves to be potent quality parameters (3). Nevertheless, no in vitro penetration test was used, since they need a relatively complex infrastructure, which is not available for the majority of the average boar semen quality analysis laboratories, and our work has been centered on those tests that can be carried out in such laboratories. Thus, following the point of view of an average boar semen quality analysis, we strongly support the necessity of these laboratories to introduce functional, osmotic tests as obligatory ones in their routine protocols. These tests would be complimented, of course, with other more classical tests, specially the observation of the percentage of viability of fresh samples, which has shown itself to be another statistically potent test (see Table 6). It is worth noting, however, that our results have been obtained on only one farm, and with a limited number of boars and inseminated sows. Studies involving greater numbers of both might affect these conclusions somewhat. We have to remember that, in a former study, the rate of L-lactate production was marked as a statistically stronger test than the ORT Test (2), whereas in this study the result was not the same. Thus, although the great relevance of the various functional tests is undoubted, the precise importance of each one of them in boar semen quality analysis could vary with respect to the concrete conditions of the studied farms and semen samples.

Sperm motility could be considered as a functional marker in boar sperm analysis, since motility is directly related to the sperm's ability to obtain and process energy (15). Our study did not show any concrete relationship between sperm motility descriptors obtained by CASA and in vivo fertility data. This is not very surprising and, in fact, a similar lack of a strong relationship has been observed both in boar (3) and in other species, such as human (34-36). In the case of boar, this effect could be explained by the intrinsic characteristics of sperm motility. Boar sperm has low mean values of its motion parameters, especially when compared to other mammals like dog (18) and horse (19). This implies that the variation limits of data, and then the subsequent variation coefficients, from these parameters are small. This, besides the great homogeneity of the in vivo fertility results, leads us to look for correlations between two types of parameters (motion parameters and in vivo fertility results) with very small coefficient variations. This makes the appearance of such relationships very difficult. Moreover, boar sperm motility is very dependent on the environment, and factors like temperature or the type and size of the slides and coverslips utilized greatly influence it (13). Consequently, boar sperm motility can only be considered to be useful in sperm quality analysis when all of the external factors that can influence it, from temperature to the time that the motion analysis needs to be performed and the precise type of mechanical support on which the semen sample is placed, are totally controlled. Since this is a very difficult goal in standard, field conditions, we have to assume that motility will not be a good boar semen analysis parameter in the majority of cases.

The difficulties observed in the exact determination of boar motility, of course, greatly affect the analysis of the motile subpopulation structure of boar ejaculates. Thus, our observations corroborate the already published findings of separate motile subpopulations in boar ejaculates (10,16,17,21). Our study reveals the presence of 3 separate motile sperm subpopulations. These results, including the motion characteristics, were similar to those observed by Abaigar et al. (17) and Thurston et al. (21). All of these data suggest that the presence of a specific motile subpopulation structure in an ejaculate is a physiological finding, which could be related to the fertilizing ability of a sample. This can also be supported by the finding of similar structures in a wide range of separate mammalian species. However, in our study, the majority of motile sperm (about 90%) were included in a single subpopulation. This implies that differences in the specific motile subpopulation

structure among ejaculates are not very evident and, thus, the finding of any relationship between motile subpopulation structure and in vivo fertility data is very difficult. This great disproportion between the percentages of each subpopulation was not found in previous articles (10,17,20,21). This can be a consequence of two separate, but coordinated, factors. The first would be the existence of very great differences in the proportion of motile subpopulations among individuals. In this respect, it is worth noting that results from our laboratory using the same CASA analysis system and performed on semen samples for other boars rendered 4 subpopulations with a relative percentage among them of about 18%, 41%, 8.5% and 32%, respectively (37). This clearly indicates that the specific motile subpopulation structure of an ejaculate depends on the boar from which the sample came. The second factor would clearly be the use of different CASA analytical conditions, including the physical support for the semen sample (i.e., the use of a simple slide/coverslip system). This leads to the attaining of very different motile subpopulation structure. This latter could be a natural consequence of the discussed difficulties that are intrinsic in the determination of boar sperm motility. Thus, in these conditions, other functional tests, such as ORT and HRT Tests, are better for determining boar semen quality since they reflect sperm functional status in a more homogeneous, less variable manner. Concomitantly, the optimal use of CASA-obtained data of boar sperm motility for routine semen analysis will need an absolutely precise, complete and exact, international, standard protocol that will allow for a correct interpretation of these data. It is noteworthy that this protocol will be different from that already established for other species, as human (38), since functional characteristics of boar sperm motility are very specific. Finally, the assumption of such a protocol will allow for the performing of a wide study to verify the exact motile subpopulation structure of boar semen ejaculates, since the results for separate laboratories could be compared without biases due to the precise CASA analysis and, above all, CASA samples processing.

In conclusion, our data indicate that the optimization of boar semen quality analysis needs the inclusion of some functional tests. In a semen quality analysis laboratory that worked in field conditions, the functional tests that seemed to be optimal were those involving osmotic resistance of sperm, such as the ORT and HRT Tests, although other functional tests, such as the rate of L-lactate production, can be more useful in other analytical conditions. However, the maximal optimization of semen analysis as a

predictive tool for in vivo fertility in an average commercial farm was obtained when tests were statistically combined among themselves by logistic regression techniques. Moreover, our results indicate that this logistic regression can be applied to find the precise fertility rate of a single boar, although this aim has not been fully tested in this work. On this basis, ORT and HRT Tests and the percentage of viability of fresh samples are the best tests to look for this information. Finally, CASA-obtained motility data, specially that involving motile subpopulation structure, could be useful in boar semen analysis although their usefulness will be greatly improved if a strict, specific protocol for CASA utilization is applied to boar semen samples.

5. REFERENCES

1. Schilling E, Vengust M, Bajt G, Tomcic M. The osmotic resistance test (ORT) of boar spermatozoa and the relation to pregnancy rate and litter size. 9th IPVS Congress, Barcelona, 1986;p.77.

2. Rigau T, Piedrafita J, Reverter A, Canal M, Rodríguez-Gil, J.E. The rate of L-lactate production: a feasible parameter for the fresh diluted boar semen quality analysis. Anim Reprod Sci 1996;43:161-172.

3. Gadea J, Matás C, Lucas X. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. Anim Reprod Sci 1998;56:95-108.

4. Aumuller R, Willeke H. Computer controlled analysis of boar semen with the "Cellsoft" system. In: Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination. Dublin, Ireland. 1988;MS227.

5. Hammit D G, Martin PA, Callanan T. Correlation between heterospermic fertility and assays of porcine seminal quality before and after cryopreservation. Theriogenology;32:385-399.

6. Rath D, Armbrech S, Schaap P, Weitze KF. Experience with a computerized videomicrographic system for sperm analysis. In: Proceedings of the 11th International

Congress on Animal Reproduction and Artificial Insemination. Dublin, Ireland. 1988;MS288.

7. Galli A, Bosisio M. Quality of semen stored at +15˚C/16˚C as related to fertility of artificially inseminated swine. Theriogenology 1988;30:1185-1190.

8. Clark LK, Schinckel AP, Singleton W L, Einstein ME, Teclaw RF. Use of farrowing rate as a measure of fertility of boar. JAVMA. 1989;194:239-243.

9. Holt C, Holt WV, Moore HDM, Red HCB, Curnock RM. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: Results of two fertility trials. J Androl 1997;18:312-323.

10. Hirai M, Boersma A, Hoeflich A, Wolf E, Foll J, Aumuller R, Braun J. Objectively measured sperm motility and sperm head morphometry in boars (*Sus scrofa*): Relation to fertility and seminal plasma growth factors. J Androl 2001;22:104-110.

11. Pérez-Llano JL, Lorenzo JL, Yanes P, Trejo A, García-Casado PA short hypoosmotic swelling test for the prediction of boar sperm fertility. Theriogenology 2001;56:387-398.

12. Berger T, Anderson DL, Penedo MCT. Porcine sperm fertilizing potential in relationship to sperm functional capacities. Anim Reprod Sci 1996;44:231-239.

13. Sánchez R. Control de calidad espermática. Anaporc 1991;104:27-33.

14. Cardullo RA. Oxygen metabolism, motility, and the maintenance of viability of caudal epididymal rat sperm. Diss Abstr Int B Sci Eng 1986;47:892.

15. Roldán ERS. Signal transduction during mammalian sperm acrosomal exocytosis. In: Lauria A, Gandolfi F, Enne G, Gianaroli L (eds), Gametes: Development and function. Rome, Serono Symposia 1998;219-228.

16. Holt WV. Can we predict fertility rates? Making sense of sperm motility. Reprod Domest Anim 1996;31:17-24.

17. Abaigar T, Holt W, Harrison R, Del Barrio G. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments Biol Reprod 1999;60:2-41.

18. Rigau T, Farré M, Ballester J, Mogas T, Peña A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 2001;56:801-815.

19. Quintero-Moreno A, Miró J, Rigau T, Rodríguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 2003; 59:1973-1990.

20. Holt C, Holt WV, Moore HDM. Choice of operating conditions to minimize sperm subpopulation sampling bias by the assessment of boar semen by computer-assisted semen analysis. J Androl 1996;17:587-596.

21. Thurston LM, Watson P, Mileham A, Holt W. Morphologically distinct sperm subpopulation defined by fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl 2001;22:382-394.

22. Amman RP, Hammerstedt RH. *In vitro* evaluation of sperm quality: an opinion. J Androl 1993;14:397-406.

23. Bamba K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. Theriogenology 1988;29:1245-1251.

24. Rodríguez-Gíl JE, Rigau T. Effects of slight agitation on the quality of refrigerated boar semen. Anim Reprod Sci 1995;39:141-146.

25. Caiza de la Cueva FI, Rigau T, Pujol MR, Piedrafita J, Rodríguez-Gil JE. Resistance to hyperosmotic stress of boar spermatozoa: the role of the ionic pumps and the relationship with cryosurvival. Anim Reprod Sci 1997;48:301-315.

26. Rodríguez-Gil JE, Rigau T. Effects of ouabain on the response to osmotic changes in dog and boar spermatozoa. Theriogenology 1995;45:873-888.

27. Boyers SP, Davis R, Katz DF. Automated Semen Analysis. Curr Probl Obstet Gynecol Fertil 1989;12:172-200.

28. Davis RO, Siemers RJ. Derivation and reliability of kinematics measures of sperm motion. Reprd Fertil Dev 1995;7:857-869.

29. SAS. SAS/STAC Software: SAS Inst. Inc.; Carry, NC. USA. 1996.

30. Altman DG. Practical Statistics for Medical Research. London: Chapman and Hall; 1991.

31. Bostofte E, Bagger P, Michael A, Stakemann G. Fertility prognosis for infertile men: results of follow-up study of semen analysis in infertile men from two different populations evaluated by the Cox regression model. Fertil Steril 1990;54:1100-1106.

32. Davis RO, Drobnis EZ, Overstreet JW. Application of multivariate cluster, discriminate function and stepwise regression analyses to variable selection and predictive modelling of sperm cryosurvival. Fertil Steril 1995;63:1051-1057.

33. Schilling E, Vengust M. Determination of osmotic resistance of boar spermatozoa and its relationship with the storage ability of semen samples. Zuchthygiene 1985;20:61-78.

34. Polansky FF,. Lamb MJ. Do the results of semen analysis predict future fertility?. A survival analysis study. Fertil Steril 1988;49;1059-1065.

35. Grunert JH, de Geyrter CH, Bordt J. Does computerized analysis of sperm movement enhance the predictive value for semen analysis for in-vitro fertilization results? Int J Androl 1989;12:329

36. Hiting A, Comhaire F, Vermeulen L, Dhont M, Vermeulen A, Vandekerckhove D. Value of sperm characteristics and the result of in vitro fertilization for predicting the outcome of assisted reproduction. J Androl 1989;13:59-66.

37. Rivera del Álamo M, Palomo MJ, Quintero-Moreno A, Rigau T, Rodríguez-Gil JE. Sperm subpopulations in boar semen and their relationship with natural photoperiod. Proc 17th Congress of the International Pig Veterinary Society. Ames, Iowa 2002, p. 494.

38. WHO, WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4d ed. Cambridge: Cambridge University Press; 1999.

CAPÍTULO V

MULTIVARIATE CLUSTER ANALYSIS REGRESSION PROCEDURES AS TOOLS TO IDENTIFY SPERM SUBPOPULATION IN RABBIT SEMEN AND TO PREDICT SEMEN FERTILITY AND LITTER SIZE.

PROCEDIMIENTO DE AGRUPAMIENTO MULTIVARIADO DE REGRESION COMO HERRAMIENTA PARA IDENTIFICAR LAS SUBPOBLACIONES ESPERMÁTICAS EN SEMEN DE CONEJO Y PREDECIR SU CAPACIDAD DE FERTILIDAD Y PROLIFICIDAD.

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ABSTRACT

Computerized motility analysis (CASA) shows that four separate subpopulations of spermatozoa with different motility characteristics coexist in rabbit ejaculates. There were significant ($P<0.01$) differences in the distribution of these subpopulations among specific rabbit strains, total sperm abnormalities and the percentage of altered acrosomes. Furthermore, logistic and linear multivariate regressions among several parameters of rabbit semen quality analysis were tested for use as predictive tools for the fertilizing ability of a specific artificial-insemination semen sample. Logistic regression analysis rendered two mathematical, significant ($P<0.01$) models: one between sperm viability and conception rate and the other between total sperm abnormalities and conception rate. Multiple lineal regression analyses also yielded some significant relationships between both fertility ($P<0.001$) and litter size ($P<0.05$), with respect to some semen characteristics. Our results support the hypothesis that the predictive in vivo fertility use of the results obtained in a standard rabbit semen quality analysis can be reasonably achieved by applying linear and logistic regression analyses among several parameters of rabbit semen quality analysis. On the other hand, these regression analyses can only reasonably be achieved after analyzing rabbit semen motility patterns by a CASA system.

Key words: Sperm subpopulations, Motility pattern, Rabbit spermatozoa, Fertility, Litter size.

1. INTRODUCTION

Studies carried out by several researchers have demonstrated the existence of specific motile sperm subpopulation structures in mammalian fresh ejaculates. These subpopulations are defined by their specific sperm motion characteristics, and they have been found in species like the common marmoset, gazelle, boar, dog or stallion (Holt, 1996; Abaigar et al., 1999; Rigau et al., 2001, Thurston et al., 2001; Quintero-Moreno et al., 2003). The existence of such a structure in mammals from very wide phylogenetic origins could suggest the existence of a relationship between changes in the concrete subpopulation structure of an ejaculate and its fertilizing ability. Results obtained in

stallion (Quintero-Moreno et al., 2003) and boar (Hirai et al, 2001) agrees with this suggestion. Therefore, the study and characterization of these subpopulations opens up new ways to improve semen analysis techniques. With regard to rabbits, no bibliographical references about the study of identified subpopulations have been found, in spite of the potential practical interest of having such knowledge for this species. On the other hand, the existence of motile sperm subpopulations in an ejaculate is practically neglected in the classical semen quality analysis. This neglect could induce a bias into the correct evaluation of sperm quality in the case that sperm subpopulation structure and semen fertilizing ability were related. Thus, studies about sperm subpopulations could be of importance to reach a better definition of mammalian semen quality. This could also have significant economic repercussion, since better semen analysis will lead to the commercialization of mammalian semen doses for artificial insemination (AI) which offer greater guarantees of quality.

Sperm motile subpopulations can only be analyzed by using a computerized analysis system of motility (CASA). These CASA analyses are widely accepted as providing rapid and objective measurements of individual sperm parameters, such as sperm-count and sperm-movement characteristics. The technical details of the CASA system were widely described by Boyers et al. (1989), and several other studies have explained operational and practical aspects of CASA instruments (Davis and Katz, 1993; Davis and Siemers, 1995; Irvine, 1995; Krause, 1995; Mortimer, 2000; Verstegen et al., 2002). Theoretically, the CASA system allows for the study of motion characteristics in a sperm subpopulation to an unprecedented degree of sophistication and, in this way, several studies have analyzed sperm-motion parameters in cryopreservation or in vitro fertilization experiments by using statistical multivariate systems (Davis et al., 1991 and 1995; Holt, 1996). These studies suggest that the use of this methodology greatly improves the identification of sperm subpopulations with specific motion characteristics in an ejaculate (Abaigar et al., 1999 and 2001; Thurston et al., 2001). However, CASA analysis rendered a very large variety of separate motility parameters, which in most cases are highly correlated. This fact makes it difficult to objectively select parameters which explain the overall sperm movement more accurately. As a result, it is necessary to look for a more suitable statistical method to employ in the CASA data analysis in order to conserve all of the relevant information. One possibility is a multivariate analysis based on the clustering of variables and observations

which would make it possible to apply CASA in the study of sperm subpopulations (Quintero et al., 2003).

The rabbit is the smallest common laboratory mammal from which serial semen samples can be readily collected. In fact, rabbit semen analysis is most frequently used in routine reproductive toxicology studies that are applied to human medicine (Amann, 1982). However, the classical rabbit semen analysis shows very great limitations, since the visual and subjective determination of total sperm motility seems to not be a very useful tool for mammalian semen quality analysis (Rigau et al., 1996). Besides the interest in human toxicology, rabbit AI is increasing in countries where intensive rabbit raising is practiced, and AI is routinely performed in many of large rabbit farms of Italy, France, Hungary and Spain (Roca et al., 2000). In this sense, the optimization of rabbit-semen analysis is a clear necessity to improve breeding management in this species.

This study is designed to investigate three related aims. Firstly, the determination of the presence of separate sperm subpopulations, with specific motion characteristics, in diluted ejaculates. To this end, a priority consideration has been given to the optimization of the parameters of CASA by the clustering of the motion parameters which can be used to better define sperm-motion characteristics. Secondly, the analysis of relationships among sperm subpopulations in diluted semen with the rabbit genetic background and morphological sperm characteristics, as well as the sperm concentration in the whole fresh ejaculate. Thirdly, the determination of relationships between the results of semen analysis and the rate of conception, litter size, and fertility percentage in female rabbits utilized in AI. As a whole, all of this information could contribute to a greater optimization in the practical approach of rabbit semen quality analysis applied to AI.

2. MATERIALS AND METHODS

2.1. Animals and semen collection

The trial was carried out in the experimental farm of the Institut de Recerca i Tecnologia Agrària (Generalitat de Catalunya, Spain). This farm has isolated roof and walls and a control system for light ventilation and temperature, and eenvironmental

conditions were maintained as described below. Animals used belong to 4 groups corresponding to two sire lines of rabbits. The first line was the Caldes one (C), which came from the New Zealand White strain. The second one was the R line, which was obtained after crossing two generations from a pool of animals of 3 separate commercial lines. The reciprocal crossbreeds C x R and R x C were also utilized in the experiment. Lines C and R were selected for increased post-weaning daily gain by individual selection (Gomez et al., 1996; Estany et al., 1992). After weaning, fifty-nine bucks (Table 1) were housed in individual cages with a photoperiod of 16 hours light/day and a temperature ranging from 14ºC to 24.4ºC. Animals were fed "*ad libitum*" with commercial rabbit pellets (15.5% crude protein, 2.3% fat, 17.2% crude fiber) until 60 days of age. Then, they were restricted to 180 g/day of another commercial diet (16% crude protein, 4.3% fat, 17% fiber). Semen samples were collected when bucks were 9 to 10 months old by using an artificial vagina without the presence of a female. Two ejaculates per male per week were obtained. In all cases both ejaculates were collected the same day, with an interval of 30 minutes.

Table 1.

Number of males and number of ejaculates (in parentheses), of each genetic type of buck.

Pool	Genetic line			
	C	R	C x R	R x C
1	3(6)	3(6)	5(10)	3(5)
2	4(7)	4(7)	4(7)	3(5)
3	4(7)	4(6)	4(7)	3(4)
4	4(8)	4(7)	4(7)	3(6)

Genetic lines are defined in Materials and Methods.

2.2. Sperm evaluation

Ejaculates were stored at 37ºC in a water bath until evaluation 15 minutes after collection. Samples containing urine and cell debris were discarded whereas gel plugs were removed. After an initial visual inspection, useful ejaculates from bucks of the same line were pooled. The number of males and ejaculates per pool of each genetic type of the bucks are given in Table 1.

Percentages of viability, altered acrosomes and morphological abnormalities were determined after a vital Nigrosin-Eosin stain (Bamba, 1988). Samples were evaluated at

x1000 magnifications, and the number of sperm counted per sample was 200-300. After that, pools were diluted (1:5, v/v) in a commercial extender (Kubus S.A.; Majadahonda, Spain). Then, individual motility of diluted samples was evaluated at x400 in a phase-contrast microscope (Roca et al., 2000). At the same time, the concentration of diluted samples was measured using a hemocytometer chamber. Finally, aliquots of the diluted semen pools were stored at 18ºC to perform CASA analysis 4 hours after collection. For this analysis, a 1-mL aliquot of each sample was incubated for 5 min at 37°C in a water bath. After that, 3 consecutive 5-μL drops for each sample in all of the studied ejaculates in this study were observed through an optical phase-contrast microscope with a heatable (37°C) plate. This microscope was connected to a commercial CASA analysis system (Microptic S.A.; Barcelona, Spain). Three fields per drop were taken for the analysis, and the total number of spermatozoa analyzed in each semen sample (including those not motile) was 50 to 100. The CASA system is based upon the analysis of 16 consecutive, digitalized photographic images obtained from a single field at x200 augmentations on a dark field. These 16 consecutive photographs were taken in a total time lapse of 0.64 sec, which implied a velocity of image-capturing of 1 photograph each 40 millisec. After CASA analysis, total motility was defined as the percentage of motile spermatozoa with a mean velocity (VAP) >10 μm/sec.

2.3. Study of the fertilizing ability of rabbit semen "in vivo"

Aliquots (0.86 mL – 1 mL) of the semen pools were stored at 18ºC until their use for AI (6-10 hours after collection). The AI protocol was performed on 125 does from a dam line, reared on a commercial farm. The dams were treated with subcutaneous application of PMSG, 12-15 UI (Folligon^{R}, Intervet, Holland) 48h prior to AI. Ovulation of does was finally induced by intramuscular administration of 0.8 mg busereline acetate (Suprefact^{R}, Hoechst-Roussel, Germany). Sperm concentrations of the 0.5-mL insemination doses were 80 x10^{6} sperm/mL. Pregnancy diagnosis was performed 15 days after AI by abdominal palpation. The total number of dams inseminated was 125, whereas those with confirmed gestation were 112, thus indicating a fertility index of 89.6%. Immediately after parturition, the number of total newborn rabbits was recorded, in order for further use of these data in our analysis.

2.4. Statistical analysis

Data were processed by the SAS statistical package (2000). The sperm motility descriptors obtained from CASA were clustered in separate variable groups by the VARCLUS procedure. This analysis was performed to reduce the numbers of variables (21 in our specific system, data not shown) with the purpose of selecting parameters which explain the overall sperm movement more accurately. Another clustering procedure, FASTCLUS, was used for separating the spermatozoa in concrete sperm subpopulations (Quintero et al., 2003). The spermatozoa were divided into clusters such that every observation belonged to one and only one cluster. Spermatozoa that were very close to each other were assigned to the same cluster, while spermatozoa which were far apart are in different clusters.

The separation of spermatozoa into clusters was performed over the total number of spermatozoa obtained in the diluted semen samples. Globally, 2624 motile sperm from 43 samples were analyzed. From these, 27 samples were from single ejaculates, whereas the other 16 were from pooled ejaculates. Spermatozoa were distributed among the 4 genetic lines used in the following manner: Six hundred forty-two sperm were from Genetic Group 1, 618 from Genetic Group 2, 730 from Genetic Group 3 and 634 from Genetic Group 4. A General Lineal Model (PROC GLM) was used to evaluate significant differences ($P<0.05$) among clusters of sperm subpopulations, whereas the LSMEANS procedure was used to compare the obtained sperm subpopulations among them. These analyses grouped the tested motion parameters into 6 separate clusters (data not shown). Further analysis of the relationship among parameters in each cluster led to the choice of the individual parameter that, inside of each cluster, conserved the maximal information over the total parameters. Following this analysis, chosen parameters were:

Mean lateral head displacement (mean ALH): The mean head displacement along its curvilinear trajectory around the mean trajectory. Units are μm.

Linear coefficient (LIN): The percent coefficient between linear velocity and curvilinear velocity. Units are %.

Mean velocity (VAP): The mean trajectory of sperm per unit of time. Units are μm/sec.

Frequency of head displacement (BCF): The number of lateral oscillatory movements of the sperm head around the mean trajectory. Units are Hz.

Algebraic angular mean displacement (algMAD): The algebraic value of the advancing angle of the sperm trajectory, provided that negative values indicate a clockwise displacement. Units are angular degrees.

Minor harmonic oscillation of the head (HLO): The minimum value of the distance between the curvilinear trajectories with respect to the mean trajectory. Units are μm.

Logistic regression analyses were used to relate the dichotomous conception rate to the sperm parameters, and linear regression analyses (Pearson's correlation and multiple regressions) were used to examine the relationship between litter size and measured semen parameters (Holt et al, 1997). The relationship between the in vivo fertility percentage of specific genetic-type fertility and the measured semen parameters was also tested. These latter comparisons were tested by using a Chi-square test. However, a categorization of some of these variables by using the FASTCLUS clustering procedure was previously needed before the Chi-square test use. Such obtained categories were distributed in the following form:

Following sperm concentration of diluted samples:
Group A, ejaculates with: $\leq 350 \times 10^6$ spermatozoa/mL.
Group B, ejaculates with: $> 350 - \leq 500 \times 10^6$ spermatozoa/mL.
Group C, ejaculates with: $> 500 \times 10^6$ spermatozoa/ mL.

Following the percentage of in vivo fertility:
Group A, ejaculates with: $> 50\% - \leq 78\%$.
Group B, ejaculates with: $> 78\% - \leq 90\%$.
Group C, ejaculates with: $> 90\%$.

Following the percentage of viability:

Group A, ejaculates with: ≤ 85%.
Group B, ejaculates with: > 85%-≤ 90%.
Group C, ejaculates with: > 90%.

Following the percentage of total abnormalities:
Group A, ejaculates: ≤ 9%.
Group B ejaculates: > 9%-≤ 19%.
Group C ejaculates: >19%.

Following the percentage of altered acrosomes:
Group A, ejaculates with: ≤9%.
Group B, ejaculates with : >9%-≤13%.
Group C, ejaculates with: >13%-≤18%.
Group D, ejaculates with: >18%.

Finally, a GLM procedure was used again to evaluate the effect of the interaction between the obtained sperm subpopulations and the previously categorized parameters, whereas the LSMEANS procedure was used to exactly localize differences. Thus, the main objective of these categories is to have some sort of references that allows us to observe changes in the distribution of motile subpopulations depending upon genetic type, sperm concentration, in vivo fertility, viability, morphological abnormalities and altered acrosomes.

3. RESULTS

3.1 Mean semen quality analysis of rabbit ejaculates

The mean values for the overall semen-quality parameters are shown in Table 2. The presence of significant differences ($p<0.01$) in several parameter values among genetic lines is noteworthy (data not shown).

3.2. Sperm subpopulation analysis in fresh and diluted semen

The motility data set analyzed by FASTCLUS procedures rendered 4 motile sperm subpopulations. Summarized statistics for these subpopulations are shown in Table 3. Qualitative interpretations of these data are:

Subpopulation 1.

This subpopulation was characterized by the highest degree of progressiveness with highly active spermatozoa, as inferred by very high LIN and VAP values. More than 11% of the spermatozoa in the data set were assigned to this subpopulation.

Table 2.

General characteristics of fresh rabbit semen samples.

Parameters	Mean + ES	Confidence interval
Volume (mL)	0.92 ± 0.06	0.86 to 1.0
Sperm concentration in hematocytometer chamber (x 10^6 /ml)	394.6 ± 9.58	375.6 to 413.5
Total motility (%)	69.69 ± 3.52	62.18 a 77.21
Sperm viability after eosin-nigrosin staining (%)	86.11 ± 0.48	85.15 to 87.07
Sperm with structurally abnormal acrosomes (%)	11.65 ± 0.45	10.75 to 12.56
Sperm with head morphological abnormalities (%)	1.19 ± 0.09	0.99 to 1.38
Sperm with neck and midpiece abnormalities (%)	3.75 ± 0.16	3.41 to 4.08
Spermatozoon with proximal cytoplasmic droplet (%)	3.49 ± 0.42	2.65 a 4.33
Spermatozoon with distal cytoplasmic droplet (%)	4.43 ± 0.25	3.93 a 4.93
Sperm tail abnormalities (%)	3.08 ± 0.30	2.47 to 3.68
Total sperm abnormalities (%)	23.59 ± 0.88	21.85 to 25.33

Values are obtained from 16 separate pooled semen samples.

Subpopulations 2 and 3.

Both subpopulations showed sperm trajectories less straight than Subpopulations 1 and 4, since LIN and VAP were low in both subpopulations. Concomitantly, both subpopulations also had high values of mean ALH and very high BCF levels. The percentage of motile spermatozoa which were included in Subpopulation 2 was 20.5%, whereas those included in Subpopulation 3 were 16.9 % (Table 3).

Subpopulation 4.

This sperm was characterized by high progressiveness, with high LIN values, although they showed concomitant low VAP and mean ALH values. These results reflected the

existence of complex, although overall straight, sperm trajectories. About 51% of the total motile spermatozoa were included in this subpopulation.

Table 3.

Sperm subpopulations and motility descriptors in diluted rabbit semen

Sperm motility descriptors	Subpopulations			
	1	2	3	4
n	343	498	444	1339
(%)	13	19	17	51
VAP (μm/sec)	73.31 ±0.70 [a]	29.87 ± 0.58 [b]	21.84 ± 0.61 [c]	19.92 ± 0.35 [d]
LIN (%)	76.84 ± 0.78 [a]	28.62 ± 0.64 [b]	33.08 ± 0.68 [c]	74.78±0.39 [a]
Mean ALH (μm/sec)	2.63 ± 0.07 [a]	3.38 ± 0.06 [b]	2.40 ± 0.06 [a]	0.95 ±0.04 [c]
BCF (Hz)	13.12 ± 0.25 [a]	14.15 ± 0.22 [a]	15.35 ± 0.22 [b]	12.80 ± 0.12 [a]
HLO (μm)	0.45 ± 0.02 [a]	0.15 ± 0.02 [b]	0.05 ± 0.02 [c]	0.05 ± 0.01 [c]
AlgMAD (˚)	-0.49 ± 0.01[a]	6.48 ± 58 [b]	-39.48 ± 0.62 [c]	-1.74 ±0.35 [a]

Motility descriptors have been described in Table 2. Different superscripts in a row indicate significant differences ($P<0.05$). Results are expressed as means ± SE of spermatozoa from 43 semen samples analyzed (27 rabbits + 16 pool ejaculates) from 4 different genetic groups. The total number of motile spermatozoa analyzed was 2624. The spermatozoa were distributed among genetic groups in the following manner: 642 motile spermatozoa were from Genetic Group 1, 618 from Genetic Group 2, 730 from Genetic Group 3, and 634 from Genetic Group 4.

The proportion of each subpopulation significantly ($P<0.001$) varied, with respect to the genetic line where the sperm came from. Thus, the percentage of spermatozoa classified in Subpopulation 1 ranged from 9.81% to 16.25%, depending upon the individual, whereas the percentage identified in Subpopulation 2 ranged from 14.49 % to 22.17%. Subpopulation 3 varied from 15.05% to 18.63% and Subpopulation 4 from 45.90% to 59.19% among the rabbit lines (data not shown). This suggests the presence of a specific, genetic factor that was involved in the relative proportion of each motile subpopulation in a specific rabbit ejaculate.

3.3. Sperm Subpopulation Distribution in Ejaculates with Proven In vivo Fertilizing Ability

Ejaculates with proven in vivo fertilizing ability showed a sperm subpopulation structure which was not different from those which failed to achieve positive results for in vivo fertility (data not shown). In this sense, both types of ejaculates showed the presence of 4 separate subpopulations of motile spermatozoa (Table 4, and data not shown). As

stated, these subpopulations were homologous to those described in Table 3 and there was only a small difference in the percentage of distribution between Subpopulations 1 and 2.

Table 4.

Sperm subpopulations and motility descriptors in diluted rabbit semen with proven fertility

Sperm motility descriptors	Subpopulations			
	1	2	3	4
n	146	259	214	644
(%)	11.6	20.5	16.9	51.0
VAP (μm/sec)	77.79 ± 1.15 [a]	30.64 ± 0.87 [b]	23.39 ± 0.94 [c]	21.12 ± 0.55 [c]
LIN (%)	79.08 ± 1.22 [a]	26.36 ± 0.92 [b]	31.53 ± 1.00 [c]	74.01 ±0.58 [d]
Mean ALH (μm/sec)	2.45 ± 0.13 [a]	3.56 ± 0.10 [b]	2.67 ± 0.10 [a]	0.99 ±0.06 [c]
BCF (Hz)	12.09 ± 0.43 [a]	14.62 ± 0.32 [b]	15.34 ± 0.35 [b]	12.65 ± 0.20 [a]
HLO (μm)	0.56 ± 0.04 [a]	0.19 ± 0.03 [b]	0.05 ± 0.04 [c]	0.05 ± 0.02 [c]
AlgMAD (˚)	-0.48 ± 1.12 [a]	6.63 ± 0.84 [b]	-37.29 ± 0.91 [c]	-1.71 ±0.53 [a]

Motility descriptors have been described in Table 2. Different superscripts in a row indicate significant differences ($P<0.05$). Results are expressed as means ± SE of spermatozoa from 16 pool ejaculates from 4 different genetic groups. The total number of motile spermatozoa analyzed was 1263. The spermatozoa were distributed among genetic groups in the following manner: 350 motile spermatozoa were from Genetic Group 1, 350 from Genetic Group 2, 236 from Genetic Group 3, and 327 from Genetic Group 4.

It is noteworthy that the precise subpopulation structure of ejaculates with proven in vivo fertility varied according to their percentage of total morphological abnormalities. Thus, Subpopulation 3 was significantly lower (about 9%, $P<0.05$) in those ejaculates with a percentage of abnormalities above 19% in respect to those with a lower abnormality percentage (Table 5). Concomitantly, the percentage of Subpopulation 4 was also significantly ($P<0.05$) higher in ejaculates whose percentage of morphological abnormalities was above 19% (Table 5). On the contrary, Subpopulation 3 was significantly higher ($P<0.05$) in ejaculates whose percentage of altered acrosomes was above 18%, whereas a concomitant, significant ($P<0.05$) decrease of Subpopulation 4 was observed in these ejaculates (Table 6).

Sperm subpopulation structure did not change depending upon the total number of spermatozoa when the semen samples ranged between $350x10^6$ and $500x10^6$ spermatozoa/mL (data not shown). Furthermore, sperm subpopulation structure was not dependent on the percentage of viability, at least in the viability range observed in our study (between 77% and 95%, data not shown). Notwithstanding, it is worth noting that

LIN increased to values of 55.5% in samples with a percentage of viability above 90% when compared to the other ejaculates with proven in vivo fertility (data not shown). Finally, sperm subpopulation structure did not vary depending upon litter size when comparing both parameters (data not shown).

Table 5.

Relationship between the proportion of the motile sperm subpopulations in ejaculates and the total sperm abnormalities of the whole ejaculates.

Sperm Subpopulation	Total sperm abnormalities categories		
	≤9%	>9 ≤19%	>19%
Subpopulation 1 (%)	10.93 (66) [a]	11.46 (47) [a]	13.25 (33) [a]
Subpopulation 2 (%)	20.20 (122) [a]	20.00 (82) [a]	22.09 (55) [a]
Subpopulation 3 (%)	18.38 (111) [a]	19.27 (79) [a]	9.64 (24) [b]
Subpopulation 4 (%)	50.50 (305) [a]	49.27 (202) [a]	55.02 (137) [b]
Total Sperm Count (%)	47.8 (604)	32.5 (410)	19.7 (249)

Different superscripts in a row indicate significant differences (P<0.05). Results are expressed as percentages on the total motile sperm population from ejaculates with proven fertility. Total sperm count indicates the percentage of total motile sperm that were analyzed in each category. Numbers in parentheses indicate the final number of motile spermatozoa that were analyzed in each group.

Table 6.

Relationship between the proportion of the motile sperm subpopulations in ejaculates and the total abnormal acrosomes of the whole ejaculates.

Sperm Subpopulation	Total abnormal acrosomes categories			
	≤9%	>9 ≤13%	>13 ≤18%	>18%
Subpopulation 1 (%)	11.86 (46) [ab]	12.30 (53) [ab]	14.97 (25) [a]	7.94 (22) [b]
Subpopulation 2 (%)	18.30 (71) [a]	19.95 (86) [ab]	19.16 (32) [ab]	25.27 (70) [b]
Subpopulation 3 (%)	15.21 (59) [a]	14.62 (63) [a]	16.17 (27) [a]	23.47 (65) [b]
Subpopulation 4 (%)	54.64 (212) [a]	53.13 (229) [a]	49.70 (83) [ab]	43.32 (120) [b]
Total Sperm Count (%)	388 (30.7)	431 (34.1)	167 (13.2)	277 (22.0)

Different superscripts in a row indicate significant differences (P<0.05). Results are expressed as percentages on the total motile sperm population from ejaculates with proven fertility. Total sperm count indicates the percentage of total motile sperm that were analyzed in each category. Numbers in parentheses indicate the final number of motile spermatozoa that were analyzed in each group.

3.4. Relationship between Seminal Characteristics and In Vivo Fertility and Litter Size

Logistic regression analyses rendered two mathematical models with high percentages of concordance between quality analysis and in vivo fertility. The first model, with a 68.3% of concordance (P<0.01), directly related the percentage of viability and in vivo

fertility (Table 7). The second model was even better, since it had a percentage of concordance of 76.4% ($P<0.01$). This latter model was based on a mathematical combination of percentages of both viability and total morphological abnormalities which was related to in vivo fertility (Table 7). It is noteworthy that no motility parameter collaborated in the attainment of better logistic regression analyses.

On the other hand, a multiple regression model derived by backward elimination of variables for seminal parameters which could be applied to in vivo fertility yielded worse results, since the model prediction was 32.2 % (data not shown). This linear model included the percentages of viability, altered acrosomes and total morphological abnormalities, sperm concentration and VSL as significant variables ($P<0.001$; data not shown).

Table 7.

Logistic regression of seminal characteristics with artificial insemination results (conception rate) in rabbits. Details of three statistical models.

Statistical Model/Parameter	Regression Coefficient	Standard Error	Significance of individual parameters (p-value)	Significance of Statistical model (p-value)	Percent Concordance
Model 1	-	-	-	0.0148	68.3
Sperm viability	0.1755	0.08	0.0288		
Constant	-18.0255	7.22	0.0126		
Model 2			-	0.0080	76.4
Sperm viability	0.3133	0.12	0.0064		
Sperm abnormality	0.0742	0.036	0.0391		
Constant	-31.9323	11.70	0.064		

Conception rate (model 2) = $1/ [1+e^{-(-31.93 + 0.31\text{sperm viability} + 0.07\text{sperm abnormality})}]$

Finally, multiple regression model analysis derived by backward elimination of variables for seminal parameters which could be applied to litter size yielded a low relationship between them, with a prediction percentage of the best obtained model of 16.1 % (data not shown). This model included the percentage of viability, sperm concentration and the WOB, LIN, DNC, Mean ALH and BCF motility parameters (data not shown).

4. DISCUSSION

Our results demonstrate the presence of specific, well-defined sperm subpopulations in rabbit ejaculates, which can be easily defined by their motility characteristics. Sperm subpopulations have been detected on the basis of their differential CASA-derived kinematic parameters with multivariate statistical methods, most notably cluster analysis This approach has been used to identify sperm subpopulations in semen from man (Davis et al., 1992), boar (Abaigar et al., 1999), gazelle (Abaigar et al., 2001), and stallion (Quintero et al., 2003). In this sense, the combination of CASA with the multivariate statistical analysis (Abaigar et al., 1999; Quintero et al., 2003) demonstrates that functionally distinct sperm subpopulations exist within samples. Moreover, studies of boar and stallion samples have confirmed that semen subpopulations differ among themselves in their pattern of movement and, possibly, their physiological status. In the present study, four specific sperm subpopulations were identified. Group values for the individual motility descriptors provide an indication of the type of motion behavior shown by each subpopulation. Physiological interpretation of the numerical data is thereby facilitated. Thus, in the present study it was evident that Subpopulations 1 and 4 represented the most progressive spermatozoa. On the contrary, Subpopulations 2 and 3 were characterized by highly vigorous but non-linear motion, possibly representing forms of spermatozoa with uncoordinated motility. This probably indicates that these cells may have been affected by samples management or other intrinsic factors indicating the beginning of a degenerative process. Subpopulation 4, which contained the majority of motile spermatozoa, would represent active cells which would easily be detected in a subjective estimation of motility. In this sense, we have to remember that the percentage of Subpopulation 4 was inversely related to the percentage of altered acrosomes in whole ejaculates, and, in turn, Subpopulation 4 is directly related to the percentage of morphological abnormalities. On the other hand, percentages of Subpopulation 4 were related to concomitant changes in percentages of Subpopulation 3, indicating a close relationship between both subpopulations. These results seem to indicate that functional changes in whole rabbit ejaculates could be strongly related to the equilibrium between Subpopulations 3 and 4. However, since rabbit ejaculates showed very good overall quality characteristics, the observed variations in semen quality parameters, and hence motile subpopulation structure, were probably too small to be translated into clear changes in the in vivo fertilizing ability of these samples. Thus, to clarify this hypothesis it will be necessary to work with

ejaculates with poor seminal characteristics. Unfortunately, these ejaculates are immediately rejected in farm conditions, and only by working in laboratorial conditions could this rationale be tested.

Our results show that the specific subpopulation structure of an ejaculate is related to several parameters such as the genetic line, total percentage of abnormal acrosomes and total morphological abnormalities of semen samples. The relationship of rabbit genetic lines to motility sperm subpopulation were expected because other researchers have found a similar relationship between the motility and the individual rabbit on mean values and other semen characteristics (Battaglini et al., 1992; Castellini and Lattaioli, 1999). The biggest differences in distribution of sperm subpopulations were between C and R lines. Notwithstanding, semen samples in crossbred rabbits were more homogeneous in their sperm subpopulation structure. In this way, our results confirm previous observations in species such as boar and horse where movement differences exist among individuals (Abaigar et al., 2001; Quintero-Moreno et al., 2003). Furthermore, the comparison between motile subpopulation structure and total abnormalities indicates that sperm trajectories would vary according to sperm morphology and total motility. This effect would be considered logical if we assumed that the motility of a single spermatozoon would depend on its structure and cellular damage. Acrosomal damage loss must induce changes in both motility patterns and sperm subpopulation distribution. In fact, changes in the structure of sperm subpopulations were more evident when percentages of both sperm morphological abnormalities and altered acrosomes were the highest, suggesting a direct relationship between normal rabbit sperm structure and motility.

It is interesting to verify that previous observations have determined the presence of a similar motile sperm subpopulation structure in ejaculates from very phylogenetically separated mammals such as the common marmoset, gazelle, horse or boar (Holt, 1996; Abaigar et al., 1999 and 2001; Quintero et al., 2003). The observation of a great phylogenetic difference could be interpreted in the sense that the appearance of 3 to 4 subpopulations is a widespread phenomenon in mammals, thus opening a new insight in their study. However, more indepth studies involving more mammal species as well as in vitro fertility assays are necessary in order for a full comprehension of the physiological role of this subpopulation structure in mammal ejaculates.

We have been unable to find any research in rabbits involving the search of a predictive relationship between semen parameters and conception rate or litter size. Only some trials relating semen quality or number of sperm inseminated to fertility have been found (Castellini and Lattaioli, 1999; Roca et al., 2000). In this sense, our results show that, under a predictive point of view regarding fertility, the best parameters of rabbit semen analysis were percentages of viability and morphological abnormalities. It is noteworthy that logistic regression did not consider as predictive parameters those involving motility. This suggests that, whereas the sperm motility descriptors obtained by CASA analysis could not be used to predict the fertilizing ability of average ejaculates, results concerning viability and morphological abnormalities could do so at a high (76%) level of concordance. It was particularly interesting that both viability and morphological abnormalities of average ejaculates are very easy to determine and have a very low cost. This implies that both tests can be easily performed in field conditions, thus providing a fast and easy system to obtain a reasonable tool for selecting single ejaculates for their use in field AI. Complementing these tests with the determination of the percentage of altered acrosomes and semen concentration will further add more predictive information that would almost be completely achieved by determining the mean VSL values by CASA. Thus, from a field-and-laboratory-work point of view, a reasonably complete rabbit semen quality analysis applied to average ejaculates can be achieved after carrying out only the following tests: Percentages of viability, total morphological abnormalities and altered acrosomes and estimation of the sperm concentration of the ejaculate.

The poor predictive results shown by motion parameters seem to indicate that motility itself does not play a relevant role in the fertilizing ability of average ejaculates, since the minimal level of motility detected in these average samples is enough to fully achieve in vivo fertilization. In fact, this conclusion is only true in fresh, average samples, whereas in other conditions motility can play a primordial role in the predictive use of semen analysis. Thus, in cryopreserved human spermatozoa logistic regression analysis revealed that the CASA system, together with morphometric analysis, can be used as a predictive tool with a high degree of accuracy (about 87%), and the most informative variables involving motility were ALH and VAP (Macleod and Irwin, 1995). Fertilizing ability is commonly measured as the percentage of female rabbits conceiving by AI and is indicative of the semen's efficiency. Consequently, one of the main goals in spermatology is to look for new methods of sperm assays to detect alterations of sperm characteristics that show

reduced fertility or infertility (Hammerstedt, 1996). In a previous trial in boar, mean velocity was a good parameter in the seminal analysis and could be used to eliminate ejaculates with low quality and fertility, but it was not sufficiently precise to discriminate between the best and the average ejaculates, although the relationship between fertility and motility is controversial possibly due to different experimental conditions (Gadea et al., 1998). These results would agree with those obtained in our study. Going deeper into these analyses, Barratt et al. (1993) demonstrated that human sperm concentration was the most predictive factor of time to conception. As stated, our experimental model showed that the best predictive parameters following both logistic and linear multivariate regressions could account for up to 32% of the variability of fertility. Allowing for experimental error, only 68% of the variation is attributable to other factors, some of which could be associated with sperm function. Thus, these results strongly suggest that other tests of sperm quality besides viability or morphological abnormalities, especially those involving sperm motility, can realistically be utilized for the identification of poor-quality semen samples or low-fertility rabbits.

The relation between litter size and motility is controversial, because prolificacy has a great dependence on the female rabbit's physical and sanitary conditions. In our study, the combination of sperm motility descriptors (WOB, LIN, BCF, and Mean ALH) and sperm concentration and viability could be used to obtain an acceptable predictive system in order to eliminate ejaculates which rendered low values of litter size. However, the obtained model only explained 16% of the variability in litter size. This indicates that 84% of the variation in the prediction of litter size was attributable to other factors than those which were analyzed. In this way, some of these unknown factors could be associated with physiological aspects of the reproductive tract or intrinsic differences among females or differences in management.

In conclusion, rabbit ejaculates show a characteristic and specific motile sperm subpopulation structure, which is similar to that determined in other mammal species. The subtle variations in this structure observed in average ejaculates have no incidence in the fertilizing ability of those, although they could play an important role in determining this ability in samples with la ower in vivo fertilizing index. Finally, in vivo fertility of rabbit average ejaculates can be reasonably predicted by using logistic regression models involving semen quality analysis tests as percentages of viability and morphological

abnormalities, whereas litter size is more poorly predicted by linear regression models which utilize the same tests together with semen concentration and some motility parameters obtained after CASA analyses.

5. REFERENCES

Abaigar T, Holt W, Harrison R, Del Barrio G. Sperm subpopulation in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biol Reprod*. 1999; 60:32-41.

Abaigar T, Cano M, Pickard AR, Holt WV. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (*Gazella dama mhorr*): effects of body weight, electroejaculation technique and short-term semen storage. *Reproduction*. 2001; 122:265-273.

Amann RP. Use of animal models for detecting specific alterations in reproduction. *Fundam Appl. Toxicol.* 1982; 2. 13-26.

Bamba K. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology.* 1988; 29:1245-1251.

Barrat CLR, Tomlinson MJ, Cooke ID. Prognostic significance of computerized motility analysis for in vivo fertility. *Fertil Steril*. 1993; 60:520-525.

Battaglini M, Castellini C, Lattaioli P. Variability of the main characteristics of rabbit semen. *J Appl Rabbit Res*. 1992; 15:439-446.

Berger T, Anderson DL, Penedo MCT. Porcine sperm fertilizing potential in relationship to sperm functional capacities. *Anim Reprod Sci*. 1996; 44:231-239.

Boyers SP, Davis R, Katz DF. Automated Semen Analysis. *Curr Probl Obstet Gynecol Fertil* 1989; 12:172-200.

Buendía P, Soler C, Paolicchi F, Gago G, Urquieta B, Pérez-Sánchez F, Bustos-Obregón E. Morphometric characterization and classification of Alpaca sperm heads using Sperm-Class Analyzer ® computer-assisted system. *Theriogenology*. 2002; 57:1207-1218.

Castellini C, Lattaioli P. Effect of number of motile sperm inseminated on reproductive performance of rabbits does. *Anim Reprod. Sci*. 1999; 57:111-120.

Davis RO, Overstreet JW, Asch RH, Ord T, Silber, SL. Movement characteristics of human epididymal sperm used for fertilization of human oocytes *in vitro*. *Fertil Steril*. 1991; 56:1128-1135.

Davis RO, Katz DF. Operational Standards for CASA Instruments. *J Androl*. 1993; 14:385-394.

Davis RO, Gravance CG. Consistency of sperm morphology classification methods. *J Androl*. 1994; 15:83-91.

Davis RO, Drobnis EZ, Overstreet JW. Application of multivariate cluster, discriminate function and stepwise regression analyses to variable selection and predictive modelling of sperm cryosurvival. *Fertil. Steril*. 1995; 63:1051-1057.

Davis RO, Siemers RJ. Derivation and reliability of kinematics measures of sperm motion. *Reprod. Fertil Dev*. 1995; 7:857-869.

Estany J, Camacho J, Baselga M, Blasco A. Selection response of growth rate in rabbits for meta production. *Genet Sel Evol*. 1992; 24:527-537.

Farrell PB, Foote RH, Simkin ME, Clegg ED, Wall R.J. Relationship of semen quality, number of sperm inseminated, and fertility in rabbits. *J. Androl*. 1993; 14:464-471.

Gadea J, Matás C, Lucas X. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. *Anim Reprod Sci.* 1998; 56:95-108.

Gómez EA, Rafael O, Ramon J, Baselga M. A genetic study of a line selected on litter size at weaning. In: Lebas F, eds. *Proc. 6th Congress of the World Rabbit Science Association*, Toulouse/ France, 1996; 2: 289-292.

Hammerstedt RH. Evaluation of sperm quality: identification of the subfertile male and courses of action. *Anim Reprod Sci*. 1996; 42:77-87.

Hirai M, Boersma A, Hoeflich A, Wolf E, Foll J, Aumuller R, Braun J. Objectively measured sperm motility and sperm head morphometry in boars (*Sus scrofa*): Relation to fertility and seminal plasma growth factors. *J Androl*. 2001; 22:104-110.

Holt WV. Can we predict fertility rates? Making sense of sperm motility. *Reprod Domest Anim.* 1996; 31:17-24.

Holt C, Holt WV, Moore HDM, Red HCB, Curnock RM. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: Results of two fertility trials. *J Androl*. 1997; 18:312-323.

Irvine DS. Computer assisted semen analysis system: sperm motility assessment. *Hum Reprod*. 1995; 10:53-59.

Krause W. The significance of computer-assisted semen analysis (CASA) for diagnosis in andrology and fertility prognosis. *Hum Reprod*. 1995; 10:60-66.

MacLeod IC, Irvine D S. The predictive value of computer-assisted semen analysis in the context of a donor insemination programme. *Hum Reprod*. 1995; 10:580-586.

Marai IFM, Habeeb, AAM, Gad AE. Rabbits' productive, reproductive and physiological performance traits as affected by heat stress: a review. *Liv Prod Sci*. 2002; 78:71-90.

Mortimer ST. CASA-Practical Aspects. *J Androl*. 2000; 21:515-524.

Pérez-Sánchez F, Tablado L, Yeung CH, Cooper TG, Soler C. Changes in the motility pattern of spermatozoa from the rabbit epididymis assessed by Computer-Aided Sperm Motion Analysis. *Mol Repro Dev*. 1996; 45:364-371.

Quintero-Moreno A, Miró J, Rigau T, Rodríguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. *Theriogenology*. 2003; 58:1973-1990.

Rigau T, Farré M, Ballester J, Mogas T, Peña A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. *Theriogenology.* 2001; 56:801-815.

Rigau, T., Piedrafita, J., Reverter, A., Canal, M., Rodríguez-Gil, J.E. The rate of L-lactate production: a feasible parameter for the fresh diluted boar semen quality analysis. Anim Reprod Sci 1996; 43: 161-172.

Roca J, Martínez S, Vázquez JM, Lucas X, Parrilla I, Martínez EA. Variability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15˚C. *Anim Reprod Sci*. 2000; 64:103-112.

SAS. SAS/STAC Software: *SAS Inst. Inc*.; Carry, NC. USA. 1996.

Thurston LM, Watson PF, Holt WV. Sources of variation in the morphological characteristics of sperm sub-population objectively assessed by a novel automated sperm morphology analysis system. *J Reprod. Fertil*. 1999; 117:271-280.

Thurston LM, Watson P, Mileham A, Holt W. Morphologically distinct sperm subpopulation defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. *J Andrology*. 2001; 22:382-394.

Verstegen J, Iguer-Ouada M, Onclin K. Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology*; 57:149-179.

Williams J, Gladen B, Schrader S, Turner T, Phelps L, Chapin R. Semen analysis and fertility assessment in rabbits: statistical power and design considerations for toxicology studies. *Fundam Appl Toxicol.* 1990; 15:651-665.

CAPITULO VI:
RESÚMEN Y DISCUSION GLOBAL DE LOS RESULTADOS

Al principio de la década de los noventa existían pocos estudios sobre el análisis computarizado de la motilidad espermática en semen de equino (Varmer y col, 1991; Jasko y col; 1990 y 1992; Palmer y Magistrini, 1992), porcino (Aumuller y Willeke, 1988; Rath y col, 1988) o de conejo (Williams y col, 1990; Farrel y col, 1993). Sin embargo, en estos últimos años se ha incrementado el número de estudios donde se utiliza este análisis para evaluar las características cinéticas de los espermatozoides en estas especies y de los mamíferos en general (Verstegen y col, 2002). La mayoría de estas investigaciones han sido realizadas mediante softwares comerciales que poseen muchos descriptores de motilidad espermática. Si la cantidad de parámetros obtenidos es muy grande, la selección se hace mediante un criterio arbitrario, no sometiéndose estas variables a los análisis estadísticos de agrupamiento que aportan los paquetes de análisis estadístico más sofisticado y que son muy útiles para realizar dicha selección. Así, existen pocas investigaciones hechas en semen de mamíferos donde se haya utilizado el análisis estadístico multivariado como herramienta para seleccionar los parámetros de motilidad que expliquen adecuadamente la motilidad espermática (Abaigar y col, 1999; Quintero y col, 2001). En cambio, en semen humano se han encontrado mayor número de experimentos (Bostofte y col, 1990; Barratt y col, 1993; Mac Leod e Irving; 1995; Davis y col; 1995; Krause, 1995).

Las variables seleccionadas por el procedimiento utilizado en esta tesis corresponden a un agrupamiento equitativo de los índices de velocidad, angularidad y los parámetros de oscilación de la cabeza del espermatozoide. Sin embargo, se ha de enfatizar que es muy difícil determinar la metodología estadística más exacta que estandarice de forma óptima las distintas trayectorias trazadas por un espermatozoide, ya que, a excepción de algunas variables de movimiento de la cabeza del espermatozoide, casi todos los parámetros presentan entre ellos correlaciones medias o altas. Esto ocurre debido a que estos parámetros son productos, cocientes y/o relaciones porcentuales derivadas de las relaciones

entre ellos. Por lo tanto, al comparar los resultados obtenidos entre las especies estudiadas se puede observar que los grados de asociación entre las variables varían en gran medida.

En nuestro caso, la aplicación del agrupamiento jerárquico en grupos numerosos de parámetros que explican un mismo fenómeno demuestra ser una herramienta poderosa para seleccionar los descriptores que mejor explican el movimiento espermático en los mamíferos estudiados. En la Tabla 1 se puede observar un resumen de las variables seleccionadas por cada especie evaluada de una población global de 21 descriptores de la motilidad espermática (capítulos III-V). Es importante comentar que muchas de las variables seleccionadas (VAP, LIN, ALHmed, BCF) fueron comunes entre las especies evaluadas, además de presentar también coeficientes de correlación muy similares. La variación explicada por los descriptores de motilidad al realizar la agrupación de variables por características comunes presentó un R^2 superior a 0,70 en todas las especies.

Tabla 1.

Descriptores de motilidad seleccionados por especie y su respectivo coeficiente de correlación.

Parámetro	Especie		
	Caballo	Cerdo	Conejo
Velocidad Lineal (VAP)	0,9450	0,9688	0,9423
Coeficiente de linealidad (LIN)	0,8762	0,9640	0,9683
Amplitud lateral del movimiento lateral de la cabeza (ALHmed)	0,9220	0,9374	0,9390
Índice de oscilación (WOB)	0,8497	-	-
Dance (DNC)	0,8905	0,8459	
Frecuencia de batida de la cabeza (BCF)	0,7841	0,7672	0,7576
Menor oscilación armónica de la cabeza espermática (HLO)	-	1,00	0,8421
Máxima oscilación armónica de la cabeza espermática (HMX)	-	0,8461	-
Oscilación armónica media de la cabeza espermática (HME)	0,9753	-	-
Desplazamiento angular medio algebraico (AlgMAD)	-	1,00	1,00
Variación total explicada por los descriptores de motilidad seleccionados	0,7298	0,8422	0,7943

Al realizar el estudio de las subpoblaciones espermáticas por especie y de acuerdo a los resultados obtenidos, se puede afirmar que la utilización del análisis estadístico de agrupamiento de observaciones (Proc Fasclus) realiza una correcta segregación y agrupamiento de los espermatozoides de acuerdo a características comunes de movimiento.

Como se ha plasmado anteriormente, en los experimentos realizados se constató la presencia de 3 o 4 subpoblaciones, las cuales se caracterizan por poseer patrones cinéticos diferentes dentro de cada una de ellas, además de las diferencias esperadas dentro de cada especie. Solo la BCF se mantuvo ligeramente constante dentro de las subpoblaciones e inclusive fue bastante similar entre las especies evaluadas (capítulos III-V).

El análisis de agrupamiento de las trayectorias espermáticas demuestra la existencia de subpoblaciones espermáticas muy bien definidas en semen de caballo cerdo y conejo (Capítulos III al V). El semen de caballo y conejo esta compuesto por 4 subpoblaciones espermáticas que poseen distintas distribuciones porcentuales en función de la evaluación de los descriptores de la motilidad espermática. La excepción fue el cerdo, el cual sólo evidencia 3 subpoblaciones espermáticas, de las cuales una de ellas es muy representativa, ya que posee el 89% de los espermatozoides del eyaculado (Tabla 2). Un detalle característico fue observar que las subpoblaciones presentaban rasgos comunes entre las especies. Así, al igual que el cerdo, el caballo (72%) y en el conejo (51%) también exhiben una subpoblación predominante (subpoblación 1). En general, esta población mayoritaria se caracteriza por poseer espermatozoides con una elevada motilidad progresiva y trayectorias muy lineales. Sin embargo, éstos no son los espermatozoides más rápidos de la muestra seminal. Así, la ALHmed indica un tipo de movimiento de baja amplitud, a pesar de que la BCF es muy similar al de las otras poblaciones espermáticas (capítulos III-V).

Tabla 2.
Distribución de las subpoblaciones espermáticas por especie.

Especie	Subpoblaciones			
	1	2	3	4
Caballo	72,4 (1917)	19,7 (523)	6,6 (174)	1,3 (35)
Cerdo	89,4 (4928)	9,2 (510)	1,4 (77)	-
Conejo	51,0 (1339)	19,0 (498)	16,9 (444)	13,1 (343)

( ): Cantidad de espermatozoides evaluados.

El cerdo y el caballo también presentan una población equivalente de espermatozoides bastante rápidos (subpoblación 2). Sin embargo, a pesar de esta rapidez sus movimientos exhiben una LIN inferior a los espermatozoides de la subpoblación predominante. De hecho, en el conejo, el LIN de los espermatozoides de la subpoblación 2 continúa siendo

bajo y similar al de la subpoblación 1. El valor de ALHmed del espermatozoide se mantiene constante y similar a la subpoblación 1 en el conejo. Sin embargo, tanto en cerdo como en caballo este valor aumenta. Esta subpoblación es la segunda más grande en cuanto a su valor porcentual y oscila entre el 9 y el 20% de la población total de espermatozoides contenidos en el semen.

La subpoblación 3 en caballos y cerdos representan un porcentaje muy bajo de la población de espermatozoides (6,6 y 1,4%, respectivamente). En ambas especies, ésta es una población muy rápida pero con un LIN muy reducido que disminuye un 45% al compararlo con la subpoblación 1. Además, estos espermatozoides exhiben valores elevados de ALHmed. La evaluación conjunta de estos resultados parece indicar la presencia de una población con espermatozoides hiperactivados, sobretodo en la especie porcina. En cambio, en conejos esta subpoblación presentó mayor valor porcentual (16,9%) del total de espermatozoides de la muestra seminal. Sin embargo, estos espermatozoides son lentos, con escaso movimiento lineal y con una ALHmed baja, muy inferior a la presentada por los caballos y cerdos. Todo esto hace pensar que esta población es absolutamente distinta en la especie cunícola (Capítulos III-V).

Las mayores discrepancias entre especies se observaron en la subpoblación 4. Así esta subpoblación no esta presente en cerdos. En semen de conejo, la subpoblación 4 estuvo representada por espermatozoides sumamente veloces y muy lineales. En cambio, en caballos, a pesar de ser una población espermática sumamente veloz, muestra un LIN muy bajo asociado con la ALHmed bastante alta, lo cual es representativo de un patrón de movimiento de hiperactivación.

Al realizar el análisis de las subpoblaciones espermáticas en semen refrigerado de caballo fue evidente que el almacenamiento a 4˚C por 24 horas modifica la distribución porcentual de las subpoblaciones espermáticas (capítulo III), lo cual se evidencia con la disminución porcentual de la población predominante en un 9% y con un aumento en las subpoblaciones de espermatozoides con trayectorias menos lineales. Todo esto se debe a que disminuye la linealidad y la progresividad de las trayectorias espermáticas, aumentando además la BCF. Estos resultados también están asociados a una disminución de la viabilidad en un 12% y al incremento de un 7% de los acrosomas alterados en comparación a las muestras evaluadas en semen fresco. Es evidente que el estrés térmico

ocasiona cambios funcionales sobre la célula espermática, lo cual es apreciable al observar la pérdida de viabilidad y del acrosoma, además de la redistribución subpoblacional durante el proceso de almacenamiento a 4˚C, lo cual es señal de un progresivo deterioro seminal.

En caballos y conejos no se encontraron experimentos previos donde se estructure el análisis de motilidad espermática y separen los espermatozoides en subpoblaciones. En cambio, en cerdo existen experimentos recientes con los cuales se pueden contrastar nuestros resultados. Nuestros resultados avalan los obtenidos por Abaigar y col (1999) y Thurston y col (2002), los cuales también observaron 3 subpoblaciones espermáticas en eyaculados de porcinos. Por otra parte, todas las especies estudiadas muestran poblaciones espermáticas con trayectorias lineales y con movimientos muy cambiantes. Además, aparecen siempre otras subpoblaciones con trayectorias espermáticas muy veloces, pero no lineales. En mucha menor proporción, y solo en el caballo y el cerdo, se evidenciaron trayectorias con velocidad media muy alta, LIN bajo y ALHmed elevado, lo que podría indicar la presencia de espermatozoides hiperactivados. Este hecho se ha descrito en humanos, en donde un espermatozoide de estas características presenta una VCL de 150 μm/s, un LIN <50% y una ALHmed alrededor de los 7,0 μm (Mortimer y col, 1998). En cambio, en equinos un espermatozoide con estas características posee valores de VCL superiores a 180 μl/s y ALHmed superior a 12 μl (Rathi y col, 2001). Esta distribución, sin duda, se debe a las características de motilidad inherente a cada especie. Por otra parte, el hecho de la aparición de subpoblaciones razonablemente equiparables en las especies estudiadas parece indicar que estas subpoblaciones representan espermatozoides en diferentes estados de desarrollo que provienen de diferentes partes del epidídimo (Abaigar y col, 1999). Otra conjetura podría ser la de células con diferentes morfologías que difieren en características funcionales y/o fisiológicas. Sea como fuese, nuestros resultados sugieren que el estudio de las subpoblaciones espermáticas puede llegar a ser una poderosa herramienta para la mejora del examen de análisis seminal en los eyaculados de mamíferos en general.

Al observar el efecto propio del animal (caballos, cerdo) o de la línea genética (conejo) sobre la distribución de estas subpoblaciones los resultados varia de acuerdo a la especie evaluada. Así, no se detectaron grandes variaciones en la distribución porcentual de las subpoblaciones espermáticas entre verracos. Esta observación indica que la distribución

subpoblacional de los espermatozoides fue similar en cada cerdo. Sin embargo, sí se observaron grandes variaciones en algunos parámetros de motilidad individual dentro de cada subpoblación, lo cual indica la existencia de diferencias individuales en función del movimiento espermático. En equinos se puede apreciar diferencias entre los animales utilizados para el experimento. En este sentido, la relación existente entre cada caballo y las subpoblaciones espermáticas fue un hecho esperado, debido a que otros investigadores han encontrado relaciones bastante estrechas entre el semental equino y las características seminales generales, entre las cuales se incluye la motilidad (Palmer y Magistrini, 1992; Varmer y col, 1991; Jasko y col, 1992). Por lo tanto, nuestros resultados confirman estas observaciones previas. En conejos, al igual que en el caballo las variaciones individuales entre la línea genética utilizada fueron también una realidad. De hecho, otros investigadores también han encontrado una relación similar entre los conejos evaluados en función de muchos parámetros de calidad seminal (Battaglini y col, 1992; Castellini y col, 1999).

La motilidad espermática esta directamente relacionada con la funcionalidad del espermatozoide. Por lo tanto, el patrón de movimiento espermático estará estrechamente relacionado con las pruebas de calidad seminal, especialmente con aquellas relacionadas con la funcionalidad espermática. Entre estas pruebas destacamos la producción de L-lactato, el test de resistencia osmótica (ORT) o el test de choque hiperosmótico (HRT). En nuestros estudios, estas 3 pruebas sólo fueron realizadas en muestras seminales de cerdos, por lo que la determinación de la relación motilidad/pruebas funcionales sólo es aplicable a esta especie. Así, respecto al ritmo de formación de L-lactato se observó que la VAP y el LIN se relacionaron de forma directa con los niveles de producción de L-lactato. Además, la estructura de cada subpoblación parece estar asociada al ritmo de producción de L-lactato, ya que la estructura subpoblacional variaba en cuanto a su porcentaje y a las características de motilidad (ver capítulo IV). Este hecho indica que la motilidad y el porcentaje de cada subpoblación espermática podrían estar relacionados con la capacidad de producción de L-lactato del eyaculado en sí. De manera similar, se observó la presencia de variaciones específicas de los parámetros de motilidad en muestras con respuesta diferente al ORT. Así, los valores de LIN fueron menores en muestras con ORT por encima de 59%, disminuyendo aún más esta velocidad cuando los valores de ORT se incrementaron. Por el contrario, los valores medios de ALHmed y la BCF entre otros, se incrementaron al elevarse también los resultados del ORT. Al relacionar los resultados de

ORT con las subpoblaciones espermáticas, es importante mencionar que la subpoblación 3 desaparece prácticamente en muestras con valores de ORT inferiores al 59%, mientras que, se incrementa la subpoblación 1 en muestras seminales con valores de ORT superiores a 78%. Tomando en cuenta que la ORT es una de las pruebas de calidad que muestran mayor correlación negativa con la capacidad fertilizante de un eyaculado en vivo (Shilling y Vengust, 1985; Shilling y col, 1986), estos resultados sugieren alguna posible relación entre las subpoblaciones, especialmente la 1 y la capacidad fertilizante del eyaculado.

El HRT presenta resultados cualitativos similares al ORT, por lo que, las conclusiones son semejantes (ver capítulo III). De todas maneras, hay que insistir en la importancia que tiene la correlación de las pruebas funcionales. En este sentido, el ORT ha sido una de las pruebas de funcionalidad espermática más utilizadas para medir calidad seminal en el cerdo (Schilling y Vengust, 1985; Schilling y col, 1984 y 1986, Caiza de la Cueva y col, 1997[a]). En cambio el HRT ha sido desarrollado por nosotros, y según los resultados presentados en esta tesis y en investigaciones previas, podría utilizarse como herramienta efectiva para la determinación de la funcionalidad espermática (Rodríguez-Gil y Rigau, 1995; Caiza de la Cueva y col, 1997[b]; Quintero y col, 2003). La principal diferencia entre el ORT y el HRT test radica en que el ORT determina la resistencia de los espermatozoides a un medio con una sola condición osmótica, mientras que el HRT test evalúa la habilidad de estas células de resistir a los cambios súbitos de osmolaridad, por lo que determina la capacidad de los espermatozoides de adaptarse a 2 condiciones osmolares diferentes. Un aspecto muy importante a destacar es que ambas pruebas fueron seleccionadas por los test estadísticos como las más importantes a incluir en una analítica de calidad seminal precisa. Por otro lado, e insistiendo en pruebas funcionales asociadas al análisis seminal, Rigau y col (1996) demostraron que la producción de L-lactato es una prueba que posee una fuerte correlación con algunos parámetros de calidad seminal, inclusive con el ORT. Además, al realizar el análisis de componentes principales se demostró que el ritmo de producción de L-lactato posee gran fuerza estadística, relacionándose con la capacidad fecundante "in vivo" del eyaculado (Rigau y col, 1996). Por lo tanto, en su conjunto, las pruebas funcionales en su conjunto son muy relevantes dentro de la analítica seminal porcina, si bien su importancia variará seguramente en función de las condiciones de estudio de las granjas y de las muestras seminales.

El estudio de la regresión logística entre la calidad seminal y la fertilidad en granja realizado en porcinos proporcionó 2 modelos estadísticamente significativos con porcentajes de concordancia entre 67% y 69%. Estas regresiones logísticas incluyeron los ORT y HRT y la viabilidad espermática como parámetros predictivos de la fertilidad seminal, excluyendo así todos los descriptores individuales de la motilidad espermática y las anormalidades morfológicas. Estos resultados contrastan con los obtenidos por otros autores. Así, los modelos estadísticos del experimento de Gadea y col (1998) seleccionaron el porcentaje de penetración espermática y la motilidad como los parámetros más adecuados para predecir la fertilidad seminal en un 27%. Incluso Holt y col (1997) no encontraron ningún modelo significativo que relacionen la motilidad y la fertilidad "in vivo", si bien esta carencia de relación entre los descriptores de motilidad medidos por CASA y la fertilidad ha sido observada tanto en esta tesis como en los estudios llevados a cabo por otros autores (Aumuller y Wileke, 1988; Rath y col, 1988; Berger y col, 1996).

El estudio de la relación entre análisis seminal y fertilidad "in vivo" en la especie porcina ha mostrado más resultados importantes. Así, hay que destacar que parámetros como las anormalidades morfológicas no han sido variables importantes en el análisis de los factores que pueden afectar la fertilidad, a pesar de que muchas muestras utilizadas para las inseminaciones artificiales presentaban porcentajes de anomalías como gotas citoplasmáticas proximales (GCP) muy elevadas. Sorprendentemente, porcentajes de GCP superior al 50% no afectaron la fertilidad ni tampoco la prolificidad de las muestras (Tabla 3), probablemente debido que estos eyaculados con porcentaje similar de concentración espermática, obtuvieron valores de motilidad y de viabilidad espermática similar, e inclusive superior en algunos casos a aquellos obtenidos por los cerdos con porcentajes de GCP menores al 20%. En relación con este punto, se ha descrito que en el semen refrigerado durante 4 días, las gotas citoplasmáticas disminuyen los índices de fertilidad y prolificidad, pero la motilidad total no es afectada, oscilando entre 83,5 y 89,9 % (Waberski y col, 1994). Nuestros resultados muestran que la presencia de GCP no afecta la velocidad individual de los espermatozoides, lo cual se comprueba al no modificarse la VAP ni el LIN de los espermatozoides observados. De hecho, estos espermatozoides pueden ser más veloces que los que no presentan esta anormalidad. Por otro lado, los movimientos de la cabeza presentan mayor frecuencia y oscilación en los espermatozoides que presentan GCP, lo cual revela que su presencia afecta la movilidad de esta parte de la célula. La presencia masiva de esta anomalía no se relaciona tampoco con un incremento

en el porcentaje de acrosomas alterados ni tampoco se observa un efecto global de los cambios de presión osmótica sobre la totalidad de espermatozoides de la muestra. En conclusión, la presencia masiva de la GCP en espermatozoides no afecta significativamente los parámetros más importantes de calidad seminal.

Tabla 3.

Características de motilidad y viabilidad del semen con presencia masiva de gota citoplasmática proximal (GCP) y su efecto sobre la fertilidad y prolificidad en cerdas inseminadas con semen de estas características.

Parámetro	Porcentaje de Gota citoplasmática proximal (GCP, μ± ES)		
	GCP≤20 (32)	GCP>20≤50 (45)	GCP>50 (38)
VAP (μm/sec)	37,33 ± 1,39 [b]	47,26 ± 1,92 [a]	49,72 ± 2,10 [a]
LIN (%)	50,46 ± 1,71 [b]	57,76 ± 2,36 [a]	55,60 ± 2,59 [ab]
ALHmed (μm)	2,49 ± 0,06 [ab]	2,33 ± 0,09 [b]	2,64 ± 0,10 [a]
BCF (Hz)	14,42 ± 0,20 [ab]	13,92 ± 0,29 [b]	15,10 ± 0,31 [a]
Viabilidad (%)	88,39 ± 2,11 [a]	87,26 ± 1,60 [a]	89,12 ± 1,80 [a]
ORT (%)	79,20 ± 1,81 [a]	77,12 ± 1,66 [a]	83,05 ± 1,92 [a]
Fertilidad (%)	84,0 ± 8,0 [a]	82,2 ± 11,0 [a]	81,5 ± 12,0 [a]
Prolificidad (n)	9,55 ± 0,74 [a]	11,56 ± 0,66 [a]	12,68 ± 0,74 [a]

VAP: Velocidad lineal, LIN: Índice de linealidad, ALHmed: Amplitud media del desplazamiento lateral de la cabeza, BCF: frecuencia de batida de la cabeza, ORT: Test de resistencia osmótica.
( ): Los números entre paréntesis corresponden al número de cerdas inseminadas.
(a, b): Letras distintas en la misma fila implican diferencias significativas.

En referencia a la prolificidad, los modelos de regresión múltiple empleados en nuestro estudio con el fin de relacionar el tamaño de la camada con los parámetros de calidad seminal sólo dieron una predectividad del 16%. De hecho, el modelo más idóneo solo presentó una propensión estadística (0,05>P<0,10) capaz de explicar la variación en cuanto a la prolificidad, escogiendo la viabilidad, la GCP y sobretodo, algunos parámetros de motilidad (VAP, LIN, HLO). En una experiencia previa realizada mediante regresión múltiple se seleccionaron variables como el porcentaje de penetración y la motilidad como los parámetros más apropiados que predicen en un 29,9% el tamaño de la camada, excluyendo el ORT y el HOST. Pero a pesar de esto ambos parámetros presentaron una correlación significativa de 0,35 y 0,30, respectivamente (Gadea y col, 1998). Otros estudios han mostrado varios modelos que incluyeron solo parámetros de motilidad

individual, los cuales explicaron entre un 12 y 24% de variación el tamaño de la camada (Holt y col, 1997). A pesar de ello, alguna relación existe entre la motilidad seminal y la prolificidad, puesto que eyaculados con motilidades totales superiores al 93,2% son más propensos de tener tamaños de camadas superiores a 10 lechones por parto, mientras que muestras con motilidades del 80,9% presentaron tamaños de camadas inferiores (Hirai y col, 2001). Esta relación, más bien laxa entre la motilidad y la prolificidad estaba inserta en la base del porcentaje de predicción obtenido en nuestros modelos de regresión. En este estudio algunos parámetros de motilidad parecen ser buenos parámetros en el análisis seminal y podrían ser utilizados para eliminar eyaculados con bajo potencial para producir camadas óptimas. Sin embargo, hay que recordar el número de lechones nacidos por parto depende mucho de las características intrínsecas de la cerda.

En conejos, los análisis de regresión logística basados en la creación de variable dicotómica (0: no gestante, 1: gestante) aportaron 2 modelos matemáticos con un porcentaje de concordancia mayor que en cerdos que osciló entre 68,3 y 76,4%. Hay que destacar que los mejores modelos seleccionaron la viabilidad espermática y el porcentaje de anormalidades espermáticas como los parámetros que mejor predicen la fertilidad del semen de conejo. Sin embargo, estos modelos matemáticos no incluyeron la motilidad como un parámetro de importancia, aunque otros autores si han encontrado correlaciones significativas entre los descriptores de motilidad aportados por el CASA y la fertilidad en conejas (Farrel y col, 1993).

En semen de conejos no se realizaron las pruebas de funcionalidad espermática, lo cual imposibilita comparar adecuadamente estos resultados con los obtenidos en cerdos. Por otro lado, la utilización de la regresión múltiple seleccionó a los parámetros de viabilidad, anormalidades morfológicas, acrosomas alterados, concentración espermática y la VSL del espermatozoide como los más adecuados, prediciendo en su conjunto un 32,2% la fertilidad de la muestra seminal. Finalmente, y utilizando de nuevo la regresión lineal múltiple, el porcentaje de predicción del tamaño de la camada fue bajo (16,1%), pero, a diferencia de los resultados en cerdas, el modelo resultante del análisis fue estadísticamente significativo y escogió los parámetros de viabilidad y concentración espermática, además de algunos descriptores de la motilidad individual. Siguiendo con los estudios de fertilidad, se pudo apreciar que los eyaculados con probada fertilidad presentaron una distribución porcentual de las subpoblaciones muy similar a aquellas

muestras que no fecundaron a una hembra apta. Sólo se puede destacar que existen diferencias en cuanto al porcentaje de anormalidades espermáticas y acrosomas alterados en los eyaculados fértiles, así la subpoblaciones 3 y 4 presentaron mayor variación en aquellos eyaculados con porcentaje de anormalidades superiores al 19% y en porcentaje de acrosomas alterados por debajo del 18%.

El estudio de fertilidad seminal equina se llevó a cabo de una manera descriptiva al comparar la distribución de las subpoblaciones espermáticas en las muestras seminales que tuvieron al menos una fertilidad probada con la motilidad total y concentración espermática de la muestra. Nuestros resultados sugieren que motilidades totales superiores al 80% podrían garantizar la fertilidad de la muestra equina. Sin embargo, no hay mucha diferencia si inseminamos yeguas con dosis seminales con concentraciones espermáticas de 10 a 20 x 10^9 espermatozoides/ml. Por otro lado, se apreció mayor homogeneidad en los eyaculados con al menos una fertilidad probada, ya que se observaron 3 subpoblaciones espermáticas en lugar de 4 y con presencia de espermatozoides con movimiento lineal y pocas características degenerativas. No existen muchas publicaciones donde relacionan la fertilidad con los parámetros de calidad seminal y la inmensa mayoría solo establece análisis de correlación. Otros autores han descrito una correlación significativa entre la fertilidad y variables como la motilidad total y la velocidad media del espermatozoide determinada por CASA, si bien no observaron ninguna relación con el índice de linealidad (Jasko y col, 1992). Otros estudios de correlación demuestran que la fertilidad esta influenciada por el número de espermatozoides motiles depositados en el tracto reproductivo de la yegua (Demick y col, 1976); y por las características morfológicas de los espermatozoides, sobretodo por las anormalidades de cabeza y por las GCP (Jasko y col, 1990).

Nuestros resultados se basan en métodos novedosos de análisis estadístico que censan y dividen la muestra seminal en subpoblaciones, al tiempo que explican la asociación existente entre la calidad seminal y la fertilidad o prolificidad. Muchas investigaciones muestran una falta de asociación entre movimiento espermático y fertilidad, tanto en el hombre como en muchas especies de interés zootécnico (Polansky y Lamb, 1988; Hiting y col, 1989; Holt y col, 1997). A pesar de esto, la motilidad espermática siempre es un parámetro muy importante a tomar en cuenta y debe de estar presente en los análisis de calidad seminal, ya que es una manera muy útil de medir la funcionalidad del

espermatozoide en relación a su habilidad para obtener y procesar la energía (Cardullo, 1986). Nuestros estudios no demuestran una relación estrecha entre los descriptores individuales de la motilidad espermática obtenidos por CASA y la fertilidad en granja. De todas maneras, este hecho no nos sorprende, ya que resultados similares han sido descritos por otros investigadores (Polansky y Lamb, 1988; Hiting y col, 1989). En el caso del cerdo, el efecto podría explicarse por las características intrínsecas de la motilidad espermática. El semen de cerdo presenta espermatozoides más lentos que otras especies, Este hecho, se comprueba en esta tesis, al comparar las trayectorias espermáticas del cerdo con la del caballo. Además, la motilidad del semen porcino es extraordinariamente sensible a múltiples factores externos (Sánchez, 1991), lo que dificulta en grado sumo su correcta evaluación. Estos fenómenos, aunque en menor medida, también se observaron en las otras especies estudiadas Consecuentemente, la motilidad espermática sólo puede ser considerada como un parámetro primordial en el análisis de la calidad seminal de caballos, conejos y sobretodo de cerdos, cuando la mayoría los factores extrínsecos que puedan influenciar sobre este parámetro estén totalmente controlados, lo cual es bastante improbable bajo condiciones de granja. Otro factor importante a destacar son las diferentes condiciones de análisis que acompañan al CASA, incluyendo sobretodo el soporte físico utilizado para analizar la muestra seminal (uso de sobre-cubreobjetos, cámaras especiales, etc). Esto dificulta la determinación precisa de la estructura de las subpoblaciones y podría ser una consecuencia de las dificultades intrínsecas en la determinación de la motilidad espermática, sobretodo en el semen de cerdo. Así, bajo estas condiciones, las pruebas funcionales como el ORT o el HRT son más indicadas para determinar la calidad del semen, ya que, están sujetas a menores errores metodológicos. Por otro lado, en un futuro muy próximo y en función de las crecientes exigencias que impera sobre la comercialización del semen porcino, el análisis seminal del cerdo necesitará de un protocolo estándar que permita una correcta interpretación de los resultados derivados del análisis seminal. Sin embargo, debido al movimiento específico que posee el espermatozoide del cerdo, este protocolo debe variar al establecido para humanos (WHO, 1999). Finalmente, este protocolo universal debe ser amplio y permitir el estudio de la estructura espermática poblacional, lo que permitirá comparar los resultados de los distintos laboratorios dentro de las tendencias propias del CASA.

REFERENCIAS BIBLIOGRÁFICAS

Abaigar T; Holt W; Harrison R; Del Barrio G. 1999. Sperm subpopulation in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod; 60: 32-41.

Aumuller R; Willeke H. 1988. Computed controlled analysis of boar semen with the "Cellsoft" system. In: Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination. Dublin/Ireland; 3: MS227.

Barratt CLR; Tomlison MJ; Cooke ID. 1993. Prognostic significance of computerized motility analysis for in vivo fertility. Fertil Steril; 60 (3): 520-525.

Battaglini M ; Castellini C ; Lattaioli P. 1992. Variability of the main characteristics of rabbit semen. J Appl Rabbit Res; 15: 439-446.

Berger T; Anderson DL; Penedo MCT. 1996. Porcine sperm fertilizing potential in relationship to sperm functional capacities. Anim Reprod Sci; 44: 231-239.

Bostofte E; Bagger P; Michael A; Stakemann G. 1990. Fertility prognosis for infertile men: results of follow-up study of semen analysis in infertile men from two different populations evaluated by the cox regression model. Fertil Steril; 54 (6): 1100-1106.

Caiza de la Cueva F; Rigau T; Pujol MR; Piedrafita J; Rodríguez-Gil JE. 1997[a]. Resistance to hyperosmotic stress of boar spermatozoa: the role of the ionic pumps and the relationship with cryosurvival. Anim Reprod Sci; 48: 301-315.

Caiza de la Cueva F; Pujol MR; Rigau T; Bonet S; Miró J; Briz M; Rodríguez-Gil JE. 1997[b]. Resistance to osmotic stress of horse spermatozoa: The role of ionic pumps and their relationship to cryopreservation success. Theriogenology; 48: 947-968.

Cardullo RA. 1986. Oxygen metabolism, motility, and the maintenance of viability of caudal epididymal rat sperm. Diss Abstr Int B Sci Eng; 47: 892.

Castellini C; Lattaioli P. 1999. Effect of number of motile sperm inseminated on reproductive performance of rabbits does. Anim Reprod Sci; 57: 111-120.

Davis RO; Drobnis EZ; Overstreet JW. 1995. Application of multivariate cluster, discriminate function and stepwise regression analyses to variable selection and predictive modelling of sperm cryosurvival. Fertil Steril; 63: 1051-1057.

Demick DS; Voss JL; Pickett BW. 1976. Effect of coling, storage, glycerolization and spermatozoal numbers on equine fertility. J Anim Sci; 43: 633-637.

Farrel PB; Foote RH; Simkin ME; Clegg ED; Wall RJ. 1993. Relationship of semen quality, number of sperm inseminated, and fertility in rabbits. J Androl; 14: 464-471.

Gadea J; Matás C; Lucas X. 1998. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. Anim Reprod Sci; 56: 95-108.

Hirai M; Boersma A; Hoeflich A; Wolf E; Foll J; Aumuller R; Braun J. 2001. Objectively measured sperm motility and sperm head morphometry in boars (*Sus scrofa*): Relation to fertility and seminal plasma growth factors. J Androl; 22: 104-110.

Hiting A; Comhaire F; Vermeulen L; Dhont M; Vermeulen A; Vandekerckhove D. 1989. Value of sperm characteristics and the result of in vitro fertilization for predicting the outcome of assisted reproduction. J Androl; 13:59-66.

Holt C; Holt W; Moore HDM; Reed HCB; Curnock RM. 1997. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: Results of two fertility trials. J Andrology; 18: 312-323.

Jasko DJ; Lein DH; Foote RH. 1990. Determination of the relationship between sperm morphologic classifications and fertility in stallion: 66 cases (1987-1988). JAVMA; 197: 389-394.

Krause W. 1995. Computer-assisted semen analysis system: comparison with routine evaluation and prognostic value in male infertility and assisted reproduction. Hum Reprod; 10: 61-66.

Jasko DJ; Little TV; Lein DH; Foote RH. 1992. Comparison of spermatozoa movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). JAVMA; 200: 979-985.

MacLeod IC; Irvine DS. 1995. The predictive value of computer assisted semen analysis in the context of a donor insemination programme. Hum Reprod; 10: 580-586.

Mortimer ST; Swan MA; Mortimer D. 1998. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. Hum Reprod; 13: 2139-2146.

Palmer E; Magistrini M. 1992. Automated analysis of stallion semen post-thaw motility. Acta Vet Scand; 88 (suppl): 137-152.

Polansky FF; Lamb MJ. 1988. Do the results of semen analysis predict future fertility? A survival analysis study. Fertil Steril; 49: 1059-1065.

Quintero-Moreno, A., Rivera del Álamo, M., Rigau T., Rodríguez-Gil, J. E. 2001. Optimización de los parámetros de motilidad espermática porcina mediante agrupamiento jerárquico. ITEA; 22 (II): 832-834.

Quintero-Moreno A; Rigau T; Rodríguez-Gil JE. 2003. The hyperosmotic resistance test: a feasible parameter for boar spermatozoa function. Vth International Conference on Boar Semen Preservation, Utrecht/The Netherlands, August 24-27. In press.

Rath D; Armbrecht S; Schaap P; Weitze KF. 1988. Experiences with a computerized videomicrographic system for sperm analysis. In: Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination. Dublin/Irland; 3: MS288.

Rathi R; Colenbrander B; Bevers MM; Gadella BM. 2001. Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprd; 65: 462-470.

Rigau T; Piedrafita J; Reverter A; Canal M; Rodríguez-Gil JE. 1996. The rate of L-lactate production: A feasible parameter for the fresh diluted boar semen quality analysis. Ani Reprod Sci; 43: 161-172.

Rodríguez-Gil, JE; Rigau T. 1995. Effects of slight agitation on the quality of refrigerated boar semen. Anim Reprod Sci; 39: 141-146.

Sánchez R. 1991. Control de calidad espermática. Anaporc; 104: 27-33.

Schilling E; Vengust M; Smidt D. 1984. ORT: Un nuevo sistema para predecir la congelabilidad y la capacidad de almacenamiento de los espermatozoides de verraco. Proc 8th IPVS Congress, Belgica, pp. 346 (abstr.).

Schilling E; Vengust M. 1985. Determination of osmotic resistance of boar spermatozoa and its relationship with the storage ability of semen samples. Zuchthygiene; 20: 61-78.

Schilling E; Vengust M; Bajt G; Tomcic M. 1986. The osmotic resistance (ORT) of boar spermatozoa and the relation to pregnancy rate and litter size. Proc 9th IPVS Congress, Barcelona, pp. 77 (abstr.).

Thurston LM; Watson P; Mileham A; Holt W. 2001. Morphologically distinct sperm subpopulation defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Andrology; 22 (3): 382-394.

Varmer D; Vaughan S;, Johnson L. 1991. Use of computerised system for evaluation of equine spermatozoa motility. Am J Vet Res; 52: 224-230.

Verstegen J; Iguer-Ouada M; Onclin K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology; 57: 149-179.

Waberski D; Meding S; Dirksen G; Weitze KF; Leiding C; Hahn R. 1994. Fertility of long-term-stored boar semen: Influence of extender (Androhep and Kiev), storage time and plasma droplets in the semen. Anim Reprod Sci;, 36: 145-151.

Williams J; Gladen B; Schrader S; Turner T; Phelps L; Chapin R. 1990. Semen analysis and fertility assessment in rabbits: Statistical power and design considerations for toxicology studies. Fundam Appl Toxicol; 15: 651-665.

WHO. 1999. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4^{d} ed. Cambridge: Cambridge University Press.

CAPÍTULO VII:
CONCLUSIONES

1. La utilización de los procedimientos estadísticos de agrupamientos de observaciones en primer lugar y de variables en segundo lugar son una buena alternativa para garantizar la separación de la población espermática seminal en subpoblaciones, además de optimizar el número de descriptores que describen la motilidad espermática.

2. El semen del caballo y el de conejo presentan cuatro subpoblaciones espermáticas, con patrones de movimientos propios y distintos entre una subpoblación y otra, mientras que el cerdo presenta el semen más homogéneo con presencia de tres subpoblaciones espermáticas.

3. La distribución porcentual de las subpoblaciones dentro del eyaculado es muy desproporcionada en todas las especies evaluadas, existiendo en todos los casos una población predominante.

4. Tanto en el cerdo como en el caballo, existe una población muy pequeña que de acuerdo a su patrón de movimiento podría corresponder a espermatozoides hiperactivados.

5. El semen de caballos que presentaron al menos una fertilización probada no presentaron la subpoblación 4, la cual esta representada por espermatozoides con patrones de movimientos que podrían representar estados degenerativos.

6. Las distintas pruebas de calidad seminal realizadas en semen de cerdo y conejo predicen muy poco la capacidad fecundante y el potencial para producir camadas grandes de un eyaculado, mientras que los descriptores de la motilidad espermática carecen de poder predictivo en el semen de cerdo.

7. Las pruebas de funcionalidad espermática son la herramienta más efectiva para evaluar y predecir la calidad seminal y la fertilidad y prolificidad del semen de cerdos y a falta de estas pruebas en el semen de conejos, la viabilidad y las anormalidades espermáticas presentan el mayor valor predictivo.

CAPÍTULO VIII:

ANEXOS

Anexo 1.
Hierarchical clustering of the sperm motility descriptors in boars.

Hierarchical clustering	Sperm motility descriptors	R^2 with own cluster (OC)	R^2 with next closest (NC)	Proportion (1- R^2_{oc} /1-R^2_{nc})
Cluster 1	ALHmed *	0.9374	0.0565	0.0664
	ALHmax	0.9120	0.0030	0.0883
	DNM	0.9032	0.0171	0.0984
	HME	0.8499	0.1898	0.1853
	HY	0.5349	0.2905	0.6556
Cluster 2	LIN *	0.9640	0.3689	0.0570
	STR	0.6335	0.1660	0.4395
	WOB	0.8782	0.4508	0.2217
	AI	0.7151	0.4246	0.4950
Cluster 3	VSL	0.8911	0.4697	0.2054
	VAP *	0.9688	0.4211	0.0539
	AV	0.9049	0.4775	0.1820
Cluster 4	MADABS	0.7672	0.5596	0.5286
	BCF *	0.7672	0.1617	0.2777
Cluster 5	HLO *	1.0000	0.0587	0.0000
Cluster 6	MADALG *	1.0000	0.2039	0.0000
Cluster 7	VCL	0.8256	0.5321	0.3727
	DNC *	0.8459	0.3332	0.2311
	HHI	0.7580	0.1753	0.2935
Cluster 8	HMX *	0.8461	0.3064	0.2219
	HBS	0.8461	0.4029	0.2577

[a] n= 6.297 spermatozoas

* Selected variables.

For a definition of the motility descriptors, see p. 58. The cluster summary gives the number of sperm motility descriptors in each cluster and the variation explained by the cluster component. Two squared correlation are printed for each cluster; the column labeled “own cluster” give the squared correlation of the sperm motility descriptor with own cluster component. The larger the squared correlation is the better. The column labeled “next closest” contain the next highest squared correlation of the sperm motility descriptor with a cluster component. This value is low if the clusters are well separated. The column headed “1- R^2_{oc} /1-R^2_{nc}” gives the ratio of one minus the “own cluster” R^2 to one minus the “next closest” R^2. A small “1- R^2_{oc} /1-R^2_{nc}” indicates a good clustering.

Anexo 2.
Hierarchical clustering of the sperm motility descriptors in rabbits.

Hierarchical clustering	Sperm motility descriptors	R^2 with own cluster (OC)	R^2 with next closest (NC)	Proportion $(1- R^2_{oc} /1-R^2_{nc})$
Cluster 1	VCL	0.8082	0.6591	0.5627
	ALHmed*	0.9390	0.3183	0.0894
	ALHmax	0.8747	0.2771	0.1734
	DNC	0.9057	0.2928	0.1334
	DNM	0.6117	0.3274	0.5773
	HHI	0.7081	0.3461	0.4464
Cluster 2	LIN*	0.9683	0.3085	0.0459
	STR	0.6732	0.3367	0.4926
	WOB	0.8927	0.4087	0.1815
	AI	0.7276	0.3724	0.4340
Cluster 3	VSL	0.7766	0.1800	0.2725
	VAP*	0.9423	0.3120	0.0838
	AV	0.8823	0.4353	0.2084
	HMX	0.5658	0.4326	0.7653
	HBS	0.7250	0.3684	0.4355
	HY	0.5662	0.2193	0.5557
Cluster 4	MADabs	0.7576	0.5476	0.5358
	BCF*	0.7576	0.0745	0.2619
Cluster 5	MADalg*	1.0000	0.0868	0.0000
Cluster 6	HLO*	0.8421	0.0820	0.1721
	HME	0.8421	0.4193	0.2720

n=2645 spermatozoa obtained in 43 semen diluted samples. From these, 27 ejaculates were from different rabbits and 16 of them pertaining a pool ejaculates. The spermatozoa were distributed among four genetic groups in the following manner: 642 motile spermatozoa were from genetic group 1, 618 from genetic group 2, 730 from genetic group 3, and 634 from genetic group 4. For a definition of the motility descriptors, see p.58. The cluster summary gives the number of sperm motility descriptors in each cluster and the variation explained by the cluster component. Two squared correlations are printed for each cluster; the column labeled "R^2 with own cluster" gives the squared correlation of the sperm motility descriptor with its own cluster component. The larger the squared correlation is the better. The column labeled "R^2 with next closest" contains the next highest squared correlation of the sperm motility descriptor with a cluster component. This value is low if the clusters are well separated. The column headed "$1- R^2_{oc} /1-R^2_{nc}$" gives the ratio of one minus its own cluster R^2 to one minus the next closest R^2. A small "$1- R^2_{oc} /1-R^2_{nc}$" indicates a good clustering.
* Selected variables.

Anexo 3.

Relationship between the mean of sperm motion characteristics in boar semen with respect to the ORT Test in whole samples.

Sperm motility descriptors	ORT Test Groups		
	>45-≤59	>59-≤78	>78
VAP (μm/sec)	36.4 ± 3.6[a]	42.4 ± 1.7[a]	40.6 ± 1.4[a]
LIN (%)	74.0 ± 3.2[a]	56.4 ± 1.5[b]	50.0 ± 1.2[c]
mean ALH (μm)	1.5 ± 0.1[a]	2.4 ± 0.1[b]	2.5 ± 0.1[b]
DNC (μm^2/sec)	136.0 ± 23.2[a]	176.2 ± 10.9[b]	190.8 ± 9.1[b]
BCF (Hz)	12.5 ± 0.5[a]	14.2 ± 0.2[b]	14.6 ± 0.2[b]
HLO (μm)	0.01 ± 0.01[a]	0.10 ± 0.01[b]	0.10 ± 0.01[b]
HMX (μm)	2.00 ± 0.10[a]	2.33 ± 0.05[b]	2.34 ± 0.04[b]
algMAD (angular degrees)	-13.1 ± 2.9[ab]	-10.5 ± 1.4[a]	-16.6 ± 1.8[b]

Results are shown as means ± S.D. of 5,515 spermatozoa from 28 ejaculates.

Motility descriptors have been described in page 58.

Different superscripts in a row indicate significant ($P<0.05$) differences among groups.

ORT Test is expressed as percentage.

Anexo 4.

Relationship between the mean of sperm motion characteristics in boar semen with respect to the HRT Test in whole samples.

Sperm motility descriptors	HRT Test Groups		
	>0.01-≤0.35	>0.35-≤0.7	>0.7
VAP (μm/sec)	38.5 ± 2.6^{a}	44.2 ± 1.8^{a}	45.2 ± 2.0^{a}
LIN (%)	63.2 ± 2.6^{a}	50.6 ± 2.0^{b}	$47.6 \pm 1.b^{b}$
mean ALH (μm)	2.2 ± 0.1^{a}	2.4 ± 0.1^{b}	2.7 ± 0.1^{b}
DNC (μm^2/sec)	145.5 ± 16.3^{a}	172.9 ± 12.7^{a}	214.1 ± 11.6^{b}
BCF (Hz)	14.3 ± 0.3^{ab}	13.8 ± 0.2^{a}	14.6 ± 0.2^{b}
HLO (μm)	0.04 ± 0.02^{a}	0.14 ± 0.01^{b}	0.12 ± 0.01^{b}
HMX (μm)	2.26 ± 0.06^{a}	2.31 ± 0.05^{b}	2.49 ± 0.04^{b}
AlgMAD (angular degrees)	-10.6 ± 1.4^{a}	-15.6 ± 1.1^{b}	-15.7 ± 1.0^{b}

Results are shown as means ± S.D. of 4,097 spermatozoa from 21 ejaculates. Motility descriptors have been described in page 58. Different superscripts in a row indicate significant ($P<0.05$) differences among groups. The Hyperosmotic Resistance Test is expressed as a relation between viability of the disrupted and undisturbed samples (VHIPER), as explained in the section 2, capitol IV.

Anexo 5.

Linear regression of seminal characteristics in boar with average litter size in sows.

Statistical model	Regression Coefficient	SE	Significance of individual parameters (P)	Significance of Statistical model (P)	R-square
Model	-	-	-	0.0899	0.1672
VAP	0.28506	0.11174	0.0131		
LIN	-0.18645	0.08335	0.0287		
HLO	-13.54193	5.90645	0.0251		
VIABLES	-0.30774	0.10008	0.0031		
ACROSOM	-0.19223	0.10581	0.0739		
PCD	0.05081	0.01720	0.0044		
LACTATO	0.14111	0.09412	0.1386		
Constant	35.91915	9.19298	0.0002		

Only the most significant effects ($P<0.10$) are shown. Others parameters of seminal characteristics were removed by linear regression (Backward elimination).

Motility descriptors and general characteristics of diluted semen have been described in page 58.

VIABLES: Percentage of viability by Eosin/ nigrosin staining.

PCD: Immature spermatozoon with proximal cytoplasmic droplet (%)

Anexo 6.

Linear regression of seminal characteristics in rabbits with average litter size in female rabbits.

Statistical model	Regression Coefficient	Standard Error	Significance of individual parameters	Significance of Statistical model (P)	R-square
Model	-	-	-	0.0298	0.1610
WOB	0.66063	0.22548	0.0042		
LIN	-0.47867	0.17419	0.0071		
DNC	-0.03956	0.02216	0.0772		
ALHmed	8.26987	4.1256	0.0477		
BCF	-1.74422	0.58282	0.0035		
Sperm concentration	-0.01456	0.00436	0.0012		
Sperm viability	0.31836	0.14239	0.0275		
Constant	-21.93833	16.57573	0.1886		

Average litter size= -21.93 + 0.66*WOB - 0.47*LIN – 0.03*DNC + 8.26*ALHmed – 1.74*BCF – 0.014*Sperm concentration + 0.31*Sperm viability.

Others parameters of seminal characteristics were removed (VSL, VCL, VAP, sperm with structurally abnormal acrosomes, total sperm abnormalities) by linear regression (Backward elimination).

Motility sperm descriptors have been described in page 58.