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TESIS DOCTORAL

Obtención, almacenamiento y morfometría de espermatozoides aviares: aplicación para la caracterización y criopreservación de espermatozoides de especies silvestres

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Silvia Villaverde Morcillo

Directores

Julián Santiago Moreno
Milagros C. Esteso Díez

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Silvia Villaverde Morcillo

GOBIERNO DE ESPAÑA
MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD
INIA
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria

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Memoria presentada por

Silvia Villaverde Morcillo

Para optar al grado de Doctor

por la Universidad Complutense de Madrid

VºBº de los Directores

Dr. Julián Santiago Moreno

Dra. Milagros C. Esteso Díez

Departamento de Reproducción de Animal
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (I.N.I.A.)

A mis padres,

Enseñarás a volar,
pero no volarán tu vuelo.
Enseñarás a soñar,
pero no soñarán tu sueño.
Enseñarás a vivir,
pero no vivirán tu vida.
Sin embargo…
en cada vuelo,
en cada vida,
en cada sueño,
perdurará siempre la huella
del camino enseñado.
Madre Teresa de Calcuta

“Imposible, es solo una opinión.”
Paulo Coelho

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Y es cierto que es difícil empezar, por una vez, a escribir sin referencias ni formatos, porque en esta página hay que resumir cientos de sentimientos y recuerdos desde la perspectiva más personal, y agradecer a todas las personas que han dejado una parte de sí mismos en este trabajo, y son muchos, espero no olvidarme de nadie.

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ÍNDICE

RESUMEN

Esta tesis ha sido pensada y estructurada para estudiar los aspectos básicos de la espermatología aviar y desarrollar las técnicas de criopreservación de semen en diferentes especies de aves. Estas técnicas, que forman parte de las denominadas técnicas de reproducción asistida o TRA están ganando cada vez más protagonismo en la mejora y preservación tanto de razas cómo de especies. El desarrollo de estas técnicas ha permitido la proliferación de proyectos de cría en cautividad apoyando los programas de conservación de especies autóctonas y han supuesto una importante mejora en el manejo genético de las poblaciones. Aún así, a pesar del éxito obtenido con estas técnicas en algunas especies de aves, son técnicas apenas desarrolladas en aves silvestres, en gran parte debido a la falta de conocimientos sobre los aspectos fisiológicos y las características bilógicas de los espermatozoides de estas especies.

El estudio de los aspectos biofísicos de los gametos y del tejido gonadal de las diferentes especies es el método más efectivo para desarrollar protocolos apropiados de criopreservación. Además en las diferentes especies de aves silvestres el acceso al material genético y las muestras viables de semen es difícil, consecuencia de su anatomía y fisiología, y muy reducida en el tiempo, debido a la breve estación reproductiva que presentan y por lo tanto las técnicas de criopreservación representan, además, una posibilidad de mejorar el aprovechamiento de los recursos disponibles. Es por ello que en el trabajo se planteó en primer lugar el desarrollo y evaluación de las técnicas de recolección *post-mortem* de semen, empleando como modelo una especie de ave doméstica, el gallo (Capítulo 1). En este capítulo se comparan dos técnicas de obtención de espermatozoides en aves, lavado del conducto deferente y flotación. De las

muestras obtenidas mediante ambas técnicas se han evaluado parámetros de concentración y motilidad espermática empleando analisis computerizado (CASA) mediante un microscopio Nikon Eclipse (model 50i) en contraste de fases y empleando el software Sperm Class Analyzer SCA® v.4.0. (Microptic S.L., Barcelona, Spain). Se evaluaron los porcentajes de espermatozoides con motilidad progresiva y no progresiva y los espermatozoides inmótiles. La calidad del movimiento espermático también se evaluó mediante los parámetros de velocidad, velocidad curvilínea (VCL), velocidad rectilínea (VSL), la velocidad para la trayectoria corregida (VAP) y los parámetros de linealidad (LIN), desplazamiento lateral de la cabeza (ALH) y la frecuencia de golpe flagelar (BCF).

La viabilidad de los espermatozoides se evaluó mediante tinción vital con ioduro de propidio (PI) y SYBR-14 como fluorocromos que permiten el examen de la integridad de membrana. Esta tinción, examinada con microscopio de epifluorescencia (40x; longitud de onda 450-490nm), permite diferenciar los espermatozoides vivos o con membrana intacta, teñidos de verde, y los muertos o con membrana dañada, teñidos de color rojo. El método de lavado fué con el que se obtuvieron mejores resultados lacanzando el doble de la concentración espermática (596,5±75,4 millones de espermatozoides/mL/conducto deferente) que las muestras procedentes de eyaculado (622±72,5 millones d espermatozoides/mL) y menor contaminación de las muestras con cristales de uratos y células sanguíneas. Se evaluaron también, de manera paralela a los métodos de extracción, dos diluyentes diferentes Lake-Ravie y Lake 7.1 y su efecto sobre las muestras a las 0h a temperatura corporal y a las 24h y 48h tras haber sido refrigeradas a 5ºC. Los resultados demostraron que la refrigeración afecta negativamente a los parámetros de calidad del semen aunque el diluyente Lake 7.1

preservó mejor la calidad espermática tanto de muestras eyaculadas cómo de muestras obtenidas *post-mortem*.

En el Capítulo 2 se planteó la optimización del método de tinción y evaluación de la morfometría de los espermatozoides aviares. En este caso se emplearon muestras procedentes de eyaculados naturales de dos especies modelo, el gallo y la perdiz roja *(Alectorix rufa),* y se compararon las dos tinciones más frecuentemente empleadas para evaluación morfométrica de espermatozoides, la tinción de Hemacolor® y la tinción de azul de anilina. Ambas tinciones se evaluaron con microscopio óptico de campo claro (x100, objetivo de inmersión, MOTIC BA 210) y se analizaron con un software de captura y análisis de imagen (MOTIC Spain S.L. Barcelona, Spain). Para ambas especies la tinción con Hemacolor® proporcionó mayor porcentaje de células medibles (893,7±11,7% en gallos y 71,9±15,3% en perdiz roja) y además presentó una mayor repetitividad en la medición de la longitud y el área en los gallos y de la anchura en la perdiz. Por lo que se concluyó que la tinción de Hemacolor® puede ser considerada la mas apropiada para el análisis morfométrico computerizado de los espermatozoides aviaries.

En el Capítulo 3 se describen los parámetros básicos del semen de águila real obtenido a través de falsas cópulas, se realizó; un espermiograma básico y se analizaron los valores de motilidad y morfometría. Además se evaluó la capacidad protectora del diluyente L&R-84 frente a los daños causados por la refrigeración y se procedió a la congelación de las muestras con dos crioprotectores diferentes Glicerol (11%) y DMA (6%). No se observaron diferencias significativas entre ambos crioprotectores. En ambos casos la motilidad y viabilidad de las muestras descendió tras la descongelación.

En el Capítulo 4 se empleó la técnica desarrollada en el Capítulo 2 para estudiar los parámetros morfométricos medios, las características morfológicas básicas de los eyaculados y la descripción de las subpoblaciones espermáticas, según la morfometría, presentes en los eyaculados de tres especies diferentes de halcón *(Falco peregrinus brookei, Falco peregrinus peregrinus y Falco rusticolus).* Los resultados apoyan la conclusión de que el pleimorfismo es una de las características de los eyaculados de las aves rapaces ya que en todas las especies se observa un elevado porcentaje de células inmaduras (halcón peregrino brookei 55,50%, halcón peregrino escocés 65,46% y gerifalte 60,66%). El análisis de clusters realizado identificó cuatro subpoblaciones con diferentes características morfométricas. Dichas subpoblaciones se han hallado en las tres especies de halcón en proporciones similares.

SUMMARY

This thesis has been design and structured the basic aspects of avian spermatology and developing cryopreservation protocols y different avian species. These techniques, which are part of the artifitial reproduction techniques or ART, are increasing their importance for improving and preserving varities and species. The developed of these techniques has allowed the growth of captive breeding projects backing the efforts to native species conservation, with an important improvement of the population management subserving the genetic variability. Therefore, apart from the punctual success with some species, these are techniques not completely developed in wild species, in part due to the lack of knowledge about physiology, and biology characteristics of the sperm of these species. The study of the biophysical aspects of gametes and gonadal tissue of the different species is the most effective method for developing of suitable cryopreservation protocols. Moreover in wild species the access to genetic material and viable spermatozoa samples is challenging, due to their anatomy, physiology characteristics, and the short breeding season, so cryopreservation techniques represent an improvement of resources management. For this reason in this work was structured in two phases; the first part, the developing and evaluation of different *post mortem* collection techniques for spermatozoa in a domestic species as a model (Chapter 1). In this chapter two *post mortem* collection techniques, flushing and floating, are compared. Samples obtained by both methods are evaluated measuring concentration and motility parameters by computerized analyses (CASA) by an epifluorescence phase contrast microscope Nikon Eclipse (model 50i) together with the software Sperm Class Analyzer SCA® v.4.0. (Microptic S.L., Barcelona, Spain). We

also analysed the percentages of motile and inmotile spermatozoa and movement quality. Spermatozoa movement and quality were evaluated by the parameters of velocity velocidad curvilínea (VCL) and velocidad rectilínea (VSL). The percentage of immotile sperm, the percentage of sperm showing non-progressive motility and the percentage showing progressive motility (sperm swimming forward quickly in a straight line) were recorded. Sperm movement characteristics - curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF) - were also analysed.

Propidium iodide (PI) and SYBR-14 were used as fluorochromes for the examination of membrane integrity (sperm viability), samples were then examined by epifluorescence microscopy (40×; wavelength 450–490 nm). Sperm showing a green colour (no PI staining) were deemed alive, while red coloured sperm (PI-positive) and red-and-green coloured cells were considered dead (red coloration means that the membrane has been damaged and has lost its function). Better results were accomplished by the flushing method obtaining double concentration (596,5±75,4 millions of espermatozoa/mL/*vas deferent*) than ejaculated samples (622±72,5 millions of espermatozoa/mL) and less urates and blood contamination.

At the same time two extenders were compared Lake-Ravie and Lake 7.1, their effects in the sampels were assessed at 0h, 24h and 48h. Results showed tht refrigeration affects negatively to sperm quality although the extender Lake 7.1 preserved better the sperm quality of refrigerated samples both *post-mortem* and ejaculated.

In the second chapter optimization of staining and morphometric methods was performed. In this case samples from natural ejaculates of two avian species, domestic

rooters and red-legged partridge *(Alectorix rufa),* were evaluated stained with two different staining methods Hemacolor® and aniline blue. Both staining methods were evaluated by optical microscope light (x100, objetivo de inmersión, MOTIC BA 210) and a images' capture and analyses software (MOTIC Spain S.L. Barcelona, Spain). In both species Hemacolor® provided higher percentage of measurable cells (893,7±11,7% en gallos y 71,9±15,3%) and also showed greater repeatability for length and area in roosters and for width in red-legged partridge. Hemacolor® technique might be deemed the more appropriate for computerised avian sperm assessment.

In chapter 3, basic sperm parameters of the natural ejaculate in Golden eagle are described. We also evaluated the protective capacity of the extender Lake-Ravie for the for the refrigeration effects and compared two different cryoprotectants glycerol (11%) and DMA (6%). No significant differences were observed between both cryoprotectants, inboth cases motility and viability decreased defore thawing.

In Chapter 4, the morphometric evaluation technique described in Cahpter 2 was applied to study basic morphometric, morphologic and spermatozoa subpopulations in three different falcon species. Results show that pleimorphy is one of the characteristics of raptors ejaculates as the percentages of immature cells were similarly high in all the species (halcón peregrion brookei 55,50%, halcón peregrino escocés 65,46% y gerifalte 60,66%). Clusters' analyses identified four spermatozoa subpopulations with different morphometric characterictics presents in all the species in similar proportions.

INTRODUCCIÓN GENERAL

Historia de las técnicas de reproducción asistida (TRA) en especies animales.

Las técnicas de reproducción asistida junto con los métodos de criopreservación de germoplasma han sido significativamente implementadas en las últimas décadas tanto en la medicina humana como en la medicina veterinaria. Estas técnicas han supuesto grandes avances en la reproducción animal que han repercutido muy positivamente en la conservación y producción animal. Se han convertido en una parte esencial de la cría de animales de producción, principalmente en vacuno de leche y el ganado lanar, cambiando drásticamente los métodos de selección, predicción genética y mejora de las razas, facilitando la evaluación y selección de los reproductores (Harris, 1998), mejorando los resultados y con un importante retorno económico (Madan, 2005).

La historia de la reproducción asistida podría remontarse hasta mucho antes. Existen antiguas, y no documentadas, historias de origen árabe en las que se cuenta cómo en el siglo XIV se recogía el semen de yeguas que habían sido cubiertas pertenecientes a tribus rivales para inseminar las suyas e igualar así la calidad de los caballos que, entonces, eran esenciales para la defensa y el ataque (Foote, 2002).

Sin embargo, las primeras referencias documentadas sobre el estudio de la espermatología datan del año 1678 cuando Leeuwenhoek observó por primera vez los espermatozoides, a los que denominó animáculos, a través de una lente de aumento fabricada por él mismo (Leeuwenhoek, 1678). A partir de este momento fueron necesarios varios siglos hasta la primera inseminación exitosa documentada, realizada en 1784 por Spallanzani en un perro, que dio lugar al nacimiento de tres cahorros (Walton, 1933). No fue hasta la primera mitad del siglo XX, cuando las técnicas de

inseminación se convirtieron en objeto directo de investigación, primero en Rusia y Japón a través de los trabajos de Ivanoff (1922), y más tarde extendiéndose por toda Europa occidental (Foote, 2002).

A lo largo de la década de los 40, las técnicas de reproducción asistida tomaron un auge importante en los Estados Unidos, favoreciendo su desarrollo e implantación en la producción animal mediante la colaboración directa entre ganaderos e investigadores de la Universidad de Cornell (Salisbury, 1978). Fue entonces cuando se establecieron conceptos cómo la selección de parentales, la evaluación testicular, la recogida, evaluación y procesado del semen e incluso la evaluación de la capacidad fecundante (Foote, 1998). Con el tiempo las técnicas desarrolladas en Estados Unidos fueron extendiéndose a nivel mundial generando un gran impacto en el modelo de producción animal debido a razones obvias tales como las ventajas económicas y las asociadas a la conservación de la variedad genética (Prieto et al., 2014), ya que facilitaron el manejo y la conservación de ejemplares genéticamente valiosos (Comizzolli and Holt, 2014), combatiendo así la pérdida de la diversidad genética.

Una de las claves en el desarrollo de la reproducción asistida ha sido la evaluación espermática que, aún hoy, sigue siendo esencial en el desarrollo de estas técnicas. La evaluación y análisis del volumen del eyaculado, la concentración, la motilidad y la morfología espermática han sido correlacionadas con la capacidad fecundante y la calidad seminal en diferentes especies (Sieme et al., 2004; Muiño et al., 2008; Vicente-Fiel et al., 2013), y es considerada una parte indispensable para el desarrollo de estas técnicas en nuevas especies.

Evolución y aplicación de las técnicas de reproducción asistida (TRA) en animales silvestres y aves.

En la actualidad existe un incremento en el interés general, tanto de la población cómo del ámbito científico, hacia la preservación de especies o razas de animales cuyas poblaciones han disminuido de manera relevante. El empeño en conservar la información genética de dichas especies ha generado la creación de bancos de germoplasma representativos de las mismas, enfocando la atención de los investigadores en la recuperación, evaluación y criopreservación de material espermático *post-mortem* (Holt, 2000; Hishinuma et al., 2003; Martínez-Pastor et al., 2005; Santiago-Moreno et al., 2006; Pukazhenthi et al., 2006). La recogida *post-mortem* de material espermático puede resultar interesante ya que posibilita la obtención de germoplasma en especies domésticas (Hinrichs, 2012; Turri et al., 2013), cinegéticas (Martínez et al., 2008; Pradiee et al., 2014) y de ejemplares de especies silvestres hallados muertos (Gunn et al., 2008; Gañán et al., 2010; Keeley et al., 2012; Santiago-Moreno et al., 2015).

La viabilidad del semen obtenido *post-mortem* está estrechamente relacionada con el tiempo y las condiciones desde la muerte del animal hasta la obtención, análisis y preservación del esperma entre el momento de la muerte y la recogida, procesado y almacenado del esperma (Soler et al., 2003; Soler et al., 2005; Fernández-Santos et al., 2011). La mayoría de los estudios realizados sobre la obtención y descripción de espermatozoides no eyaculados se ha desarrollado en especies de mamíferos. Los resultados obtenidos han revelado que la viabilidad de los espermatozoides epididimarios es sorprendentemente alta incluso después de haber sido congelados y descongelados (Fernandez-Santos et al., 2009).

La evolución del desarrollo de las tecnologías reproductivas en aves se inició con el uso industrial de la inseminación artificial en pavos a mediados del siglo pasado (Cooper and Rowell, 1958), y recientemente se ha empezado a realizar criopreservación de material seminal con fines de conservación de razas autóctonas de gallinas (Blesbois et al., 2007; Santiago-Moreno et al., 2011) y en diferentes especies de aves silvestres amenazadas (Blanco et al., 2000; Blanco et al., 2012). En este sentido cabe destacar los trabajos de Graham en la década de los 70 y 80 sobre diluyentes y almacenamiento de semen mediante la técnica de "ultracooling", que permitía mantener el semen viable durante 24 horas a temperaturas de hasta -45ºC, mediante la disminución del punto crioscópico utilizando crioprotectores (Graham et al., 1982). Tras el establecimiento de la capacidad de crioprotección del glicerol (GLY) en la congelación de semen de gallo (Polge et al., 1949), los trabajos encaminados a la mejora y estandarización de los métodos de criopreservación han sido numerosos, destacándose los desarrollados por Lake y Stewart (1978) y Sexton (1980) como los primeros con resultados realmente exitosos.

La información generada sobre preservación de semen aviar se fundamenta, principalmente, en trabajos realizados con gallinas. De forma general, el proceso de congelación suele determinar una supervivencia, en el mejor de los casos, de un 60% de las células espermáticas (Woelders et al., 2006), aunque sólo el 1,6-19,7% de los espermatozoides preservarán su capacidad fecundante (Wishart, 1982; Tajima et al., 1989). Diferentes factores de carácter medioambiental, como la estación del año en que se realice la recogida seminal y la congelación (Santiago-Moreno et al., 2011a), también pueden incidir en la congelabilidad de las células espermáticas y, por tanto, en la capacidad fecundante tras la descongelación. El porcentaje medio de fertilidad obtenido

con inseminación artificial en gallinas con esperma congelado se sitúa en torno a un 40%, pero con un rango muy variable que oscila del 2,8% al 94%. El GLY y la dimetil-acetamida (DMA) son los agentes crioprotectores más utilizados y que han alcanzado mejores resultados en la criopreservación espermática del gallo (Graham et al., 2007). A diferencia del GLY, la DMA no requiere de un lavado previo a la inseminación artificial, pero como característica negativa se destaca su alta citotoxicidad. Por tanto, es necesario que la inseminación se realice de forma inmediata tras la descongelación.

La utilización de DMA, a concentraciones del 6%-9%, ha permitido obtener tasas de fertilidad altas en distintas especies aviares, incluida la gallina (Tselutin et al., 1999; Chalah et al., 1999; Tai et al., 2001; Santiago-Moreno et al., 2011b). Se requiere de una técnica "rápida" de congelación que, generalmente, implica periodos de equilibrado tan cortos como 1 minuto (Tselutin et al., 1999, Chalah et al., 1999) ó 10 minutos (Tai et al., 2001; Santiago-Moreno et al., 2011b), seguidos de velocidades muy rápidas de congelación: 200ºC/min (Woelders et al., 2006), o hasta de 600ºC/min (semen directamente sumergido en nitrógeno) (Tselutin et al., 1999). Se ha descrito una interacción entre el tipo de almacenamiento (*pellets* o pajuelas) y el crioprotector utilizado. Para el GLY los mejores resultados se obtienen con semen almacenado en pajuelas, mientras que para el DMA, los mejores resultados se han obtenido con el uso de *pellets* (Tselutin et al., 1999).

La extensión en el uso de la criopreservación espermática en aves domésticas determinó importantes avances en la adecuación de diluyentes y almacenamiento seminal a corto plazo, que ha repercutido, con éxito variable, en otras especies silvestres cómo el halcón peregrino *(Falco peregrinus)*, el cernícalo americano *(Falco sparverius)* (Bird et al., 1976), algunas especies de buitres como el buitre leonado *(Gyps fulvus)*

(Madeddu et al., 2009) o el buitre dorsiblanco bengalí *(Gyps bengalensis)* (Umapathy et al., 2005), la grulla *(Grus canadensis)* (Gee et al., 2004), hubaras y en algunas especies de psitácidas (Samour et al., 2004).

En la actualidad, la aplicación de técnicas de reproducción asistida y criopreservación de gametos en rapaces silvestres presenta importantes limitaciones. En primer lugar la marcada estacionalidad en su comportamiento reproductor, con un breve periodo reproductivo de entre 30-120 días (Blanco et al., 2002) que conlleva un periodo igualmente breve para la obtención, procesado y almacenamiento de muestras (Birkhead and Fletcher, 1995). Otro factor determinante es el estrés que sufren estas especies, asociado al manejo, cuando se realiza la recogida de semen *in vivo*. Se han descrito varias técnicas de obtención de semen *in vivo*. En primer lugar, la cópula voluntaria de aves sexualmente improntadas, ampliamente utilizada en cetrería (Hammerstrom, 1970). Consiste en trabajar con animales que copulan voluntariamente sobre sombreros adaptados, cimbeles o sobre el propio cuidador. Al trabajar con animales sexualmente improntados disminuye considerablemente el estrés del individuo y permite la obtención de muestras de gran calidad y sin contaminación por orina (Blanco et al., 2009). Otros métodos empleados son la electro-eyaculación y el masaje abdominal, estos métodos requieren un mayor manejo de las aves e incluso la utilización de anestesia y con frecuencia el semen se encuentra contaminado.

En los últimos años, se han descrito métodos de obtención de espermatozoides *post-mortem* en aves, aunque sin buenos resultados en cuanto a calidad espermática (Gunn et al., 2008). Si bien las técnicas de obtención de muestras espermáticas epididimarias en mamíferos están bien desarrolladas, en las aves todavía se encuentra en una fase inicial.

Caracterización gamética en especies silvestres.

El estudio morfo-funcional del espermatozoide, unido a la criobiología espermática, representan pilares básicos del conocimiento para una óptima implementación de técnicas de reproducción asistida, como la inseminación artificial. La espermatología comparada en distintas especies de aves silvestres, facilita el conocimiento de procesos fisiológicos como el ciclo del epitelio seminífero y la espermatogénesis, y la influencia que sobre esta última pueden ejercer diferentes factores medioambientales, tanto naturales (ej. fotoperiodo) como artificiales (pesticidas, contaminantes atmosféricos, etc.) (Kumar and Holt, 2014). Esto determina que las líneas germinales espermáticas hayan sido propuestas como útiles marcadores biológicos de los efectos medioambientales y del cambio climático.

La morfometría espermática ha sido empleada durante mucho tiempo para evaluar la calidad y la viabilidad de los espermatozoides en diferentes especies cómo la caprina (Gravance et al., 1995), la equina (Ball and Mohammed, 1995; Brito, 2007), la bovina (Sekoni and Gustafsson, 1987) e incluso la humana (Soler et al., 2003). Las técnicas convencionales de evaluación de la morfología y la morfometría espermáticas se han basado en análisis subjetivos de diferentes parámetros (Baker and Clarke, 1987; Verstegen et al., 2002). Esta subjetividad ha condicionado la rigurosidad de los resultados ya que existen diferencias evidentes entre laboratorios y técnicos que desarrollan dicho análisis (Ombelet et al., 1997; Boersma and Braun, 1999; Cooper, 1999). Actualmente existen diferentes sistemas automáticos de evaluación espermática con la suficiente precisión y exactitud en la valoración de los parámetros seminales como para considerarse una herramienta fundamental en la evaluación de la calidad de una muestra dada. Estos sistemas se denominan sistemas ASMA (*Automated Sperm*

Morphometry Analysis) (Gravance et al., 1996; Esteso et al., 2003, 2015). Estos sistemas de análisis computerizados han superado la subjetividad de los métodos visuales tradicionales (Hidalgo et al., 2006). El uso de estos métodos objetivos de evaluación se ha ido estandarizando para su utilización en una gran variedad de especies (Sancho et al., 1998; Vicente-Fiel et al., 2013). El proceso de estandarización de estas técnicas incluye los métodos de obtención y tinción de las muestras más adecuadas para asegurar la precisión, objetividad y repetitividad del método (Davis and Gravance, 1993; García-Herreros et al., 2006; Esteso et al., 2015).

Estas técnicas se han validado en diferentes especies, pero es en mamíferos en los que la forma y el tamaño de la cabeza espermática han sido directamente relacionados con la capacidad fecundante (Sekoni and Gustafsson; 1987; Chandler et al., 1988). En especies de peces, como la anguila, se ha empleado como una herramienta para evaluar los efectos de los diferentes métodos de criopreservación (Asturiano et al., 2007). Sin embargo en aves, el empleo de las técnicas computerizadas es prácticamente inexistente, no está estandarizado y necesita ser desarrollado desde el inicio. Las características morfométricas de los espermatozoides aviares han hecho que el uso de los sistemas ASMA sea más complicado que en otras especies. Este sistema de análisis de imagen no reconoce la forma filiforme de su cabeza espermática con suficiente exactitud como para arrojar datos exactos sobre las medidas y características morfométricas.

Los parámetros morfológicos del esperma aviar han sido muy estudiados en diferentes especies. Existen diversos estudios en los que se detallan aspectos morfológicos, tanto normales como anormales, de los espermatozoides aviares

evaluados con microscopía óptica y electrónica (Bakst and Howarth, 1975; Bakst, 1987; Tabatabaei et al., 2009; du Plessis and Soley, 2014). En dichos estudios la morfología de la cabeza espermática es considerada como uno de los factores de mayor importancia en la evaluación de la calidad espermática, aunque lo abordan desde una perspectiva descriptiva sin llegar a establecer rangos en las diferentes dimensiones de la misma. Tan solo en gallos existe bibliografía donde se describe detalladamente la morfología normal y anormal de los espermatozoides evaluados a través de microscopía electrónica (Grigg, et al, 1949) pero no hay apenas resultados sobre los aspectos morfológicos del esperma aviar avaluados mediante microscopía óptica con excepción de la perdiz roja, especie en la que se ha realizado una caracterización de la morfometría de la cabeza espermática en muestras teñidas con azul de anilina (Santiago-Moreno et al., 2015). En dicho estudio se empleó la evaluación morfométrica para evaluar los cambios asociados a la estación y valorar las variaciones relacionados con la pureza genética.

En otras especies de aves silvestres, como las falconiformes, los estudios mofo-funcionales espermáticos son escasos e insuficientes para afrontar, con ciertas garantías de éxito, la criopreservación de espermatozoides y su aplicación mediante técnicas de reproducción asistida.

El presente trabajo surge de la necesidad de disponer de técnicas para la obtención y almacenamiento de material espermático en situaciones *post-mortem*, y la estandarización de un método computerizado de análisis morfométrico de espermatozoides aviares que supongan un pilar básico para profundizar en la espermatología de estas especies, con especial referencia a falconiformes, y para la

aplicación en programas de conservación que impliquen el desarrollo de bancos de germoplasma.

REFERENCIAS

Asturiano, J.F., Marco-Jiménez, F., Peñaranda, D.S., Garzón, D.L., Pérez, L., Vicente, J.S., Jover, M., 2007. Effect of sperm cryopreservation on the European eel sperm viability and spermatozoa morphology. Reprod. Domest. Anim. 42, 162–166.

Baker, H.W., Clarke, G.N., 1987. Sperm morphology: consistency of assessment of the same sperm by different observers. Clin. Reprod. Fertil. 5, 37–43.

Bakst, M.R., Howarth, B., 1975. The head, neck and midpiece of cock spermatozoa examined with the transmission electron microscope. Biol. Reprod. 12, 632–640.

Bakst, M.R., 1987. Anatomical basis of sperm-storage in the avian oviduct. Scanning Microsc. 1, 1257–1266.

Ball, B.A., Mohammed, H.O., 1995. Morphometry of stallion spermatozoa by computer-assisted image analysis. Theriogenology 44, 367–377.

Bird, D.M, Lagüe, P.C., Buckland, R.B., 1976. Artificial insemination vs natural mating in American kestrel. Can. J. Zool. 58, 1183-1191.

Birkhead, T. R. 1995. Sperm competition: Evolutionary causes and consequences. Reprod. Fert. Dev. 7, 755-775.

Birkhead, T.R., Fletcher, F., 1995. Male phenotype and ejaculate quality in the zebra finch *Taeniopygia guttata*. Proc. Biol. Sci. 262, 329–334.

Blanco, J.M., Gee, G., Wildt, D.E., Donoghue, A.M., 2000. Species variation in osmotic, cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon spermatozoa. Biol. Reprod. 63, 1164-1171.

Blanco, J.M., Gee, G.F., Wildt, D.E., Donoghue, A.M., 2002. Producing progeny from endangered birds of prey: treatment of urine-contaminated semen and a novel intramagnal insemination approach. J Zoo Wildl Med. Mar; 33(1), 1-7.

Blanco, J.M., Long, J.A., Gee, G., Wildt, D.E., Donoghue, A.M., 2012. Comparative cryopreservation of avian spermatozoa: Effects of freezing and thawing rates on turkey and sandhill crane sperm cryosurvival. Anim. Reprod. Sci. 131(1-2), 1-8.

Blesbois, E., Seigneurin, F., Grasseau, I., Limouzin, C., Besnard, J., Gourichon, D., Coquerelle, G., Rault, P., Tixier-Boichard, M., 2007. Semen cryopreservation for ex situ management of genetic diversity in chicken: creation of the French avian cryobank. Poult. Sci. 86, 555–564.

Boersma, A., Braun, J., 1999. Computer-assisted analysis of sperm morphology in veterinary medicine. Berl. Munch. Tierarztl. Wochenschr. 112, 81–85.

Brito, L.F.C., 2007. Evaluation of Stallion Sperm Morphology. Clin. Tech. Equine Pract. 6, 249–264.

Chandler, J.E., Painter, C.L., Adkison, R.W., Memon, M.A., Hoyt, P.G., 1988. Semen quality characteristics of dairy goats. J. Dairy Sci. 71, 1638–1646.

Comizzolli, P., Holt, W. V., 2014. Reproductive Science in Animal Conservation, in: Reproductive Science in Animal Conservation. pp. 331–356.

Cooper, D., Rowell, J., 1958. Relations between fertility, embryonic survival and some semen characteristics in the chicken. Poult. Sci.37, 699-707

Cooper, T.G., 1999. Experience with external quality control in spermatology. Hum. Reprod. 14, 765–769.

Davis, R.O., Gravance, C.G., 1993. Standardization of specimen preparation, staining, and sampling methods improves automated sperm-head morphometry analysis. Fertil. Steril. 59, 412–417.

du Plessis, L., Soley, J.T., 2014. Light microscopic features and morphometry of sperm in the emu *(Dromaius novaehollandiae)*. Theriogenology 81, 203–209.

Esteso, M.C., Rodríguez, E., Toledano-Díaz, A., Castaño, C., Pradiee, J., López-Sebastián, A., Santiago-Moreno, J., 2015. Descriptive analysis of sperm head morphometry in Iberian ibex *(Capra pyrenaica)*: Optimum sampling procedure and staining methods using Sperm-Class Analyzer®. Anim. Reprod. Sci. 155, 42-49.

Esteso, M.C., Fernández-Santos, M.R., Soler, A.J., Montoro, V., Martínez-Pastor, F., Garde, J.J., 2009. Identification of sperm-head morphometric subpopulations in iberian red deer epididymal sperm samples. Reprod. Domest. Anim. 44, 206–211.

Esteso, MC, Fernández-Santos, MR, Soler, AJ, Garde, J.J., 2003. Head dimensions of cryopreserved red deer spermatozoa are affected by thawing procedure. Cryoletters 24, 261–268.

Fernández-Santos, M.R., Soler, A.J., Ramón, M., Ros-Santaella, J.L., Maroto-Morales, A., García-Álvarez, O., Bisbal, A., Garde, J.J., Coloma, M.A., Santiago-Moreno, J., 2011. Effect of post-mortem time on post-thaw characteristics of Spanish ibex *(Capra pyrenaica)* spermatozoa. Anim. Reprod. Sci. 129, 56-66.

Foote, R. H. 1998. Artificial Insemination to Cloning: Tracing 50 Years of Research. Published by the author, Ithaca, New York.

Foote, R., 2002. The history of artificial insemination: Selected notes and notables. J. Anim. Sci.80, 1-10.

Gañán, N., Sestelo, A., Garde, J.J., Martínez, F., Vargas, A., Sánchez, I., Pérez-Aspa, M.J., López-Bao, J.V., Palomares, F., Gomendio, M., Roldan, E.R.S., 2010. Reproductive traits in captive and free-ranging males of the critically endangered Iberian lynx *(Lynx pardinus)*. Reproduction 139, 275–285.

García-Herreros, M., Aparicio, I.M., Barón, F.J., García-Marín, L.J., Gil, M.C., 2006. Standardization of sample preparation, staining and sampling methods for automated sperm head morphometry analysis of boar spermatozoa. Int. J. Androl. 29, 553–563.

Gee, G.F., Bertschinger, H., Donoghue, A.M., Blanco, J., Soley, J., 2004. Reproduction in nondomestic birds: physiology, semen collection, artificial insemination and cryopreservation. Avian Poult. Biol. Rev. 15, 47-101.

Graham, E.F., Nelson, D.S., Schmehl, M.K., 1982. Development of extender and techniques for frozen turkey semen. 2. Fertility trials. Poult. Sci. 61, 558–63.

Gravance, C.., Lewis, K.M., Casey, P.J., 1995. Computer automated sperm head morphometry analysis (ASMA) of goat spermatozoa. Theriogenology 44, 989–1002.

Gravance, G.G., Vishwanath, R., Pitt, C., Casey, P.J., 1996. Computer automated morphometric analysis of bull sperm heads. Theriogenology 46, 1205–15.

Grigg G.W., A.J., Hodge, 1949. Electron microscopic studies of spermatozoa: I. The morphology of the spermatozoon of the common domestic fowl *(Gallus domesticus)*. Aust J Sci Res Ser B. 2, 271–286.

Gunn, M.R., Champion, Z., Casey, M.E., Teal, P., Casey, P.J., 2008. Testicular and spermatozoan parameters in the pukeko (*Porphyrio porphyrio melanotus*). Anim. Reprod. Sci. 109, 330–342.

Harris, D.L., 1998. Livestock improvement: art, science, or industry? J. Anim. Sci. 76, 2294–2302.

Hidalgo, M., Rodríguez, I., Dorado, J., 2006. Influence of staining and sampling procedures on goat sperm morphometry using the Sperm Class Analyzer. Theriogenology 66, 996–1003.

Hinrichs, K., 2012. Assisted reproduction techniques in the horse. Reprod. Fertil. Dev. 25, 80–93.

Hishinuma, M., Suzuki, K., Sekine, J., 2003. Recovery and cryopreservation of sika deer *(Cervus nippon)* spermatozoa from epididymides stored at 4 °C. Theriogenology 59, 813–820.

Holt, W. V., 2000: Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53, 47-58.

Ivanoff, E. I. 1922. On the use of artificial insemination for zootechnical purposes in Russia. J. Agric. Sci. 12:244–256.

Keeley, T., McGreevy, P.D., O'Brien, J.K., 2012. Cryopreservation of epididymal sperm collected post-mortem in the Tasmanian devil *(Sarcophilus harrisii)*. Theriogenology 78, 315–325.

Kumar, E., Holt, W.V., 2014. Impacts of endocrine disrupting chemicals on reproduction in wildlife. Adv. Exp. Med. Biol. 753, 55–70.

Lake, P.E., Stewart, J.M., 1978. Preservation of fowl semen in liquid nitrogen--an improved method. Br. Poult. Sci. 19, 187–194.

Leeuwenhoek, A. 1678. De natis e` semine genitali animalculis. R. Soc. (Lond.) Philos. Trans. 12, 1040–1043.

Madan, M.L., 2005. Animal biotechnology: applications and economic implications in developing countries. Rev. Sci. Tech. 24, 127–139.

Madeddu, M., Berlinguer, F., Ledda, M., Leoni, G.G., Satta, V., Succu, S., Rotta, A., Pasciu, V., Zinellu, A., Muzzeddu, M., Carru, C., Naitana, S., 2009. Ejaculate collection efficiency and post-thaw semen quality in wild-caught Griffon vultures from the Sardinian population. Reprod. Biol. Endocrinol. 7, 18.

Martínez, A.F., Martínez-Pastor, F., Álvarez, M., Fernández-Santos, M.R., Esteso, M.C., de Paz, P., Garde, J.J., Anel, L., 2008. Sperm parameters on Iberian red deer: Electroejaculation and post-mortem collection. Theriogenology 70, 216–226.

Martinez-Pastor, F., Guerra, C., Kaabi, M., Diaz, A.R., Anel, E., Herraez, P., de Paz, P., Anel, L., 2005. Decay of sperm obtained from epididymes of wild ruminants depending on post-mortem time. Theriogenology 63, 24–40.

Muiño, R., Tamargo, C., Hidalgo, C.O., Peña, A.I., 2008. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: effects of cryopreservation and between-bull variation. Anim. Reprod. Sci. 109, 27-39.

Ombelet, W., Pollet, H., Bosmans, E., Vereecken, A., 1997. Results of a questionnaire on sperm morphology assessment. Hum. Reprod. 12, 1015–1020.

Polge, C., Smith A.U., Parkes A.S., 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature (Lond.) 164:666.

Pradiee, J., Esteso, M.C., Castaño, C., Toledano-Díaz, A., López-Sebastián, A., Santiago-Moreno, J., 2014. Cryopreservation of epididymal sperm from ibexes *(Capra pyrenaica)* using short equilibration time with glycerol. Theriogenology 82, 525-528.

Prieto, M.T., Sánchez-Calabuig, M.J., Hildebrandt, T.B., Santiago-Moreno, J., Saragusty, J., 2014. Sperm cryopreservation in wild animals. Eur. J. Wildl. Res. 60, 851–864.

Pukazhenthi, B., Comizzoli, P., Travis, A.J., Wildt, D.E., 2006. Applications of emerging technologies to the study and conservation of threatened and endangered species. Reprod. Fertil. Dev. 18, 77–90.

Tabatabaei, S., Batavani, R.A., Talebi, A.R., 2009. Comparison of semen quality in indigenous and Ross broiler breeder roosters. J. Anim. Vet. Adv. 8, 90–93.

Salisbury, G. W., VanDemark, N.L., Lodge J.R., 1978. Physiology of Reproduction and Artificial Insemination of Cattle. 2nd ed. W. H. Freeman Co., San Francisco.

Samour, J.H., 2004. Semen Collection, Spermatozoa Cryopreservation, and Artificial Insemination in Nondomestic Birds. J. Avian Med. Surg. 18(4), 219-223.

Sancho, M., Pérez-Sánchez, F., Tablado, L., de Monserrat, J., Soler, C., 1998. Computer assisted morphometric analysis of ram sperm heads: Evaluation of different fixative techniques. Theriogenology 50, 27-37.

Santiago-Moreno, J., Castaño, C., Toledano-Díaz, A., Coloma, M.A., López-Sebastián, A., Prieto, M.T., Campo, J.L., 2011. Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: optimization of freezing rate and equilibration time. Poult. Sci. 90, 2047-2053.

Santiago-Moreno, J., Castaño, C., Toledano-Díaz, A., Esteso, M.C., López-Sebastián, A., Gañán, N., Hierro, M.J., Marchal, F., Campo, J.L., Blesbois, E., 2015. Characterization of red-legged partridge *(Alectoris rufa)* sperm: Seasonal changes and influence of genetic purity. Poult. Sci. 94, 80-87.

Sekoni, V.O., Gustafsson, B.K., 1987. Seasonal variations in the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial insemination. Br. Vet. J. 143, 312-317.

Sieme, H., Katila, T., Klug, E., 2004. Effect of semen collection practices on sperm characteristics before and after storage and on fertility of stallions. Theriogenology 61, 769-784.

Soler, C., de Monserrat, J.J., Gutierrez, R., Nunez, J., Nunez, M., Sancho, M., Perez-Sanchez, F., Cooper, T.G., 2003. Use of the Sperm-Class Analyser® for objective assessment of human sperm morphology. Int. J. Androl. 26, 262-270.

Soler, C., Gadea, B., Soler, A.J., Fernández-Santos, M.R., Esteso, M.C., Núñez, J., Moreira, P.N., Núñez, M., Gutiérrez, R., Sancho, M., Garde, J.J., 2005. Comparison of three different staining methods for the assessment of epididymal red deer sperm morphometry by computerized analysis with ISAS®. Theriogenology 64, 1236-1243.

Tajima, A., Graham, E.F., Hawkins, D.M., 1989. Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermic competition method. J. Reprod. Fertil. 85, 1–5.

Tselutin, K., Seigneurin, F., Blesbois, E., 1999. Comparison of cryoprotectants and methods of cryopreservation of fowl spermatozoa. Poult. Sci., 78, 586-590.

Turri, F., Madeddu, M., Gliozzi, T.M., Gandini, G., Pizzi, F., 2013. Effect of testicle post-mortem storage on goat frozen-thawed epididymal sperm quality as a tool to improve genebanking in local breeds. Animal 8(3), 440-447.

Umapathy, G., Sontakke, S., Reddy, A., Ahmed, S., Shivaji, S., 2005. Semen characteristics of the captive Indian white-backed vulture (*Gyps bengalensis*). Biol. Reprod. 73, 1039-1045.

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

Vicente-Fiel, S., Palacín, I., Santolaria, P., Hidalgo, C.O., Silvestre, M.A., Arrebola, F., Yániz, J.L., 2013. A comparative study of the sperm nuclear morphometry in cattle, goat, sheep, and pigs using a new computer-assisted method (CASMA-F). Theriogenology 79, 436–442.

Walton, A. 1933. The Technique of Artificial Insemination. Imperial Bureau Anim. Genetics. Oliver and Boyd, Edinburgh.

Woelders, H., Zuidberg, C.A., Hiemstra, S.J., 2006. Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. Poult. Sci. 85, 216-22.

Wishart, G., 1982. Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa in vitro. J. Reprod. Fertil. 66, 457-462.

OBJETIVOS

El objetivo general del presente trabajo es el desarrollo y puesta a punto de una serie de técnicas que permitan la recogida *post-mortem* y almacenamiento de células espermáticas para su caracterización gamética en distintas especies de aves, y aplicación como marcadores biológicos de influencias medioambientales (ej. cambio climático, pesticidas, metales pesados, etc.), o para programas de conservación (criopreservación en bancos de recursos zoogenéticos) en aves amenazadas.

Los objetivos específicos son:

1. Desarrollo de un método eficaz de recogida *post-mortem* de espermatozoides en aves.

2. Comparación de la eficacia de diferentes diluyentes para el almacenamiento de espermatozoides en condiciones de refrigeración.

3. Evaluación de diferentes técnicas de tinción para la estandarización del análisis computerizado morfométrico de espermatozoides aviares.

4. Aplicación de técnicas de evaluación morfométrica para la caracterización gamética en eyaculados de falconiformes (águila real y halcones).

5. Determinación de la influencia de la criopreservación espermática en el tamaño de la cabeza del espermatozoide y en la existencia y distribución de subpoblaciones espermáticas en halcones.

CAPÍTULO 1

Influence of post mortem sperm recovery method and extender on fresh and chilled rooster sperm variables

S. Villaverde-Morcillo, M.C. Esteso, C. Castaño, J. Santiago-Moreno**. Influence of post mortem sperm recovery method and extender on fresh and chilled rooster sperm variables.** Reproduction in Domestic Animal. Manuscript ID: RDA-OA-Jul-2015-0276.R2

Influencia del método y del diluyente en las variables espermáticas en muestras frescas y refrigeradas de semen de gallo obtenido post-mortem.

RESUMEN

Varios tipos de técnicas de obtención de espermatozoides *post-mortem* han sido descritas en mamíferos, sin embargo su uso en aves es poco común. Este artículo compara la eficacia de dos técnicas de recogida *post-mortem* en gallos, lavado y flotación, y se evalúa la eficacia de dos diluyentes, Lake-Ravie y Lake 7.1. Con el objetivo de determinar si las capacidades protectoras frente a la refrigeración de estos diluyentes son diferentes para semen eyaculado u obtenido *post-mortem* se analizaron muestras de semen eyaculado, obtenido mediante masaje, diluidos en ambos diluyentes. Las variables de las muestras, tanto de los eyaculados cómo de las muestras *post-mortem* fueron evaluadas inmediatamente a temperatura ambiente (0h), y tras la refrigeración a 5ºC durante 24 h y 48 h. Mediante el método de lavado del conducto deferente se obtuvieron más espermatozoides que mediante el método de flotación (596,5 ± 75,4 x 10^6 espermatozoides/ml *vs*. 341,0 ± 87,6 x 10^6 espermatozoides/ml; $P<0,05$); incluso el número de espermatozoides recuperados por el primer método fue similar al número de espermatozoides obtenido en el eyaculado por el método de masaje (630,3 ± 78,2 x 10^6 espermatozoides/ml). Para todas las muestras, obtenidas en cualquiera de los métodos descritos, el diluyente Lake-Ravie proporcionó mejores valores en las variables de motilidad espermática a las 0h. Sin embargo, en las muestras refrigeradas fue el diluyente Lake 7.1. el que proporcionó mejor viabilidad espermática y presentó mayor capacidad protectora ($P<0,05$) con respecto a la mayoría de variables de motilidad. En conclusión, el método de lavado se considera el más adecuado para la

obtención de espermatozoides *post-mortem* en aves. En caso de que las muestras requieran ser refrigeradas a 5ºC hasta su análisis, el diluyente Lake 7.1. es el diluyente recomendado.

Palabras clave: Esperma aviar; diluyentes; Post-mortem; Almacenamiento espermático

ABSTRACT

Many post mortem sperm collection techniques have been described for mammalian species, but their use in birds is scarce. This paper compares the efficacy of two post mortem sperm retrieval techniques - the flushing and float-out methods - in the collection of rooster sperm, in conjunction with the use of two extenders, i.e., Lake-Ravie medium and Lake 7.1 medium. To determine whether the protective effects of these extenders against chilling are different for post mortem and ejaculated sperm, pooled ejaculated samples (procured via the massage technique) were also diluted in the above extenders. Post mortem and ejaculated sperm variables were assessed immediately at room temperature (0 h), and after refrigeration at 5ºC for 24 h and 48 h. The flushing method retrieved more sperm than the float-out method (596.5 ± 75.4 million sperm/ml *vs.* 341.0 ± 87.6 million sperm/ml; $P<0.05$); indeed, the number retrieved by the former method was similar to that obtained by massage-induced ejaculation (630.3 ± 78.2 million sperm/ml). For sperm collected by all methods, the Lake-Ravie medium provided an advantage in terms of sperm motility variables at 0 h. In the chilled sperm samples, however, the Lake 7.1 medium was associated with higher percentages of viable sperm, and had a greater protective effect ($P<0.05$) with respect to most motility variables. In conclusion, the flushing method is recommended for collecting sperm from dead birds. If this sperm needs to be refrigerated at 5ºC until analysis, Lake 7.1 medium is recommended as an extender.

Keywords: Avian sperm; Diluents; Post-mortem; Sperm storage.

INTRODUCTION

Germplasm preservation and assisted reproduction techniques are of interest in the conservation and economic exploitation of many species (Prieto et al., 2014); they facilitate the management and conservation of genetically valuable individuals (Comizzolli and Holt, 2014) and help prevent the decline of genetic diversity. Several works on the germplasm banking of sperm from endangered mammalian species have been particularly focused on the recovery, assessment and cryopreservation of epididymal sperm retrieved post mortem (Hishinuma et al., 2003; Martínez-Pastor et al., 2005; Santiago-Moreno et al., 2006). The viability of such gametes depends on the time elapsed between the death of the donor animal and sperm collection and processing (Fernández-Santos et al., 2011). Most of the research performed on the collection and assessment of non-ejaculated sperm has involved that of mammalian species, in which the viability of epididymal sperm is surprisingly high, even after cryopreservation (Soler et al., 2005). However, very little such work has been performed in birds.

Germplasm banks for endangered avian species are still being set up. Unfortunately, sperm collection from birds is not without its difficulties, and our knowledge regarding avian spermatology and sperm cryopreservation needs to improve. Assisted reproduction techniques have been used with variable success in certain wild species such as the peregrine falcon (*Falco peregrinus*), the American kestrel (*Falco sparverius*) (Bird et al., 1976), the Griffon vulture (*Gyps fulvus*) (Madeddu et al., 2009) and Indian white-backed vulture (*Gyps bengalensis*) (Umapathy et al., 2005), cranes (*Grus Canadensis*) (Sharlin et al., 1979; Blanco et al., 2012), Canada geese (*Branta canadensis*) (Gee et al., 2004), houbaras (*Chlamydotis undulata*) and a number of psittacine species (Samour, 2004). None of these studies involved the use of post

mortem-collected sperm; ejaculated and cryopreserved sperm was always used. Sperm from dead birds, could, however, be a useful resource when trying to set up germplasm banks. It might also provide useful information regarding the physiological reproductive status of the donor, and could even be used for examining the impact of environmental pollutants on reproductive function (Kumar and Holt, 2014).

In birds, sperm motility in the testes is minimal. It increases somewhat in the rudimentary epididymis, but only reaches a maximum when in the vas deferens (Munro, 1935, 1938). In roosters (*Gallus gallus domesticus*), post mortem sperm recovery has been performed by squeezing the vas deferens using a forceps (Ahammad et al., 2011), while in the Japanese quail (*Coturnix coturnix*) it has been achieved via the homogenisation of sections of the vas deferens in a sucrose solution (Clulow and Jones, 1982). Gunn et al. (2008) described and compared different techniques for post mortem sperm collection in wild pukekos (*Porphirio porphirio*), although none of the cells obtained was motile. It was suggested that either pukeko sperm in the vas deferens are not motile, become activated only after ejaculation, or that those retrieved were simply dead (these authors did not evaluate sperm viability or extender effectiveness).

The choice of extender can have a great impact on sperm quality - almost as much as the retrieval method. Moreover, since there is often a delay between sperm recovery and sperm analysis and/or freezing (e.g., when the laboratory is a long way from the place of semen collection), the use of an adequate extender becomes a priority. It may be that extenders commonly used with ejaculated avian sperm might also be usable with post mortem-collected sperm.

The aim of the present work was to assess the efficacy of two recovery techniques in conjunction with two extenders in post mortem rooster sperm collection and storage.

The protective effect of these extenders on post mortem-collected and ejaculated rooster sperm was also compared.

MATERIAL AND METHODS

Experimental birds

The birds used in this study were of two Spanish chicken breeds (White Prat and Black-Red Andaluza) housed at the El Encín Research Station (Madrid, Spain, 40º31'N). All birds were 2 years old. Procedures were performed using sperm obtained post mortem from the vas deferens of 30 adult roosters humanely euthanized by cervical dislocation during the breeding season, or using ejaculated sperm obtained from 12 live roosters. All handling procedures were approved by the INIA Ethics Committee (reference number CEEA 2011/017) and performed in accordance with the Spanish Policy for Animal Protection RD1201/2005, which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

Experimental design

In the first part of the study, a 2x2 factorial design involving the two post mortem sperm collection methods (flushing and float-out) and the two extenders (the Lake-Ravie and Lake 7.1 media) was followed. Thirty roosters were randomly assigned to have their sperm diluted with one or the other extender (n=15 for each extender). For each bird, sperm was collected from the vas deferens of one testis via the flushing method, and from the other by the float-out method (Fig. 1). Following sperm collection, sperm variables were assessed at room temperature (0 h) and then after refrigeration at 5ºC for 24 h and 48 h.

To determine whether the protective effect of the extenders differed depending on whether the sperm was post mortem-collected or ejaculated, the pooled ejaculated samples were divided into two aliquots and diluted 1:1 (V:V) with Lake-Ravie or Lake 7.1 medium. The sperm variables were then similarly assessed at room temperature (0 h) and after refrigeration at 5ºC for 24 h and 48 h.

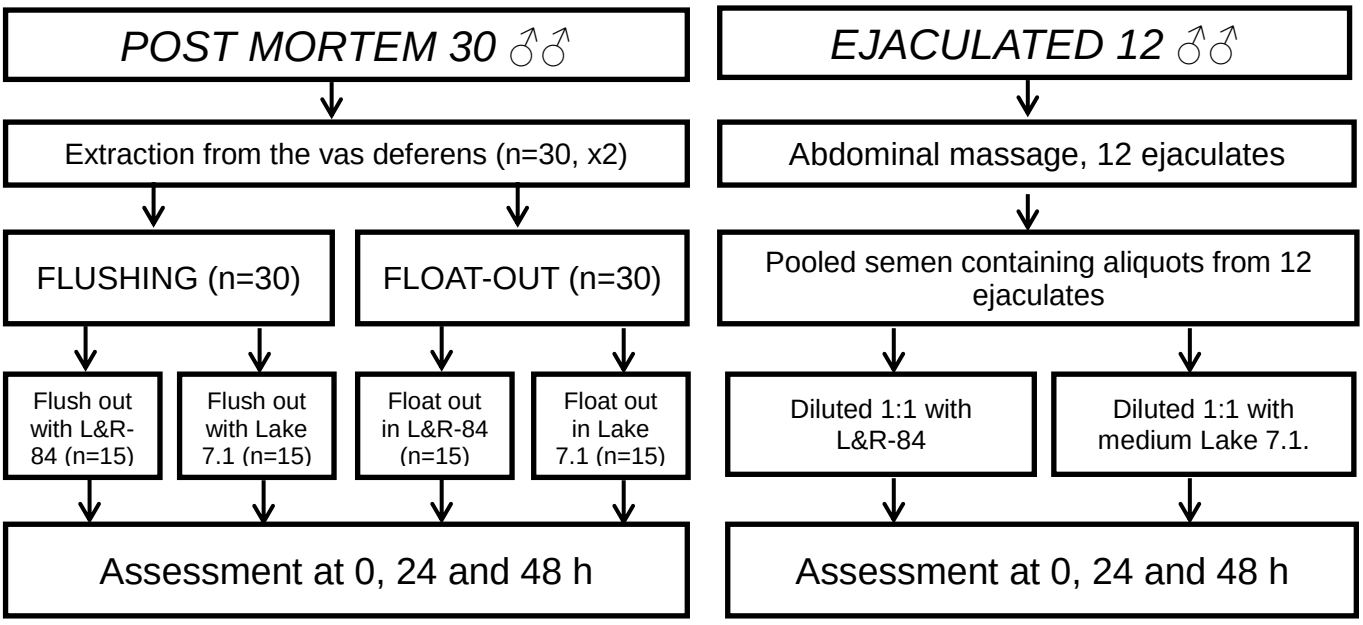

Fig. 1. Experimental design: Procedures were performed using sperm obtained post mortem from the vas deferens of 30 adult roosters or using ejaculated sperm obtained from 12 live roosters. Following sperm collection, sperm variables were assessed at room temperature (0 h) and then after refrigeration at 5ºC for 24 h and 48 h.

Sperm collection

Extenders: Two different extenders were used to retrieve the sperm by all the above methods and to dilute the ejaculates: 1) Lake and Ravie medium (Lake and Ravie, 1984), composed of sodium glutamate (1.92 g; 0.1 M), glucose (0.8 g; 0.04 M), magnesium acetate $4H_2O$ (0.08 g; 0.004 M), potassium acetate (0.5 g; 0.05 M), polyvinyl pyrrolidone (M_r10 000; 0.3 g; 0.0003 M) and 100 mL H_2O (343 mOsm/kg, pH 7.08); and 2) Lake 7.1 medium, composed of magnesium acetate tetrahydrate $4H_2O$ (0.08 g; 0.004 M), potassium citrate tribasic monohydrate (0.128 g; 0.004 M), sodium-L-glutamate (1.52 g; 0.08 M), glucose (0.6 g; 0.03 M), BES (3.05 g; 0.14 M), 5.8 ml of 1N sodium hydroxide, and 100 ml H_2O (370 mOsm/kg, pH = 7.1) (Lake and Stewart, 1978). Both diluents were prepared in the laboratory using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co.

Post mortem sperm collection: Testes with their corresponding vas deferens were collected within 1 h of euthanisation by opening the carcass along the mid line. All the viscera were removed and the testes extracted together with the ureters and renal vasculature, thus avoiding damage to the vas deferens. The ureter and renal vasculature were carefully dissected out to minimize blood and urine contamination of the sperm sample. Once the vas deferens was isolated from the testes and adjacent tissues, sperm collection was performed either by the flushing or float-out method.

Flushing method: Samples were obtained by injecting 1.5 mL of an extender (see below) at 38ºC into the proximal extreme of the vas deferens using a 27G needle attached to a 2 mL syringe. The entire volume of the extender and the collected sperm

was placed in a sterile plastic Petri dish (Sterilin®, Sterilin Ltd., Newport, UK), from which it was transferred to a *polystyrene* tube (2 mL). The samples were then incubated at 38ºC for 15 min.

Float-out method: The vas deferens was cut into 0.5 cm-long pieces which were submerged in 1.5 mL of an extender (see below) at 38ºC in a 2 ml tube. These samples were then incubated at 38ºC for 15 min.

Collection of ejaculated sperm: Ejaculated sperm samples from live birds were obtained using the massage technique of Burrows and Quinn (1937). This requires the abdominal massage in birds by gripping the base of their copulatory organ after its being made to protrude by mild stimulation. The collected samples were then pooled, diluted 1:1 (V:V) and cooled at 5ºC during the transport to the lab. Once in the lab, samples were incubated at 38ºC during 15 minutes previous their analysis.

Sperm assessment

Sperm concentration and sperm motility were assayed as previously described (Santiago-Moreno et al., 2012) using a computer-aided sperm analysis (CASA) system coupled to a Nikon Eclipse (model 50i) phase contrast microscope (in negative contrast mode), and employing Sperm Class Analyzer SCA® v.4.0. software (Microptic S.L., Barcelona, Spain). The total volume retrieved after flushing or float-out was considered the final sperm volume to calculate sperm concentration. Sperm samples were diluted as required for ease of analysis over the range 1:10 to 1:50 (V:V) in the Lake-Ravie or Lake 7.1 medium, and loaded onto a warmed slide (38ºC). The percentage of immotile

sperm, the percentage of sperm showing non-progressive motility and the percentage showing progressive motility (sperm swimming forward quickly in a straight line) were recorded. Sperm movement characteristics - curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF) - were also analysed.

Propidium iodide (PI) and SYBR-14 were used as fluorochromes for the examination of membrane integrity (sperm viability), counting 200 cells (Chalah and Brillard, 1998). For this, 4 µl of SYBR-14 and 10 µl of semen sample were placed in an Eppendorf tube containing 200 µl of HEPES medium (20 mM Hepes, 197 mM NaCl, 2.5 mM KOH, and 10 mM glucose; pH 7.0, osmolality 400 mOsm/kg), and incubated at 5ºC for 10 min in the dark. Two microlitres of PI were then added and the solution incubated for 2 min at 5ºC. The samples were then examined by epifluorescence microscopy (40×; wavelength 450–490 nm). Sperm showing a green colour (no PI staining) were deemed alive, while red coloured sperm (PI-positive) and red-and-green coloured cells were considered dead (red coloration means that the membrane has been damaged and has lost its function).

Statistical analyses

Data were expressed as means ± SE. Sperm variables with non-normal distributions (as determined by the Kolmogorov-Smirnov test) were arcsine-transformed before statistical analysis. The influence of the recovery method and the extender on the sperm variables at different refrigeration times was assessed by repeated-measures ANOVA following the statistical model $x_{ijk} = m + A_i + B_j + AB_{ij} +$

e_{ijk}, where x_{ij} = the value of the measured sperm variable, m = the overall mean, A_i = the effect of the recovery method (i = 1-2; flushing/float-out), B_j = the effect of the extender (j = 1-2; Lake-Ravie/Lake 7.1), AB_{ij} = the interaction between A and B, and e_{ijk} = the residual (k = 1-60). The protective effect of the extender on the post mortem and ejaculated sperm at different refrigeration times was compared by repeated-measures ANOVA following the statistical model $x_{ijk} = m + A_i + B_j + C_k + ABC_{ijk} + e_{ijkl}$, where x_{ij} = the measured sperm variable, m = the overall mean for the variable x, A_i = the effect of sperm origin (i = 1-2; post mortem or ejaculated); B_j= the effect of the extender (j = 1-2; Lake-Ravie/Lake 7.1); AB_{ij} = the interaction between A, B, and e_{ijk} = the residual (l=1-60 for the post-mortem samples and 12 pooled ejaculated samples). Significance was set at $P<0.05$. All calculations were made using Statistica software for Windows v.12 SP3 (StatSoft Inc., Tulsa, OK, USA).

RESULTS

The interaction between recovery methods and sperm diluents didn't exert significant influence on sperm variables. Irrespective of the extender used, significantly more sperm were retrieved by the flushing method than by the float-out method (596.5 ± 75.4 million sperm per vas deferens vs. 341.0 ± 87.6 million sperm per vas deferens; $P<0.05$); indeed, the number was similar to that recorded for the ejaculated sperm (630.3 ± 78.2 million sperm) (Fig. 2).

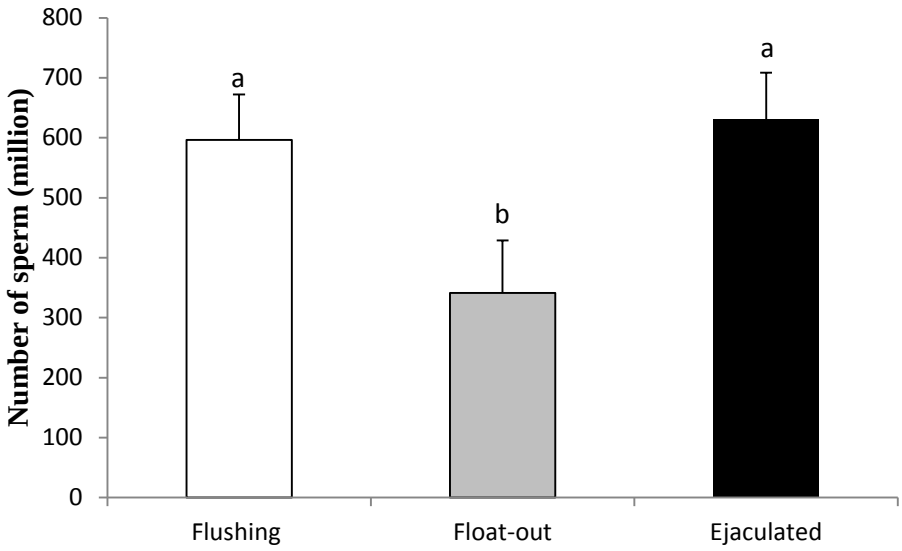

Fig. 2. Number of sperm (mean ± S.E.) collected by the two post mortem recovery methods and by massage-induced ejaculation. Different letters in figure represent significant differences. Values of flushing and float-out are expressed as number of sperm recovered in one vas deferens.

All sperm variables were negatively affected with increasing refrigeration time. No interactions were seen between the method of sperm extraction and the extender used; the effects of both were therefore analysed independently.

Compared to the float-out method, the flushing method more negatively affected the percentage of sperm showing progressive motility, the ALH and the BCF at 0 h, and the percentage showing non-progressive motility at 48 h ($P<0.05$). However, with the flushing method, the percentage of sperm showing progressive motility after 24 h at 5ºC was significantly higher ($P<0.05$), as was the BCF value, than that obtained with the float-out method (Table 1).

Table 1. Influence of post mortem sperm collection technique (flushing and float-out) on sperm variables.

Variable	0 hours		24 hours		48 hours	
	Flushing	**Float-out**	**Flushing**	**Float-out**	**Flushing**	**Float-out**
Concentration (mill sperm/mL)	596.5±75.4 [a]	341± 87.6 [b]	596.5±75.4 [a]	341± 87.6 [b]	596.5±75.4 [a]	341± 87.6 [b]
VS (%)	49.2 ± 13.6[a]	58.5 ± 22.1[a]	41.0 ± 24.5[b]	46.2 ± 27.2[b]	29.2 ± 24.8[c]	36.9 ± 25.2[c]
PM (%)	3.8 ± 7.2[b]	7.3 ± 11.6[a]	2.2 ± 6.2[b]	0.4 ± 0.7[c]	0.1 ± 0.2[c]	1.7 ± 6.8[c]
NPM (%)	24.2 ± 17.3[a]	27.3 ± 17.7[a]	11.6 ± 11.1[b]	11.2 ± 15.6[b]	4.7 ± 4.8[c]	11.9 ± 12.9[b]
IM (%)	69.7 ± 21.5[a]	65.3 ± 27.6[a]	84.8 ± 19.1[b]	84.2 ± 20.0[b]	92.2 ± 13.2[c]	79.4 ± 22.7[c]
VCL (µm/s)	21.8 ± 11.1[a]	24.1 ± 12.2[a]	15.9 ± 10.8[b]	10.1 ± 7.7[b]	10.5 ± 6.8[b]	11.6 ± 7.6[b]
VSL (µm/s)	8.2 ± 8.3[a]	11.6 ± 9.1[a]	5.3 ± 5.9[b]	3.8 ± 4.4[b]	2.6 ± 4.6[b]	3.9 ± 3.6[b]
VAP (µm/s)	12.2 ± 9.5[a]	15.5 ± 10.2[a]	8.6 ± 7.8[b]	5.8 ± 5.3[b]	4.6 ± 3.3[b]	5.9 ± 4.7[b]
ALH (µm)	0.9 ± 0.9[b]	3.5 ± 4.1[a]	0.4 ± 0.7[b]	0.1 ± 0.2[b]	0.1 ± 0.2[b]	0.1 ± 0.3[b]
BCF (Hz)	2.5 ± 3.1[b]	6,6 ± 5.2[a]	1.2 ± 1.9[b]	0.4 ± 0.9[c]	0.2 ± 0.5[c]	0.4 ± 1.0[c]

Different letters (a, b) within rows indicate significant differences (P<0.05).
VS (%): % viable sperm; PM (%): % sperm showing progressive motility; NPM (%), % sperm showing non-progressive motility; IM (%): % immotile sperm; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

For both post mortem collection methods, the use of Lake-Ravie extender returned better ALH and BCF values, but only at 0 h. After refrigeration, the use of the Lake 7.1 medium was associated with higher percentages of viable sperm and sperm showing non-progressive motility, and better VCL and VAP values (Table 2).

The type of extender also had a significant effect on the ejaculated sperm variables at different refrigeration times. The percentages of sperm showing non-progressive motility, and the VSL and BCF values, were higher (P<0.05) at 0 h when extended with Lake-Ravie medium. However, during refrigeration, the Lake 7.1 medium seemed to provide a greater protective effect, as shown by the values of most of the variables examined (Table 3).

Table 2. Influence of sperm extenders on post mortem-recovered sperm variables.

Variable	0 h		24 h		48 h	
	L&R-84 Medium	**Lake 7.1 Medium**	**L&R-84 Medium**	**Lake 7.1 Medium**	**L&R-84 Medium**	**Lake 7.1 Medium**
VS (%)	55.2 ± 23.5^{a}	52.5 ± 20.9^{a}	34.4 ± 29.3^{b}	52.8 ± 18.9^{a}	21.7 ± 25.4^{c}	44.2 ± 19.2^{b}
PM (%)	4.8 ± 5.7^{a}	6.2 ± 12.4^{a}	1.4 ± 3.7^{b}	1.2 ± 5.1^{b}	1.7 ± 6.9^{b}	0.1 ± 0.3^{b}
NPM (%)	27.4 ± 18.6^{a}	24.3 ± 16.4^{a}	7.2 ± 10.8^{c}	15.4 ± 14.5^{b}	5.9 ± 8.2^{c}	10.6 ± 11.7^{b}
IM (%)	65.1 ± 26.4^{a}	69.7 ± 25.0^{a}	85.6 ± 23.1^{b}	83.4 ± 15.5^{b}	84.7 ± 22.7^{b}	86.9 ± 16.4^{b}
VCL (µm/s)	23.5 ± 11.6^{a}	22.5 ± 11.8^{a}	12.6 ± 11.6^{b}	13.4 ± 7.7^{b}	9.2 ± 5.7^{c}	12.8 ± 8.0^{b}
VSL (µm/s)	10.9 ± 7.7^{a}	8.9 ± 9.7^{a}	4.5 ± 6.5^{b}	4.6 ± 3.9^{b}	2.7 ± 2.9^{b}	3.8 ± 2.9^{b}
VAP (µm/s)	14.8 ± 9.1^{a}	13.0 ± 10.6^{a}	7.1 ± 8.2^{b}	7.3 ± 5.3^{b}	4.3 ± 3.6^{c}	6.2 ± 4.3^{b}
ALH (µm)	1.1 ± 1.1^{a}	0.7 ± 0.9^{b}	0.3 ± 0.7^{b}	0.2 ± 0.3^{b}	0.1 ± 0.2^{b}	0.1 ± 0.3^{b}
BCF (Hz)	3.5 ± 3.4^{a}	2.1 ± 3.3^{b}	0.9 ± 1.9^{c}	0.7 ± 1.2^{c}	0.2 ± 0.6^{c}	0.3 ± 0.68^{c}

Different letters (a,b,c) within rows indicate significant differences (P<0.05).
VS (%): % viable sperm; PM (%): % sperm showing progressive motility; NPM (%), % sperm showing non-progressive motility; IM (%): % immotile sperm; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

Table 3. Influence of sperm extenders on ejaculated sperm stored at 5ºC.

Variable	0 hours		24 hours		48 hours	
	L&R-84 Medium	**Lake 7.1 Medium**	**L&R-84 Medium**	**Lake 7.1 Medium**	**L&R-84 Medium**	**Lake 7.1 Medium**
Concentration (mill sperm/mL)	622 ± 72.5^{a}	638.6 ± 77^{a}	622 ± 72.5^{a}	638.6 ± 77^{a}	622 ± 72.5^{a}	638.6 ± 77^{a}
VS (%)	68.3 ± 10.3^{a}	61.9 ± 13.9^{a}	65.3 ± 7.2^{a}	57.0 ± 11.1^{b}	46.7 ± 15.2^{c}	50.7±10.2^{b}
PM (%)	5.5 ± 6.2^{a}	5.3 ± 8.6^{a}	0.3 ± 0.3^{c}	1.0 ± 1.8^{b}	0.0 ± 0.0^{c}	0.3 ± 0.3^{c}
NPM (%)	41.6 ± 10.5^{a}	18.0 ± 11.4^{b}	8.5 ± 13.4^{c}	15.9 ± 7.2^{b}	1.8 ± 2.3^{d}	11.6 ± 6.4^{c}
IM (%)	52.8 ± 11.8^{b}	69.1 ± 28.3^{a}	91.2 ± 13.7^{d}	83.0 ± 8.3^{c}	98.2 ± 2.3^{d}	88.1 ± 6.7^{c}
VCL (µm/s)	27.4 ± 12.3^{a}	21.9 ± 18.1^{a}	11.9 ± 11.8^{b}	17.6 ± 5.6^{b}	6.9 ± 3.1^{c}	17.8 ± 5.8^{b}
VSL (µm/s)	12.6 ± 7.6^{a}	8.9 ± 12.5^{b}	5.7 ± 6.9^{c}	4.5 ± 3.8^{c}	1.3 ± 0.9^{d}	3.9 ± 3.0^{c}
VAP (µm/s)	17.3 ± 8.5^{a}	12.7 ± 14.7^{a}	7.8 ± 8.5^{b}	7.9 ± 4.7^{b}	2.3 ± 1.9^{c}	7.1 ± 4.3^{b}
ALH (µm)	1.9 ± 0.8^{a}	1.2 ± 1.2^{a}	0.4 ± 0.0^{b}	0.3 ± 0.4^{b}	0.0 ± 0.0^{c}	0.6 ± 0.7^{b}
BCF (Hz)	5.9 ± 2.7^{a}	3.4 ± 3.1^{b}	1.3 ± 1.7^{c}	1.4 ± 1.9^{c}	0.0 ± 0.0^{d}	2.1 ± 2.6^{b}

Different letters (a,b,c,d) within rows indicate significant differences (P<0.05).
VS (%): % viable sperm; PM (%): % sperm showing progressive motility; NPM (%): % sperm showing non-progressive motility; IM (%): % immotile sperm; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency.

DISCUSSION

This study is the first comparing two extenders and two post mortem sperm retrieval techniques of rooster sperm. The results showed the float-out method to recover, in one vas deferens, some 54% of the sperm obtained by massage-induced ejaculation. The float-out method, which has been used with the pukeko (Gunn et al., 2008) and mice (Mohammadzadeh et al., 2011), was easier to perform and faster than the flushing method, but the sperm sample were usually contaminated with blood cells and urates. The presence of white blood cells may increase the production of reactive oxygen species (ROS), adversely affecting the long-term storage of the sperm (Aitken and Bennetts, 2006). The flushing method was more effective, however, recovering almost the same number of sperm in only one vas deferens as in the ejaculates. The flushing method reduces contamination by blood cells and urates, but at 0 h (i.e., before chilling) it was associated with a lower percentage of sperms showing progressive motility, and lower ALH and BCF values compared with the float out. The better motility variables at 0 h obtained with the float-out method might be explained in that only cells which are free in the lumen, i.e., those that are capable of swimming, are recovered; with the flushing method, most of the cells in the lumen, including dead and those cells located near the vasa's walls would be dragged out with the extender. These should include all the immature cells that could be less motile just after the recollection of the sample. The pressure and the shearing forces wielded on the sperm cells by the flushing method should be taken into account. Negative effect of pressure have been described in previous report on different types of cell (Bouaziz et al., 1998; Fioravanti et al., 2005; Santiago-Moreno et al., 2007), perhaps caused by changes in ion fluxes through membranes (Podolsky, 1956) and the perturbation of membrane-bound proteins

(Scarlata, 2005). However, no such differences were seen between samples after cooling at 5ºC for 24 or 48 h, suggesting that any harmful effects are transitory and are more probably due to the activation of the less mature spermatozoa.

All the sperm variables examined were negatively affected with increasing refrigeration time. Avian seminal plasma, which is produced in the testis, epididymis and vas deferens (no accessory sexual glands are present in birds) is functionally important since it stimulates sperm motility (Marzoni et al., 2013). However, at low temperature (4ºC) it has been reported to have a harmful effect *in vitro*, which has been associated with the activation of phospholipase (Douard et al., 2000). ROS production (Aitken and Bennetts, 2006) and changes in pH during cooling (Fennema et al., 1973) may also contribute towards a reduction in sperm quality. The Lake 7.1 medium includes a zwitterion buffer, BES, in its composition; the pH therefore varies less during cooling. This might explain the better results obtained with this medium for the chilled samples.

The ejaculated semen responded similarly to sperm recovered post mortem, with sperm variables worsening with refrigeration time; the Lake 7.1 medium was also more protective of this sperm than the Lake and Ravie medium. At 0 h, only slight differences were detected with respect to post mortem-collected sperm (by either method); the percentage of ejaculated sperm showing non-progressive motility, and the VSL value, were both better with the Lake-Ravie than the Lake 7.1 medium. However, unlike that seen for the post mortem sperm, the viability of the ejaculated sperm was better maintained at 24 h of refrigeration by the Lake-Ravie medium. In contrast, at 48 h of refrigeration, the Lake 7.1 medium better preserved both the ejaculated and post

mortem-collected sperm. Nonetheless, these findings suggest that the differences between ejaculated sperm and sperm recovered post mortem are less marked than those observed in mammalian species, in which the extender used, the post mortem/ejaculated origin of the semen, and the cooling and freezing protocol followed, all influence the response of the sperm (Holt, 2000; Holt et al., 2005). In mammals, epididymal sperm are more resistant to cooling and freezing than ejaculated sperm. Non-ejaculated sperm have a different membrane structure as a consequence of not having been in contact with accessory sex gland secretions (Leahy and de Graaf, 2012). Seminal plasma in mammalian species seems to induce changes related to sperm capacitation and involving membrane stabilization (Harkema et al., 2004).

Resistance to low temperatures has been reported for the epididymal sperm of several mammalian species, including impala (*Aepyceros melampus*), lion (*Panthera leo*), bull (*Bos taurus*), buffalo (*Syncerus caffer*) and ibexes (*Capra pyrenaica*) (Gilmore et al., 1998; Herold et al., 2004; Muiño et al., 2007; Pradiee et al., 2014). Because the same volume of extender (1.5 mL) was used in both flushing and float-out method, the final sperm concentration was related to the effectiveness of the method. In addition, the ejaculated samples were diluted 1:1 (V:V) for an optimal maintenance and transport to the lab. Hence, final sperm concentrations varied according the method, and this may also affect the results.

The scarce functional differences between the ejaculated rooster sperm and that collected post mortem, explains their similar responses to chilling. Avian sperm motility is minimal in testis, increases somewhat in the rudimentary epididymis, and reaches a

maximum in the vas deferens (Munro, 1935, 1938). These sperm attain fertilizing capacity gradually during their passage through the epididymis and vas deferens (Ahammad et al., 2011). Avian sperm seem to undergo neither capacitation nor the motility hyperactivation process within the female reproductive tract prior to fertilization, despite the long time they spend there before oocyte penetration (Howart, 1971; Lemoine et al., 2008). However, recent report doesn't discard possible capacitation process inside the sperm storage tubules (SSTs) (Bakst and Bauchan 2015).

In conclusion, this study describes two methods for harvesting rooster sperm post mortem from the vas deferens of roosters, and its successful chilled storage. The flushing method allowed obtaining higher numbers of sperm. This technique can be easily performed in wild and domestic birds found dead or critically injured, which could be interest in breeding and conservation programs. Lake-Ravie medium provided better sperm variable results with fresh semen, while in the refrigerated samples the Lake 7.1 medium returned the best results, regardless of the sperm being ejaculated or post mortem in origin.

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REFERENCES

Ahammad, M.U., Nishinoa, C., Tatemoto, H., Okura, N., Kawamoto, Y., Okamoto, S., Nakada, T., 2011. Maturational changes in motility, acrosomal proteolytic activity, and penetrability of the inner perivitelline layer of fowl sperm, during their passage through the male genital tract. Theriogenology 76, 1100-1109.

Aitken, R.J., Bennetts, L., 2006. Reactive oxygen species: friend or foe, in de Jonge C., Barrat C. (Eds), The Sperm Cell: Production, Maturation, Fertilization, Regeneration. Cambridge University Press, UK, 170-193.

Bird DM, Lague PC, B., land RB, 1976. Artificial insemination vs natural mating in American kestrel. Can J Zool 58, 1183-1191.

Blanco, J.M., Long, J.A., Gee, G., Wildt, D.E., Donoghue, A.M., 2012. Comparative cryopreservation of avian spermatozoa: Effects of freezing and thawing rates on turkey and sandhill crane sperm cryosurvival. Anim. Reprod. Sci. 131, 1-8.

Bouaziz, A., de Ficquelmont-Loizos, M.M., Richert, A., Caprani, A., 1998. Direct physical factors and PGI2 and TXA2 secretions by a human endothelial cell line: in vitro investigation of pressure and shear stress applied independently or in synergy. Thromb. Res. 90, 279-289.

Burrows W.H., Quinn J.P. 1937. The Collection of Spermatozoa from the Domestic Fowl and Turkey. Poult. Sci. 16, 19–24.

Carter, J.E., Rich, S.J., Graham, E.F., Lillehei, R.C., 1973. The effect of pressure and cooling rate on spermatozoa, kidney tissue, and the whole kidney. Cryobiology 10, 263-270.

Chalah, T., Brillard, J.P., 1998. Comparison of assessment of fowl sperm viability by eosin-nigrosin and dual fluorescence (SYBR-14PI). Theriogenology 50, 487-493.

Comizzolli, P., Holt, W. 2014. Recent advances and prospects in germplasm. Preservation of rare and endangered species. In: Reproductive Science in Animal Conservation. (Holt, W., Brown, J.L., Comizzoli, P. (Eds.). pp 331-356. Springer, NY, USA.

Clulow, J., Jones, R.C., 1982. Production, transport, storage and survival of spermatozoa in the male Japanese quail, Coturnix coturnix. J. Reprod. Fertil. 64, 259-66.

Douard, V., Hermier, D., Blesbois, E., 2000. Changes in turkey semen lipids during liquid in vitro storage. Biol. Reprod. 63, 1450-1456.

Fennema, O.R., Powrie, W.D., Marth, E.H., 1973. Low-temperature preservation of foods and living matter. Fennema O.R. Ed., Marcel Dekker, Inc., New York.

Fernández-Santos, M.R., Soler, A.J., Ramón, M., Ros-Santaella, J.L., Maroto-Morales, A., García-Álvarez, O., Bisbal, A., Garde, J.J., Coloma, M.A., Santiago-Moreno, J., 2011. Effect of post-mortem time on post-thaw characteristics of Spanish ibex (*Capra pyrenaica*) spermatozoa. Anim. Reprod. Sci. 129, 56-66.

Fioravanti, A., Benetti, D., Coppola, G., Collodel, G., 2005. Effect of continuous high hydrostatic pressure on the morphology and cytoskeleton of normal and osteoarthritic human chondrocytes cultivated in alginate gels. Clin. Exp. Rheumatol. 23, 847-853.

Gee, G.F., Bertschinger, H., Donoghue, A.M., Blanco, J., Soley, J., 2004. Reproduction in nondomestic birds: physiology, semen collection, artificial insemination and cryopreservation. Avian Poult. Biol. Rev. 15, 47-101.

Gilmore, J.A., McGann, L.E., Ashworth, E., Acker, J.P., Raath, J.P., Bush, M., Critser, J.K., 1998. Fundamental cryobiology of selected African mammalian spermatozoa and its role in biodiversity preservation through the development of genome resource banking. Anim. Reprod. Sci. 53, 277-97.

Gunn, M.R., Champion, Z., Casey, M.E., Teal, P., Casey, P.J., 2008. Testicular and spermatozoan parameters in the pukeko (*Porphyrio porphyrio melanotus*). Anim. Reprod. Sci. 109, 330-342.

Harkema, W., Visser, I., Soede, N.M, Kemp, B., Woelders, H., 2004. Capacity of boar spermatozoa to bind zona pellucida proteins in vitro in relation to fertilization rates in vivo. Theriogenology 61, 227-238.

Herold, F.C., Aurich, J.E., Gerber, D., 2004. Epididymal sperm from the African buffalo (Syncerus caffer) can be frozen successfully with AndroMed and with Triladyl but the addition of bovine seminal plasma is detrimental. Theriogenology 61, 715-24.

Hishinuma M., Suzuki K., Sekine J. 2003. Recovery and cryopreservation of sika deer (*Cervus nippon*) spermatozoa from epididymides stored at 4 °C. Theriogenology 59, 813-820.

Holt, W. V., 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53, 47-58.

Holt, W. V., Medrano, A., Thurston, L.M., Watson, P.F., 2005. The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Theriogenology 15, 370-382.

Howart, B. Jr., 1971. An examination for sperm capacitation on the fowl. Biol. Reprod. 3, 338-341.

Kumar, E., Holt, W. V, 2014. Impacts of endocrine disrupting chemicals on reproduction in wildlife. Adv. Exp. Med. Biol. 753, 55-70.

Lake, P.E., Ravie, O., 1984. An exploration of cryoprotective compounds for fowl spermatozoa. Br. Poult. Sci. 25, 145-150.

Lake, P.E., Stewart, J.M., 1978. Preservation of fowl semen in liquid nitrogen--an improved method. Br. Poult. Sci. 19, 187-194.

Leahy, T., de Graaf, S.P., 2012. Seminal plasma and its effect on ruminant spermatozoa during processing. Reprod. Domest. Anim. 47, 207-213.

Lemoine, M., Grasseau, I., Brillard, J.P., Blesbois, E., 2008. A reappraisal of the factors involved in in vitro initiation of the acrosome reaction in chicken spermatozoa. Reproduction 136, 391-399.

Madeddu, M., Berlinguer, F., Ledda, M., Leoni, G.G., Satta, V., Succu, S., Rotta, A., Pasciu, V., Zinellu, A., Muzzeddu, M., Carru, C., Naitana, S., 2009. Ejaculate collection efficiency and post-thaw semen quality in wild-caught Griffon vultures from the Sardinian population. Reprod. Biol. Endocrinol. 7, 18.

Martínez-Pastor, F., Guerra, C., Kaabi, M., Diaz, A.R., Anel, E., Herraez, P., De Paz, P., Anel, L., 2005. Decay of sperm obtained from epididymes of wild ruminants depending on post-mortem time. Theriogenology 63, 24-40.

Marzoni, M., Castillo, a, Sagona, S., Citti, L., Rocchiccioli, S., Romboli, I., Felicioli, a, 2013. A proteomic approach to identify seminal plasma proteins in roosters (*Gallus gallus domesticus*). Anim. Reprod. Sci. 140, 216-223.

Mohammadzadeh, S., Maksudov, G.Y., Doronin, Y.K., 2011. Survival of spermatozoa in the genital tract of mice post mortem. Dokl. Biol. Sci. 436, 62-64.

Muiño, R., Fernández, M., Peña, A.I., 2007. Post-thaw survival and longevity of bull spermatozoa frozen with an egg yolk-based or two egg yolk-free extenders after an equilibration period of 18 h. Reprod. Domest. Anim. 42, 305-11.

Munro, S.S., 1935. Motility and fertilizing capacities of fowl sperm in the excretory ducts. Proc. Soc. Exp. Biol. Med. 33, 255–7.

Munro, S.S., 1938. Functional changes in fowl sperm during their passage through the excurrent ducts of the male. J. Exp. Zool. 79, 71-92.

Podolsky, R.J., 1956. A mechanism for the effect of hydrostatic pressure on biological systems. J. Physiol. 132, 38-39P.

Pradiee, J., Esteso, M.C., Castaño, C., Toledano-Díaz, A., López-Sebastián, A., Santiago-Moreno, J., 2014. Cryopreservation of epididymal sperm from ibexes (*Capra pyrenaica*) using short equilibration time with glycerol. Theriogenology 82, 525-8.

Prieto, MT., Sánchez-Calabuig, MJ., Hildebrandt, TB.,Santiago-Moreno, J., Saragusty, J., 2014. Sperm cryopreservation in wild animals. Eur. J. Wildl. Res. 60, 851–864.

Samour, J.H., 2004: Semen collection, spermatozoa cryopreservation, and artificial insemination in nondomestic birds. J. Avian Med. Surg. 18, 219-223.

Santiago-Moreno, J., Toledano-Díaz, A., Pulido-Pastor, A., Gómez-Brunet, A., López-Sebastián, A., 2006. Birth of live Spanish ibex (*Capra pyrenaica hispanica*) derived from artificial insemination with epididymal spermatozoa retrieved after death. Theriogenology 66, 283-291.

Santiago-Moreno, J., Toledano-Díaz, A., Dorado, J., Pulido-Pastor, A., Coloma, M.A., López-Sebastián, A., 2007. Sperm recovery and cryopreservation of Spanish ibex epididymal spermatozoa. Arch. Androl. 53, 309-316.

Santiago-Moreno, J., Castaño, C., Toledano-Díaz, A., Coloma, M.A., López-Sebastián, A., Prieto, M.T., Campo, J.L., 2012. Cryoprotective and contraceptive properties of egg yolk as an additive in rooster sperm diluents. Cryobiology 65, 230-234.

Scarlata, S., 2005: The effect of hydrostatic pressure on membrane-bound proteins. Braz. J. Med. Biol. Res. 38, 1203-1208.

Sharlin, J.S., Shaffner, C.S., Gee, G.F., 1979. Sperm head length as a predictor of fecundity in the sandhill crane, Grus canadensis. J. Reprod. Fertil. 55, 411-413.

Soler, A.J., Esteso, M.C., Fernández-Santos, M.R., Garde, J.J., 2005. Characteristics of Iberian red deer *(Cervus elaphus hispanicus)* spermatozoa cryopreserved after storage at 5°C in the epididymis for several days. Theriogenology 64, 1503-1517.

Umapathy, G., Sontakke, S., Reddy, A., Ahmed, S., Shivaji, S., 2005. Semen characteristics of the captive Indian white-backed vulture (*Gyps bengalensis*). Biol. Reprod. 73, 1039-1045.

CAPÍTULO 2

Influence of staining method on the values of avian sperm head morphometric variables

S. Villaverde-Morcillo, M.C. Esteso, C. Castaño, A. Toledano Díaz, A. López-Sebastián, J.L. Campo, J. Santiago-Moreno, 2015. **Influence of staining method on the values of avian sperm head morphometric variables.** Reproduction in Domestic Animals. 50, 750-755.

Influencia del método de tinción en los valores de las variables morfométricas de la cabeza del espermatozoide aviar.

RESUMEN

Los sistemas computerizados para la evaluación de la morfometría espermática (sistemas ASMA) han sido empleados satisfactoriamente con varias especies de mamíferos. Desafortunadamente, han sido muy poco empleados para la evaluación de semen aviar, como consecuencia de la forma filiforme del espermatozoide aviar. Este estudio compara dos técnicas de tinción (Hemacolor® y azul de anilina) para el análisis morfométrico del gallo y de la perdiz roja como parte de un método de captura a través de microscopía óptica asistida por computador. Para ambas especies, la tinción con Hemacolor® proporcionó un porcentaje significativamente mayor de células medibles (93,7±11,7% en gallos y 71,9±15,3% en perdiz roja). Hemacolor® además mostró mayor repetitividad (coeficientes de variación menores) para la longitud y el área en gallos y para la anchura en la perdiz roja. En los gallos, con la técnica de tinción Hemacolor® las cabezas espermáticas fueron significativamente ($p<0,05$) más grandes en anchura y área que las teñidas con azul de anilina. En la perdiz roja, no se observaron diferencias en las mediciones obtenidas en anchura y área con las dos tinciones aunque la tinción de azul de anilina fue asociada con cabezas espermáticas más largas. En conclusión, los valores morfométricos obtenidos fueron diferentes dependiendo de la tinción empleada y de la especie. Aun así, la tinción de Hemacolor® podría ser considerada la más apropiada para la captura de imágenes y su análisis computerizado ya que proporciona mayor porcentaje de células medibles y presenta mayor repetitividad de la técnica.

Palabras clave: gallos; perdiz roja; morfometría de la cabeza espermática; Hemacolor®; azul de anilina.

ABSTRACT

Computer-assisted systems for the assessment of sperm morphometry (ASMA systems) have been used successfully with several mammalian species. Unfortunately, they have so far been of little use for assessing bird semen, a consequence of the filiform shape of avian spermatozoa. This study compares two staining techniques (Hemacolor® and aniline blue staining) for the morphometric analysis of rooster and red-legged partridge spermatozoa as part of a computer-assisted light microscopy method. For both species, Hemacolor® staining provided a significantly higher percentage of measurable cells (93.7±11.7% in roosters and 71.9±15.3% in red-legged partridges). Hemacolor® also showed greater repeatability (lower coefficients of variation) for length and area in roosters' sperm and for width in the case of red-legged partridge's sperm. In the roosters, the Hemacolor® technique returned significantly ($p<0.05$) larger sperm head width and area values than did the aniline blue technique, while the latter resulted in greater sperm head length values ($p<0.05$). In the red-legged partridge, no differences were seen in the results for sperm head width and area provided by the two techniques, but aniline blue staining was associated with longer length measurements. In conclusion, the morphometric values recorded differed depending on the staining method and species. However, the Hemacolor® technique might be deemed the more appropriate for computerised sperm assessment systems since it provides larger percentages of measureable cells and shows greater repeatability.

Keywords: chicken; red-legged partridge; sperm head morphometry; Hemacolor®; aniline blue.

INTRODUCTION

Sperm morphometry and viability have long been employed to asses male fertility in goats (Gravance et al., 1995), stallions (Ball and Mohammed, 1995; Brito, 2007), bulls (Sekoni and Gustafsson, 1987) and humans (Soler et al., 2003). Conventional techniques based on the subjective assessment of sperm variables (Baker and Clarke, 1987; Verstegen et al., 2002) can, however, return significantly different results when performed at different laboratories or by different operators (Ombelet et al., 1997; Cooper, 1999; Boersma and Braun, 1999). To overcome this, a number of computerised systems have been developed that provide high levels of accuracy and reliability in the assessment of sperm morphometry (ASMA) (Gravance et al., 1996; Esteso et al., 2003, 2015). The problem of subjectivity associated with visual assessment methods have been significantly reduced with ASMA systems (Hidalgo et al., 2006). Moreover, these computerized systems have been standardised for use with semen from different animals, including humans (Sancho et al., 1998; Vicente-Fiel et al., 2013). This standardization establishes the most appropriate staining and sampling techniques to use in each case (Davis and Gravance, 1993; García-Herreros et al., 2006).

ASMA systems are commonly used to examine mammalian sperm since they provide good head morphometry results, which can then be used to predict fertilization rates (Sekoni and Gustafsson, 1987; Chandler et al., 1988) and sperm freezability (Peña et al., 2005; Esteso et al., 2006). In birds, however, ASMA systems have been little used; the filiform morphology of avian spermatozoa is not recognized by the majority of software systems, because they are designed to capture the usual elliptical head of mammal's sperm, and certainly techniques have not been standardised. However,

morphology is among the most important factors in avian sperm evaluation, and numerous light and electron microscopy studies have detailed the morphological features of normal and abnormal sperm in a number of bird species (Bakst and Howarth, 1975; Bakst, 1987; Tabatabaei et al., 2009; Du Plessis and Soley, 2014). Indeed, detailed electron microscopy-based information is available on the classification, measurement and description of normal and defective rooster sperm (Grigg and Hodge, 1949). However information is very scarce on its *morphometric* assessment at the level of light microscopy (Du Plessis and Soley, 2014). The availability of computer-assisted light microscopic methods that could reliably and quickly determine avian sperm morphometry would be an important advance. With this in mind, the present study compares the performance of two staining techniques - Hemacolor® and aniline blue staining - as part of a computer-assessed, light microscopic method for measuring avian sperm head characteristics.

MATERIALS AND METHODS

Animals

The semen samples used in this study were collected twice a week from 12 roosters *(Gallus gallus domesticus)* and 18 red-legged partridges *(Alectoris rufa)* kept at the El Encín Research Station (Madrid, Spain, 40º31'N). All birds were mature and considered healthy. All handling procedures were approved by the INIA Ethics Committee (reference number CEEA 2011/017) and performed in accordance with the Spanish Policy for Animal Protection RD1201/2005, which conforms to European

Union Directive 86/609 regarding the protection of animals used in scientific experiments.

Sperm collection

Ejaculated sperm samples were collected from March to August in both species, using the massage technique of Burrows and Quinn (1937) for the roosters, and a technique adapted to red-legged partridges (Santiago-Moreno et al., 2015). After collection, the volume of each sample was recorded. The samples were then immediately diluted 1:1 (v:v) with Lake-Ravie medium (Lake and Ravie, 1984) (composed of sodium glutamate 1.92 g, glucose 0.8 g, magnesium acetate $4H_2O$ 0.08 g, potassium acetate 0.5 g, polyvinylpyrrolidone [M_r10 000] 0.3 g, and 100 mL H_2O; 343 mOsm/kg, pH 7.08) at ambient temperature. This was prepared using reagent-grade chemicals purchased from Panreac Quimica S.A. (Barcelona, Spain) and Sigma Chemical Co. The diluted samples were then refrigerated at 5°C and transported to the laboratory for analysis.

Staining procedures

Smears were prepared in duplicate by spreading 5 µL drops of diluted sperm samples onto glass slides and allowing them to air-dry. The two replicates per sample were then stained, one with Hemacolor® (Merck KGaA, Darmstadt, Germany) and one with aniline blue (methyl blue for microscopy) (Fluka, Sigma-Aldrich, St. Louis, MO) (Table 1).

Table 1. Hemacolor® and aniline blue staining procedures.

Staining technique	Step	Reagent	Time
Hemacolor®	1	Fixative solution	2 min
	2	Stain solution I	2 min
	3	Stain solution II	2 min
Aniline blue	1	2% glutaraldehyde solution	30 min
	2	Aniline blue	5 min

For Hemacolor® staining, the smears were fixed and stained with corresponding kit's acid and basic stains for 2 min each, according to the manufacturer's recommendations (Soler et al., 2005).

For aniline blue staining, the dry smears were fixed at room temperature in buffered 2% glutaraldehyde in PBS (i.e., 5 g of aniline blue in 100 ml PBS, filtered and adjusted to pH 3.5 with 2% glacial acetic acid (Merck, Germany) (Santiago-Moreno et al. 2009) for 30 min and air-dried. The slides were then stained with 5% aqueous aniline blue mixed with 2% acetic acid (pH=3.5) for 5 min, washed with distilled water, and air-dried once more.

Once stained and dried, all slides were sealed with Eukitt mounting medium (Panreac Quimica S.L.U., Barcelona, Spain) and a coverslip.

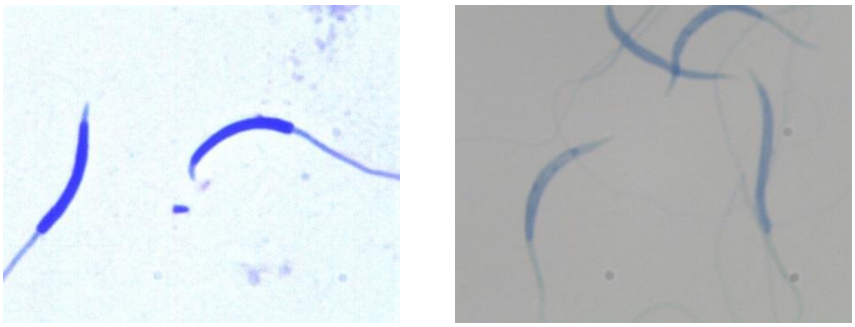

Fig. 1. Sperm from rooster stained with Hemacolor® (a) and aniline blue (b).

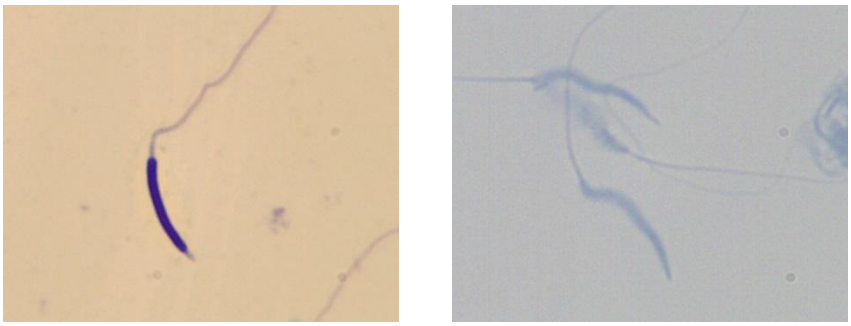

Fig 2. Sperm from red-legged partridge stained with Hemacolor® (a) and aniline blue (b).

Morphometric analysis

Sperm head morphometry was assessed in 50 rooster and 50 red-legged partridge sperm smears (25 stained by each method). The slides were subjected to computerised morphometric analysis using Motic Image Advanced V.3.0 software (Motic Spain, S.L.U. Barcelona, Spain) and Motic BA 210 optical microscope (Motic Spain, S.L.U. Barcelona, Spain) with a 100x oil immersion objective (bright field). Video signals were acquired using a 1SP 1.3 MP Moticam camera (Motic Spain S.L., Barcelona, Spain) attached to the microscope and connected to a computer. Twenty five spermatozoa per sample were randomly captured under the software's manual acquisition mode. The system detected the boundary of the sperm heads; their outlines were displayed as green overlays superimposed on the video image. Head boundaries were traced manually by the operator using the editing tool provided by the system. Both stains clearly separated the end of the head from the midpiece and the flagellum: accurate measurements of the sperm head were therefore guaranteed. Each sperm head was measured for length (including the acrosome), width and area. The area was

calculated as the sum of the pixellated area within the head boundary. The length and width were determined using the callipers provided by the program.

Statistical analysis

The Shapiro–Wilk test showed the collected data to follow a normal distribution; no transformation was therefore required. Differences in morphometric sperm head variables were assessed by two way ANOVA, followed by a *post hoc* Tukey test for multiple comparisons. The statistical model used was: $x_{ijk} = m + A_i + B_j + AB_{ij} + e_{ijk}$, where x_{ij} = the measured sperm head variable, m = the overall mean of variable x, A_i = the effect of the staining method (i = 1-2), B_j = the effect of species (j=1-2), AB_{ij} = the interaction between the staining method and species, and e_{ijk} = the residual (j = 1-25). Differences in the number of measurable sperms produced by the techniques were analysed by one way ANOVA following the model: $x_{ij} = m + A_i + e_{ijk}$, where x_{ij} = the accuracy of the assay, m = the overall mean percentage of measurable sperms, A_i = the effect of species (i = 1-2), and e_{ij} = the residual (j = 1-25). Coefficients of variation (CVs) were calculated as the standard deviation divided by the mean of the morphometric variables measured, expressed as a percentage. Coefficients of variation for 10 repeated measures of the same-sperm head morphometric variables were calculated to determine the repeatability of the results provided by the staining procedures. All statistical analyses were performed using STATISTICA software for Windows v.12.0 (StatSoft Inc., Tulsa, OK, USA). Data are expressed as means ± standard deviations. Where applicable, significance was set at $p<0.05$.

RESULTS

ANOVA revealed the interaction *species x staining technique* to have a significant effect on the values returned for sperm head width ($p<0.001$) and sperm head area ($p<0.05$), but not for sperm head length.

The percentage of measurable spermatozoa was higher ($p<0.05$) with the Hemacolor® staining technique than with the aniline blue technique, both for the rooster and partridge sperm (Table 2). The reasons why some spermatozoa could not be measured included: 1) background particles around the sperms preventing the identification of the cells (this occurred with both the Hemacolor® and aniline blue techniques), and 2) the stain being too light to differentiate cells from the background (this occurred with the aniline blue technique).

Table 2. Percentage of measurable spermatozoa in chicken and red-legged partridge semen smears, obtained via the Hemacolor® and aniline blue staining techniques.

Species	**Hemacolor stain**	**Aniline blue stain**
	Mean ± SD (CV)	**Mean ± SD (CV)**
Chicken	93.7 ± 11.7 % (13%)[a]	70.5 ± 16.8 % (24%)[b]
Red-legged partridge	71.9 ± 15.3 % (21.3%)[a]	55.4 ± 10.3 (18.6%)[b]

Different letters (a, b) within rows indicate significant ($p<0.05$) differences between staining techniques.

The values recorded for head width and area for the rooster sperm were significantly greater with the Hemacolor® than with the aniline blue technique. In contrast, the aniline blue technique was associated with larger sperm head lengths (Table 3). In the red-legged partridge, no differences were recorded between the two

techniques in terms of sperm head width and area, but the heads were again measured as longer by the aniline blue technique (Table 4).

Table 3. Rooster sperm head length, width and area, as determined by the two staining techniques (N=total spermatozoa measured). Coefficients of variation (CV) for repeated measures (n=10 replicates) of rooster sperm head morphometric variables, after staining with the two techniques, are shown inside parentheses.

Staining technique	Length (µm)	Width (µm)	Area (µm^2)	N
	Mean ± SD (CV)	Mean ± SD (CV)	Mea n± SD (CV)	
Hemacolor®	13.5 ± 3.5 ^{b} (0.9% ^{b})	1.3 ± 1.7 ^{a} (5.9% ^{a})	13.9 ± 0.7 ^{a} (0.0% ^{b})	625
Aniline blue	14.2 ± 3.4^{a} (1.4% ^{a})	1.0 ± 1.5 ^{b} (5.8% ^{a})	11.6 ± 0.2^{b}(1.5% ^{a})	625

Different letters (a, b) within columns indicate significant differences between means ($p<0.05$).

Table 4. Red-legged partridge sperm head length, width and area, as determined by the two staining techniques (N=total spermatozoa measured). Coefficients of variation (CV) for repeated measures (n=10 replicates) of red-legged partridge sperm head morphometric variables, after staining with the two techniques, are shown inside parentheses.

Staining technique	Length (µm)	Width (µm)	Area (µm^2)	N
	Mean ± SD (CV)	Mean ± SD (CV)	Mean ± SD (CV)	
Hemacolor®	14.2±3.0^{b} (1.0% ^{a})	1.3±0.3^{a} (4.7% ^{b})	15.9±5.6 ^{a} (0.4% ^{a})	625
Aniline blue	15.4±5.0^{a} (0.6% ^{b})	1.3±0.4^{a} (6.1% ^{a})	15.1±4.9^{a} (0.4% ^{a})	625

Different letters (a, b) within columns indicate significant differences between means ($p<0.05$).

The repeatability of the techniques differed according to the species and variable measured. For sperm head length and area in roosters, the aniline-blue staining showed a greater CV than the Hemacolor® staining and similar in the width value (Table 5). In the red-legged partridge, the Hemacolor® technique had a greater CV with respect to

sperm head length, while aniline blue staining resulted in the highest CV with respect to the measurement of sperm head width, CVs related to area remained similar (Table 6).

DISCUSSION

This is the first report to compare sperm head morphometry values obtained by two techniques in two avian species: the domestic chicken and red-legged partridges. The best performing staining technique for computer-assisted assessment of these avian species' sperm would appear to be the Hemacolor® technique.

ASMA systems offer accuracy and repeatability in the measurement of sperm head morphometric variables when they are properly adapted for use with each species. This includes the choice of staining method, digitization and computer analysis calibration (Gago et al., 1998; Sancho et al., 1998; Hidalgo et al., 2005, 2006). The ASMA systems designed for use with mammalian sperm are, however, largely unsuitable for use with avian spermatozoa, probably because of the latters' filiform (or sometimes helical) shape (Calhim et al., 2009). Morphological - but not morphometric - assays in birds have therefore largely been based on non-computerised light microscopy techniques (Wakely and Kosin, 1951; Hemberger et al., 2001; Gee et al., 2004) or even electronic microscopy techniques (Grigg and Hodge 1949; Lake et al., 1968; Thurston et al., 1982).

Different staining methods for ASMA systems have been tested for measuring mammalian spermatozoa. Those most habitually used are Hemacolor®, Diff-Quick®, Haematoxylin and SpermBlue® (Gago et al., 1998; Soler et al., 2005; Esteso et al., 2015). Hemacolor® is the stain most suitable for sperm head morphometry assessment in ibexes (*Capra pyrenaica*) (Esteso et al., 2015) and humans (Maree et al., 2010). In

the present work, the Hemacolor® stain provided a higher percentage of measurable spermatozoa for both bird species. This might be explained in that Hemacolor® produces a stronger stain than aniline blue, thus providing higher contrast images (in which cells better stand out against the background) for automatic analysis. Unfortunately, in those samples with large numbers of particles, the Hemacolor® technique runs into difficulties since these particles are also stained, making it difficult to recognise the sperm cells.

Aniline blue staining has been used to assess chromatin condensation status in human spermatozoa (Terquem and Dadoune, 1983; Dadoune et al., 1988; Auger et al., 1990) since the stain indicates the persistence of histones. However, it has never been used for morphometric purposes. It is commonly used to stain avian spermatozoa, since it reveals morphological abnormalities well and renders acrosome integrity easy to examine (Santiago-Moreno et al., 2009). Although its stain intensity is quite mild, it was sufficient to be picked up by the present computerised system. However, the percentage of measurable sperms provided was lower than with the Hemacolor® technique. The better definition of the acrosome provided by the aniline blue technique may explain why it resulted in longer sperm head lengths than the Hemacolor® technique, both in the roosters and partridges. Although aniline blue staining allows the identification of discrete alterations to sperms and better acrosome imaging when using phase contrast microscopy (Santiago Moreno, 2009), the present computerised assay was faster and more reliable when Hemacolor® staining was used.

The effects of staining on sperm morphometric values when using ASMA have been reported by several authors (Boersma and Braun, 1999; Gravance et al., 1998). In the present study, the two different staining techniques involved two different fixatives:

methanol for the Hemacolor® stain, and a 2% glutaraldehyde solution for the aniline blue stain. Some authors suggest that different fixatives dehydrate or swell sperm cells to different extents (Soler et al., 2005; Esteso et al., 2015), and sperm head size would seem subject to such effects (Hidalgo et al., 2006). Formaldehyde (Alkan et al., 2002), Hancock's solution (Ferdinand, 1992) and glutaraldehyde (du Plessis and Soley, 2014) have all been used in sperm assessment, the latter being reported to reduce the cell shrinkage observed in air-dried semen smears and allowing for better structural detailing under phase contrast microscopy (Gee et al., 2004; Bertschinger et al., 1992). In the present work, the rooster sperm heads were recorded as wider and as having a larger area when examined under Hemacolor® staining. This may reflect a species-specific response to its methanol fixative. Similar results have been observed in other species (Buendía et al., 2002; Gago et al., 1998; Wakely and Kosin, 1951).

Both staining methods showed good reproducibility. However, in roosters, the higher CVs obtained for the sperm head length and area measured after staining with aniline blue suggest the Hemacolor® technique to be more reliable.

The lengths of the rooster sperm heads obtained with both techniques were similar to those obtained by other authors using electron microscopy (13-14 µm). However, the widths recorded with both staining techniques (1.0-1.3 µm) were higher than those obtained via the latter method (0.5-0.7µm) (Grigg and Hodge, 1949; Thurston and Hess, 1987). Differences in the required fixation methods may explain these differences. Moreover, it is known that a high degree of shrinkage may be associated with the preparation of biological material for scanning electron microscope (Van der Horst et al. 1991).

In conclusion, the present work shows that the values of the morphometric variables recorded depended on the staining method and species. Of the two techniques tested, Hemacolor® staining might be considered the more appropriate for computerised sperm assays since it provides higher percentages of measurable cells and shows better repeatability.

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Conflict of interest

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

REFERENCES

Alkan S, Baran A, Özdas¸ ÖB, Evecen M. 2002. Morphological defects in turkey semen. Turk J Vet Anim Sci 26,1087-92.

Auger J, Mesbah M, Huber C, Dadoune JP, 1990. Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. Int J Androl 13, 452-462.

Baker HW, Clarke GN, 1987. Sperm morphology: consistency of assessment of the same sperm by different observers. Clin Reprod Fertil 5, 37-43.

Bakst MR, 1987. Anatomical basis of sperm-storage in the avian oviduct. Scanning Microsc 1, 1257-1266.

Bakst MR, Howarth B, 1975. The head, neck and midpiece of cock spermatozoa examined with the transmission electron microscope. Biol Reprod 12, 632–640.

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

Bertschinger HJ, Burger WP, Soley JT, de Lange JH, 1992. Semen collection and evaluation of the male ostrich. Grahamstown, Proc Biennial Congr S Afr Vet Assoc, 154-8.

Boersma A, Braun J, 1999. Computer-assisted analysis of sperm morphology in veterinary medicine. Berl. Munch. Tierarztl. Wochenschr. 112, 81–85.

Brito LFC, 2007: Evaluation of Stallion Sperm Morphology. Clin Tech Equine Pract 6, 249-264.

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

Burrows WH, Quinn JP, 1937: The collection of the spermatozoa from the domestic fowl and turkey. Poult Sci 16, 1, 19-24.

Calhim S, Lampe HM, Slagsvold T, Birkhead TR, 2009. Selection on sperm morphology under relaxed sperm competition in a wild passerine bird. Biol Lett 5, 58-61.

Chandler JE, Painter CL, Adkison RW, Memon MA, Hoyt PG, 1988. Semen quality characteristics of dairy goats. J Dairy Sci 71, 1638-1646.

Cooper TG, 1999. Experience with external quality control in spermatology. Hum Reprod 14, 765-769.

Dadoune JP, Mayaux MJ, Guihard-Moscato ML, 1988. Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. Andrologia 20, 211-217.

Davis RO, Gravance CG, 1993. Standardization of specimen preparation, staining, and sampling methods improves automated sperm-head morphometry analysis. Fertil Steril 59, 412-417.

Du Plessis L, Soley JT, 2014. Light microscopic features and morphometry of sperm in the emu *(Dromaius novaehollandiae)*. Theriogenology 81, 203-209.

Esteso MC, Fernández-Santos MR, Soler AJ, Garde, J.J., 2003. Head dimensions of cryopreserved red deer spermatozoa are affected by thawing procedure. Cryoletters 24, 261-268.

Esteso MC, Soler AJ, Fernández-Santos MR, Quintero-Moreno AA, Garde JJ, 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red Deer *(Cervus elaphis hispanicus)* epididymal sperm samples. J Androl 27, 662-670.

Esteso MC, Rodríguez E, Toledano-Díaz A, Castaño C, Pradiee J, López-Sebastián A, Santiago-Moreno J, 2015. Descriptive analysis of sperm head morphometry in Iberian ibex *(Capra pyrenaica)*: Optimum sampling procedure and staining methods using Sperm-Class Analyzer(®). Anim Reprod Sci 155, 42–49.

Ferdinand A. 1992. Licht- und elektronenmikroskopische Untersuchungen zur Morphologie von Ganterspermatozoen. PhD Thesis. Hannover: University of Veterinary Medicine.

Gago C, Pérez-Sánchez F, Yeung CH, Tablado L, Cooper TG, Soler C, 1998. Standardisation of sampling and staining methods for the morphometric evaluation of sperm heads in the Cynomolgus monkey (Macaca fascicularis) using computer-assisted image analysis. Int J Androl 21, 169–176.

García-Herreros M, Aparicio IM, Barón FJ, García-Marín LJ, Gil MC, 2006. Standardization of sample preparation, staining and sampling methods for automated sperm head morphometry analysis of boar spermatozoa. Int J Androl 29, 553–563.

Gee GF, Bertschinger H, Donoghue AM, Blanco J, Soley J, 2004. Reproduction in nondomestic birds: physiology, semen collection, artificial insemination and cryopreservation. Avian Poult Biol Rev 15, 47-101.

Gravance C, Lewis KM, Casey PJ, 1995: Computer automated sperm head morphometry analysis (ASMA) of goat spermatozoa. Theriogenology 44, 989-1002.

Gravance CG, Vishwanath R, Pitt C, Casey PJ, 1996. Computer automated morphometric analysis of bull sperm heads. Theriogenology 46, 1205-1215.

Gravance C, Champion Z, Casey P, 1998. Computer-assisted sperm head morphometry analysis (ASMA) of cryopreserved ram spermatozoa. Theriogenology 49, 1219-1230.

Grigg GW, Hodge AJ 1949. Electron microscopic studies of spermatozoa: I. The morphology of the spermatozoon of the common domestic fowl *(Gallus domesticus)*. Aust J Sci Res Ser B 2, 271-86.

Hemberger MY, Hospes R, Bostedt H, 2001. Semen collection, examination and spermiogram in ostriches. Reprod Dom Anim 36, 241-3.

Hidalgo M, Rodriguez I, Dorado J, Sanz J, Soler C, 2005. Effect of sample size and staining methods on stallion sperm morphometry by the Sperm Class Analyzer. Vet Med (Praha) 50, 24-32.

Hidalgo M, Rodríguez I, Dorado J, 2006. Influence of staining and sampling procedures on goat sperm morphometry using the Sperm Class Analyzer. Theriogenology 66, 996-1003.

Lake PE, Ravie O, 1984. An exploration of cryoprotective compounds for fowl spermatozoa. Br Poult Sci 25, 145-150.

Lake PE, Smith W, Young D, 1968. The ultrastructure of the ejaculated fowl spermatozoon. Q J Exp Physiol Cogn Med Sci 53, 356-66.

Maree L, du Plessis SS, Menkveld R, Van der Horst G, 2010. Morphometric dimensions of the himan sperm head depend on the staining method used. Hum Reprod 25, 1369-1382.

Ombelet W, Pollet H, Bosmans E, Vereecken A, 1997. Results of a questionnaire on sperm morphology assessment. Hum Reprod. 12, 1015-1020.

Peña FJ, Saravia F, García-Herreros M, Núñez-Martín I, Tapia JA, Johannisson A, Wallgren M, Rodríguez-Martinez H, 2005. Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. J Androl 26, 716-723.

Sancho M, Pérez-Sánchez F, Tablado L, de Monserrat J, Soler C, 1998. Computer assisted morphometric analysis of ram sperm heads: Evaluation of different fixative techniques. Theriogenology 50, 27-37.

Santiago-Moreno J, Castaño C, Coloma MA, Gómez-Brunet A, Toledano-Díaz A, López-Sebastián A, Campo JL, 2009. Use of the hypo-osmotic swelling test and aniline blue staining to improve the evaluation of seasonal sperm variation in native Spanish free-range poultry. Poult Sci 88, 2661-2669.

Santiago-Moreno J, Castaño C, Toledano-Díaz A, Esteso MC, López-Sebastián A, Gañán N, Hierro MJ, Marchal F, Campo JL, Blesbois E, 2015. Characterization of red-legged partridge *(Alectoris rufa)* sperm: Seasonal changes and influence of genetic purity. Poult Sci 94, 80–87.

Sekoni VO, Gustafsson BK, 1987. Seasonal variations in the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial insemination. Br Vet J 143, 312-317.

Soler C, de Monserrat JJ, Gutierrez R, Nunez J, Nunez M, Sancho M, Perez-Sanchez F, Cooper TG, 2003. Use of the Sperm-Class Analyser R for objective assessment of human sperm morphology. Int J Androl 26, 262-270.

Soler C, Gadea B, Soler AJ, Fernández-Santos MR, Esteso MC, Núñez J, Moreira PN, Núñez M, Gutiérrez R, Sancho M, Garde JJ, 2005. Comparison of three different staining methods for the assessment of epididymal red deer sperm morphometry by computerized analysis with ISAS®. Theriogenology 64, 1236-1243.

Tabatabaei S, Batavani RA, Talebi AR, 2009. Comparison of semen quality in indigenous and Ross broiler breeder roosters. J. Anim. Vet. Adv. 8, 90-93.

Terquem A, Dadoune JP, 1983. Aniline blue staining of human spermatozoa chromatin. Evaluation of nuclear maturation. In: The sperm cell (André J., ed.). Nijhoff Publishers, The Hague 3, 249-252.

Thurston RJ, Hess RA, 1987. Ultrastructure of spermatozoa of domesticated birds: comparative study of turkey, chicken and guinea fowl. Scan microscopy 1,1829-1838

Thurston RJ, Hess RA, Hughes BL, Froman DP, 1982. Ultrastructure (*Numidia meleagris*) of the guinea fowl spermatozoon. Poult Sci 61, 1738-1743.

Van der Horst G, Curry PT, Kitchin RM, Burgess W, Thorne ET, Kwiatkowski D, Parker M, Atherton RW.1991. Quantitative light and scanning electron microscopy of ferret sperm. Mol Reprod Dev 30, 232-240.

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

Vicente-Fiel S, Palacín I, Santolaria P, Hidalgo CO, Silvestre MA, Arrebola F, Yániz JL, 2013. A comparative study of the sperm nuclear morphometry in cattle, goat, sheep, and pigs using a new computer-assisted method (CASMA-F). Theriogenology 79, 436-442.

Wakely WJ, Kosin IL, 1951. A study of the morphology of the turkey spermatozoa--with special reference to the seasonal prevalence of abnormal types. Am. J. Vet. Res. 12, 240–245.

CAPÍTULO 3

Characterization of natural ejaculates and sperm cryopreservation in a golden eagle *(Aquila chrysaetus)*

S. Villaverde-Morcillo, R. García-Sánchez, C. Castaño, E. Rodríguez, F. Gonzalez, M. Esteso, J. Santiago-Moreno. 2015. **Characterization of natural ejaculates and sperm cryopreservation in a golden eagle *(Aquila chrysaetus)*.** Journal of Zoo and Wildlife Medicine. 46(2), 335-338.

Caracterización de los eyaculados naturales y la criopreservación del esperma de águila real (Aquila chrysaetus)

RESUMEN

Este trabajo describe las características espermáticas y la respuesta a la refrigeración del semen eyaculado de un ejemplar adulto de águila real *(Aquila chrysaetus)* mantenido en cautividad y entrenado para permitir la recogida de semen mediante falsas cópulas. Se realizó el espermiograma básico, y se analizaron los valores de motilidad y morfometría utilizando un método computerizado. Para el almacenamiento del semen, los efectos del diluyente Lake-Ravie, con polivinilpirrolidona (PVP) fueron evaluados a 5ºC. El mismo diluyente fue empleado para la congelación de las muestras con glicerol (GLY) (11%) y dimetilacetamida (DMA) (6%) que fueron comparados como crioprotectores. El diluyente mantuvo la viabilidad espermática en periodos de hasta 6 días. Aunque la motilidad espermática y el porcentaje de espermatozoides vivos fue menor en las muestras descongeladas (5,8-14,6% y 44,0-42,0% respectivamente) que en las frescas (46,7% y 74,6%, respectivamente), no se observaron diferencias entre ambos crioprotectores. Estos resultados podrían emplearse en el almacenamiento de semen de águila real y de otras especies de rapaces.

Palabras clave: Aquila chrysaetus, criopreservación, eyaculado, águila real, semen, espermatozoide.

ABSTRACT

This paper describes the sperm characteristics and response to cooling and freezing of naturally ejaculated semen from a captive, adult golden eagle *(Aquila chrysaetus)*, trained to allow sperm recovery via cooperative copulation. A basic spermiogram was prepared, and sperm motility and morphometric variables recorded using a computer-aided system. For sperm storage, the effects of a polyvinylpyrrolidone (PVP)-based extender were evaluated at 5ºC. The same extender was also used in freezing procedures in which glycerol (11%) and dimethylacetamide (DMA) (6%) were compared as cryoprotectants. The extender preserved sperm viability over storage periods of up to six days. Although sperm motility and percentage live sperm values were poorer for frozen-thawed (5.8-14.6% and 44-42%, respectively) than fresh samples (46.7% and 74.6%, respectively), no differences were seen between the effects of the two cryoprotectants. These results could be of use when attempting to store the sperm of golden eagles and other raptors.

Keywords: Aquila chrysaetus, cryopreservation, ejaculate, golden eagle, semen, spermatozoa.

INTRODUCTION

The golden eagle *(Aquila chrysaetus)* is found in Eurasia, North America and some parts of Africa, and overall is catalogued as of least concern by the IUCN. Artificial breeding techniques have been used with rather variable success in some wild birds (Birkhead, 1995; Blanco et al., 2002; Blanco et al., 2012; Gee et al., 1985). Further development of these techniques is, however, required: knowledge of the biological characteristics and physiology of wild bird sperm is still lacking, and sperm cryopreservation protocols need to be optimised. Studying the sperm variables of wild avian species is the best way to develop appropriate cryopreservation protocols. To the best of our knowledge, the literature contains no reports on the baseline characteristics of golden eagle semen.

MATERIAL AND METHODS

Over a period of two years, an adult (more than 30 years of age), male golden eagle kept at the GREFA Wildlife Rehabilitation Centre (Madrid, Spain) (recovered from injuries but unable to return to the wild) was trained to allow cooperative semen collection. Since the bird was already used to human presence, although not imprinted, successful mating were obtained after four months of training. The bird was housed on its own in an outdoor pen and was fed one day-old chicks, quails and rabbits. Semen collection consisted of voluntary false copulation (over the bird's trainer's back) from the onset of the breeding season until regular azoospermic samples were obtained. The procedure was undertaken in the eagle's normal pen and always by the same trainer (Gee et al., 1985; Stunden et al., 1998). Ejaculated semen was collected in a gloved

hand, placed in a 1.5 mL microcentrifuge tube (Eppendorf®, Eppendorf Ibérica SLU, San Sebastián de los Reyes, Madrid 28703, Spain), and diluted 1:1 (v/v) with a tempered (38ºC) medium composed of sodium glutamate (1.92 g), glucose (0.8 g), magnesium acetate $4H_2O$ (0.08 g), potassium acetate (0.5 g) and polyvinylpyrrolidone (M_r10 000; 0.3 g) dissolved in 100 ml H_2O (final osmolarity 343 mOsm/kg) (Lake and Ravie 1984). The extender was prepared in our laboratory using reagent-grade chemicals purchased from Sigma Chemical Co. (St. Louis, Missouri 63103, USA). The diluted semen was then immediately cooled to 5ºC, introducing the vial directly in the fridge and the evaluation of sperm variables performed at 0 h, 24 h and ≥72 h (range 3-6 days) following incubation at 38ºC for 10 minutes at each sampling. Sperm motility and concentration were determined using a SCA® (Microptic SL**,** Barcelona 08029, Spain) computer-aided image analysis system coupled to a phase contrast microscope, with settings adjusted to detect avian spermatozoa (A^2=5µm^2: VCL (10-100µm/sg). The percentages of motile spermatozoa, and spermatozoa showing progressive motility were recorded. Sperm movement characteristics - curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF) - were also analysed. Three progression ratios, expressed as percentages, were calculated from the three velocity measurements described above: linearity (LIN = VSL/VCL x 100), straightness (STR = VSL/VAP x 100), and wobble (WOB = VAP/VCL x 100). Sperm viability was tested by adding 4 µl of SYBR-14 diluted 1/19 in DMSO and incubating 10 min in a dark box at 5ºC, and then adding 2 µl of propidium iodide (PI) (final proportions (v/v) of SYBR-14 and PI: 1/20.5 and 1/41, respectively) and incubating for 2 min at 5ºC in complete darkness; 200 spermatozoa were then evaluated using an epifluorescence microscope at 40X

(wavelength 490 nm). Green-stained spermatozoa were deemed alive while red- or red-and-green-stained spermatozoa were considered dead. Sperm morphometry (area, perimeter, height and length) was evaluated by analysing 75 cells from three different smears (25 cells per smear) stained with aniline blue. A drop of 10 µL of each diluted semen sample was spread on a glass slide and allowed to dry. These smears were then fixed at room temperature in buffered 2% glutaraldehyde in PBS for 30 min and air-dried. The slides were then stained with 5% aqueous aniline blue mixed with 2% acetic acid (pH=3.5) for 5 min, washed with distilled water, and air-dried once more. Briefly, the staining solution was prepared by adding 5 g of aniline blue (Water Blue, Fluka, USA) to 100 ml PBS, filtering, and adjusting to a pH of 3.5 with a solution of 2% glacial acetic acid (Merck, Germany); morphometric measures were performed using a Motic Images Advanced 3.0 (Motic Spain SL, Barcelona 08349, Spain) computer-aided image analysis system coupled to a phase contrast microscope. The percentage of spermatozoa with an intact acrosome was determined by phase contrast microscopy, examining 200 aniline blue-stained cells (Lake & Ravie, 1984). The diluted sample was transferred to a refrigerator for 1h at 5ºC and then divided into two aliquots. Glycerol (final concentration 11%) or dimethylacetamide (DMA, final concentration 6%), both cooled to 5ºC, was then added to each aliquot respectively. This mixture was equilibrated for 10 min. These mixtures were then frozen by allowing 70 µl droplets ejected from a Gilson pipette (Gilson, Inc**.,** Middleton, Wisconsin 53562-0027, USA) to fall directly into liquid nitrogen. The pellets were recovered using a small sieve and stored in cryovials. After 2-3 months the pellets were thawed at 60-65ºC using a patented DDP-70® (INIA, 28040 Madrid, Spain) device and pooled for sperm analysis (in duplicate); this device consisted in a thermo-regulated conical hotplate that allowed

an ultra-fast warming of pellet. The influence of storage time on sperm variables was analysed by ANOVA followed by a Bonferroni *post-hoc* test. All calculations were performed using SPSS software v.20 for Windows (IBM SPSS Statistics; IBM Corp., Armonk, New York 10504-1722, USA).

RESULTS

A total of 112 dummy copulations occurred between February and March (the breeding season for this species). Sperm-containing ejaculates were recovered in 57.14% (n=64) of these 'matings'; azoospermic ejaculates were observed at the beginning and the ending of the breeding season. Ejaculate volume varied (range 1-115 µl; mean 42.2±31.8 µl). The mean sperm concentration was 467.7±392 $x10^6$ sperm/ml. The mean motility was 46.7±14%. The morphological abnormalities detected included coiled tails (11.4±13%), bent tails (5.9±4.6%), abnormal heads (19.1±11.8%) and loose heads (7.8±6.2%).

Sperm morphology was very heterogeneous within and among the ejaculates. The most common abnormal sperm morphology was a rounded head with a triangular acrosome, although a stinger-shaped acrosome was also commonly observed and deemed normal based on intactness. Sperm cell mean total surface area was 6.8±1.7 μm^2; the head was 7.4±0.8 µm long and 1.1±0.2 µm wide. No differences were seen in main sperm characteristics between samples stored at 5ºC for 0 h and 24 h; however, the VSL, VAP, LIN, STR, and WOB values were significantly lower ($p<0.05$) in samples stored for ≥72 h (Table 1), probably due to oxidative damage and loss of mitochondrial membrane potential associated to apoptosis.

Table 1.
Sperm variables (mean ± SD) at different storage times (5ºC).

	Storage time at 5ºC		
	0 h (n = 7)	**24 h (n = 10)**	**≥72 h (n= 4)**
Total motility (%)	46.7±14.6	48.8±6.1	40.3±10.1
Progressive motility (%)	20.1±16.0	12.0±5.0	8.7±5.0
VCL	58.1±22.6	45.1±6.2	33.8±12.8
VSL	30.5±11.8[a]	21.3±3.7[a,b]	12.3±7.4[b]
VAP	41.3±13.7[a]	29.9±4.6[a,b]	20.1±9.5[b]
LIN	9.0±1.9[a]	9.5±0.6[a]	6.8±1.9[b]
STR	74.5±3.3[a]	71.0±4.3[a]	59.2±7.1[b]
WOB	70.7±3.2[a]	66.3±3.3[a]	58.2±5.2[b]
ALH	2.5±0.67	2.8±0.30	2.0±0.8
BCF	8.4±2.3	9.4±0.6	7.7±2.3
Live spermatozoa (%)	74.6±19.8	80.7±5.1	75.0±7.2
NAR (%)	86.1±4.4	87.9±1.6	83.0±4.3
Abnormal spermatozoa (%)	38.3±12.9	39.1±25.5	42.6±10.4

Means in the same line with different superscripts [a,b] are significantly different ($p<0.05$).
CASA adjustments for sperm motility variables were: Static spermatozoa, spermatozoa with VCL < 10 µm/s; Non- progressive motility, spermatozoa with LIN < 50%; Progressive motility, spermatozoa with STR > 70%. VCL(µm/s): curvilinear velocity; VSL(µm/s): straight-line velocity; VAP(µm/s): average path velocity; LIN(%): linearity of track; WOB(µm): wobble; ALH(µm): amplitude of lateral head displacement; NAR (%): acrosome integrity.

Table 2. Sperm variables (mean ± SD) for frozen-thawed sperm extended with a PVP-based extender plus glycerol or dimethylacetamide (DMA) as a cryoprotectant.

	Cryoprotectant	
	11% Glycerol	**6% DMA**
Total motility (%)	5.8±0.2	14.6±0.1
Progressive motility (%)	0.4±0.1	2.6±0.1
VCL	35.5±6.5	47.4±6.0
VSL	10.1±6.7	21.6±6.1
VAP	18.6±8.9	30.8±5.6
LIN	28.5±11.0	45.5±9.0
STR	54.3±6.0	70.0±0.0
WOB	52.4±12.4	65.0±10.3
ALH	1.8±0.3	2.9±0.1
BCF	7.0±0.0	10.3±0.1
Live spermatozoa (%)	44.0±9.2	42.0±8.1
NAR (%)	70.0±9.2	66.0±8.1
Abnormal sperm (%)	86±11.3	42.0±10.2

CASA adjustments for sperm motility variables were: Static spermatozoa, spermatozoa with VCL < 10 µm/s; Non- progressive motility, spermatozoa with LIN < 50%; Progressive motility, spermatozoa with STR > 70%. VCL(µm/s): curvilinear velocity; VSL(µm/s): straight-line velocity; VAP(µm/s): average path velocity; LIN(%): linearity of track; WOB(µm): wobble; ALH(µm): amplitude of lateral head displacement; NAR (%): acrosome integrity.

DISCUSSION

Although sperm motility and percentage live sperm values were poorer for frozen-thawed (5.8-14.6% and 44-42%, respectively) than fresh samples (46.7% and 74.6%, respectively), no differences were seen between the effects of the two cryoprotectants. Earlier studies recorded urine contamination as one of the main problems when collecting semen samples via the massage technique (Blanco et al., 2000; Gee et al., 1985). In the present study, no urine contamination was detected, although dead cells were common. However, while dead cell contamination hinders sperm analysis it does not seem to directly affect semen quality. Although the massage technique provides

good semen samples (Lake & Ravie, 1984; Temple, 1972), cooperative semen collection would appear to provide very good quality sperm in the golden eagle. The advanced age and individual characteristics of the eagle providing sperm in the present study may have influenced the high percentage of abnormal spermatozoa, although the effect of age on avian semen characteristics and production is not well known.

The ejaculate volume varied widely, as has been described in other species such as the Indian white-backed vulture and the Houbara bustard (Temple, 172; Umapathy et al., 2005). The average sperm concentration was lower than that recorded for mallard ducks and pigeons, perhaps because the golden eagle is a predominantly monogamous species while the latter two are polygamous; the golden eagle would therefore need less competitive spermatozoa (Birkhead et al., 1995; Santiago-Moreno et al., 2009; Sontakke et al., 2004). The percentage of abnormal sperm recorded was also higher than that noted for ostriches and the white-backed vulture (Temple, 1972; Grier et al., 1973; Umapathy et al., 2005), perhaps due to the present animal's age. However, the mean sperm motility recorded (46.7%) was higher than that reported for the Spanish imperial eagle and the peregrine falcon, and indeed higher than that described for the golden eagle by other authors (Blanco et al., 2002). This difference might be due to the method used to obtain sperm samples, which was by massage in these other studies. This suggests that the quality of cooperatively-obtained semen could be higher than that obtained by massage.

In conclusion, this is the first report of cooperative copulation for the collection of golden eagle semen, and to our knowledge the first to describe the morphometric characteristics of golden eagle spermatozoa. The PVP-based medium used in this study is recommended for storing sperm from this species for periods of up to 24 h; it may

even be of some use for storage periods of a few days. Because the results are the traits of this particular animal, further work is needed to analyse more individuals and to determine the average of golden eagle sperm variables and which cryoprotectant is the most appropriate for freezing golden eagle sperm.

REFERENCES

Birkhead, T. R. 1995. Sperm competition: Evolutionary causes and consequences. Reprod. Fert. Dev. 7, 755-775.

Blanco JM, Gee G, Wildt DE, Donoghue AM. 2000. Species variation in osmotic, cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon spermatozoa. Biology of Reproduction. 63, 1164-1171.

Blanco JM1, Gee GF, Wildt DE, Donoghue AM. 2002. Producing progeny from endangered birds of prey: treatment of urine-contaminated semen and a novel intramagnal insemination approach. J Zoo Wildl Med. Mar; 33(1), 1-7.

Blanco, J. M, J. A. Long, G. Gee, D. E. Wildt, and A. M. Donoghue. 2012. Comparative cryopreservation of avian spermatozoa: Effects of freezing and thawing rates on turkey and sandhill crane sperm cryosurvival. Anim. Reprod. Sci. 131, 1-8.

Gee, G.F., M. R. Bakst, and T. J. Sexton. 1985. Cryogenic preservation of semen from the greater sandhill crane. J. Wildlife Manage. 49, 480-484.

Grier J.W. 1973. Techniques and results of artificial insemination with Golden Eagles. Raptor Res. 7,1-12.

Santiago-Moreno, J., C. Castaño, M. A. Coloma, A. Gómez-Brunet, A. Toledano-Díaz, A. López-Sebastián, and J. L. Campo. 2009. Use of the hypo-osmotic swelling test and aniline blue staining to improve the evaluation of seasonal sperm variation in native Spanish free-range. Poult. Sci. 88, 2661-2669.

Sontakke, S.D., G. Umapathy, V. Sivaram, S. D. Kholkute, and S. Shivaji. 2004. Semen characteristic, cryopreservation and successful artificial insemination in the Rock blue pigeon *(Columba livia)*. Theriogenology 2004, 139-153.

Stunden C.E., C. K. Bluhm, K. M. Cheng, and R. Rajamahendran. 1998. Plasma testosterone profiles, semen characteristics, and artificial insemination in yearling and adult captive Mallard ducks *(Anas platyrhynchos)*. Poult. Sci. 77, 882-887.

Temple, S. A. 1972. Artificial insemination with imprinted birds of prey. Nature 287-288.

Umapathy, G., S. Sontakke, A. Reddy, S. Ahmed, and S. Shivaji. 2005. Semen characteristics of the captive Indian white-backed vulture *(Gyps bengalensis)*. Biol. Reprod. 73, 1039-1045.

Wishart, G.J., C. Lindsay, H. J. Staines, and P. McCormick. 2002. Semen quality in captive Houbara bustard, *Chlamydotis undulataundulata*. Reprod. Fertil. Dev. 14, 401-405.

CAPÍTULO 4

Sperm morphology, sperm head morphometry, and sperm subpopulations in falcon ejaculates

S. Villaverde-Morcillo, A.J. Soler, M.C. Esteso, C. Castaño, A. Miñano-Bernal, F. Gonzalez, J. Santiago-Moreno. 2015. **Sperm morphology, sperm head morphometry, and sperm subpopulations in falcon ejaculates.** Enviado a Zoo Biology (ZOO 15-160).

Morfología espermática, morfometría de la cabeza del espermatozoide y subpoblaciones espermáticas en eyaculados de halcón.

RESUMEN

La morfología fue estudiada en tres especies de halcones: halcón peregrino brookei (*Falco peregrinus brookei),* halcón peregrine escocés (*Falco peregrinus peregrinus*) y halcón gerifalte (*Falco rusticolus).* Las características morfométricas del semen de estas especies fueron también examinadas y las subpoblaciones identificadas empleando los descriptores morfométricos de la cabeza del espermatozoide. Las muestras espermáticas fueron obtenidas mediante masaje y falsas cópulas voluntarias y diluídas 1:1 (v:v) con diluyente Lake-Ravie. Las extensiones fueron preparadas de las muestras diluidas, teñidas con Hemacolor® y sometidos a: 1) análisis morfológico (microscopio óptico de campo claro), y 2) análisis morfométrico computerizado; de cada cabeza de espermatozoide se obtuvieron medidas de longitud, anchura, área y perímetro. Además, las muestras de gerifalte se mezclaron en pool que fueron congelados en pellets empleando DMA como crioprotector, el análisis se repitió en las muestras descongeladas. La media del porcentaje de células inmaduras (espermatocitos y espermátidas) fue igualmente elevada en las tres especies: Halcón peregrino brookei 55,50%, Halcón peregrino escocés 65,46% y gerifalte 60,66%. El análisis de clusters identificó cuatro subpoblaciones con diferentes características morfométricas (P<0,001). La proporción relativa de estas subpoblaciones fue similar en las tres especies. Las medias de los valores obtenidos para las variables morfométricas para las cuatro subpoblaciones fueron menores (P<0,001) en las muestras descongeladas que en las muestras frescas. Los resultados apoyan la idea de que el pleimorfismo es una

característica del semen de las aves rapaces. Este resultado, junto con la existencia de cuatro subpoblaciones espermáticas con características morfométricas diferentes podría ser importante para el desarrollo de las técnicas y protocolos de criopreservación de semen de halcón.

Palabras clave: rapaces; halcón peregrino; halcón gerifalte; morfología espermática; morfometría de la cabeza espermática; Hemacolor®

ABSTRACT

Sperm morphology was studied in three falcon species: the Brookei peregrine falcon *Falco peregrinus brookei*, the Scottish peregrine falcon *Falco peregrinus peregrinus*, and the gyrfalcon *Falco rusticolus.* The morphometric characteristics of their sperm were also examined and sperm subpopulations identified using sperm head morphometric descriptors. Sperm samples were collected by massage and voluntary false copulation and diluted 1:1 (v:v) with Lake and Ravie medium. Smears were prepared of the diluted samples, stained with Hemacolor®, and subjected to: 1) morphological analysis (bright field optical microscopy), and 2) computerised morphometric analysis; each sperm head was measured for length, width, area and perimeter. In addition, in the gyrfalcon, pooled semen was frozen in pellets using DMA as a cryoprotectant and the analyses repeated after thawing. The mean percentage of immature sperm (spermatocytes and spermatids) was similarly high in all three species: Brookei falcon peregrine 55.50%, Scottish peregrine falcon 65.46% and gyrfalcon 60.66%. Clustering analyses identified four subpopulations of spermatozoa with different morphometric characteristics ($P<0.001$). The relative proportions of these subpopulations were similar in all three species. The mean values recorded for the morphometric variables of the four subpopulations were, however, lower ($P<0.001$) in the thawed gyrfalcon samples than in fresh samples. The results further support the idea of pleiomorphy as a characteristic of raptor sperm. This finding, plus that of the existence of four sperm subpopulations with different morphometric characteristics, may be important in the future development of cryopreservation protocols for falcon sperm.

Keywords: raptors; peregrine falcon; gyrfalcon; sperm morphology; sperm head morphometry; Hemacolor®

INTRODUCTION

The family Falconidae is represented by 66 species around the world, of which 18 now appear on the IUCN Red List as facing some threat of extinction (IUCN 2015). Habitat fragmentation (Benscoter et al., 2013), pollution (Duke, 2008) and climate change (Sanz-Elorza et al., 2003) remain constant threats. While habitat protection, species protection and threat management strategies all have their place in the conservation of these falcons, captive breeding and the banking of genetic resource appear as increasingly important options (see Holt et al., 1996; Gilmore et al., 1998). Indeed, establishing self-sustaining captive populations of different falcon species is a major conservation goal (Prieto et al., 2014). However, preserving the genetic variation of a species in this manner requires large numbers of animals be maintained (Reed and Bryant, 2000; Brook et al., 2006; Flather et al., 2011), and the space available in zoological gardens, animal parks and research centres is limited. In contrast, the cryopreservation of germplasm offers a means of preserving genetic variation without large space requirements (Holt et al., 1996; Holt and Pickard, 1999; Holt, 2008). Further, the cryopreservation of germplasm would allow the controlled "breeding" of individuals of genetic interest (Holt and Pickard, 1999; Holt and Lloyd, 2009; Behr et al., 2009) even after their death (Roldan et al., 2006).

Artificial reproduction techniques have been widely used in human and domestic animal species and are now being transferred to wild species (Silber et al., 2013). Artificial insemination (AI) using fresh or cryobanked semen is the method most commonly employed (Pukazhenthi et al., 2006). For many species, however, basic spermatological

and cryobiological studies still need to be performed if the best results are to be achieved (Leibo and Songsasen, 2002; Gee et al., 2004).

In pheasants (Immler et al., 2007), passerines (Lüpold et al., 2009), goats (Gravance et al., 1995), stallions (Ball and Mohammed, 1995; Brito, 2007), bulls (Sekoni and Gustafsson, 1987) and humans (Soler et al., 2003), the freezability and fertilizing capacity of spermatozoa is strongly influenced by their morphological and morphometric characteristics (Esteso et al., 2003; Peña et al., 2005; Esteso et al., 2006). The same may be true for raptor sperm. In addition, the morphometric alterations caused by freezing might affect the quality of thawed raptor sperm.

With a view to understanding more about the optimum conditions required for the successful cryobanking of falcon sperm, the aims of the present work were to determine the proportions of mature and immature sperm cells (morphological analysis) and to identify sperm subpopulations according to sperm head morphometric descriptors (morphometric analysis) in ejaculates of the Brookei peregrine falcon *Falco peregrinus brookei*, the Scottish peregrine falcon *Falco peregrinus peregrinus*, and the gyrfalcon *Falco rusticolus*. In addition, the influence of freezing-thawing on sperm head morphological and morphometric variables was studied using pooled gyrfalcon semen as a model.

MATERIALS AND METHODS

Sperm was donated by three Brookei peregrine falcons, two Scottish peregrine falcons (the property of Sevilla Falcons S.L. Seville, Spain and the *Centro de Halcones de Madrid*, Madrid, Spain), and 23 gyrfalcons (housed at the Rocfalcon Breeding Centre in Lleida, Spain). All birds were mature and healthy. Handling procedures were performed in accordance with the Spanish Policy for Animal Protection RD53/2013, which

conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

Thirty semen samples were collected from the three Brookei peregrine falcons (10 samples per bird), 20 from the two Scottish peregrine falcons (10 samples per bird) and 23 from the 23 gyrfalcons (one sample per bird). All samples were collected in 1.5 mL Eppendorf® microcentrifuge tubes (Eppendorf Ibérica SLU, San Sebastián de los Reyes, Madrid, Spain) using either cloacal massage (Burrows and Quinn 1937; Samour 2004) or voluntary false copulation (trained birds only: 2 Brookei, 1 Scottish and 3 gyrfalcons) (Temple 1972) over the breeding seasons (March and April) of 2014 and 2015. The volume of each sample was immediately recorded. They were then diluted 1:1 (v:v) with Lake-Ravie medium (Lake and Ravie, 1984) (sodium glutamate 1.92 g, glucose 0.8 g, magnesium acetate $4H_2O$ 0.08 g, potassium acetate 0.5 g, polyvinylpyrrolidone [M_r10 000] 0.3 g, and 100 mL H_2O; 343 mOsm/kg, pH 7.08) at ambient temperature. This extender was prepared using reagent-grade chemicals purchased from Panreac Quimica S.A. (Barcelona, Spain) and Sigma Chemical Co (St. Louis, Missouri, USA).

Sperm morphology and head morphometry were examined as previously described (Villaverde-Morcillo et al., 2015) using a Motic BA 210 optical microscope (Motic Spain, S.L.U. Barcelona, Spain) with a 100x oil immersion objective lens (bright field) and Motic Image Advanced v.3.0 software (Motic Spain, S.L.U. Barcelona, Spain). Briefly, diluted semen samples were stained with Hemacolor® (Merck KGaA, Darmstadt, Germany) and dried. The slides were then sealed with Eukitt mounting medium (Panreac Quimica S.L.U., Barcelona, Spain).

The percentage of immature cells in every stained sample was assessed in 200 randomly selected cells (morphological analysis). Spermatogonia, spermatocytes and spermatids were identified using as references those previously identified as such in testes from individuals of the different falcon species euthanized at two wildlife rehabilitation centres (CRFS El Valle, Murcia, Spain, and GREFA, Majadahonda, Madrid, Spain). Testis sections were fixed in formaldehyde, stained with haematoxylin and eosin, and examined by bright field optical microscopy at 20x and 40x (Bellvé et al. 1977; Millette and Bellvé 1977).

Twenty five spermatozoa per sample were then randomly captured using Motic Image Advanced v.3.0 software in manual acquisition mode (Motic S.L.U., Barcelona, Spain), and subjected to computerised morphometric analysis. Each sperm head was measured for length (excluding the acrosome), width, area and perimeter.

Pooled semen samples (n=9) from the 23 gyrfalcons were used to examine the influence of freezing-thawing on sperm morphometric variables. Each pool was diluted 1:1 (v:v) in Lake and Ravie medium at ambient temperature and cooled to 5ºC. Dimethylacetamide (DMA) (final concentration 6%) at 5ºC was then added. This mixture was allowed to equilibrate for 10 min, and then frozen by allowing 50 µl droplets ejected from a Gilson pipette (Gilson, Inc., Middleton, Wisconsin 53562-0027, USA) to fall directly into liquid nitrogen. The pellets were recovered using a small sieve and stored in cryovials. After 1 month the pellets were thawed at 60-65ºC using a DDP-70® thermoregulated conical hotplate (INIA, Madrid, Spain). Once thawed, pooled samples were washed with Lake and Ravie medium to remove the DMA (Santiago-Moreno et al., 2011), which prevents the adequate staining of sperm cells. Twenty five

spermatozoa per pool were subjected to pre-freezing and post-thawing computerised morphometric analysis as above.

All statistical analyses were performed using STATISTICA software for Windows v.12.0 (StatSoft Inc., Tulsa, OK, USA). The Shapiro–Wilk test showed the collected data to follow a normal distribution; no transformation was therefore required. Differences in the percentage of immature cells between species, and in morphometric sperm head variables within and between species, were assessed by one way ANOVA followed by a *post hoc* Tukey test for multiple comparisons, adhering to the statistical model $x_{ij} = m + A_i + e_{ijk}$, where x_{ij} = the value of the measured sperm variable, m = the overall mean of variable x, A_i = the effect of species (i = 1-3) or freezing-thawing process (1-2), and e_{ij} = the residual (j = 1-25). Coefficients of variation (CVs) (expressed as a percentage) were calculated as the standard deviation divided by the mean of the morphometric variable in question. For all species, sperm head length, width, area and perimeter values were subjected to *k*-means cluster analysis to identify four subpopulations. The morphometric descriptors for the subpopulations were identified and compared by general linear model analysis of variance (GLM-ANOVA). The influence of species and freezing-thawing (performed only with gyrfalcon sperm) on the size of each subpopulation was analysed via the Chi-squared test. Where applicable, significance was set at $P<0.05$.

RESULTS

The immature sperm cells in the ejaculates were classified morphologically as either 1) spermatocytes, i.e., rounded cells with a large basophilic nucleus and a diameter

dependent on their stage of development (type I or type II), 2) spermatids in different phases of development, i.e., ranging from spherical cells with a very basophilic nucleus to cells with an incipient flagellum but retaining most of their cytoplasm, or 3) quasi-mature spermatozoa still with the residual body attached to the head. No spermatogonia were observed in the ejaculates. The mean percentage of immature sperm was similar in all three species (Brookei peregrine falcon 55.50±20%, Scottish falcon 65.46±31% and gyrfalcon 60.66±19.4%).

Significant differences were detected between the species in terms of the morphometric variables measured ($P<0.001$) (Table 1). The heterogeneity was high in all ejaculates (particularly in fresh gyrfalcon sperm) as revealed by the large CVs recorded.

Table 1 Mean length, width, area and perimeter of spermatozoa in semen samples from each falcon species. Values are expressed as mean±SD (% coefficient of variation).

Variable	**Species**			
	Brookei peregrine falcon (fresh)	**Scottish peregrine falcon**	**Gyrfalcon**	**Gyrfalcon**
Type of sample	**Fresh**	**Fresh**	**Fresh**	**Frozen-thawed**
Length (µm)	7.0±1.2(17.0)[a]	6.3±1(16.2)[b]	6.3±1.4(22.7)[b]	6.0±1.2(20.5)[c]
Width (µm)	1.7±0.3(16.3)[a]	1.6±0.2(14.3)[a]	2.1±0.5(22.0)[b]	2.2±0.3(15.2)[c]
Area (μm^2)	10.0±2.4(24.0)[a]	8.5±1.4(24.0)[b]	11.6±4.0(35.0)[c]	11.4±2.7(24.3)[c]
Perimeter (µm)	16.5±2.8(17.0)[a]	14.8±2.1(17.0)[b]	16.0±3.2(20.0)[a]	15.5±2.7(17.4)[a]

Different letters (a, b, c) within rows reflect significant differences.

Tables 2-5 show the proportions of each subpopulation in the sperm of the different species examined. For all species, subpopulation (Sp) 1 cells had the longest and widest heads, those of Sp 2 also had long heads but were narrower, those of Sp 3 had a very clear fusiform shape, and those of Sp 4 were oval shaped.

Table 2. Mean sperm head length, width, area and perimeter in the four spermatozoa subpopulations in Brookei peregrine falcon fresh semen samples (mean±SD), and subpopulation proportions (%).

Variable	**Subpopulation**			
	Sp1	**Sp2**	**Sp3**	**Sp4**
Length (µm)	9.6±0.8[a]	8.0±0.6[b]	6.8±0.5[c]	6.0±0.6[d]
Width (µm)	1.8±0.3[a]	1.7±0.3[b]	1.7±0.3[b]	1.7±0.2[b]
Area (µm^2)	15.3±2.5[a]	11.7±1.2[b]	9.6±1.0[c]	8.0±0.7[d]
Perimeter (µm)	22.4±1.6[a]	18.6±1.0[b]	16.1±0.8[c]	13.5±1.8[d]
Proportion (%)	8.8	24.8	37.7	28.4

Different letters (a, b, c) within rows reflect significant differences.

Table 3. Mean sperm head length, width, area and perimeter in the four spermatozoa subpopulations in Scottish peregrine falcon fresh semen samples (mean±SD), and subpopulation proportions (%).

Variable	**Subpopulation**			
	Sp 1	**Sp 2**	**Sp 3**	**Sp 4**
Length (µm)	7.5±0.6[a]	6.6±0.4[b]	5.8±0.3[c]	5.1±0.5[d]
Width (µm)	1.6±0.3[a]	1.7±0.2[b]	1.7±0.2[b]	1.7±0.2[b]
Area (µm^2)	10.5±1.2[a]	9.0±0.8[b]	8.0±0.6[c]	7.0±0.5[d]
Perimeter (µm)	17.7±1.2[a]	15.5±0.6[b]	14.0±0.5[c]	12.2±0.6[d]
Proportion (%)	13.3	29.3	33.3	24.0

Different letters (a, b, c) within rows reflect significant differences.

The sperm cell heads of the frozen-thawed gyrfalcon samples were shorter and wider than those seen in fresh samples ($P<0.05$). No differences were seen, however, in terms of overall head area, or perimeter length (Table 1). The mean values of the

morphometric variables for all four subpopulations were smaller in the frozen-thawed than in the fresh samples (P<0.001) (Tables 4 and 5).

Table 4. Mean sperm head length, width, area and perimeter in the four spermatozoa subpopulations in gyrfalcon fresh semen samples (mean±SD), and subpopulation proportions (%).

Variable	Subpopulation			
	Sp 1	**Sp 2**	**Sp 3**	**Sp 4**
Length (µm)	9.3±1.0[a]	7.4±1.2[b]	6.0±0.7[c]	5.0±0.7[d]
Width (µm)	2.6±0.5[a]	2.4±0.4[b]	2.0±0.4[c]	2.0±0.4[c]
Area (µm^2)	20.7±3.1[a]	15.2±1.5[b]	10.3±1.2[c]	7.7±1.0[d]
Perimeter (µm)	23.0±2.0[a]	18.7±1.5[b]	15.1±1.0[c]	12.5±1.1[d]
Proportion (%)	11.5	18.5	37.0	33.0

Different letters (a, b, c) within rows reflect significant differences.

Table 5 Mean sperm head length, width, area and perimeter in the four spermatozoa subpopulations in gyrfalcon frozen-thawed semen (mean±SD), and subpopulation proportions (%).

Variable	Subpopulation			
	Sp 1	**Sp 2**	**Sp 3**	**Sp 4**
Length (µm)	8.6±1.3[a]	6.7±0.6[b]	5.8±0.5[c]	4.6±0.5[d]
Width (µm)	2.4±0.3[a]	2.3±0.3[b]	2.2±0.2[c]	2.1±0.3[d]
Area (µm^2)	17.7±2.0[a]	13.1±1.0[b]	10.4±1.0[c]	8.5±1.0[d]
Perimeter (µm)	21.4±2.6[a]	17.0±1.0[b]	14.7±0.7[c]	12.3±1.0[d]
Proportion (%)	26.6	44.8	19.5	8.8

Different letters (a, b, c) within rows reflect significant differences.

DISCUSSION

The results show the existence of sperm pleiomorphy (within-male variation in sperm morphology) with a high incidence of spermatocytes and spermatids in all ejaculates in all three species. This supports the idea that sperm pleiomorphy is a characteristic of raptors. The high percentage of immature cells agrees with that reported earlier for

peregrine falcon ejaculates (Blanco et al., 2001), although the latter authors identified the immature sperm cells they saw as spermatogonia. In the present work, however, comparisons with identified reference cells clearly identified the immature cells as spermatocytes and spermatids, i.e., cells in later phases of spermatogenesis (Kirby and Froman, 2000; Deviche et al., 2011).

The degree of sperm pleiomorphy shown by a species differs depending on the degree of sperm competition its reproductive behaviour entails. For example, in polygamous species that practise extensive polyandry (e.g., the chicken), sperm morphology is homogenous and there are few head abnormalities (Santiago-Moreno et al., 2009). In contrast, the natural ejaculates of the golden eagle (*Aquila chrysaetos*), a monogamous species, contain morphologically heterogeneous spermatozoa (including large numbers of spermatocytes and spermatids), and over 37% may show head abnormalities (Villaverde-Morcillo et al., 2015). The present results for falcons reveal an even higher incidence of immature sperm (55-65%). This suggests falcons may show a degree of monogamy unusual even among birds. Differences in the degrees of pleiomorphy are also seen between smaller birds. In the Eurasian bullfinch (*Pyrrhula pyrrhula*), a more monogamous species in which sperm competition is low, 8-18% of sperms may be immature/show head abnormalities, while in the polygamous dunnock (*Prunella modularis*), in which sperm competition is intense, only 4-5% may be so affected (Birkhead and Immler, 2007).

It may be that the high percentage of spermatocytes and spermatids in falcon ejaculates has a physiological role in the reproduction of these species. In certain invertebrates, such as *Bombyx mori,* it has been suggested that non-fertile sperm aid in the successful transfer of fertile sperm to the site of sperm storage or fertilization in the

female (Sahjara and Takemura, 2003). It has even been suggested that some morphological forms of human sperm have evolved to perform different roles in relation to sperm competition (e.g., kamikaze and egg-getter sperms) (Baker and Bellis, 1995). More work is required to determine what the physiological role of pleiomorphy may be.

This paper is the first to describe sperm morphometric variables in falcon species. Significant differences were seen between the three species examined, perhaps due to slight variation in mating system, reproduction strategy (Albrechtová et al., 2014; Anderson et al., 2005; Kleven et al., 2008), phylogeny or population dynamics. More work will be needed to determine the exact causes.

Four sperm subpopulations (Sp 1-4) were identified, each with different morphometric characteristics. The proportion of each subpopulation was similar in all three species. However, while in fresh gyrfalcon sperm Sp 3 and 4 were larger, Sp 1 and 2 were larger in the frozen-thawed samples. Further, the values of the morphometric variables recorded for all four subpopulations were smaller for the frozen-thawed gyrfalcon spermatozoa than for the fresh samples. This effect of freezing and thawing has been observed in some mammalian species (Gravance et al., 1998; Esteso et al., 2003; Esteso et al., 2006). One hypothesis suggests the reduction in sperm head dimensions after freezing-thawing can be explained by acrosome damage or loss (Thomas et al., 1998), or even due to changes in sperm chromatin structure (Gravance et al. 1998; Blottner et al., 2001).

Some authors have associated mammalian sperm head morphology with their ability to overcome a mucus barrier in migration tests, and report the relative size of the non-migrating subpopulation to be negatively related to male fertility (Martínez-Rodríguez

et al., 2015). Studies in various taxa have suggested that an increase in the size of all sperm components, plus sperm head elongation, are associated with better sperm migration and faster swimming speeds (Briskie et al., 1992; Gomendio and Roldan, 2008; Fitzpatrick et al., 2009; Tourmente et al., 2011). It is often assumed that spermatozoa with larger heads swim faster because they can store more energy, but no such direct relationship has been observed in *in vitro* studies in birds (Rowe et al., 2013). Other studies have shown that larger spermatozoa are more hydrodynamic, and therefore faster (Malo et al., 2006).

The collection of basic knowledge on falcon sperm morphology and morphometry is the first step in designing optimised protocols for the cryopreservation of gametes and artificial reproduction programmes. Further work is needed to determine whether sperm morphology and morphometry change over the breeding season and whether this might affect fertilization capacity. This might indicate the best time to collect sperm for use in cryobanking and conservation programmes that involve artificial reproduction.

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REFERENCES

Albrechtová J, Albrecht T, Ďureje L, Pallazola VA, Piálek J. 2014. Sperm morphology in two house mouse subspecies: do wild-derived strains and wild mice tell the same story? PLoS One 9:e115669.

Anderson MJ, Nyholt J, Dixson AF. 2005. Sperm competition and the evolution of sperm midpiece volume in mammals. J Zool 267,135–142.

Baker RR, Bellis MA. 1995. Human sperm competition: Copulation, masturbation and infidelity. Chapman & Hall. London, UK.

Baker HW, Clarke GN. 1987. Sperm morphology: consistency of assessment of the same sperm by different observers. Clin Reprod Fertil. 5,37–43.

Bakst MR. 1987. Anatomical basis of sperm-storage in the avian oviduct. Scanning Microsc. 1,1257–1266.

Bakst MR, Howarth B. 1975. The head, neck and midpiece of cock spermatozoa examined with the transmission electron microscope. Biol Reprod. 12,632–640.

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

Behr B, Rath D, Mueller P, et al. 2009. Feasibility of sex-sorting sperm from the white and the black rhinoceros *(Ceratotherium simum, Diceros bicornis)*. Theriogenology. 72,353–64.

Bellvé AR, Cavicchia JC, Millette CF, et al. 1977. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol. 74,68-85.

Benscoter AM, Reece JS, Noss RF, et al. 2013. Threatened and endangered subspecies with vulnerable ecological traits also have high susceptibility to sea level rise and habitat fragmentation. PLoS One 8:e70647.

Birkhead TR, Immler S. 2007. Making sperm: design, quality control and sperm competition. Soc Reprod Fertil Suppl. 65, 175-181.

Blanco J, Wildt D, Gee G, Donoghue A. 2001. Spermatogonia in eagle and peregrine falcon ejaculates: interspecies variation, cryopreservation tolerance and implications to cryobanking. 4th Eurasian Congress on Raptors, Abstract book. Raptor Research Foundation, Sevilla, Spain, 25-29 September, 2001; 24.

Blottner S, Warnke C, Tuchscherer A, et al. 2001. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. Anim Reprod Sci. 65, 75-88.

Briskie J V, Montgomerie R. 1992. Sperm size and sperm competition in birds. Proc Biol Sci. 247, 89-95.

Brito LFC. 2007. Evaluation of Stallion Sperm Morphology. Clin Tech Equine Pract. 6, 249-264.

Brook BW, Traill LW, Bradshaw CJA. 2006. Minimum viable population sizes and global extinction risk are unrelated. Ecol Lett. 9,375-382.

Burrows WH, Quinn JP. 1937. The collection of the spermatozoa from the domestic fowl and turkey. Poult Sci. 16(1), 19-24.

Deviche P, Hurley LL, Fokidis HB. 2011. Avian testicular structure, function and regulation. In: Hormones and Reproduction of Vertebrates. Elsevier, pp. 20-70.

Du Plessis L, Soley JT. 2014. Light microscopic features and morphometry of sperm in the emu *(Dromaius novaehollandiae)*. Theriogenology. 81,203-209.

Duke G. 2008. The EU environmental policy context for monitoring for and with raptors in Europe. Ambio. 37, 397-400.

Esteso MC, Fernández-Santos MR, Soler AJ, Garde, JJ. 2003. Head dimensions of cryopreserved red deer spermatozoa are affected by thawing procedure. Cryoletters 24, 261-268.

Esteso MC, Soler AJ, Fernández-Santos MR, Quintero-Moreno AA, Garde JJ. 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red Deer (*Cervus elaphus hispanicus*) epididymal sperm samples. J Androl. 27, 662-670.

Esteso MC, Rodríguez E, Toledano-Díaz A, Castaño C, Pradiee J, López-Sebastián A, Santiago-Moreno J. 2015. Descriptive analysis of sperm head morphometry in Iberian ibex *(Capra pyrenaica)*: Optimum sampling procedure and staining methods using Sperm-Class Analyzer ®. Anim Reprod Sci. 155.42-49.

Fitzpatrick JL, Montgomerie R, Desjardins JK, et al. 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. Proc Natl Acad Sci U S A. 106, 1128-32.

Flather CH, Hayward GD, Beissinger SR, Stephens PA. 2011. Minimum viable populations: Is there a "magic number" for conservation practitioners? Trends Ecol. Evol. 26, 307-316.

Gee GF, Bertschinger H, Donoghue AM, Blanco J, Soley J. 2004. Reproduction in nondomestic birds: physiology, semen collection, artificial insemination and cryopreservation. Avian Poult Biol Rev. 15, 47-101.

Gilmore JA, McGann LE, Ashworth E, et al. 1998. Fundamental cryobiology of selected African mammalian spermatozoa and its role in biodiversity preservation through the development of genome resource banking. Anim Reprod Sci. 53,277-297.

Gomendio M, Roldan ERS. 2008. Implications of diversity in sperm size and function for sperm competition and fertility. Int J Dev Biol. 52,439-447.

Gravance C, Lewis KM, Casey PJ. 1995. Computer automated sperm head morphometry analysis (ASMA) of goat spermatozoa. Theriogenology. 44,989-1002.

Gravance CG, Vishwanath R, Pitt C, Casey PJ. 1996. Computer automated morphometric analysis of bull sperm heads. Theriogenology. 46, 1205-1215.

Gravance C, Champion Z, Casey P. 1998. Computer-assisted sperm head morphometry analysis (ASMA) of cryopreserved ram spermatozoa. Theriogenology. 49, 1219-1230.

Holt W V, Bennett PM, Volobouev V, Watson PF. 1996. Genetic resource banks in wildlife conservation. J Zool. 238, 531-544.

Holt W V. 2008. Cryobiology, wildlife conservation & reality. Cryo-Letters. 29, 43-52

Holt W V., Lloyd RE. 2009. Artificial insemination for the propagation of CANDES: the reality! Theriogenology. 71, 228-235.

Holt W V, Pickard AR. 1999. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod. 4, 143-150.

Immler S, Saint-Jalme M, Lesobre L, et al. 2007. The evolution of sperm morphometry in pheasants. J Evol Biol. 20, 1008-1014.

Kimball RT, Parker PG, Bednarz JC. 2003. Occurrence and evolution of cooperative breeding among the diurnal raptors *(Accipitridae and falconidae)*. The Auk. 120(3), 717.

Kirby JD, Froman DP. 2000. Reproduction in male birds. In: Sturkie's Avian Physiology, 5th edn. Elsevier, pp 597-615.

Kleven O, Laskemoen T, Fossøy F, et al. 2008. Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. Evolution. 62, 494-499.

Lake PE, Ravie O. 1984. An exploration of cryoprotective compounds for fowl spermatozoa. Br Poult Sci. 25, 145-150.

Leibo SP, Songsasen N. 2002. Cryopreservation of gametes and embryos of non-domestic species. Theriogenology. 57, 303-326

Lüpold S, Calhim S, Immler S, Birkhead TR. 2009. Sperm morphology and sperm velocity in passerine birds. Proc Biol Sci. 276,1175-1181.

Malo AF, Gomendio M, Garde J, et al. 2006. Sperm design and sperm function. Biol Lett. 2, 246-9.

Maree L, du Plessis SS, Menkveld R, Van der Horst G. 2010. Morphometric dimensions of the human sperm head depend on the staining method used. Hum Reprod. 25, 1369-1382.

Martínez-Rodríguez C, Alvarez M, López-Urueña E, Gomes-Alves S, Anel-López L, Tizado J E, Anel L and de Paz P. 2015. Head morphology of ram spermatozoa is associated with their ability to migrate in vitro and correlates with fertility. Reprod Fertil Dev. 27, 852-864.

Millette CF, Bellvé AR. 1977. Temporal expression of membrane antigens during mouse spermatogenesis. J Cell Biol. 74, 86-97.

Peña FJ, Saravia F, García-Herreros M, Núñez-Martín I, Tapia JA, Johannisson A, Wallgren M, Rodríguez-Martinez H. 2005. Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. J Androl. 26, 716-723.

Prieto MT, Sánchez-Calabuig MJ, Hildebrandt TB, Santiago-Moreno J, Saragusty J 2014. Sperm cryopreservation in wild animals. Eur J Wildl Res. 60, 851-864.

Pukazhenthi B, Comizzoli P, Travis AJ, Wildt DE. 2006. Applications of emerging technologies to the study and conservation of threatened and endangered species. Reprod. Fertil. Dev. 18, 77-90.

Reed D, Bryant E. 2000. Experimental tests of minimum viable population size. Anim Conserv. 5000, 7-14.

Roldan ERS, Gomendio M, Garde JJ, et al. 2006. Inbreeding and reproduction in endangered ungulates: Preservation of genetic variation through the organization of genetic resource banks. Rep Dom Anim. 41, 82-92

Rowe M, Laskemoen T, Johnsen A, Lifjeld JT. 2013. Evolution of sperm structure and energetics in passerine birds. Proc Biol Sci 280:20122616.

Sahjara K, Takemura N. 2003. Application of artificial insemination technique to eupyrene and/or apyrene sperm in Bombyx mori. J Exp Zool 297A, 196-200.

Samour JH. 2004. Semen Collection, Spermatozoa Cryopreservation, and Artificial Insemination in Nondomestic Birds. J. Avian Med Surg. 18, 219-223.

Santiago-Moreno J, Castaño C, Coloma MA, Gómez-Brunet A, Toledano-Díaz A, López-Sebastián A, Campo JL. 2009. Use of the hypo-osmotic swelling test and aniline blue staining to improve the evaluation of seasonal sperm variation in native Spanish free-range poultry. Poult Sci. 88, 2661-2669.

Santiago-Moreno J, Castaño C, Toledano-Díaz A, et al. 2011. Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: optimization of freezing rate and equilibration time. Poult Sci. 90, 2047-53.

Santiago-Moreno J, Castaño C, Toledano-Díaz A, Esteso MC, López-Sebastián A, Gañán N, Hierro MJ, Marchal F, Campo JL, Blesbois E. 2015. Characterization of red-legged partridge *(Alectoris rufa)* sperm: Seasonal changes and influence of genetic purity. Poult Sci. 94, 80-87.

Sanz-Elorza M, Dana ED, González A, Sobrino E. 2003. Changes in the high-mountain vegetation of the Central Iberian Peninsula as a probable sign of global warming. Ann Bot. 92, 273-80.

Sekoni VO, Gustafsson BK. 1987. Seasonal variations in the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial insemination. Br Vet J. 143, 312-317.

Silber SJ, Barbey N, Lenahan K, Silber DZ. 2013. Applying clinically proven human techniques for contraception and fertility to endangered species and zoo animals: a review. J Zoo Wildl Med. 44, S111-22.

Soler C, de Monserrat JJ, Gutierrez R, Nunez J, Nunez M, Sancho M, Perez-Sanchez F, Cooper TG. 2003. Use of the Sperm-Class AnalyserR for objective assessment of human sperm morphology. Int J Androl. 26, 262-270.

Tabatabaei S, Batavani RA, Talebi AR. 2009. Comparison of semen quality in indigenous and Ross broiler breeder roosters. J Anim Vet Adv. 8, 90-93.

Temple SA. 1972. Artificial insemination with imprinted birds of prey. Nature: 237(5353), 287-288.

Thomas CA, Garner DL, DeJarnette JM, Marshall CE. 1998. Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry. Biol Reprod. 58,786-93.

Tourmente M, Gomendio M, Roldan ERS. 2011. Sperm competition and the evolution of sperm design in mammals. BMC Evol Biol 11:12.

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

Villaverde-Morcillo S, García-Sánchez R, Castaño C, Rodríguez E, González F, et al. 2015 Characterization of natural ejaculates and sperm cryopreservation in a Golden Eagle (Aquila chrysaetos). J Zoo Wildl Med. 46,335-338

Weick F. 1980. Birds of prey of the world. Collins, London.

DISCUSIÓN GENERAL

El principal método de recogida de material espermático en las aves se basa en la técnica de masaje sacro-abdominal-cloacal que ha sido ampliamente desarrollado en gallos (Burrows and Quinn, 1937), y posteriormente extrapolado, con sus respectivas variantes, a otras especies (Łukaszewicz et al., 2011; Dogliero et al., 2015). En especies silvestres, la recogida de semen mediante falsas cópulas ofrece una calidad seminal mejor, evitándose la contaminación por uratos y otro tipo de detritus (ver Capitulos 3 y 4 de la presente Tesis). No obstante, puede ser frecuente la observación de gran cantidad de células muertas, que puede dificultar el análisis de las muestras, como se observó en las muestras obtenidas del águila real. En este estudio se obtuvieron las muestras mediante falsas cópulas del águila con su cuidador, tras un periodo de manejo y entrenamiento del ave (Temple, 1972). A priori, los resultados sugieren que la calidad del semen obtenido a través de la técnica de falsas cópulas voluntarias es significativamente mejor que las obtenidas a través del masaje abdominal en el águila real. No obstante la edad del ejemplar con el que se ha llevado a cabo este estudio ha podido influir en las características de los eyaculados y en su calidad, aunque el efecto de la edad en las características del semen aviar no está apenas estudiado.

Dado que no es fácil disponer de animales troquelados entrenados para la recogida mediante falsas cópulas o por masaje abdominal, la puesta a punto de esta técnica de recogida *post-mortem* puede ofrecer excelentes oportunidades de obtención de material espermático en diferentes ejemplares de especies silvestres que se encuentren muertas tanto en colecciones y núcleos cautivos como en su medio natural. Las técnicas de obtención *post-mortem* de espermatozoides, ampliamente descritas en mamíferos,

apenas están descritas en aves. En mamíferos la técnica más extendida consiste en el lavado del epidídimo con un catéter utilizando el diluyente adecuado para cada especie (Martínez-Pastor et al., 2005; Prieto et al., 2014). Las diferencias anatómicas entre los testículos aviares y los testículos de los mamíferos, además de la amplia variación en tamaños de las aves hace necesario adaptar las técnicas empleadas en estos últimos. Otras técnicas de recogida incluyen la realización de cortes seriados a nivel de la cola del epidídimo, o mediante la aplicación de presión de aire desde el conducto deferente (Santiago-Moreno et al., 2007). Sin embargo, estas últimas parecen ejercer un efecto más perjudicial en los espermatozoides, siendo muy superior la incidencia de contaminación con células sanguíneas y/o epiteliales del conducto epididimario (Santiago-Moreno et al., 2007, 2009). En el caso de las aves se han descrito varias técnicas de obtención *post-mortem* de muestras espermáticas (Gunn et al., 2008) entre las que destacan la técnica de flotación y la técnica de lavado del conducto deferente. Sin embrago, a pesar de ser efectivas en la recogida de espermatozoides, todos los espermatozoides obtenidos en la especie aplicada (calamón, *Porphyrio porphyrio melanotus*) están estáticos. Una hipótesis de esta falta de motilidad puede ser derivada del uso de medios de lavado y mantenimiento no adecuados. En nuestro estudio comparamos estas técnicas de recogida *post-mortem* y dos tipos de diluyente (Lake 70.1, Lake-Ravie) (Lake and Ravie, 1984; Lake and Stewart, 1978), utilizando los gallos, como modelo de especie aviar.

Ambas técnicas resultaron eficaces para la obtención de espermatozoides viables del conducto deferente, aunque fue con la técnica del lavado con la que se obtuvieron unas muestras con mayor concentración de espermatozoides, aproximándose al doble de la concentración de una muestra eyaculada, mientras que las muestras obtenidas por cortes

obtuvieron una concentración similar a la de las muestra eyaculada. Además la contaminación por células sanguíneas, epiteliales y cristales de uratos, de las muestras obtenidas por lavado fue significativamente menor que en las obtenidas por cortes del conducto.

En cuanto a los valores de viabilidad en la muestras en fresco (sin refrigerar) procedentes de lavado, presentaban menor motilidad progresiva, ALH y BCF. La disminución de estos valores puede estar posiblemente causada por los efectos derivados de la propia técnica, principalmente por el efecto de la presión ejercida por el diluyente en la luz del conducto deferente. Efectos similares se han descrito en otras líneas celulares (Bouaziz et al., 1998; Fioravanti et al., 2005; Santiago-Moreno et al., 2007a) como consecuencia de los cambios en el flujo de iones de la membrana plasmática (Podolsky, 1956) y la alteración sufrida por las proteínas de membrana (Scarlata, 2005). En estudios realizados en bóvidos se ha observado que un incremento de la presión produjo una reducción en su motilidad, alteraciones acrosómicas y un incremento en la liberación de enzimas (Carter et al., 1973). Sin embargo las diferencias descritas en muestras en fresco, sin refrigerar, no se observan en las muestras refrigeradas durante 24 y 48 horas, lo que sugiere que estos efectos negativos derivados de la técnica de recolección puedan ser temporales. Además hay que tener en cuenta que en la técnica del lavado del conducto deferente se obtienen la mayoría de los espermatozoides presentes en la luz del conducto, siendo indiferente la fase de desarrollo en la que se encuentren, mientras que en la recolección mediante cortes del conducto deferente se obtienen principalmente los espermatozoides capaces de nadar fuera de la luz del conducto. Esto explica la diferencia en la concentración espermática y en la motilidad progresiva, ALH y BCF.

El tiempo de refrigeración afectó de forma negativa a todas las variables espermáticas de las muestras obtenidas *post-mortem* de forma similar que a las muestras eyaculadas. Asimismo, se observó que los diluyentes tuvieron diferentes efectos en relación al tiempo de refrigeración. El diluyente Lake-Ravie resultó más apropiado en las muestras sin refrigerar y analizadas poco después de la recogida, mientras que el diluyente Lake 7.1. preservó mejor las variables seminales en condiciones de refrigeración. Efectos deletéreos del plasma seminal han sido observados con anterioridad en estudios *in vitro* a bajas temperaturas (4ºC) asociadas con la activación de las fosfolipasas (Douard et al., 2000), producción de ROS (Reactive Oxyden Species) (Fennema et al., 1973) y cambios en el pH asociados al proceso de refrigerado (Aitken and Bennetts, 2006). Todos estos efectos pueden contribuir activamente a la pérdida de la calidad espermática. El hecho que el diluyente Lake 7.1. incluya en su composición un compuesto anfótero que actúa cómo tampón estabilizando el pH durante el proceso de refrigeración (BES) puede ser una de las causas de los mejores resultados obtenidos con el material espermático refrigerado. Estos resultados ponen de manifiesto que para la recogida *post-mortem*, así como para el mantenimiento y traslado del material espermático (*post-mortem* o eyaculado) en refrigeración, se recomiende utilizar el diluyente Lake 7.1. Esto permitirá preservar los espermatozoides en las mejores condiciones hasta su llegada al laboratorio para la realización de los pertinentes estudios sobre caracterización gamética o su procesado para la crioconservación.

El uso de los sistemas computerizados para análisis de la morfometría espermática (ASMA) cuando el método está adaptado a las características específicas es la técnica más fiable y eficaz para el análisis de la morfometría espermática de las diferentes especies. Para conseguir la precisión y repetitividad necesarias es imprescindible que la

tinción, la digitalización de la imagen y la calibración del análisis esté adaptado a la especie objeto del estudio (Gago et al., 1998; Sancho et al., 1998; Hidalgo et al., 2005, 2006). Los sistemas ASMA empleados en mamíferos han dado muy buenos resultados en diferentes especies (Sancho et al., 1998; Hidalgo et al., 2005; Esteso et al., 2006, 2015), ya que permiten evaluar hasta cuatro variables morfológicas en un elevado número de espermatozoides con objetividad. Sin embargo, estos sistemas no son capaces de identificar correctamente los espermatozoides aviares debido a sus características morfométricas ya que, en lugar de presentar una forma ovalada son filiformes (perdiz y gallo), fusiformes (halcones y águila) e incluso helicoidales (paseriformes) (Calhim et al., 2009).

Por lo tanto, hasta ahora la evaluación morfológica de los espermatozoides de ave se ha realizado a través de microscopía óptica (Wakely and Kosin, 1951; Hemberger et al., 2001; Gee et al., 2004) o electrónica (Lake et al., 1968), pero sin el apoyo de los programas de digitalización y análisis de imagen computerizados que permitan una estandarización de la técnica. Los métodos de tinción que se utilicen deben ajustarse no sólo a las caraterísticas de las células que se tiñen, sino tambien a los métodos de observación de las mismas. Para la evaluación morfométrica de los espermatozoides en mamíferos se han empleado diferentes tinciones cómo Hemacolor®, Diff-Quick®, Hematoxilina y SpermBlue® (Gago et al., 1998; Soler et al., 2005; Esteso et al., 2015), siendo el Hemacolor® la más frecuentemente empleada en especies como la cabra montés (*Capra pyrenaica*) (Esteso et al., 2015) y en humanos (Maree et al., 2010). Para la puesta a punto de la técnica analítica de la morfometría de espermatozoides aviares se ha utilizado en nuestro trabajo, como modelo animal, el gallo y la perdiz roja. Los resultados entre la comparación de las tinciones de Hemacolor® y azul de anilina

indican que el Hemacolor® representa la tinción más adecuada, ya que ofrece un porcentaje de espermatozoides correctamente analizados significativamente mayor que en las muestras teñidas con azul de anilina, siendo además, la repetitividad de las mediciones significativamente mayor.

La tinción azul anilina es usada habitualmente en especies aviares para determinar anomalías en el acrosoma, mediante microscopía óptica de contraste de fases (Santiago-Moreno et al., 2009). Sin embargo, su capacidad de tinción de la cabeza del espermatozoide es mucho menor, tiñéndola en un tono demasiado tenue para una correcta detección por parte del software. La tinción de Hemacolor®, sin embargo, tiñe por completo la cabeza del espermatozoide de un color uniforme e intenso lo que permite una delineación fácil de la célula que facilita la medición de sus dimensiones, aunque no tiñe correctamente el acrosoma y por lo tanto, empleando esta tinción no es posible evaluar alteraciones acrosómicas.

Además de las diferencias en la capacidad de tinción, ambas técnicas difieren en el método de fijación de la muestra. Hemacolor® emplea un fijador basado en alcohol (metanol) mientras que la tinción de azul de anilina se fija con glutaraldehído al 2%. Los efectos de diferentes fijadores en las extensiones han sido evaluados con anterioridad (Alkan et al., 2002; Ferdinand et al., 1992; du Plessis and Soley, 2014) siendo considerado el glutaraldehído, según algunos autores, el método de elección ya que produce una menor disminución del volumen celular que secando las células al aire (Gee et al., 2004; Bertschinger, 1992). Las diferencias morfométricas observadas en la muestras teñidas utilizando distintas tinciones han sido estudiadas con anterioridad (Boersma and Braun, 1999; Gravance et al., 1998). La morfometría espermática parece

estar afectada por los efectos osmóticos y de deshidratación asociados a los diferentes compuestos de la tinciones (Hidalgo et al., 2006).

Nuestros datos muestran además, diferencias interespecíficas en el comportamiento ante los diferentes tratamientos, lo que sugiere una respuesta diferente a los métodos de tinción.

En el caso de los gallos, los valores de anchura y área fueron mayores en las muestras teñidas con Hemacolor®. Resultados similares han sido observados en otras especies, en las que se han observado células más grandes en muestras teñidas con Hemacolor® en comparación con otras tinciones (Wakely and Kosin, 1951; Gago et al., 1998; Buendía et al., 2002). En algunos estudios, la diferencia en el tamaño de las cabezas espermáticas con diferentes métodos de tinción ha sido explicado como una posible consecuencia del efecto de deshidratación celular de los fijadores (Soler et al., 2005; Esteso et al., 2015). Sin embargo, las diferencias observadas en las cabezas espermáticas de los gallos no han sido observadas en la perdiz roja. En esta especie los valores de anchura y área se mantenían en valores similares con ambas tinciones. Esta observación apoya la teoría de que pudieran existir diferencias entre especies en la respuesta a los agentes químicos de las diferentes tinciones.

Ambas tinciones permitieron visualizar la cabeza espermática a través del microscopio óptico. La repetitividad de ambas tinciones es alta, ya que no se observaron diferencias en las medidas repetidas de un mismo espermatozoide. Aunque en el caso del gallo, los valores de CV obtenidos en las medidas repetidas sugieren que la tinción Hemacolor® tiene mayor repetitividad que la tinción azul de anilina.

La puesta a punto de la técnica de análisis morfométrico ha permitido la caracterización gamética de eyaculados de águila real, halcón peregrino brookei, halcón peregrino escocés y halcón gerifalte. En todas estas falconiformes, los eyaculados se caracterizaban por una alta heterogeneidad y pleomorfismo celular. Se destaca un alto porcentaje de células espermáticas inmaduras (espermatocitos, espermátidas), siendo esta presencia superior en los halcones que en el águila real. La heterogeneidad y pleomorfismo celular en el eyaculado es una característica de especies con estructuras sociales donde predomina la monogamia. Esto contrasta con las características de los eyaculados de especies más polígamas en las que existe un fuerte componente de poliandria como ocurre en el gallo, y también, aunque en menor medida, en la perdiz. Las especies poliándricas o aquellas que, pese a ser monógamas, presentan una elevada incidencia de cópulas con machos fuera de la pareja, requieren una mayor capacidad de competición espermática. Algunas de las características de estas especies son una mayor homogeneidad entre espermatozoides y un tamaño espermático mayor (Birkhead et al., 1998; Santiago-Moreno et al., 2009). Como contrapunto, en especies de comportamiento reproductor claramente monógamo, cómo el águila real (*Aquila chrysaetos*) o los halcones, presentan espermatozoides heterogéneos y con una elevada presencia de células inmaduras (Villaverde-Morcillo et al., 2015).

La presencia de células inmaduras en semen de halcón y águila ya se había mencionado anteriormente (Blanco et al., 2001), aunque en este caso los autores describen dichas células cómo espermatogonias. En este estudio la comparación de las células observadas con otras previamente identificadas en cortes histológicos nos permitieron identificarlas cómo espermatocitos y espermátidas, que son fases posteriores en la espermatogénesis (Kirby and Froman, 2000; Deviche et al., 2011).

Podría ser que la presencia de este tipo de células tenga un papel fisiológico en la reproducción de falconiformes tal y cómo se ha sugerido en otras especies. Por ejemplo en el gusano de la seda *(Bombyx mori)* se ha sugerido que las células infértiles presentes en el semen podrían ayudar a la trasferencia del semen fértil del macho a la hembra (Sahjara and Takemura, 2003). Sin embargo, en falconiformes es necesario realizar más estudios para poder determinar la existencia de un papel asociado a este tipo de células.

Los volúmenes de los eyaculados del águila real variaron en un intervalo de entre 1-115 µl con un volumen medio de 42,20±31,8 µl. En los halcones, el volumen medio de los eyaculados fue mayor 67,21±49,67 µl (rango 15-215 µl), sin embargo los volúmenes obtenidos en las muestras mediante masaje fueron más bajos que los eyaculados 38±21,9 µl (5-109 µl). Estos volúmenes son similares a los descritos en otras especies de aves silvestres cómo el buitre de espalda blanca o la hubara (Umapathy et al., 2005; Wishart et al., 2002). Sin embargo, en el caso del águila real, sí se observaron diferencias en la concentración espermática (467,75 x 10^6 ml^{-1}) con la contabilizada en los eyaculados de gallo (630,3 x 10^6 ml^{-1}) y especies cómo el ánade real o la paloma. Estas diferencias pueden apreciarse también en los parámetros de velocidad y motilidad. En el águila real observamos una motilidad media de 43,1%, más elevada que los descritos en otras especies de rapaces cómo el águila imperial ibérica o el halcón peregrino (Blanco et al., 2000), pero menor que la observada en el gallo (68,3±10,3%). Las diferencias entre el águila y las otras especies silvestres pueden ser consecuencia del método de obtención de las muestras, masaje *vs.* eyaculado, ya que todos los resultados mencionados se han obtenido mediante masaje. Sin embargo, las diferencias con el gallo pueden ser debidas a las diferentes estrategias reproductoras

de dichas especies, y a su relación con la competición espermática descrita con anterioridad (Birkhead et al., 1995; Stunden et al., 1998; Sontakke et al., 2004).

El capítulo correspondiente al análisis morfométrico de muestras seminales de halcón reveló, por primera vez en una especie aviar, la existencia de subpoblaciones espermáticas. Concretamente se describen cuatro subpoblaciones (Sp 1-4) con diferentes características morfométricas. Las proporciones de cada subpoblación fueron similares en las tres especies. Sin embargo, en el caso de los halcones gerifaltes se vieron diferencias entre las proporciones de las subpoblaciones entre las muestras frescas y las descongeladas. Mientras que en semen fresco las Sp3 y 4 eran las más grandes, en semen descongelado fueron la Sp1 y 2 las de mayores proporciones. Además, en los valores medios de las diferentes subpoblaciones se observa que en las muestras descongeladas las cabezas espermáticas presentaban tamaños más pequeños que las muestras frescas. Este efecto, resultado del proceso de congelación y descongelación se ha descrito en otras especies de mamíferos (Gravance et al. 1998; Esteso et al., 2003, 2006). Esta disminución del tamaño podría ser consecuencia de lesiones o pérdida de los acrosomas (Thomas et al., 1998), o cambios estructurales en la cromatina espermática (Gravance et al., 1998; Blottner et al., 2001).

La morfometría y la morfología de la cabeza de los espermatozoides ha sido relacionada con su capacidad para atravesar la barrera mucosa en test de migración *in vitro*, y han reseñado que el tamaño relativo de la población no migradora puede estar negativamente relacionada con la fertilidad del macho (Martínez-Rodríguez et al., 2015). Otros estudios en diferentes taxones han sugerido que un incremento en la cabeza espermática, implica un incremento del tamaño de todos sus componentes y está asociado con mejores resultados en la migración espermática y en la velocidad natatoria

de los espermatozoides (Briskie et al., 1992; Gomendio and Roldan, 2008; Fitzpatrick et al., 2009; Tourmente et al., 2011) o con su hidrodinamia (Malo et al., 2006).

REFERENCIAS

Aitken, R.J., Bennetts, L., 2006. Reactive oxygen species: friend or foe, in de Jonge C., Barrat C. (Eds), The Sperm Cell: Production, Maturation, Fertilization, Regeneration. Cambridge University Press, UK, 170-193.

Albrechtová J, Albrecht T, Ďureje L, et al 2014. Sperm morphology in two house mouse subspecies: do wild-derived strains and wild mice tell the same story? PLoS One 9:e115669.

Alkan S, Baran A, Özdas¸ ÖB, Evecen M. 2002. Morphological defects in turkey semen. Turk J Vet Anim Sci 26,1087–92.

Anderson MJ, Nyholt J, Dixson AF 2005. Sperm competition and the evolution of sperm midpiece volume in mammals. J Zool 267:135–142.

Birkhead, T. R. 1995. Sperm competition: Evolutionary causes and consequences. Reprod. Fert. Dev. 7, 755-775.

Birkhead TR, Immler S. 2007. Making sperm: design, quality control and sperm competition. Soc Reprod Fertil Suppl 65, 175-181.

Blanco, J.M., Gee, G., Wildt, D.E., Donoghue, A.M. 2000. Species variation in osmotic, cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon spermatozoa. Biology of Reproduction. 63, 1164-1171.

Blanco, J., Wildt, D., Gee, G., Donoghue, A., 2001. Spermatogonia in eagle and peregrine falcon ejaculates: interspecies variation, cryopreservation tolerance and implications to cryobanking. 4th Eurasian Congr. Raptors.

Bouaziz, A., de Ficquelmont-Loizos, M.M., Richert, A., Caprani, A., 1998. Direct physical factors and PGI2 and TXA2 secretions by a human endothelial cell line: in vitro investigation of pressure and shear stress applied independently or in synergy. Thromb. Res. 90, 279-289.

Briskie J V, Montgomerie R, 1992. Sperm size and sperm competition in birds. Proc Biol Sci 247, 89–95.

Bertschinger HJ, Burger WP, Soley JT, de Lange JH, 1992. Semen collection and evaluation of the male ostrich. Grahamstown, Proc Biennial Congr S Afr Vet Assoc, 154–8.

Blottner S, Warnke C, Tuchscherer A, et al 2001. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. Anim Reprod Sci 65, 75–88.

Boersma, A., Braun, J., 1999. Computer-assisted analysis of sperm morphology in veterinary medicine. Berl. Munch. Tierarztl. Wochenschr. 112, 81–5.

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

Calhim S, Lampe HM, Slagsvold T, Birkhead TR, 2009. Selection on sperm morphology under relaxed sperm competition in a wild passerine bird. Biol Lett 5, 58–61.

Carter, J.E., Rich, S.J., Graham, E.F., Lillehei, R.C., 1973. The effect of pressure and cooling rate on spermatozoa, kidney tissue, and the whole kidney. Cryobiology 10, 263-270.

Deviche P, Hurley LL, Fokidis HB, 2011. Avian testicular structure, function and regulation. In: Hormones and Reproduction of Vertebrates. Elsevier, pp. 20-70.

Dogliero, A. 2015. Use of computer-assisted semen analysis for evaluation of Rosy-faced lovebird *(Agapornis roseicollis)* semen collected in different periods of the year. Theriogenology 83, 103-6.

Douard, V., Hermier, D., Blesbois, E., 2000. Changes in turkey semen lipids during liquid in vitro storage. Biol. Reprod. 63, 1450-1456.

Du Plessis L, Soley JT, 2014. Light microscopic features and morphometry of sperm in the emu *(Dromaius novaehollandiae)*. Theriogenology 81, 203–209.

Esteso MC, Fernández-Santos MR, Soler AJ, Garde, JJ, 2003. Head dimensions of cryopreserved red deer spermatozoa are affected by thawing procedure. Cryoletters 24,261–268.

Esteso MC, Soler AJ, Fernández-Santos MR, Quintero-Moreno AA, Garde JJ, 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red Deer (Cervus elaphis hispanicus) epididymal sperm samples. J Androl 27, 662-670.

Esteso MC, Rodríguez E, Toledano-Díaz A, Castaño C, Pradiee J, López-Sebastián A, Santiago-Moreno J, 2015. Descriptive analysis of sperm head morphometry in Iberian ibex *(Capra pyrenaica)*: Optimum sampling procedure and staining methods using Sperm-Class Analyzer(®). Anim Reprod Sci 155,42–49.

Fennema, O.R., Powrie, W.D., Marth, E.H., 1973. Low-temperature preservation of foods and living matter. Fennema O.R. Ed., Marcel Dekker, Inc., New York.

Ferdinand A. 1992. Licht- und elektronenmikroskopische Untersuchungen zur Morphologie von Ganterspermatozoen. PhD Thesis. Hannover: University of Veterinary Medicine.

Fitzpatrick JL, Montgomerie R, Desjardins JK, et al, 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. Proc Natl Acad Sci U S A. 106,1128–32.

Gago C, Pérez-Sánchez F, Yeung CH, Tablado L, Cooper TG, Soler C, 1998. Standardisation of sampling and staining methods for the morphometric evaluation of sperm heads in the Cynomolgus monkey (Macaca fascicularis) using computer-assisted image analysis. Int J Androl 21, 169–176.

Gee, G.F., M. R. Bakst, and T. J. Sexton. 1985. Cryogenic preservation of semen from the greater sandhill crane. J. Wildlife Manage. 49, 480-484.

Gee, G.F., Bertschinger, H., Donoghue, A.M., Blanco, J., Soley, J., 2004. Reproduction in nondomestic birds: physiology, semen collection, artificial insemination and cryopreservation. Avian Poult. Biol. Rev. 15, 47-101.

Gomendio M, Roldan ERS, 2008. Implications of diversity in sperm size and function for sperm competition and fertility. Int J Dev Biol 52:439–447.

Gravance C, Champion Z, Casey P, 1998. Computer-assisted sperm head morphometry analysis (ASMA) of cryopreserved ram spermatozoa. Theriogenology 49, 1219–1230.

Grigg G.W., A.J., H., 1949. Electron microscopic studies of spermatozoa: I. The morphology of the spermatozoon of the common domestic fowl (Gallus domesticus). Aust J Sci Res Ser B. 2, 271–86.

Gunn, M.R., Champion, Z., Casey, M.E., Teal, P., Casey, P.J., 2008. Testicular and spermatozoan parameters in the pukeko (*Porphyrio porphyrio melanotus*). Anim. Reprod. Sci. 109, 330-342.

Hemberger MY, Hospes R, Bostedt H, 2001. Semen collection, examination and spermiogram in ostriches. Reprod Dom Anim 36, 241–3.

Hidalgo M, Rodriguez I, Dorado J, Sanz J, Soler C, 2005. Effect of sample size and staining methods on stallion sperm morphometry by the Sperm Class Analyzer. Vet Med (Praha) 50, 24–32.

Hidalgo M, Rodríguez I, Dorado J, 2006. Influence of staining and sampling procedures on goat sperm morphometry using the Sperm Class Analyzer. Theriogenology 66, 996–1003.

Kirby JD, Froman DP, 2000. Reproduction in male birds. In: Sturkie's Avian Physiology, 5th edn. Elsevier, pp 597-615.

Kleven O, Laskemoen T, Fossøy F, et al, 2008. Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. Evolution 62, 494–499.

Lake PE, Smith W, Young D. 1968. The ultrastructure of the ejaculated fowl spermatozoon. Q. Jl exp. Physiol. 53, 356-366.

Łukaszewicz E, Kowalczyk A, Rzonca Z., 2011. Characteristics of Fresh Semen of Captive-Bred Capercaillie Tetrao urogallus L. Zoo Biology 30, 672–680.

Malo AF, Gomendio M, Garde J, et al, 2006. Sperm design and sperm function. Biol Lett 2:246–9.

Maree L, du Plessis SS, Menkveld R, Van der Horst G, 2010. Morphometric dimensions of the himan sperm head depend on the staining method used. Hum Reprod 25,1369-1382.

Martinez-Pastor, F., Guerra, C., Kaabi, M., Diaz, A.R., Anel, E., Herraez, P., De Paz, P., Anel, L, 2005. Decay of sperm obtained from epididymes of wild ruminants depending on post-mortem time. Theriogenology 63, 24–40.

Martínez-Rodríguez C, Alvarez M, López-Urueña E, Gomes-Alves S, Anel-López L, Tizado J E, Anel L and de Paz P, 2015. Head morphology of ram

spermatozoa is associated with their ability to migrate in vitro and correlates with fertility. Reprod Fertil Dev 27:852-864.

Podolsky, R.J., 1956. A mechanism for the effect of hydrostatic pressure on biological systems. J. Physiol. 132, 38-39P.

Prieto, MT., Sánchez-Calabuig, MJ., Hildebrandt, TB.,Santiago-Moreno, J.,saragusty, J. 2014. Sperm cryopreservation in wild animals. Eur. J. Wildl. Res. 60, 851–864.

Rowe M, Laskemoen T, Johnsen A, Lifjeld JT, 2013. Evolution of sperm structure and energetics in passerine birds. Proc Biol Sci 280, 201-226.

Sahjara K, Takemura N, 2003. Application of artificial insemination technique to eupyrene and/or apyrene sperm in Bombyx mori. J Exp Zool 297A, 196-200.

Sancho, M., Pérez-Sánchez, F., Tablado, L., de Monserrat, J.., Soler, C., 1998. Computer assisted morphometric analysis of ram sperm heads: Evaluation of different fixative techniques. Theriogenology 50, 27–37.

Santiago-Moreno, J., Toledano-Díaz, A., Dorado, J., Pulido-Pastor, A., Coloma, M.A., López-Sebastián, A., 2007. Sperm recovery and cryopreservation of Spanish ibex epididymal spermatozoa. Arch. Androl. 53, 309-316.

Santiago-Moreno J, Castaño C, Coloma MA, Gómez-Brunet A, Toledano-Díaz A, López-Sebastián A, Campo JL, 2009. Use of the hypo-osmotic swelling test and aniline blue staining to improve the evaluation of seasonal sperm variation in native Spanish free-range poultry. Poult Sci 88,2661–2669.

Santiago-Moreno J., Astorga R. J., Luque I., Coloma M. A., Toledano-Díaz A., Pulido-Pastor A., et al. 2009. Influence of recovery method and microbial contamination on the response to freezing–thawing in ibex *(Capra pyrenaica)* epididymal spermatozoa. Cryobiology, 59, 357-362.

Scarlata, S., 2005: The effect of hydrostatic pressure on membrane-bound proteins. Braz. J. Med. Biol. Res. 38, 1203-1208.

Soler, C., Gadea, B., Soler, A.J., Fernández-Santos, M.R., Esteso, M.C., Núñez, J., Moreira, P.N., Núñez, M., Gutiérrez, R., Sancho, M., Garde, J.J., 2005. Comparison of three different staining methods for the assessment of epididymal red deer sperm morphometry by computerized analysis with ISAS®. Theriogenology 64, 1236–1243.

Sontakke, S.D., G. Umapathy, V. Sivaram, S. D. Kholkute, and S. Shivaji. 2004. Semen characteristic, cryopreservation and successful artificial insemination in the Rock blue pigeon *(Columba livia)*. Theriogenology 2004: 139-153.

Stunden C.E., C. K. Bluhm, K. M. Cheng, and R. Rajamahendran. 1998. Plasma testosterone profiles, semen characteristics, and artificial insemination in yearling and adult captive Mallard ducks *(Anas platyrhynchos)*. Poult. Sci. 77: 882-887.

Temple, S. A. 1972. Artificial insemination with imprinted birds of prey. Nature 287-288.

Thurston RJ, Hess RA, 1987: Ultrastructure of spermatozoa of domesticated birds: comparative study of turkey, chicken and guinea fowl. Scan microscopy 1,1829-1838.

Tourmente M, Gomendio M, Roldan ERS, 2011. Sperm competition and the evolution of sperm design in mammals. BMC Evol Biol 11:12.

Umapathy, G., Sontakke, S., Reddy, A., Ahmed, S., Shivaji, S., 2005. Semen characteristics of the captive Indian white-backed vulture (Gyps bengalensis). Biol. Reprod. 73, 1039-1045.

Villaverde-Morcillo S, García-Sánchez R, Castaño C, Rodríguez E, González F, et al. 2015. Characterization of natural ejaculates and sperm cryopreservation in a Golden Eagle *(Aquila chrysaetos)*. J Zoo Wildl Med 46:335-338

Wakely WJ, Kosin IL, 1951: A study of the morphology of the turkey spermatozoa--with special reference to the seasonal prevalence of abnormal types. Am J Vet Res 12, 240-245

Wishart, G.J., 2007. Cryopreservation of avian spermatozoa. Methods Mol. Biol. 368, 219–225.

CONCLUSIONES

1. El método de obtención de semen *post-mortem* mediante lavado permite obtener mayor número de espermatozoides que utilizando la técnica de flotación y casi el doble que los obtenidos en muestras eyaculadas.

2. El diluyente Lake 7.1. proporciona los mejores resultados para la preservación de semen, tanto eyaculado como obtenido *post-mortem*, en condiciones de refrigeración.

3. La tinción de Hemacolor® es más apropiada para el análisis morfométrico de las cabezas de espermatozoides aviares, ya que permite un mayor número de células capturadas medibles y mejores valores de repetitividad.

4. Las falconiformes se caracterizan por presentar un elevado pleomorfismo y heterogenicidad celular en los eyaculados, siendo esta característica superior en los halcones que en el águila real.

5. Se han identificado diferentes subpoblaciones espermáticas en los eyaculados de halcón en función del tamaño de la cabeza de los espermatozoides.

6. El análisis morfométrico de espermatozoides de halcón indica que el proceso de criopreservación determina una disminución del tamaño de la cabeza espermática.

ANEXO FOTOGRÁFICO

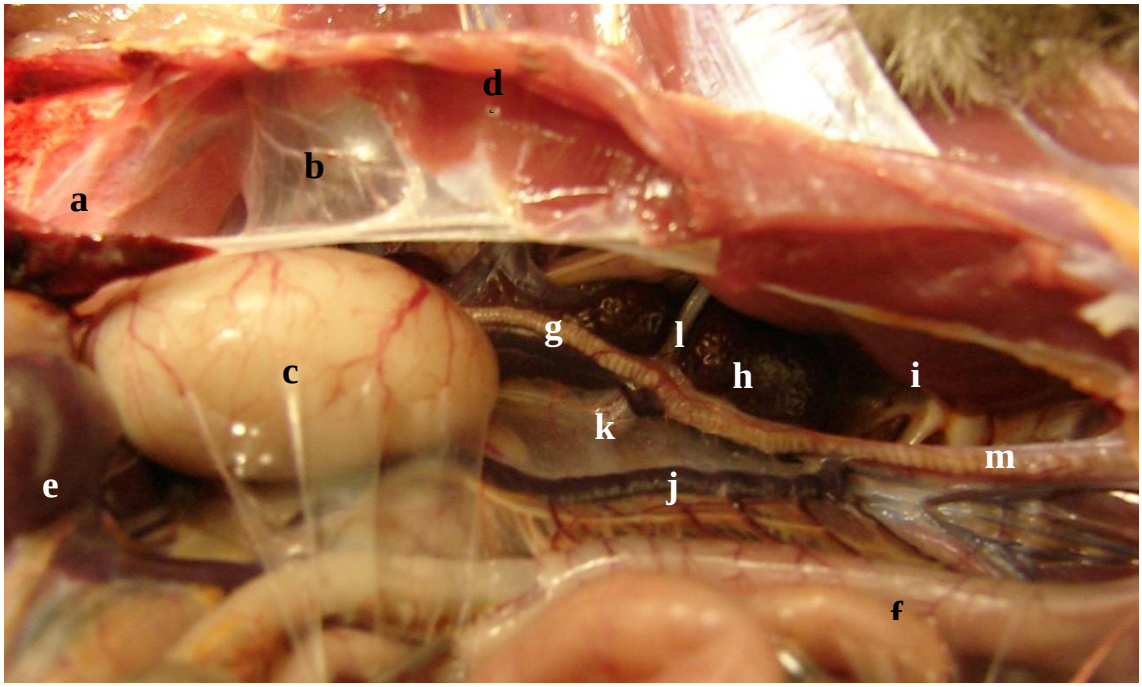


Fig. 1.- Detalle anatómico del aparato reproductor tras la retirada del tracto digestivo. Pulmón (a); saco aéreo (b); testículo (c); pared costal (d); bazo (e); colon (f); conducto deferente (g); riñón (polo caudal) (h); pared abdominal (i); vena mesentérica caudal (j); arteria aorta caudal (k); arteria isquiática (l); uréter (m).

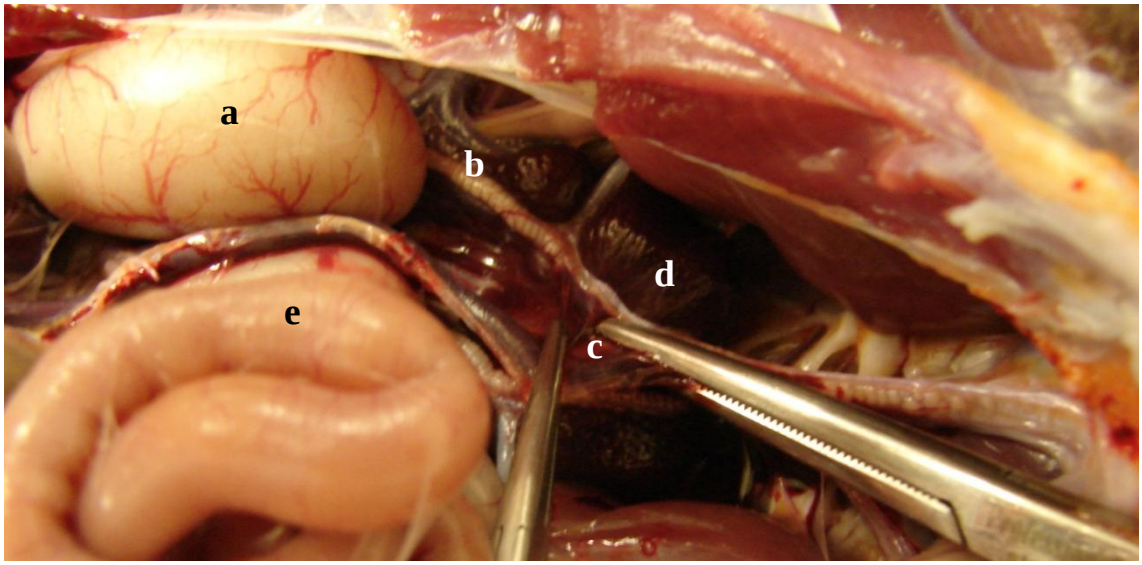


Fig. 2.- Clampado de la vena mesentérica caudal para evitar sangrado. Testículo izquierdo (a); conducto deferente (b); vena mesentérica caudal (c); riñón (d); colon (e).

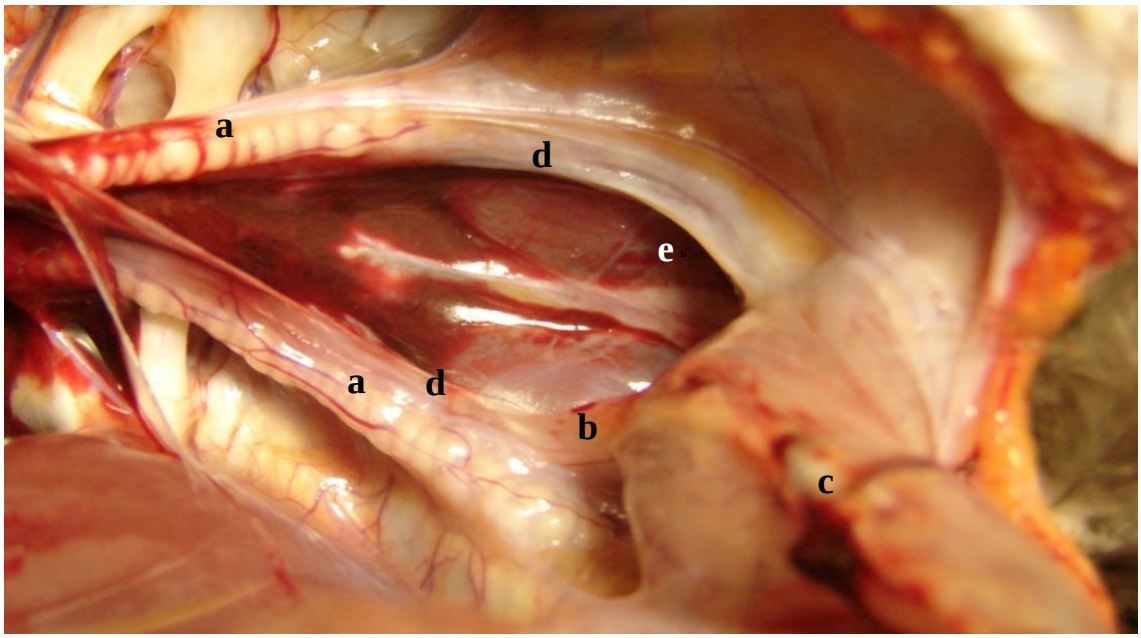


Fig. 3.- Detalle de la desembocadura del conducto deferente en la cloaca. Conducto deferente (a); cloaca (b); recto (c); uréter (d); entrada del conducto deferente en la cloaca (e).

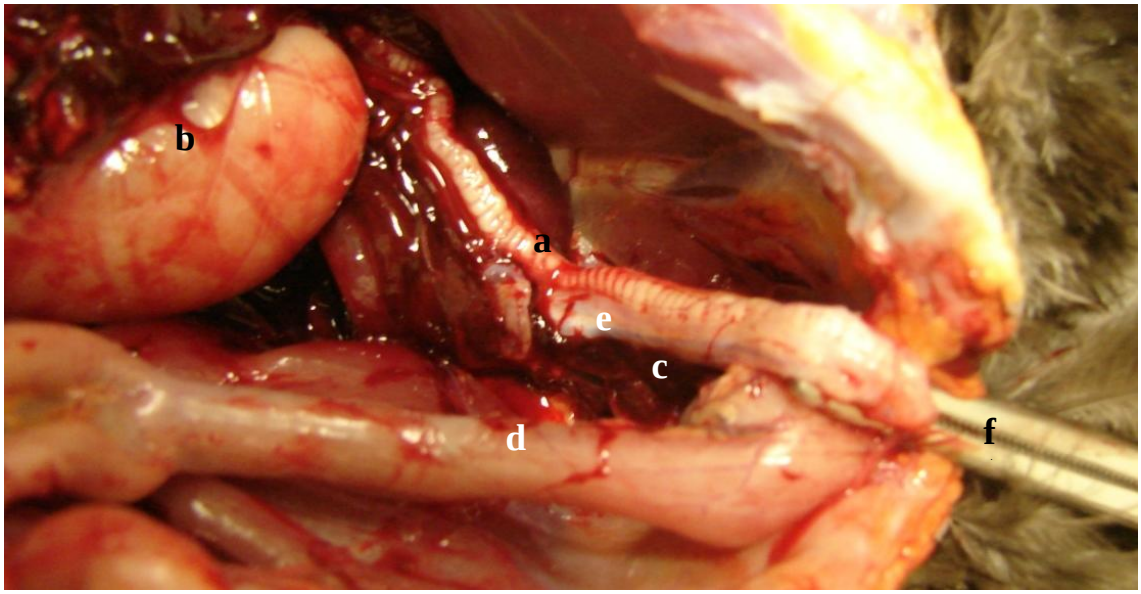
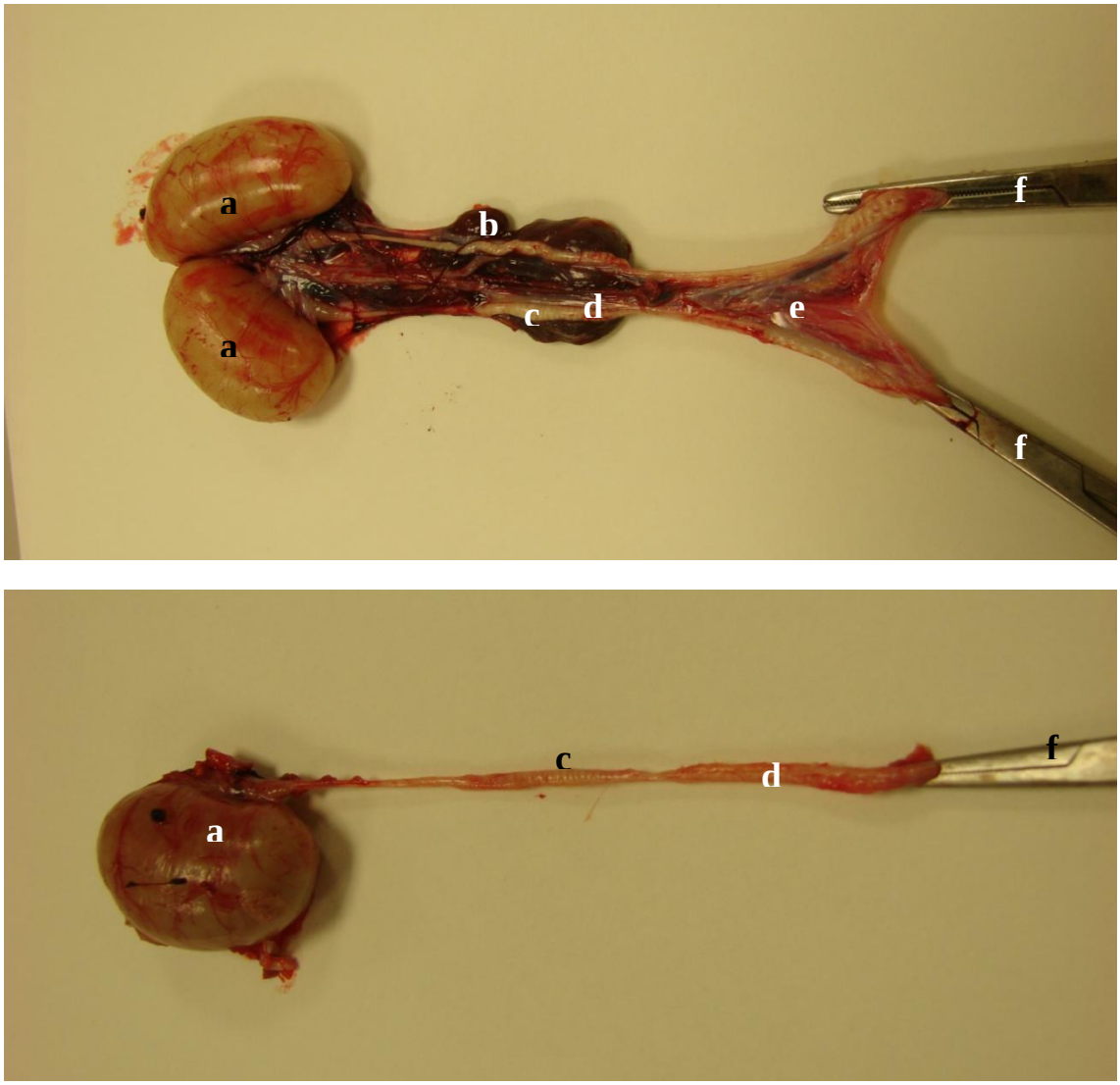


Fig. 4.- Detalle del clampado de la papila o ampolla del conducto deferente para evitar pérdidas de semen. Conducto deferente (a); testículo (b); cloaca (c); recto (d); uréter (e); pinza hemostática (f).



Figs. 5 y 6.- Sistema génito-urinario del gallo extraído de la carcasa para su disección. Testículos (a); fragmento de riñón (b); conducto deferente (c); uréter (d); mesentéreo (e); pinza hemostática (f).

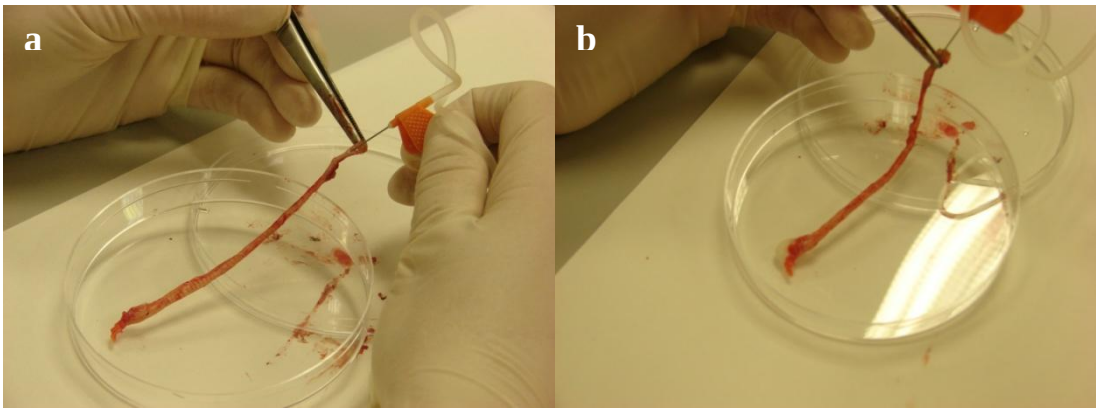


Fig. 7.- Canulación del conducto deferente para realizar la recogida de esperma por el método de lavado (flushing) (a); salida del esperma por el polo caudal del conducto deferente (b).

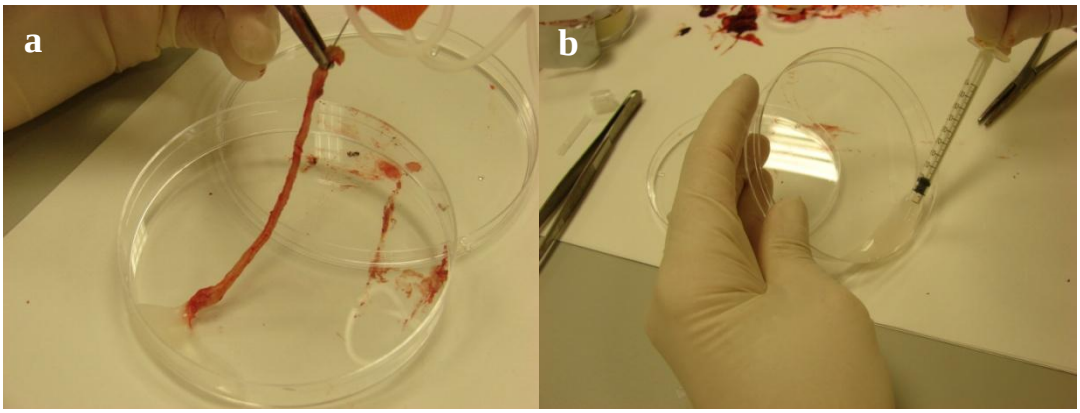


Fig. 8.- Salida de los espermatozoides diluidos del conducto deferente a la placa de Petri (a) y la posterior recogida con jeringa de 1mL (b).

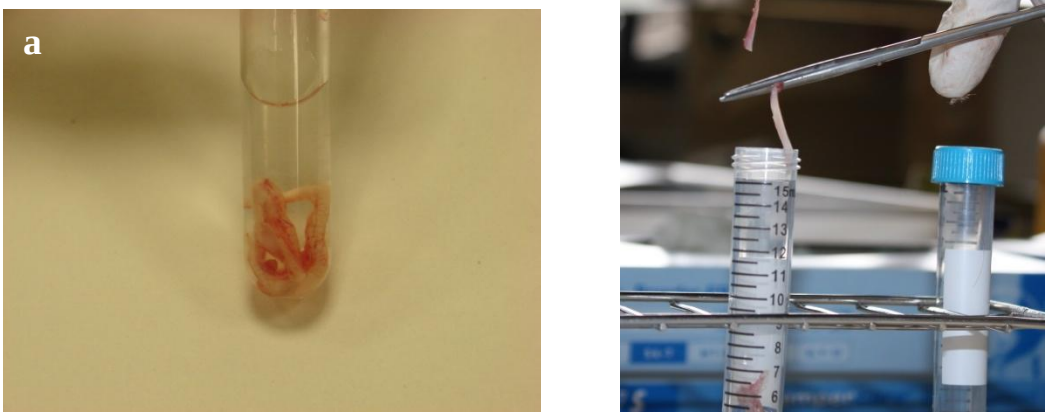


Fig. 9.- Conducto deferente cortado en fragmentos para recogida de esperma por el método de cortes (a y b).

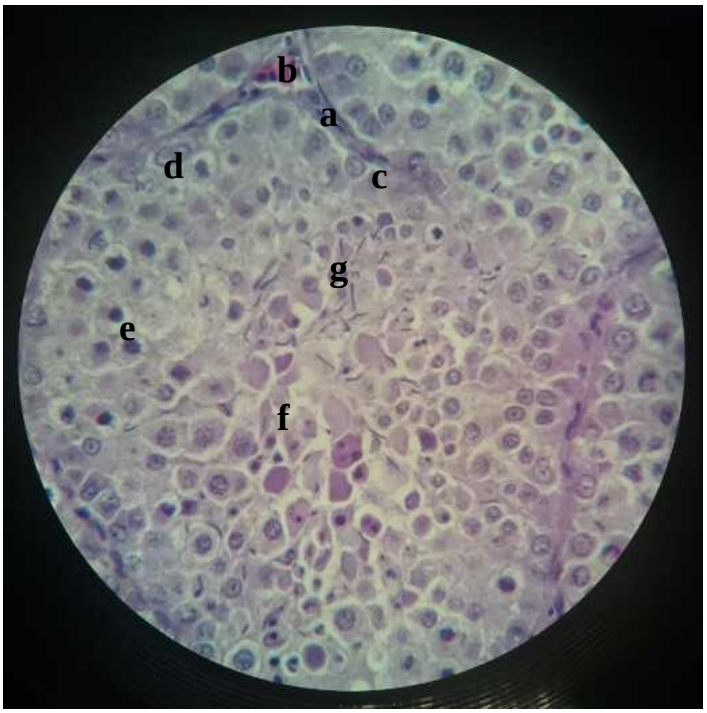


Fig. 10.- Corte histológico de testículo de halcón peregrino adulto en época de celo en el que se observa espermatogénesis activa. Intersticio (a); vascularización testicular (b); lámina basal (c); espermatogonias (d); espermatocitos (e); espermátidas (f); espermatozoides (g). Foto realizada con microscopía óptica (x100 y objetivo de inmersión).

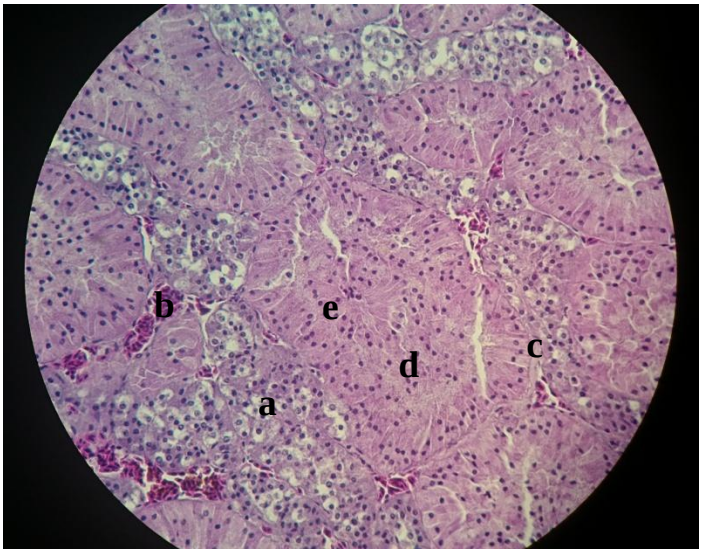


Fig. 11.- Corte histológico de testículo de halcón peregrino adulto fuera de la época de celo en el que no se observa espermatogénesis. Intersticio (a); vascularización testicular (b); lámina basal (c); espermatogonias (d); células de Sertoli (e) (x50).

Figs. 13-16.- Recogida de muestras de semen eyaculado en gallo.

Figs. 17-18.- Recogida de semen en perdiz roja.

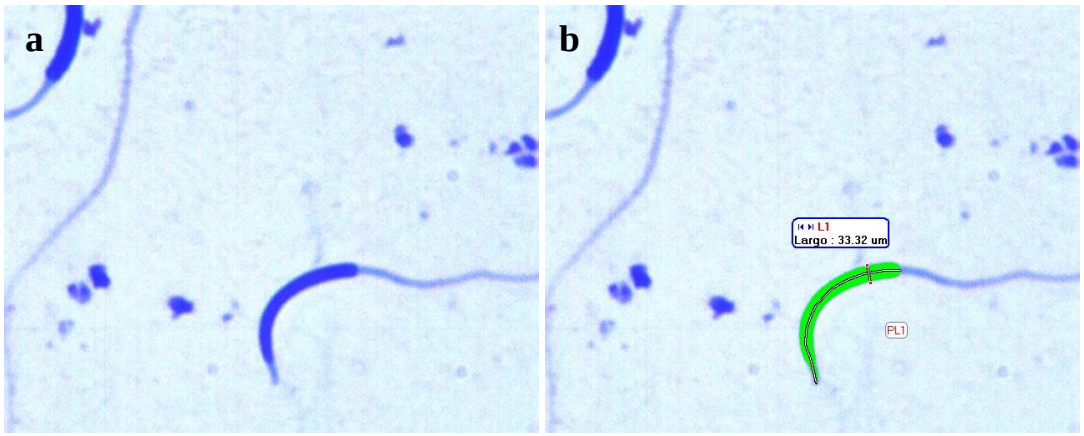


Fig. 12.- Espermatozoides de gallo obsevados con microscopía óptica (x100). Tinción de Hemacolor® (a); espermatozoide marcado para las mediciones (b).

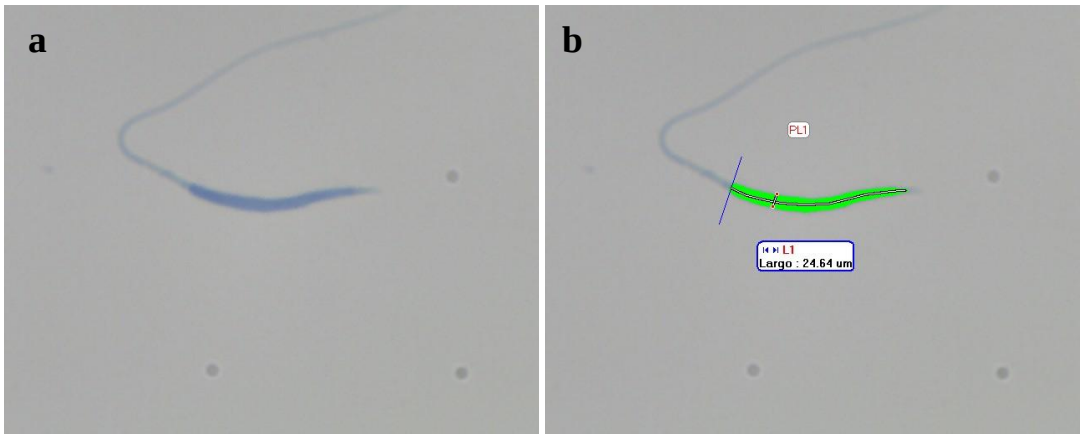


Fig. 13.- Espermatozoides de gallo observados con microscopía óptica (x100). Tinción azul de anilina (a); espermatozoide marcado para las mediciones (b).

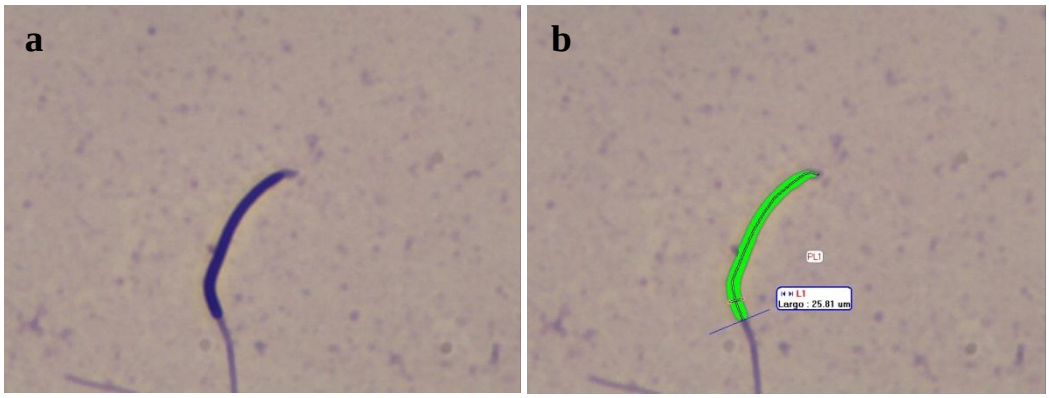


Fig. 14.- Espermatozoide de perdiz observado con microscopía óptica (x100). Tinción Hemacolor® (a); espermatozoide marcado para las mediciones (b).

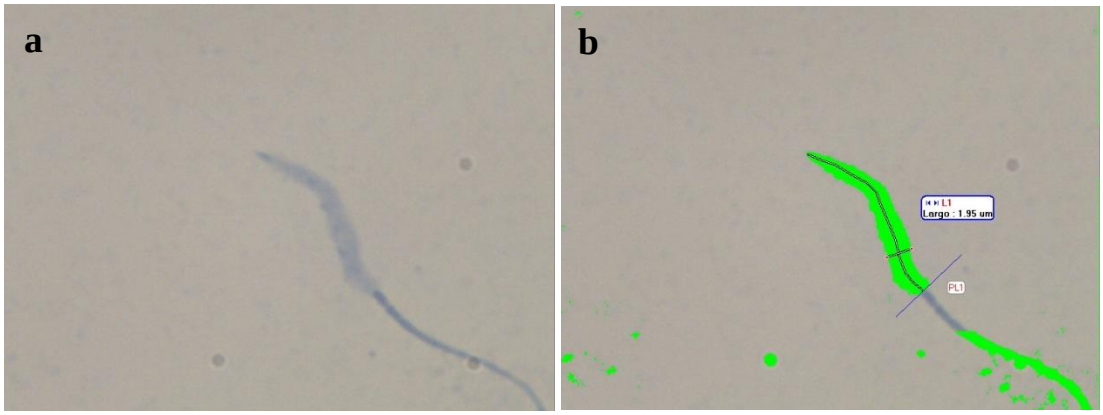


Fig. 15.- Espermatozoide de perdiz observados con microscopía óptica (x100). Tinción azul de anilina (a); espermatozoide marcado para las mediciones (b).

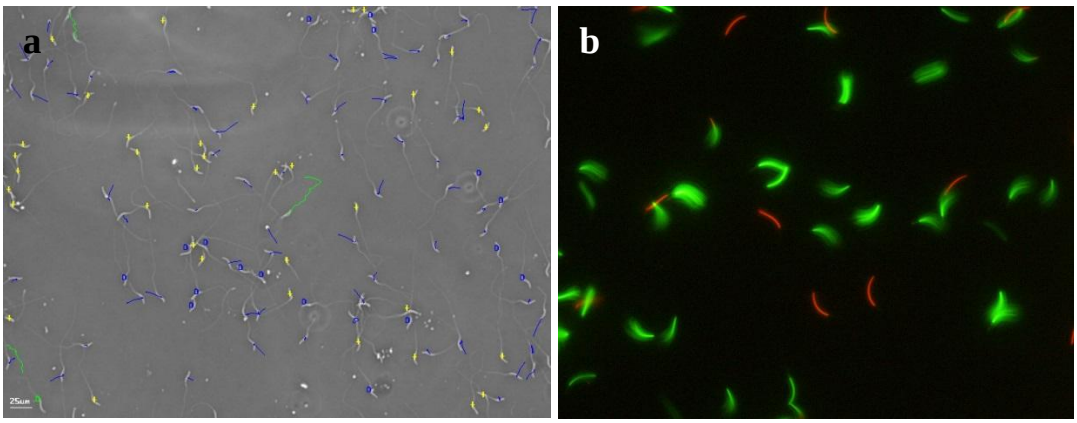


Fig. 16.- Imágenes de los análisis de motilidad con CASA (a) y viabilidad con SYBR-14/PI (b) en semen de gallo.

Fig. 17.-Halcón gerifalte en pose de saludo previo a la cópula (a) y sombrero adaptado a la recogida de semen (b).

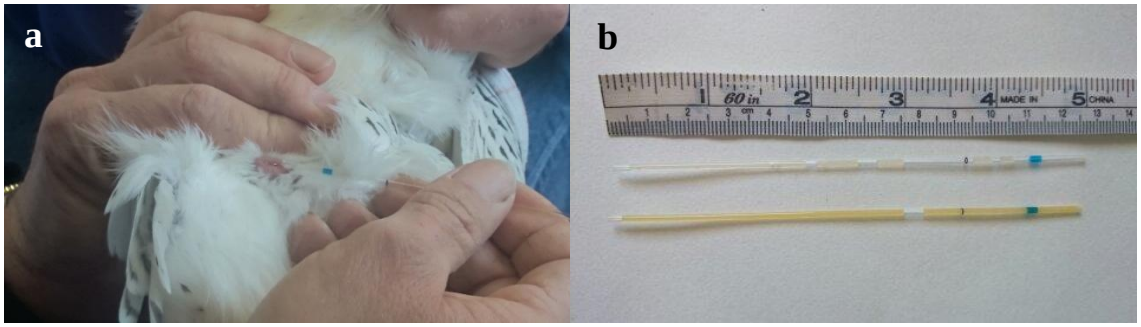


Fig. 18.- Recogida de semen en capilar mediante masaje (a) y capilares con muestras de semen de diferentes individuos (b).

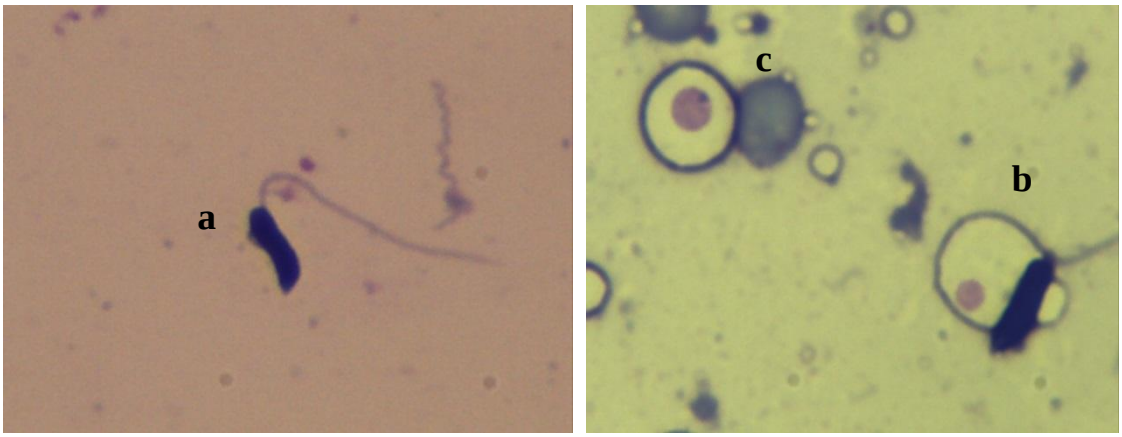


Fig. 19.- Espermatozoide y formas inmaduras observadas en eyaculado de halcón peregrino brokei; espermatozoide (a); espermátida (b); cuerpo residual (c).

Fig. 20.- Secuencia de cópula de águila real con el cuidador: fase inicial (a); salto (b); cópula (c, d); salida (e); recogida del semen del guante con capilar (f).

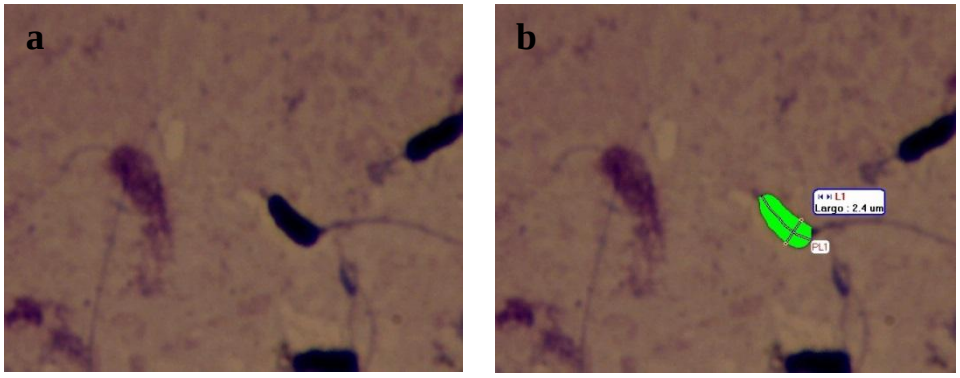


Fig. 21.- Espermatozoide de águila real teñido con Hemacolor® (a) y marcado para su medición (b). Microscopía óptica (x100).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