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GENETICS OF FRESH AND FROZEN-THAWED SEMEN TRAITS AND THEIR RELATIONSHIP WITH GROWTH RATE IN RABBITS

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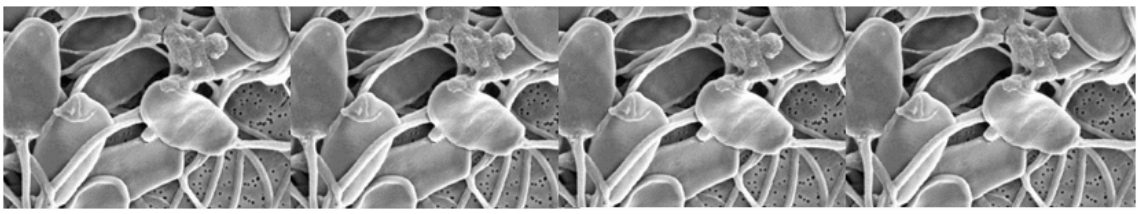
ABSTRACT-RESUMEN-RESUM



ABSTRACT

The general aim of this thesis was to study the genetic determinism for some traits related to artificial insemination (AI) dose production of fresh and frozen-thawed semen, in order to explore the interest and limitation of different strategies for their genetic improvement in a paternal line of rabbits selected for growth rate during the fattening period (28-63 days).

In chapter 1, genetic parameters of sperm production traits are estimated as well as the genetic relationship with daily gain (DG). The heritabilities (h^2) of the semen traits were 0.13±0.05, 0.08±0.04 and 0.07±0.03 for ejaculate volume (V), sperm concentration (CN) and sperm production (PROD) per ejaculate, respectively. A favourable and moderate genetic correlation was observed between V and DG (0.36±0.34). From this chapter it may be concluded that if a seminal trait is to be included as a selection objective, a useful one could be sperm production, as it is a trait in which both volume and concentration are included.

Moreover, there is currently no evidence to suggest that selection for DG in rabbits will affect sperm production adversely.

The aim of chapter 2 was to explore the genetic determinism of some sperm quality traits and their genetic relation with the selection criteria of the paternal rabbit line. The heritabilities (h^2) of semen quality traits commonly evaluated in a classic spermiogram were 0.18, 0.19 and 0.12 for NAR (%, percentage of sperm with intact acrosome), ANR (%, percentage of sperm abnormalities) and MOT (%, percentage of total motile sperm cells) respectively. We also estimated the

h^2 of some motion CASA parameters 0.09, 0.11, 0.10, 0.11, 0.11 and 0.11 for VAP (µm/s; average path velocity), VSL (µm/s; straight-line velocity), VCL (µm/s; curvilinear velocity), LIN (%, linearity index), ALH (µm; amplitude of the lateral head displacement), STR (%, straightness). Genetic correlations between DG and semen traits showed a high $HPD_{95\%}$ (interval of highest density of 95%). However there is some consistent evidence of the negativity of the genetic correlations of DG with NAR and MOT (-0.40 and -0.53, respectively).

Chapter 3 aims to determine the repeatability and heritability of sperm head characteristics: width (W, µm), area (A, µm2),length (L, µm) and perimeter (P, µm), and explore the relationships between them and with the selection objective (DG). The results obtained showed that sperm head dimensions are heritable (ranged between 0.2 and 0.29). The genetic correlations between sperm traits were always high and positive (between 0.72 and 0.90), with the exception of L-W genetic correlation, which was moderate. Regarding the genetic correlations between DG and sperm head characteristics, the resulting means ranged from -0.09 for L-DG to -0.43 for W-DG, showing consistent evidence of the negativity of the genetic correlations.

The environmental and male effects that could have an influence on sperm freezability are studied in Chapter 4. Six different traits were evaluated: sperm concentration (CONC, 10^6spermatozoa/mL), acrosome integrity in fresh (NAR, %) and frozen-thawed semen (Nar-FT, %), sperm motility in fresh (MOT, %) and frozen-thawed semen (Mot-FT, %) and the percentage of viable sperm in frozen-thawed semen (Live-FT, %). In addition, two synthetic traits were computed: the relative reduction of acrosome integrity (Rnar, %) and relative reduction of motility (Rmot, %) after the freezing-thawing process. A multiple-

trait recursive model was used to analyse the relationships between the semen traits considered. For the fixed effects studied, the season had the highest impact on post-thaw semen characteristics. Results of the analysis of recursive coefficients showed that fresh semen concentration and motility influence the future freezability of the semen. All traits studied presented moderate repeatabilities, ranging from 0.11 to 0.38. These results provide conclusive evidence that sperm freezability in rabbits could be heritable. Regarding male correlations, there were large positive male correlations between fresh traits (r_m=0.77-0.57), as well as between direct frozen-thawed traits (r_m=0.72-1). Male effects on fresh and direct frozen-thawed traits were generally positively correlated. This correlation was moderate to high for MOT with all frozen-thawed traits (r_m=0.41-0.74) and for Mot-FT and all fresh traits (r_m=0.5-0.74); these results suggest that these traits could be genetically related.

The final chapter of this thesis focused on estimating the heritability of semen freezability traits and estimating the genetic correlation between frozen-thawed sperm traits and the growth rate in a paternal rabbit line. Estimated heritabilities showed that frozen-thawed semen traits are heritable (ranged between 0.08 and 0.15). In the case of Live-FT, the estimated heritability is the highest and suggests the possibility of effective selection. After the study of genetic correlations, it seems that DG was negatively correlated with sperm freezability, but due to the high HPD95% no further conclusions could be drawn. More data should be included in order to obtain better accuracy for the estimates of these genetic correlations. If the results obtained in the present study were confirmed, it would imply that selection for DG could alter sperm cell membranes or seminal plasma composition, both components related to sperm cryoresistance.

RESUMEN

El objetivo principal de la tesis ha sido estimar los parámetros genéticos de variables relacionadas con la producción y calidad de dosis seminales para su uso en inseminación artificial (en fresco y tras un proceso de crioconservación), con la finalidad de explorar el posible interés y las limitaciones del uso de diferentes estrategias de selección para su mejora genética en una línea paternal de conejos seleccionada por velocidad de crecimiento durante el periodo de engorde (28-63 días).

En el capítulo 1, se estimaron los parámetros genéticos de las variables relacionadas con la producción seminal así como su relación con la ganancia media diaria (GMD). Las heredabilidades (h^2) de las características seminales fueron 0,13 ± 0,05, 0,08 ± 0,04 y 0,07 ± 0,03 para el volumen eyaculado (V), la concentración de espermatozoides (CN) y la producción espermática (PROD) por eyaculado, observándose además una correlación genética favorable y moderada entre el V y la GMD (0,36 ± 0,34). De este capítulo se puede concluir que si se incluyese una característica seminal como objetivo de selección, esta sería la producción espermática, ya que es una variable que engloba de manera indirecta tanto el volumen y la concentración espermática.

El objetivo del capítulo 2 fue explorar el determinismo genético de algunas variables relacionadas con la calidad espermática y su relación genética con el criterio de selección de la línea paternal de conejos. Las h^2 de los caracteres de calidad espermática evaluados mediante un espermiograma clásico fueron 0,18, 0,19 y 0,12 para la NAR (%, el porcentaje de espermatozoides con acrosoma intacto), ANR (%, el porcentaje de espermatozoides anormales) y

MOT (%, el porcentaje de espermatozoides mótiles), respectivamente. También se estimaron las h^2 de algunas variables relacionadas con la calidad del movimiento espermático medidas con ayuda de un sistema CASA. Las estimas fueron: 0,09, 0,11, 0,10, 0,11, 0,11 y 0,11 para la VAP (µm/s, la velocidad media), VSL (µm/s, velocidad rectilínea), VCL (µm/s; velocidad curvilínea), LIN (%, índice de linealidad), ALH (µm; amplitud del desplazamiento lateral de la cabeza), STR (%, índice de rectitud). Las correlaciones genéticas entre la GMD y las características del semen mostraron un amplio $HPD_{95\%}$ (intervalo de densidad de 95%). Obteniéndose evidencias de la negatividad de las correlaciones genéticas de la GMD con NAR y MOT (-0,40 y -0,53, respectivamente).

El capítulo 3 tiene como objetivo determinar la repetibilidad y heredabilidad de las dimensiones de la cabeza del espermatozoide: anchura (W, µm), área (A, μm^2), longitud (L, µm) y perímetro (P, µm), y explorar la relación genética entre ellas y con el objetivo de selección (GMD). Los resultados obtenidos muestran que las dimensiones de la cabeza espermática son heredables (valores comprendidos entre 0,2 y 0,29). Las correlaciones genéticas entre las variables espermáticas fueron altas y positivas (entre 0,72 y 0,9), con la excepción de la correlación genética L-W que fue moderada. Respecto a la correlación genética entra GMD y las dimensiones de la cabeza espermática, los resultados obtenidos están comprendidos entre -0,09 para L-GMD y -0,43 para W-GMD sugiriendo la existencia de una correlación genética de carácter negativo.

En el capítulo 4 se estudiaron efectos ambientales y asociados al individuo que podrían ejercer influencia en la congelabilidad del semen. Para ello se evaluaron seis variables espermáticas: concentración espermática (CONC,

10^6espermatozoides/mL), integridad acrosómica en fresco (NAR, %) y tras la congelación (Nar-FT, %), motilidad en fresco (MOT, %) y tras la congelación (Mot-FT, %) y el porcentaje de espermatozoides viables tras la congelación (Live-FT, %). Además, se evaluaron dos variables sintéticas: la reducción relativa de la normalidad acrosómica (Rnar, %) y la reducción relativa de la motilidad (Rmot, %) tras el proceso de congelación. La relación existente entre las variables seminales consideradas se analizó mediante un modelo multivariante recursivo. De los efectos fijos estudiados, la estación del año fue el que mayor influencia presentó sobre las características seminales tras el proceso de congelación. Por otro lado, el resultado del análisis de los coeficientes de recursividad mostró que la concentración espermática y la motilidad del semen fresco influyen en la futura congelabilidad del semen. Todas las variables estudiadas mostraron una repetibilidad moderada (comprendida entre 0,11 y 0,38). Estos resultados evidencian que la congelabilidad del semen de conejo podría ser heredable. Con respecto a la correlación entre efectos asociados al macho (r_m), se observaron valores elevados para las variables medidas en el semen fresco (r_m=0,77-0,57), así como entre las variables medidas tras la congelación (r_m=0,72-1). El estudio de las r_m entre variables en fresco y tras la congelación mostraron una relación positiva entre ellas, siendo en el caso particular de MOT y Mot-FT una correlación moderada-alta con las características tras la congelación (r_m=0,41-0,74) y con las variables en fresco (r_m=0,5-0,74), respectivamente, sugiriendo que estas variables podrían estar genéticamente correlacionadas.

El capítulo final de la tesis está enfocado en estudiar la heredabilidad de la congelabilidad del semen y en estimar la correlación genética entre las variables seminales tras la congelación y la GMD en una línea paternal de

conejos. Los valores de heredabilidad estimados muestran que las variables seminales tras un proceso de congelación son heredables (valores comprendidos entre 0,08 y 0,15). El porcentaje de espermatozoides vivos tras el proceso de congelación presentó la heredabilidad más elevada, sugiriendo la posibilidad de una futura selección efectiva a favor del carácter. En relación con el estudio de las correlaciones genéticas, parece que el carácter GMD esté negativamente relacionado con la congelabilidad espermática, pero debido a los amplios $HPD_{95\%}$ no podemos realizar ninguna conclusión. Sería necesario disponer de un número mayor de datos para poder obtener estimas más precisas de las correlaciones genéticas. Si los resultados obtenidos en este trabajo se confirmasen en un futuro, implicaría que la selección por GMD podría alterar las membranas espermáticas o la composición del plasma seminal, ambos relacionados directamente con la resistencia del espermatozoide al proceso de crioconservación

RESUM

L'objectiu principal de la tesis ha sigut estimar els paràmetres genètics de variables relacionades amb la producció i la qualitat de dosis seminals per al seu ús en inseminació artificial (tant en fresc com després d'un procés de crioconservació), amb la finalitat d'explorar el possible interès i les limitacions de l'ús de diferents estratègies de selecció per a la seua millora genètica en una línia paternal de conills seleccionada per velocitat de creixement durant el període d'engreix (28-63 dies).

En el capítol 1, s'estimaren els paràmetres genètics de les variables relacionades amb la producció seminal així com la seua relació amb el guany mig diari (GMD). Les heretabilitats (h^2) de les característiques seminals foren 0,13 ± 0,05, 0,08 ± 0,04 y 0,07 ± 0,03 per al volum ejaculat (V), la concentració d'espermatozous (CN) i la producció espermàtica (PROD) per ejaculat, observant-se a més una correlació genètica favorable i moderada entre el V i la GMD (0,36 ± 0,34). D'aquest capítol es pot concloure que si s'inclogués una característica seminal com objectiu de selecció, aquesta seria la producció espermàtica, ja que es tracta d'una variable que engloba de manera indirecta tant el volum com la concentració espermàtica.

L'objectiu del capítol 2 fou explorar el determinisme genètic d'algunes variables relacionades amb la qualitat espermàtica i la seua relació genètica amb el criteri de selecció de la línia paternal de conills. Les h^2 dels caràcters de qualitat espermàtica avaluats mitjançant un espermiograma clàssic foren 0.18, 0.19 y 0.12 per la NAR (%, el percentatge d'espermatozous amb acrosoma

intacte), ANR (%, el percentatge d'espermatozous anormals) i MOT (%, el percentatge d'espermatozous mótils), respectivament.

També s'estimaren les h^2 d'algunes variables relacionades amb la qualitat del moviment espermàtic mesurades amb l'ajuda d'un sistema CASA. Les estimes foren: 0.09, 0.11, 0.10, 0.11, 0.11 i 0.11 per a la VAP (µm/s, la velocitat mitja), VSL (µm/s, velocitat rectilínia), VCL (µm/s; velocitat curvilínia), LIN (%, índex de linealitat), ALH (µm; amplitud del desplaçament lateral del cap), STR (%, índex de rectitud). Les correlacions genètiques entre la GMD i las característiques del semen mostraren un ampli $HPD_{95\%}$ (interval de densitat de 95%). Obtenint-se evidències de la negativitat de les correlacions genètiques de la GMD amb NAR i MOT (-0,40 y -0,53, respectivament).

El capítol 3 té com objectiu determinar la repetibilitat i heretabilitat de les dimensions del cap de l'espermatozou: amplària (W, µm), àrea (A, µm2), longitud (L, µm) y perímetre (P, µm), i explorar la relació genètica entre elles i amb l'objectiu de selecció (GMD). Els resultats obtinguts mostren que les dimensions del cap espermàtic són heretables (valors compresos entre 0.2 i 0.29). Les correlacions genètiques entre les variables espermàtiques foren altes i positives (entre 0.72 i 0.9), amb l'excepció de la correlació genètica L-W que fou moderada. Respecte a la correlació genètica entre GMD i les dimensions del cap espermàtic, els resultats obtinguts estan compresos entre -0.09 per a L-GMD i -0.43 per a W-GMD, suggerint l'existència d'una correlació genètica de caràcter negatiu.

En el capítol 4 s'estudiaren els efectes ambientals i associats a l'individu que podrien exercir influència en la congelabilitat del semen. Així, s'avaluaren sis variables espermàtiques: concentració espermàtica (CONC,

10^6espermatozous/mL), integritat acrosòmica en fresc (NAR, %) i després de la congelació (Nar-FT, %), motilitat en fresc (MOT, %) i després de la congelació (Mot-FT, %) i el percentatge d'espermatozous viables després de la congelació (Live-FT, %). A més, s'avaluaren dos variables sintètiques: la reducció relativa de la normalitat acrosòmica (Rnar, %) y la reducció relativa de la motilitat (Rmot, %) després del procés de congelació. La relació existent entre les variables seminals considerades s'analitzà mitjançant un model multivariant recursiu. Dels efectes fixes estudiats, l'estació de l'any fou el que major influència presentà sobre les característiques seminals després del procés de congelació. Per altra banda, el resultats de l'anàlisi dels coeficients de recursivitat mostrà que la concentració espermàtica i la motilitat del semen fresc influeixen en la futura congelació del semen. Totes les variables estudiades mostraren una repetibilitat moderada (compreses entre 0.11 i 0.38). Aquestos resultats evidencien que la congelabilitat del semen de conill podria ser heretable. Respecte a la correlació entre efectes deguts a mascle, s'observaren elevades correlacions entre variables del semen fresc (r_m=0.77-0.57), així com entre les variables mesurades després de la congelació (r_m=0.72-1). L'estudi de les correlacions entre efectes associats a mascle entre variables en fresc y després de la congelació mostraren una relació positiva entre elles, seent en el cas particular de MOT y Mot-FT una correlació moderada-alta amb les característiques després de la congelació (r_m=0.41-0.74) y amb les variables en fresc (r_m=0.5-0.74), respectivament, suggerint que estes variables podrien estar genèticament correlacionades.

El capítol final de la tesis està enfocat a estudiar la heretabilitat de la congelabilitat del semen y en estimar la correlació genètica entre les variables seminals després de la congelació i la GMD en una línia paternal de conills. Els

valors d'heretabilitat estimats mostren que les variables seminals després d'un procés de congelació són heretables (valors compresos entre 0.08 i 0.15). El percentatge d'espermatozous vius després del procés de congelació presentà la heretabilitat més elevada, suggerint la possibilitat d'una futura selecció efectiva a favor del caràcter. En relació amb l'estudi de les correlacions genètiques, pareix que el caràcter GMD està negativament relacionat amb la congelabilitat espermàtica, però degut als amplis $HPD_{95\%}$ no podem realitzar ninguna conclusió. Seria necessari disposar d'un nombre major de dades per a poder obtenir estimes més precises de les correlacions genètiques. Si els resultats obtinguts en aquest treball es confirmaren en un futur, implicaria que la selecció per GMD podria alterar les membranes espermàtiques o la composició del plasma seminal, ambdós relacionats directament amb la resistència de l'espermatozou al procés de crioconservació.

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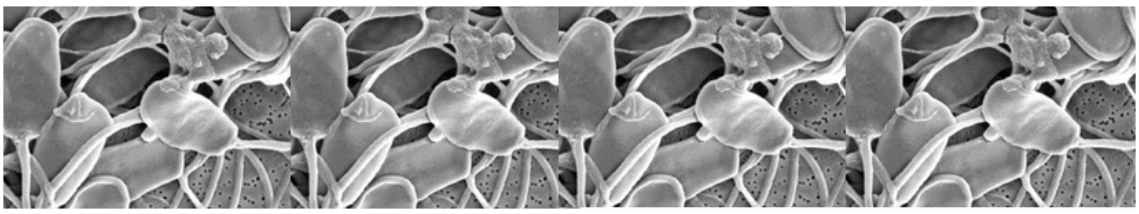
LITERATURE REVIEW



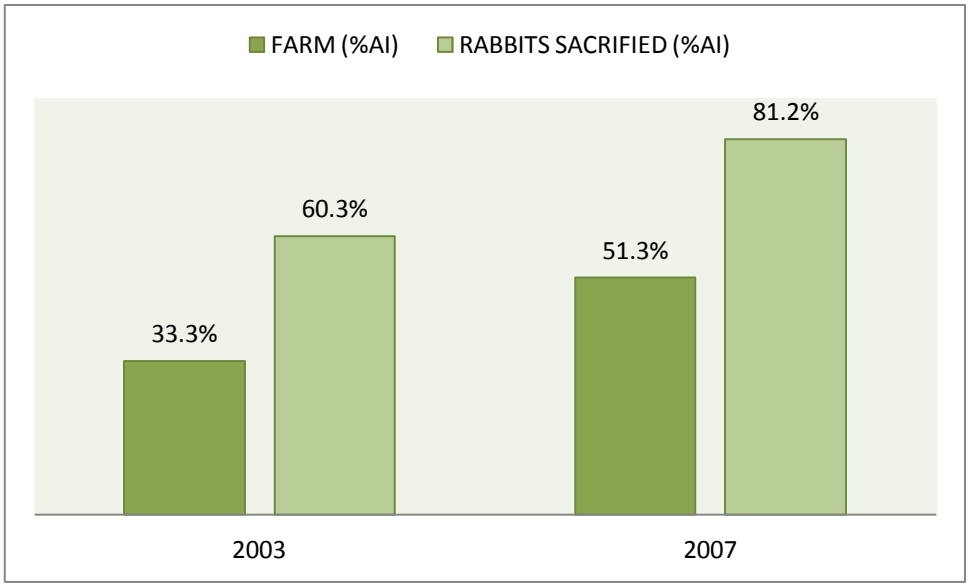
LITERATURE REVIEW

1. ARTIFICIAL INSEMINATION AND RABBIT INDUSTRY

The development of artificial insemination (AI) has changed the rabbit industry by allowing more efficient management and lowering the production cost.

Artificial insemination has eliminated the need to have a large number of males on the farm. The main advantage was the dramatic reduction in the working time needed compared to natural breeding, jointly with a significant decrease in feed and total production costs (Leyún *et al*., 1999). Since the introduction of AI in Spain, the number of farms applying AI has steadily increased, as shown in recent years (MAGRAMA, 2008; **Figure I.1**). In addition, AI utilises the semen more efficiently than natural breeding, as 20-40 doses can be collected per male and week, while a male used for natural breeding can only breed twice a week (Lavara and Vicente, 2001).

Figure I.1: Use of artificial insemination in Spanish rabbit farms



Overall, 6.5 million doses of semen are used for AI per year in Spain (Personal estimation, MAGRAMA, 2008). Today, the limited lifespan of rabbit semen doses undermines the advantages of using of stored sperm for disease control and genetic improvement. The semen must be used within a 24-48 hour period after collection (Viudes *et al*., 1999; Roca *et al*., 2000; Lavara *et al*., 2005). However, motility and overall quality decrease significantly during storage and a high sperm concentration is needed for periods of storage longer than 12 h (Viudes *et al*., 1999). In addition, there seems to be an interaction between male genotype and AI conditions (Tusell *et al*., 2010). The limited lifespan of sperm reduces the geographic locations to which it can be shipped, hampering the spread of genetic improvement, increasing the workload and diminishing the efficient use of the males.

Frozen-thawed semen could hold promise for dissemination of genetics on a global scale, banking of paternal genetics and effective biosecurity measures, resulting in a positive impact on the global rabbit industry. Storage in liquid nitrogen makes it possible to transport it over long distances, allowing the preservation of genetics as well as the banking of genetic material for use in case of emergency or for future purposes. Moreover, frozen semen could have a role in preventing future biohazards (Purdy, 2008)

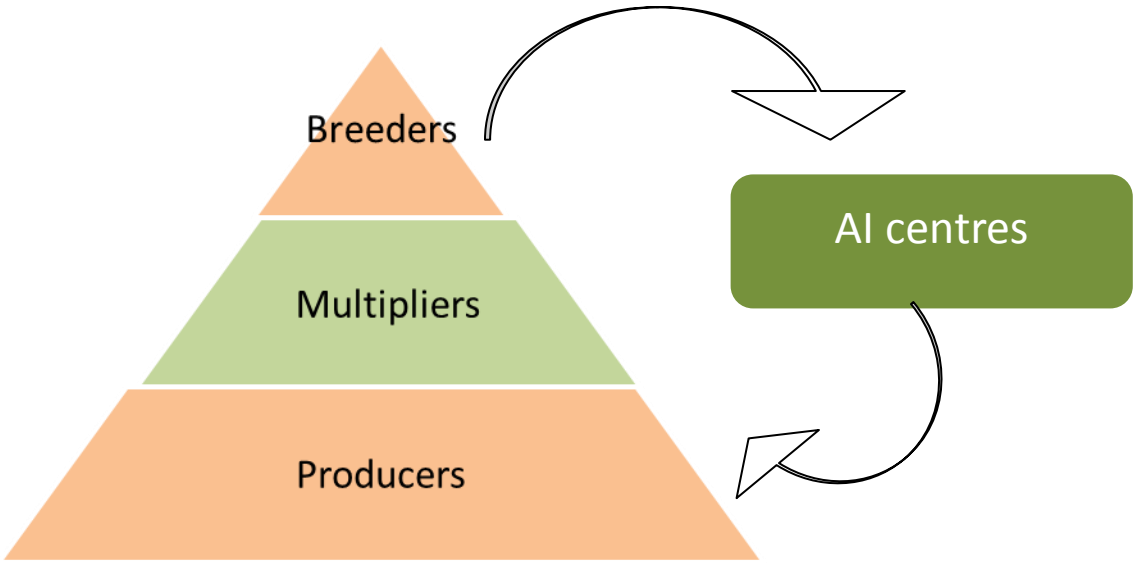
2. SELECTION OF PATERNAL RABBIT LINES

Efficiency of rabbit meat production depends on a large number of traits. These traits can be divided into reproduction and production related traits (Baselga and Blasco, 1989; Baselga, 2004). Production traits (growth, feed efficiency, and carcass and meat characteristics of rabbits) are important for the performance during the fattening period, whereas reproduction traits (kindling interval,

number of weaned rabbits, number of live young rabbits and longevity) are important for maternal lines. Rabbit breeding organisations improve both groups of traits by selection of specialised lines, paternal and maternal. Animals in the nucleus populations are selected to produce the replacement of the lines. Optimisation of the selection method in nucleus populations is important, as these populations determine the performance at all levels of the breeding pyramid.

The genetic scheme in rabbits is similar to the one used in the pig industry (pyramidal scheme, **Figure I.2**). However, if the rabbitry is large enough it may carry out the multiplication itself due to the economic, sanitary and adaptability advantages (Baselga and Blasco, 1989).

Figure I.2: Rabbit industry breeding scheme



Nowadays, paternal lines are selected for traits that have an economic impact within a farm. The most common selection criteria in paternal lines are post weaning daily gain (Rochambeau *et al*., 1989; Estany *et al*., 1992) or the weight at the end of the fattening period (Lukefahr *et al*., 1996; Larzul *et al*., 2003), but

the aim is to improve feed efficiency, a trait with a higher economic value than daily gain (Armero and Blasco, 1992) but negatively correlated with the latter (Moura *et al*., 1997; Piles *et al*., 2004). Other traits more directly related to the feed efficiency, such as residual feed intake on *ad libitum* feeding, are also being considered (Larzul and De Rochambeau, 2005).

Regarding correlated responses of selection for growth rate in meat quality and carcass composition, Hernández *et al*. (2005) found that selection for growth rate did not affect the main sensory features of meat such as tenderness and juiciness, but had a negative effect on some flavour characteristics, although it is not clear that these differences could be detected by consumers. In addition, Hernández *et al*. (2006) showed that a line selected for growth rate had a higher meat/bone ratio, higher loin percentage and higher ultimate pH of M. *Longissimus lumborum* than lines selected for litter size at weaning at the same age of maturity, but lower dressing out percentage. In contrast, in previous studies conducted with animals from similar lines that have similar weight, thus a different stage of maturity (Gómez *et al*., 1998; Pla *et al*., 1998), the paternal lines showed a lower meat/bone ratio and lower meat percentage of the carcass. In consequence, the economic advantage of having lower maintenance costs because of the earlier slaughter at the same commercial weight is partially counteracted by a discount in the price, due to a lower dressing out percentage. Nowadays rabbit carcasses are not paid for according to meat/bone ratio or their retail cuts, but breeding companies should be concerned about this (Hernández *et al*., 2006).

Artificial insemination in rabbit commercial farms is usually done with semen from males pertaining to a paternal line. In addition to having excellent growth

rate, these males should also have good semen production and quality in order to produce a high number of sperm doses. Identifying and improving the genetics of sperm production and quality will improve sperm dose production per male. Under commercial conditions, most AI centres screen ejaculates and discard those not meeting minimum standards (70% motility, less than 20% abnormal forms, low sperm concentration). Additionally, it is common practice to prepare AI doses with more sperm than needed. This practice and the mixing of ejaculates are done to compensate occasional poor quality ejaculates. For these reasons, is important to estimate the genetic correlation of sperm production and quality traits with the selection criterion in order to know if those traits could be altered by the selection process or not, and if it could be possible to improve them jointly with the main selection criterion. Regarding this last point, previous reports indicate that 58% of males selected by growth rate did not present normal sexual behaviour at the beginning of their reproductive life (Pascual *et al*., 2004). Furthermore, Rosell and De La Fuente (2009) reported that 30-40% of the global culling in male rabbits was due to low libido and low sperm quantity and/or quality.

3. FACTORS INVOLVED IN SEMEN DOSE PRODUCTION

3.1. AI Technique employed

Artificial insemination in rabbits is generally performed with 0.5 ml of fresh diluted semen within 6-24 hours after collection that contains at least 4-20 million viable, non-damaged spermatozoa (Viudes de Castro and Vicente 1997; Castellini and Lattaioli, 1999; Viudes de Castro *et al.,* 1999, Brun *et al.,* 2002a). To prepare semen doses, an ejaculate with enough quality to provide good fertility results is needed. The number of doses that can be prepared with

one ejaculate (despite its quality) depends on the kind of semen dose chosen (fresh, cooled or frozen). If the AI is performed immediately after collection, the total number of spermatozoa per dose will be less than if we inseminate 24-48 hours later, because sperm survival falls off sharply after 36 hours and its fertilising capacity tends to diminish during storage (Roca *et al*., 2000; López-Gatius *et al*., 2005). In addition, the female's prolificacy is also compromised (López and Alvariño, 1998)

3.2. Ejaculate characteristics

Male rabbits have a characteristic sexual behaviour: rapid ejaculation and capability of successive ejaculations between short intervals (up to 14 ejaculates in a time period of 3.5 h (Ambriz *et al*., 2002)). In addition, the semen can easily be collected with an artificial vagina.

Current semen evaluation is done immediately after collection and comprises a visual evaluation of ejaculate and volume. A subjective assessment of sperm motility and obvious sperm morphology defects, as an indirect measure of sperm viability and normality, is also done prior to processing. Then, the non refused ejaculates are pooled to prepare the insemination doses. Once the pool is prepared, the number of AI doses is calculated according to the concentration of the pool and the kind of storage and shipment elected (Lavara *et al*., 2003).

3.2.1. Ejaculate appearance

Rabbit semen should have a homogeneous white opalescent appearance. Debris in the ejaculate may affect the spermatozoa and may be indicative of an infection in the reproductive tract. Sometimes a plug gel may be observed

(Mukherjee *et al*., 1951). The gel must be removed to avoid spermatozoa capping.

3.2.2. Sperm quantity, traits and its evaluation

Previous reports showed the great variability evident in rabbit sperm production. The range observed for the related traits were: from 0.39 to 1.19 mL for volume, from 738 to 146x10^6 spz/mL for sperm concentration, and from 477 to 90x10^6 spz/ejaculate for total sperm per ejaculate (**Table I.1**).

Table I.1: Ejaculate characteristics

Trait		Range	References
VOL (mL)	Ejaculate volume	0.39-1.19	Bencheikh (1995); Brun *et al.* (2002a,b; 2006); Castellini *et al.* (2006a); García-Tomás *et al.* (2006a,b); Safaa *et al.* (2008); Lavara *et al.* (2010)
CON (10^6spz/mL)	Sperm concentration	146-738	
PROD (10^6spz/ejaculate)	Sperm production	90-477	

3.2.2.1. Ejaculate volume

The ejaculated semen comprises the spermatozoa suspended in the seminal plasma. Ejaculate volume can be determined by different techniques. Although it may be determined with graduated collection tubes, a more accurate measurement can be taken with the use of a calibrated micropipette (range of values presented in **Table I.1**).

Seminal plasma is a complex fluid portion and mediates the chemical function of the ejaculate. Biochemical components of seminal plasma are secreted from *rete testis*, epididymis and accessory sex glands of the male reproductive tract (Mann *et al.,* 1982). Semen plasma's basic role is as a survival medium that facilitates transport of spermatozoa, provides physical and metabolic support as an energy source for the sperm cells and influences sperm functionality.

Large dilutions of the ejaculate (subsequently large dilution of seminal plasma) lead to motility loss and a decrease in metabolic activity and fertility capacity (Maxwell and Johnson, 1999). These detrimental effects have been associated in various species with the dilution of seminal plasma factors that protect the sperm from membrane damage and premature capacitation (Maxwell *et al*., 1997; 1998)

Variations in seminal plasma composition between males, and ejaculates within males have been found (Killian *et al*., 1993; Rodríguez-Martínez *et al*., 2011)

3.2.2.2. Sperm concentration

Sperm concentration refers to the number of spermatozoa per millilitre of semen. Sperm production is the total number of spermatozoa in the ejaculate. A range of values for both characteristics is shown in **Table I.1**. Determination of the sperm concentration in a semen sample is important, because this trait is used to determine the quantity of spermatozoa that will be used if the quality of the ejaculate is good enough. Sperm concentration can be determined using a hemacytometer (Bürker, Thoma or Neubauer chamber). This method is commonly employed to determine the sperm concentration in rabbit semen from the beginning of AI (Walton, 1927). However, this methodology is not quick

enough when we need to determine a lot of samples in a brief period of time. For this reason, new technologies are developed to measure this attribute, and spectrophotometry can be used (first described by Salisbury *et al*., 1943), but this methodology fails to provide accurate measurements for samples containing non-sperm particles in the medium (as prostatic vesicles, cells, egg yolk or milk droplets). Unfortunately, due to the high content of prostatic vesicles and other kind of cells other than spermatozoa in rabbit ejaculates (Farrel *et al*., 1992; Castellini *et al*., 2006b) this technique does not provide accurate data. However, these ejaculates can be evaluated using fluorescent stains that label only sperm and not extraneous particles (Riedy *et al*., 1991). Using these fluorescent stains, the sperm concentration can be determined using fluorometry (Theau-Clément and Faliéres; 2005) and flow cytometry (Purdy and Graham, 2004). The main problem of these techniques is the cost.

3.2.3. Sperm quality, traits and evaluation

Assessment of sperm quality is an essential procedure that has to be included in the evaluation routine of AI centres.

3.2.3.1. Sperm plasma membrane integrity (Viability)

Viability test are used to determine the presence of live sperm (range of values presented in **Table I.2**). The sperm membrane is directly or indirectly related with many sperm functions (Rodríguez-Martínez, 2003), since a biochemically active membrane is required in the process of capacitation, the acrosome reaction and binding of the spermatozoa to the oocyte (Correa and Zavos, 1994). Although the sperm plasma membrane covers the entire cell, it consists of three distinct membrane compartments; one that covers the outer acrosomal

membrane, one that covers the post acrosomal portion of the sperm head and one that covers the middle and principal pieces. For this reason, different viability assays assess the integrity of different plasma membrane compartments (Mocé and Graham, 2008). Classic stains such as eosin-nigrosin or eosin-aniline blue stain, as well as fluorescent stains like propidium iodide (PI), ethidium bromide, 4'-6-diamidino-2-phenylidole (DAPI) and bisbenzimide, bind to and stain the DNA of sperm that have damage in the post acrosomal plasma membrane. The most common combination of dyes used for membrane integrity evaluations is SYBR-24/PI. In this double stain, the DNA of viable spermatozoa fluoresces green, while those with damaged plasma membrane are counterstained red, including an apoptotic (green-red) cell population (Riedy *et al*., 1991). This procedure has been successfully applied in different livestock species (Garner *et al*., 1996; Rodríguez-Martínez, 2007) and also in rabbits (Garner and Johnson, 1995). Furthermore, it may be possible to evaluate sperm cell viability together with some other attributes, such as acrosome integrity (Nagy *et al*., 2003).

3.2.3.2. Motility

Visual estimation of sperm motility is probably the most widely used test because it is simple, quick, inexpensive and can easily detect ejaculates with poor fertility (null or low motility), but is not able to classify ejaculates with minor field fertility differences (Graham, 1996).

The presence of progressively motile sperm in the ejaculate is critical to ensure adequate sperm transport and fertilisation. Furthermore, sperm motility is a good indicator of the integrity and functionality of sperm plasma membrane (Jeyendran *et al*., 1984). Visual estimation of the percentage of motile sperm in

a semen sample is a common assay performed on a routine AI dose preparation. The way in which this attribute is evaluated is important. Several systems referred to as computer-assisted semen analysers (CASA) have been developed using digital image analysis for the automated analysis of ejaculate (Mortimer, 2000). The use of this kind of systems reduced the human bias in estimating the percentage of motile sperm (Graham, 1996) when the setup procedures and object detection were properly done (Davis and Katz, 1993). In addition to the percentage of motile sperm, the CASA system can evaluate the sperm motion kinetics, which is impossible to assess using routine evaluation. Sperm kinetics includes the measure of the distance between each head point for a given sperm during the acquisition period (curvilinear velocity, VCL, μm/s), the distance between first and last head points divided by the acquisition time (straight line velocity, VSL, μm/s), and the measure of sperm head oscillation (amplitude of lateral head displacement, ALH, μm). Linearity (LIN, %) measures the departure from linear progression and is calculated as VSL/VCLx100, while the average path velocity (VAP, μm/s) is a smoothed path constructed by averaging several positions on the sperm track (Verstegen *et al*., 2002).

A relevant analysis of sperm motion should focus on the identification of normal values for a movement pattern. **Table I.2** presents the means of some sperm motion kinetics studied in the rabbit by CASA systems. Several studies in different species have shown that the quantitative assessment of sperm kinetics is valuable in identifying sperm subpopulation motility patterns, possibly related with different capacitation status (Cremades *et al*., 2005; Quintero-Moreno *et al*., 2007).

Table I.2: Rabbit sperm characteristics

Trait		Range	References
LIN (%)	Linearity index	41-74	Farrell *et al.* (1993, 1996); Brun *et al.* (2006); Lavara *et al.* (2008); Safaa *et al.* (2008); Castellini *et al*. (2011)
VAP (µm/s)	Average path velocity	40-108	
VCL (µm/s)	Curvilinear velocity	66-117	
LIVE (%)	Sperm viability	68-92	Bencheikh (1995); Brun *et al.* (2002a,b); Nizza *et al.* (2003); Roca *et al.* (2005); García-Tomás *et al.* (2006a,b); Rosato *et al.* (2011)

To date, there is no consensus regarding the relationship between sperm kinetics and field fertility, the difference in CASA instruments used, their setup, and the different sperm dilution ratios between studies have made it impossible to reach an agreement.

3.2.3.3. Sperm morphology

The morphology of spermatozoa reflects the health of the seminiferous tubules and, to some degree, the epididymis (Barth and Oko, 1989). The process of spermatozoa formation takes place over a period of 37-40 days prior to the release of the spermatozoa into the *rete testis* and *epididymis* (Swierstra and Foote, 1965). Thus, the abnormalities observed may reflect disturbances in testicular function that occurred several weeks before. Some defects of the

spermatozoa may occur during maturation in the *epididymis* and may be due to epididymal dysfunction. Since epididymal transit time in the rabbit is approximately 8-10 days (Swierstra and Foote, 1965), some sperm defects found in the ejaculate may have occurred previously, in only a few days. In general, the production of abnormal spermatozoa may be the result of the male's genetic background, environmental stresses (disease, nutritional status, temperature, etc.), or a combination of these aspects (Barth and Oko, 1989).

Table I.3: Morphology characteristics of rabbit sperm

Trait		Range	References
ANR (%)	Abnormal forms	5.4-21.6	Nizza *et al.* (2003); Roca *et al.* (2005); Lavara *et al.* (2005, 2010); García-Tomás *et al.* (2006a,b); Safaa *et al.* (2008); Rosato *et al.* (2011)
NAR (%)	Acrosome integrity	75.2-94	
L (µm)	Length of head sperm	8.02-8.51	Napier (1961); Cummins and Woodall (1985); Lavara *et al.* (2008); Marco-Jiménez *et al.* (2010)
W (µm)	Width of head sperm	4.7-4.9	

Previous reports in rabbits indicate that as the percentage of morphologically abnormal sperm increases in semen samples, the fertility decreases (Lavara *et al.*, 2005). In particular, sperm possessing abnormal forms (head, tail, midpiece) and abnormal acrosomes are associated with low fertility and prolificacy.

Table I.3 shows the mean value of some sperm morphology characteristics studied in the rabbit.

a) Abnormal forms

Different stains for sperm morphology evaluation have been described (reviewed by Kruger *et al*., 1996). Since some of the stains employed could be very hard to prepare, an alternative method is to use wet mounts. Wet mounts can be made very quickly for observation of sperm morphology without staining. Either phase-contrast or differential interference contrast can be used. Semen samples must be fixed in formaldehyde or glutaraldehyde solutions before sperm observation. Data highlight a relationship between sperm morphology and fertility (Correa *et al*., 1997; Mortimer and Menkveld, 2001; Hallap *et al*., 2004) as well as prolificacy (Gadea *et al*., 1998; Xu *et al*., 1998; Lavara *et al*., 2005) when AI is performed with a low number of sperm. Although sperm morphology affects fertility and prolificacy, this trait appears to be compensable, meaning that fertility can be improved if more sperm are included in the AI dose (Saacke *et al*., 2000). The cutoff value for sperm normality in AI rabbit centres is usually 80%, to avoid fertility-related problems.

b) Acrosome integrity

Capacitation process represents the first steps of destabilisation that spermatozoa must undergo before reaching the oocyte. Sperm capacitation provokes physiological changes in the spermatozoa and provides motility and the acrosomic reaction liberating proteolytic enzymes, such as hyaluronidase and acrosin (Flesch and Gadella, 2000). To fertilise an oocyte, the sperm must have an acrosome when it encounters the oocyte (Graham and Mocé, 2005).

If the acrosome is lost, due to membrane damage during the process or the storage of AI dose, or during its transit in the female reproductive tract, it cannot bind to the *zona pellucida* of the oocyte (Gadella, 2012).

Several techniques have been proposed to differentiate intact from reacted acrosome in spermatozoa, including phase-contrast microscopy (Casey *et al*., 1993), cytochemical staining techniques, indirect immunofluorescence using monoclonal antibodies and labelling with fluoresceinated lectins (Cross *et al*., 1986; Mortimer *et al*., 1987).

Some studies have demonstrated that male fertility is directly related with the percentage of sperm with intact acrosome (Saacke and White, 1972; Zhang *et al*., 1990, Courtens *et al*., 1994). In addition, the percentage of sperm with intact acrosome in a sample has been related with the prolificacy. In pigs, Holt *et al*. (1997) demonstrated that ejaculates with a lower percentage of reacted acrosomes after a resistance test gave higher litter size at birth.

c) Sperm morphometry

One of the principal functions of the sperm is to protect and transport the paternal haploid genotype to the oocyte. Genetic information is located inside the head of the sperm (Ward and Coffey, 1991). Traditional methods for evaluating sperm morphology are based on a subjective way, classifying the sperm as normal or abnormal depending on its morphological appearance, Sperm abnormalities, including tail defects as well as unusually small or large heads, and even extreme oddities such as sperm lacking a head or tail, or possessing two heads or two tails, are easily detected during sperm morphology assessment (Barth and Oko, 1989). In this way, morphometric analysis is more

effective to visualise differences in size and shape than routine evaluation (Gravance *et al*., 1996) and has been recommended as part of the spermiogram for domestic animals (Rodríguez-Martínez, 2007).

Microscopic techniques such as scanning electron microscopy, transmission electron microscopy and cryo-electron microscopy have been used to examine sperm head morphometry (Holt *et al*., 1999; Marco-Jiménez *et al*., 2006). But these techniques are costly and time consuming, especially to obtain large sample sizes from several preparations in a short period of time. Therefore, conventional microscopic techniques (such as light and phase contrast microscopy) for stain and wet mounts, in combination with automated sperm morphology analysis (ASMA) software, have been developed to process samples providing faster assessment of head sperm morphometry in animal species (Gravance *et al*., 1996; Hirai *et al*., 2001; Hidalgo *et al*., 2007), including rabbits (Gravance and Davis, 1995; Marco-Jiménez *et al*., 2010). Although in previous studies head sperm morphometry has been related with chromatin status (Álvarez *et al*., 2008) and cryopreservation sensibility (Thurston *et al*., 2001; Gravance *et al*., 2009), the relationship with field fertility is far from clear.

4. FACTORS AFFECTING EJACULATE CHARACTERISTICS

4.1 Environmental factors

There are many factors influencing proper sperm production and quality, such as age (Gogol *et al*., 2002; García *et al*., 2004), collection rhythms (Arroita *et al*., 2000; Mocé *et al*., 2000), environmental conditions such as temperature (Lavara *et al*., 2000) and photoperiod (Theau-Clément *et al*., 1995; Roca *et al*., 2005),

season (Safaa *et al*., 2008), nutrition (Pascual *et al*., 2004; Castellini *et al*., 2007) and sanitary status (O'Bryan *et al*., 2000).

Some of the most important factors regarding male management techniques could be those related to ejaculate collection procedures, collection rhythms and feeding strategies. Previous stimulation of males, correct type of artificial vagina and its temperature can improve the efficiency of collection (Boiti *et al*., 2005). In rabbits, the most commonly frequency used in AI centres is the extensive, with two ejaculates collected once a week, on the same day or different days (Arroita *et al*., 2000). Studies regarding the effect of different collection frequencies on the total sperm produced per male and per week demonstrated that high collection frequencies lead to a reduction in volume and sperm concentration per ejaculate (Bencheikh, 1995; Nizza *et al*., 2003), and compromise the sperm motility and viability (Bencheikh, 1995). However, some other authors did not find the same tendency (Arroita *et al*., 2000; Mocé *et al*., 2000). Moreover, a high collection frequency increased the presence of droplets of prostatic origin in the ejaculates (Castellini *et al*., 2006a), whose function seems to be important in sperm capacitation and acrosome reaction of the sperm (Castellini *et al.,* 2012). Regarding feeding strategies, the *ad libitum* feeding is positively correlated with male libido and sperm production (Maertens and Luzi, 1997).

Season effects, on sperm production and quality, have been studied in different rabbit lines (Nizza *et al*., 2003; Safaa *et al*., 2008). The main results highlighted that in summer and first weeks of autumn males show lower libido, lower sperm production and less sperm quality than in winter or spring. The seasonal effect includes the photoperiod and also temperature and humidity

changes. Photoperiod influences correct spermatozoa production through the hypothalamus-pituitary axis, but this effect could be reduced using artificial photoperiods inside rabbit farms. In commercial farms, a constant 16 hours light: 8 hours darkness is recommended because of the positive effects on spermatogenesis (reviewed by: Theau-Clément *et al*., 1998)

4.2 Genetic factors

To improve production of AI semen doses, we need to have genetic variation for the traits implied. In the literature we can find differences between lines in semen production and quality traits.

Differences between maternal lines were reported in sperm production and quality (Brun *et al*., 2002b; Theau-Clément *et al*., 2003) and also in the variability of semen characteristics within males (Theau-Clément *et al*., 2003). Similar results were found between paternal rabbit lines. Brun *et al*. (2006) reported differences between two lines divergently selected for body weight at 63 days and García-Tomás *et al*. (2006a) found differences for some seminal traits in two rabbit lines selected for growth rate.

Studies on differences between paternal and maternal lines were also proposed. In this way, Vicente *et al*. (2000) found lower sperm production, less motility and more acrosomal defects in a paternal line selected for growth than in three maternal ones.

4.2.1 Genetic parameters

In general, a wide range of heritability estimates for different semen traits in several species can be found (**Table I.4**). However, whereas in other species the

heritability of semen traits has been explored, in rabbits only few studies involving low numbers of males have been carried out (Napier, 1961; Panella *et al*., 1994; Khalil *et al*., 2007; Brun *et al*., 2009), although repeatability estimates can be found more frequently.

Table I.4: Heritability estimates (h^2) of seminal traits from different domestic species

Species	Trait	h^2	Source
Bos taurus	Volume Concentration Sperm production Motility Abnormalities (%) Viability (%)	0.09-0.65 0.01-0.36 0.03-0.54 0.01-0.23 0.07-0.33 0.00-0.27	Knights *et al*. (1984); Taylor *et al*. (1989); Ducrocq and Humblot (1995); Mathevon *et al*. (1998); Sarreiro *et al*. (2002); Yilmaz *et al*. (2004); Kealey *et al*. (2006); Grendler *et al*. (2007)
Sus scropha	Volume Concentration Sperm production Motility Abnormalities (%)	0.16-0.58 0.24-0.49 0.25-0.38 0.38 0.34	Brandt and Grandjot (1998); Smital *et al*. (2005); Oh *et al*. (2006a,2006b)
Ovis aries	Volume Concentration Motility Abnormalities (%) Viability (%)	0.11-0.30 0.06-0.17 0.03-0.16 0.16 0.01	Rege *et al*. (2000); David *et al*. (2007)

For male libido, in rabbits, Panella *et al*. (1994) and also Khalil *et al*. (2007) reported different values of h^2 estimates (0.30 and 0.17, respectively). The high value obtained by Panella *et al*. (1994) is probably due to the fact that no

permanent effects other than the additive were included in the model; in addition the libido score (3 categories) was calculated in a different way than by Khalil *et al*. (2007), who classified this trait in 5 categories.

Regarding traits involved in sperm production such as ejaculate volume and sperm concentration, moderate values of repeatability were found, indicating the existence of important individual variation. Thus, Bencheikh (1995) and García-Tomás *et al*. (2006b) estimated repeatability around 0.38 for volume and 0.35 for sperm concentration.

In rabbits, sperm motility can be evaluated by different procedures as we explained previously: mass motility, individual motility evaluated subjectively or with the aid of a CASA system. In consequence, the h^2 estimates will be slightly different depending on the evaluation procedure employed. For instance, the h^2 of mass motility was estimated to be 0.05 (Brun *et al*., 2009) and the repeatability ranged from 0.24 to 0.37 (García-Tomás *et al*., 2006b; Brun *et al*., 2009). The repeatability for individual motility evaluated subjectively was estimated to be 0.35 (García-Tomás *et al*., 2006b). In contrast, h^2 of individual sperm motility estimated with CASA system provided higher estimates (0.16 to 0.18) than for mass motility (Lavara *et al*., 2007; Brun *et al*., 2009). Regarding CASA traits, most of them have shown h^2 and repeatability estimates lower than individual motility (Lavara *et al*., 2007; Brun *et al*., 2009).

Whereas genetic parameters for quantity sperm traits in rabbits were reported by different authors, no estimations for heritability of quality sperm traits as abnormal forms or acrosome status are reported; only estimates of repeatability are available. Repeatability estimates were moderate, ranged from 0.33 to 0.4 (Bencheikh, 1995; Brun *et al.,* 2002b; Garcia-Tomas *et al.,* 2006c).

The only available estimates of h^2 for sperm head morphometry traits were obtained by Napier (1961) who reported unusually high heritabilities (0.71-0.74) in a mixed population using the sire-son regression. The high h^2 obtained could be due to the unsuitable experimental design, which did not take into account the existence of two different populations in the study.

As commented, the semen used for AI comes from males selected for growth traits. Selection for growth traits is not expected to have a great genetic relationship with semen output, in parallel to the low magnitude of the genetic correlation between growth and litter size previously observed in studies involving rabbit maternal lines (reviewed by Piles *et al.,* 2012). However, in other species, selection based on growth traits shows genetic responses that are positively correlated with the weights of reproductive organs but negatively correlated with the efficiency of sperm production and parameters of semen quality (Eisen and Johnson (1981) in mice; Johnson *et al.* (1994) in pigs; Rege *et al.* (2000) in sheep; Kealey *et al.* (2006) in cattle).

Regarding genetic correlations between seminal traits, several studies in different livestock species concluded the existence of a genetic antagonism between ejaculate volume and sperm concentration; the degree of this correlation depended on the species (around -0.3 in bulls, Ducrocq and Humblot, 1995; Karoui *et al.,* 2011; -0.6 in pigs: Smital *et al.,* 2005; Wolft, 2009). In rabbits, the only reported estimate by Brun *et al.,* 2009 cannot be considered to be different from zero (0.38 ± 0.45, for concentration and ejaculate volume).

We must be aware that in order to achieve an accurate estimation of the genetic correlation among different traits we will need larger numbers of males than for the estimation of h^2 or repeatability. In addition, another important

requirement for genetic studies is to have the pedigree of the males connected to the animals of the selection nucleus.

5. LITERATURE CITED

Álvarez M., García-Macías V., Martínez-Pastor F., Matínez F., Borragán S., Mata M., Garde J., Anel L., De Paz P. Effects of cryopreservation on head morphometry and its relation with chromatin status in brown bear (*Ursus arctos*) spermatozoa. Theriogenology 2008;70:1498 –506.

Ambriz D., Rosales A.M., Sotelo R., Mora J.A., Rosado A., García AR. Changes in the quality of rabbit semen in 14 consecutive ejaculates obtained every 15 minutes. Archives of Andrology 2002;48:389-395.

Armero Q., Blasco A. Economic weights for rabbit selection indices. J Appl Rabbit Res 1992;15:637-42.

Arroita Z., Falceto M.V., Martín Rillo S., De Alba C., Moreno C., Ciudad M.J., Rafel O. Effect of collection frequency on production, quality and storage of young bucks semen. In: Proc 7th World Rabbit Congress, 2000;Vol(A):163-6.

Barth A. D., Oko R.J. Abnormal morphology of bovine spermatozoa. Ames 1989; ia: Iowa state university press.

Baselga M., Blasco A. Mejora genética del conejo de producción de carne. 1989. Ed Mundi Prensa, Madrid.

Baselga M. Genetic improvement of meat rabbits. Programmes and diffusion. In: Proc 8th World Rabbit Congress, 2004;1:1-13.

Bencheikh N. The effect of frequency of ejaculation on semen characteristics and sperm output in the rabbit. Ann Zootech 1995;44:263-79.

Boiti C., Castellini C., Theau-Clément M., Besenfelder U., Liguori L., Renieri T., Pizzi F. Guidelines for the handling of rabbit bucks and semen. World Rabbit Sci 2005;13:71-91.

Brandt H., Grandjot G. Genetic and environmental effects on male fertility of AI boars. In: Proceedings of the 6th World Congress on Genetics Applied to Livestock Production, 1998; 23:527-530.

Brun J.M., Theau-Clément M., Bolet G. The relationship between rabbit semen characteristics and reproductive performance after artificial insemination. Anim Reprod Sci 2002a;70:139-49.

Brun J.M., Theau-Clément M., Bolet G. Evidence for heterosis and maternal effects on rabbit semen characteristics. Anim Res 2002b;51:433-42.

Brun J.M., Theau-Clément M., Esparbié J., Falières J., Saleil G., Larzul C. Semen production in two rabbit lines divergently selected for 63-d body weight. Theriogenology 2006;66:2165-72.

Brun J.M., Sanchez A., Duzert R., Saleil G., Theau-Clément M. Paramètres génètiques des caractéristiques de la semence de lapin. In Proc: 13èmes Journ Rech Cunicole 2009;11: 17-18.

Casey P.J., Hillman R. B., Robertson K. R., Yudin A. I., Liu I. K., Drobnis E. Z. Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. J Androl 1993;14:289-97.

Castellini C., Lattaioli P. Effect of motile sperms inseminated on reproductive performance of rabbit does. Anim Sci 1999;57:111-20.

Castellini C., Lattaioli P., Cardinali R., Dal Bosco A. Effect of collection rhythm on spermatozoa and droplet concentration of rabbit semen. World Rabbit Sci 2006a;14:101-6.

Castellini C., Cardinali R., Dal Bosco A., Minelli A., Camici O. Lipid composition of the main fractions of rabbit semen. Theriogenology 2006b;65: 703–12.

Castellini C., Mourvaki E., Dal Bosco A., Galli F. Vitamin E Biochemistry and function: A case study in male rabbit. Reprod Dom Anim 2007;42: 248-56.

Castellini C., Dal Bosco A., Ruggeri S., Collodel G. What is the best frame rate for evaluation of sperm motility in different species by computer-assisted sperm analysis? Fertility and Sterility 2011;96:24-7.

Castellini C., Mourvaki E., Cardinali R., Collodel G., Lasagna E., Del Vecchio M.T., Dal Bosco A. Secretion patterns and effect of prostate-derived granules on the sperm acrosome reaction of rabbit buck. Theriogenology 2012;78:715-23.

Correa JR, Zavos PM. The hypoosmotic swelling test: its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology 1994;42:351-60.

Correa J.R., Pace M.M., Zavos P.M. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. Theriogenology 1997;48:721-31.

Courtens J. L., Bolet G., Theau-Clément M. Effect of acrosome defects and sperm chromatin decondensation on fertility and litter size in the rabbit: preliminary electron microscopic study. Reprod Nutr Dev 1994;34:427-37.

Cremades T., Roca J., Rodríguez-Martínez H., Abaigar T., Vázquez J.M., Martínez EA. Kinematic changes during the cryopreservation of boar spermatozoa. J Androl 2005;26:610-18.

Cross N.L., Morales P., Overstreet J.W., Hanson F.W. Two simple methods for detecting acrosome-reacted human sperm. Gam Res 1986;15:213-26.

Cummins J.M., Woodall P.F. On mammalian sperm dimensions. J Reprod Fertil 1985;75:153-75.

David I., Druart X., Lagriffoul G., Manfredi E., Robert-Gramié C., Bodin L. Genetic and environmental effects on semen traits in Lacaune and Manech tête rouse Al rams. Genet Sel Evol 2007;39:405-19.

Davis R.O, Katz D.F. Operational standards for CASA instruments. J Androl 1993;14:385-94.

Ducrocq V., Humblot P. Genetic characteristics and evolution of semen production of young Normande Bulls. Livestock Production Science 1995;41:1-10.

Eisen E.J., Johnson B.H. Correlated responses in male reproductive traits in mice selected for litter size and body weight. *Genetics* 1981;99: 513-24.

Estany J., Camacho J., Baselga M., Blasco A. Selection response of growth rate in rabbits for meat production. Genet Sel Evol 1992;24:527-37.

Farrell P.B., Foote R.H., Loomis P.R. Elimination of granule interference facilitating accurate casa analysis of rabbit sperm. J Andrology 1992; abstract 108.

Farrell P.B., Foote R.H., Simkin M.E., Clegg E.D., Wall R.J. Relationship of semen quality, number of sperm inseminated, and fertility in rabbits. J Androl 1993;14:464-71.

Farrell P.B., Foote R.H., Mc Ardle M.M., Trouern-Trend V.L., Tardif A. Media and dilution procedures tested to minimize handling effects on human, rabbit, and bull sperm for computer-assisted sperm analysis (CASA). J Androl 1996;17:293-300.

Flesch F.M., Gadella B.M. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochimica et Biophysica Acta 2000;1469:197-235.

Gadea J., Matás C., Lucas X. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. Anim Reprod Sci 1998;18: 95-108.

Gadella B.M. Dynamic regulation of sperm interactions with the zona pellucid prior to and after fertilization. Reprod Fertil Dev 2012;25:26-37.

García M.L., Andrés I., Caselles P., Lavara R. Estudio de la edad de los machos de conejo en la inseminación artificial. Boletín de cunicultura, 2004;132:17-25.

García-Tomás, M., Sánchez J., Rafel O., Ramón J., Plies M. Heterosis, direct and maternal genetic effects on semen quality traits of rabbits. Livest Sci 2006a;100:111-20.

García-Tomás, M., Sánchez J., Rafel O., Ramón J., Piles M. Variability, repeatability and phenotypic relationships of several characteristics of production and semen quality in rabbit. Anim Reprod Sci 2006b;93:88-100.

García-Tomás M., Sanchez J., Rafel O., Ramón J., Piles M. Reproductive performance of crossbred and purebred male rabbits. Livest Sci 2006c;104:233-43.

Garner D., Johnson L. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995;53:276-84.

Garner D., Dobrinsky J., Welch G., Johnson L. Porcine sperm viability, oocyte fertilization and embryo development after staining spermatozoa with SYBR-14. Theriogenology 1996;45:1103-13.

Gogol P., Bochenek M., Smoraq Z. Effect of rabbit age on sperm chromatin structure. Reprod Domest Anim 2002;37:92-5.

Gómez E.A., Baselga M, Rafel O, Ramón J. Comparison of carcass characteristics in five strains of meat rabbit selected on different traits. Livest Prod Sci 1998;55:53-64.

Graham J.K. Analysis of stallion semen and its relation to fertility. Vet Clin N AM Equine Pract 1996;12:119-30.

Graham J.K., Mocé E. Fertility evaluation of frozen/thawed semen. Theriogenology 2005;64:492–504.

Gravance C.G., Davis R. O. Automated sperm morphometry analysis (ASMA) in the rabbit. J Androl 1995;16:88-93.

Gravance C.G., Liu I. K. M., Davis R. O., Hughes J. P., Casey P. J. Quantification of normal head morphometry of stallion spermatozoa. J Reprod Fertil 1996;108:41-6.

Gravance C.G., Casey M.E., Casey P.J. Pre-freeze bull sperm head morphometry related to post-thaw fertility. Anim Reprod Sci 2009;114:81-8.

Grendler B., Fuerst C., Fuerst-Waltl B., Schwarzenbacher H., Sölkner J. Genetic parameters for semen production traits in Austrian dual-purpose Simmental bulls. Reprod Dom Anim 2007;42:326-8.

Hallap T., Haard M., Jaakma Ü., Larsso B., Rodriguez-Martinez H. Does cleansing of frozen-thawed bull semen before assessment provide samples that relate better to potential fertility. Theriogenology 2004;62:702-13.

Hernández P., Guerrero L., Ramírez J., Mekkawy W., Pla M., Ariño B., Ibánez N., Blasco A. A Bayesian approach to the effect of selection for growth rate on sensory meat quality of rabbit. Meat Science 2005;69:123-7.

Hernández P., Ariño B., Grimal A., Blasco A. Comparision of carcass and meat characteristics of three rabbit lines selected for litter size of growth rate. Meat Science 2006;73:645-50.

Hidalgo M., Rodríguez I., Dorado J.M. The effect of cryopreservation on sperm head morphometry in Florida male goat related to sperm freezability. Anim Reprod Sci 2007;100:61-72.

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

Holt C., Holt W.V., Moore H.D., Reed H.C., Curnock R.M. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J Androl 1997;18: 312-3.

Holt W.V., Penfold L.M., Johnston S.D., Temple-Smith P., McCallum C., Shaw J., Lindemans W., Blyde D. Cryopreservation of macropodid spermatozoa: new insights from the cryomicroscope. Reprod Fert Develop 1999;11:345–53.

Jeyendran R.S., Van der Van H.H., Pérez-Peláez M., Crabo B.G., Zaneverld L.J. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984;70:219-28.

Johnson R.K., Eckardt G.R., Rathje T.A., Drudik D.K. Ten generations of selection for predicted weight of testes in swine: direct response and correlated response in body weight, backfat, age at puberty, and ovulation rate. J Anim Sci 1994;72:1978-88.

Karoui S., Díaz C., Serrano M., Cue R., Celorrio I., Carabaño M.J. Time trends, environmental factors and genetic basis of semen traits collected in holstein bulls under commercial conditions. Anim Reprod Sci 2011;124:28-38.

Kealey C.G, MacNail M.D., Tess M.W., Geary T.W., Bellows R.A. Genetic parameter estimates for scrotal circumference and semen characteristics of Line 1 Hereford bulls. J Anim Sci 2006;84:283-90.

Khalil M.H., Al-Sobayil K. A., Al-Saef A. M., García M. L., Baselga M. Genetic evaluation for semen characteristics in a crossbreeding project involving Saudi and Spanish V-line rabbits. Animal 2007;1:923-28.

Killian G.J., Chapman D.A., Rogowski L.A. Fertility-associated proteins in Holstein bull seminal plasma. Biol Reprod 1993;49:1202-7.

Knights S.A., Baker R.L., Gianola D., Gibb J.B. Estimates of heritabilities and of genetic and phenotypic correlations among growth and reproductive traits in yearling Angus bulls. J Anim Sci 1984;58:887-93.

Kruger T.F., Ackerman S.B., Simmons K.F., Swanson R.J., Brugo S.S., Acosta A.A. A quick reliable staining techniques for human sperm morphology. Arch Androl 1996;18:275-7.

Larzul C., Gondret F., Combes S., De Rochambeau H. Analyse d'une expérience de sélection sur le poids á 63 jours : I- Déterminisme génétique de la croissance. In Proc: 10èmes Journées de la Recherche Cunicole, 2003.

Larzul C., De Rochambeau H. Selection for residual feed consumption in the rabbit. Livest Prod Sci 2005;95: 67-72.

Lavara R., Mocé E., Andreu E., Pascual J.J., Cervera C., Viudes De Castro M.P., Vicente J.S. Effects of environmental temperature and vitamin supplements on seminal parameters from a rabbit line selected by high growth rate. In Proc: 7th World Rabbit Congress 2000;Vol(A):167-72.

Lavara R., Vicente J.S. Estado actual de la inseminación en cunicultura. Lagomorfa 2001;113:24-32.

Lavara R., Mocé E., Vicente JS. Buenas prácticas en inseminación artificial I. Lagomorfa 2003;128:14-23.

Lavara R., Mocé E., Lavara F., Viudes de Castro M.P., Vicente J.S. Do parameters of seminal quality correlate with the results of on-farm inseminations in rabbits? Theriogenology 2005;64:1130-41.

Lavara R., Baselga M., Vicente JS., García ML. Variance components estimate for semen characteristics in rabbits. Preliminary analysis. ITEA; 2007:28:546-8.

Lavara R., Vicente JS., Marco-Jiménez F., Baselga M. Correlation among CASA and ASMA parameters in rabbit semen. In: Proc 9th World Rabbit Congress 2008;381-6.

Lavara R., Baselga M., Vicente J.S. Aneuploidy in rabbit males: semen traits and fertility. Theriogenology 2010;74:105-10.

Leyún M., Muguerza T., Hoscos A. La inseminación artificial en banda única supera a la monta natural en todos los aspectos. Lagomorpha, 1999;105:52-62.

López F.J., Alvariño J.M.R. Artificial insemination of rabbits with diluted semen stored up to 96 hours. World rabbit science 1998,6:251-3.

López-Gatius F., Sances G., Sancho M., Yániz J., Santolaria P., Gutiérrez R., Núñez M., Núñez J., Soler C. Effect of solid storage at 15ºC on the subsequent motility and fertility of rabbit semen. Theriogenology 2005;64:252-60.

Lukerfahr S.D., Odi H,B., Atakora J.K.A. Mass Selection for 70 day body weight in rabbits. J Anim Sci 1996;74:1481-9.

Maertens L., Luzi F. Effect of the diet on sexual ardour and quality of semen in male rabbits. Cuniculture 1997;135:105-8.

MAGRAMA2008.http://www.magrama.gob.es/es/estadistica/temas/estadisticasagrarias/2008_Cuniculura_Memoria_tcm7-14332.pdf

Mann T., Lutwak-Mann C., Dixon R.L. Passage of chemicals into human and animal semen: mechanisms and significance. Crit Rev Toxicol 1982;11:1-14

Marco-Jiménez F., Pérez L., Viudes de Castro M.P., Garzón D.L., Peñaranda D.S., Vicente J.S., Jover M., Asturiano J.F. Morphometry characterisation of European eel spermatozoa with computer-assisted spermatozoa analysis and scanning electron microscopy Theriogenology, 2006;65:1302–10.

Marco-Jiménez F., Vicente J.S., Lavara R., Balasch S., Viudes de Castro M.P. Poor prediction value of sperm head morphometry for fertility and litter size in rabbit. Reprod Dom Anim 2010;45:e118-e123.

Mathevon M., Buhr M.M., Dekkers J.C.M. Environmental, management and genetic factors affecting semen production in Holstein bulls. Journal of Dairy Science, 1998;81:3321-30.

Maxwell W.M.C., Welch G.R., Johnson L.A. Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Develop 1997;8:1165-78.

Maxwell W.M.C., Long C.R., Johnson L.A., Dobrinsky J.R., Welch G.R. The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Develop 1998;10:433-40.

Maxwell W.M.C., Johnson L.A. Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenology 1999;52:1353-62.

Mocé E., Lavara R., Lavara F., Vicente J.S. Effect of reproductive rhythm on seminal parameters from a rabbit line with high growth rate. In: Proc 7th World Rabbit Congress 2000;Vol(A):197-201.

Mocé E., Lavara R., Marco-Jiménez F., Vicente J.S, López M. Características seminales de la raza de conejo Gigante de España. ITEA 2005;26:350-2.

Mocé E., Graham J.K. In vitro evaluation of sperm quality Anim Reprod Sci 2008; 105:104-18.

Mortimer D., Curtis E. F., Miller R. G. Specific labelling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. J Reprod Fertil 1987;81:127-35.

Mortimer ST. CASA-practical aspects. J Andorl 2000; 21:515-24.

Mortimer D., Menkveld R. Sperm morphology assessment-historical perspectives and current options. J Androl 2001;62:192-205.

Moura A.S.A.M.T., Kaps M., Vogt D.W., Lamberson W.R. Two-Way selection for daily gain and feed conversion in a composite rabbit population. J Anim Sci 1997;75:2350-4.

Mukherjee D.P., Johari M.P., Bhattacharya P. The gelatinous mass in rabbit semen. Nature 1951;168:422-3.

Nagy S., Jansen J., Topper E., Gadella B. A triple-stain flow cytometric method to assess plasma and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol Reprod 2003;68:1828-35.

Napier R.A.N. Fertility in the male rabbit III. Estimation of spermatozoan quality by mixed insemination, and the inheritance of spermatozoan characters. J Reprod Fertil 1961;2:273-89.

Nizza A., Di Meo C., Taranto S. Effect of collection rhythms and season on rabbit semen production. Reprod Domest Anim 2003;38:436-9.

O'Bryan M.K., Schlatt S., Philips D.J., De Kretser D.M., Hedger M.P. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo. Endocrinology 2000;141:238-46.

Oh S.H., See M.T., Long T.E., Galvin J.M. Estimates of genetic correlations between production and semen traits in boar. Asian-Australasian Journal of Animal Science 2006a;19:160-4.

Oh S.H., See M.T., Long T.E., Galvin J.M. Genetic parameters for various random regression models to describe total sperm cells per ejaculate over the reproductive lifetime of boars. J Anim Sci2006b;84:538-45.

Panella F., Castellini C., Facchin E. Heritability of some male reproductive traits in rabbit. Cah Options Mediterr 1994;8:279-83.

Pascual J.J., García C., Martínez E., Mocé E., Vicente JS. Rearing management of rabbit males selected by high growth rate: the effect of diet and season on semen characteristics. Reprod Nutr Dev 2004;44:49-63.

Piles M., Gómez E.A., Rafel O., Ramón J., Blasco A. Elliptical selection experiment for the estimation of genetics parameters of the growth rate and feed conversión ration in rabbits. J Anim Sci 2004;82:654-60.

Piles M., Tusell L., Lavara R., Baselga M. Breeding programs for improving male reproductive performance and efficiency of AI dose production in paternal lines: feasibility and limitations. In: Proceedings 10th World Rabbit Congress 2012;1-17.

Pla M., Guerrero L., Guardia D., Oliver M.A., Blasco A. Carcass characteristics and meat quality of rabbit lines selected for different objetives: I. Between lines comparision. Livest Prod Sci 1998;54:115-23.

Purdy P.H., Graham J.K. Effect of colesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology 2004;48:36-45.

Purdy P.H. Swine gene banking: A quality control perspective on collection, and analysis of samples for a national repository. Theriogenology 2008;70:1304-9.

Quintero-Moreno A., Rigau T., Rodríguez-Gil J.E. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in

rabbit semen and to predict semen fertility and litter size. Reprod. Domest Anim 2007;42:312-9.

Rege J.E.O., Toe F., Mukasa-Mugerwa E., Tembely S., Anindo D., Baker R.L., Lahlou-Kassi A. Reproductive characteristics of Ethiopian highland sheep. II. Genetic parameters of semen characteristics and their relationships with testicular measurements in ram lambs. Small Ruminant Research, 2000;37:173-87.

Riedy M., Muirhead K., Jensen C., Stewart C. Use of photolabeling technique to identify nonviable cells in fixed homologous or heterologous cell populations. Cytometry 1991;12:133-9.

Roca J., Martínez S., Vázquez J.M., Lucas X., Parrilla I., Martínez E.A. Viability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15 C. Anim Reprod Sci 2000;64:103-12.

Roca J., Martínez S., Orengo J., Parrilla I., Vázquez J., Martínez E.A. Influence of constant long days on ejaculate parameters of rabbits reared under natural environment conditions of Mediterranean area. Livest Prod Sci 2005;94:169-74.

Rochambeau H. de, Fuente L.F., Rouvier R., Ouhayoun J. Selection sur la vitesse de croissance post-sevrage chez le lapin. Genet Sel Evol 1989;21:527-46.

Rodríguez-Martínez H. Laboratory semen assessment and prediction of fertility: still utopia? Reprod Domest Anim 2003;38:312-8.

Rodríguez-Martínez H. State of the art in farm animal sperm evaluation. Reprod Fertil Dev 2007;19:91-101.

Rodríguez-Martínez H., Kvist U., Ernerudh J., Sanz L., Calvete JJ. Seminal plasma proteins: what role do they play? Am J Reprod Immunol 2011;66:11-22.

Rosato M., Iaffaldano N. Effect of chilling temperature on the long-term survival of rabbit spermatozoa held either in a tris-based or a jellified extender. Reprod Domest Anim 2011;46:301-8.

Rosell J.M., De La Fuente L.F. Culling and mortality in breeding rabbits. Preventive Veterinary Medicine 2009;88:120-7.

Saacke R.G., White J.M. Semen quality and their relationship to fertility. Proceedings of the 4th NAAB technical conference on animal reproduction AI 1972:23-7.

Saacke R.G., Dalton J.C., Nadir S., Nebel R.L., Bame J.H. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim Reprod Sci 2000;60:663-77.

Safaa H.M., Vicente J.S., Lavara R., Viudes de Castro M.P. Semen evaluation of two selected lines of rabbit bucks. World Rabbit Sci 2008 ;16:141-8.

Salisbury G., Beck G., Cupps P.T., Elliott I. The effect of dilution rate on the livability and the fertility of bull spermatozoa used for artificial insemination. J Dairy Sci 1943;26:1057-69.

Sarreiro L.C, Bergmann J.A.G., Quirino C.R., Pineda N.R., Ferreira V.C.P., Silva M.A. Heritability and genetic correlation between scrotal circumference, libido and seminal traits in Nellore bulls. Arquivo Brasileiro De Medicina Veterinaria E Zootecnia 2002;54:602-8.

Smital J., Wolf J.B., De Sousa L.L. Estimation of genetic parameters of semen characteristics and reproductive traits in AI boars. Animal Production Science, 2005;86:119-30.

Swierstra E.E., Foote R.H. Duration of spermatogenesis and spermatozoan transport in the rabbit based on cytological changes, DNA synthesis and labeling with tritiated thymidine. American Journal of Anatomy, 1965;116:401-11.

Taylor J.F., Bean B., Marshall C.E., Sullivan J.J. Genetic and environmental components of semen production traits of artificial insemination Holstein bulls. 1989;68:2703-22.

Theau-Clément M., Michael N., Esparbie J., Bolet G. Effects of artificial photoperiods on sexual behaviour and sperm output in the rabbit. Anim Sci 1995 ;60:143-9.

Theau-Clément M., Castellini C., Maertens L., Boiti C. Biostimulations applied to rabbit reproduction: Theory and practice. World Rabbit Sci. 1998;6:179-84.

Theau-Clément M., Brun J.M., Sabbioni E., Castellini C., Renieri T., Besenfelder U., Falieres J., Esparbie J., Saleil G.. Comparaison de la production spermatique de trois souches de lapins: Moyennes et variabilités. In: Proc 10èmes Journ. Rech. Cunicole 2003:81-4.

Theau-Clément M., Falieres J. Evaluation de la concentration de semence de lapins selon 2 méthodes : Hématimètre et NucleoCounter SP100. In Proc.: 11èmes Journées de la Recherche Cunicole 2005:95-9.

Thurston L.M., Watson P.F., Mileham A.J., Holt W.V. Morphologically distinct sperm subpopulations defined by fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl 2001;22:382-94

Tusell L., García-Tomás M., Baselga M., Rekaya R., Rafel O., Ramón J., López-Bejar M., Piles M. Interaction of genotype x artificial insemination conditions for male effect on fertility and prolificacy. J Anim Sci 2010;88:3475-85.

Verstegen J., Iguer-Ouada M., Onclin K. Compueter assisted semen analyzers in andrology research and veterinary practice. Theriogenology 2002;57:149-79.

Vicente J. S., Viudes-de-Castro M. P., Lavara R., Lavara F. Effect of male line on prolificacy from does inseminated with low sperm doses. In: Proc 7th World Rabbit Congress 2000;A:273-7

Viudes de Castro M.P., Vicente J.S. Effect of sperm amount on the fertility and prolificity rates of meat rabbits. Anim Reprod Sci 1997;46:313-9.

Viudes de Castro M.P., Vicente J.S., Lavara R. Effet du nombe de spermatozoïdes sur la fertilité de la semence conservée 24 heures chez le lapin. Ann Zootech 1999;48:407-12.

Walton A. The relation between "density" of sperm-suspension and fertility: as determined by artificial insemination of rabbits. Proc Roy Soc B 1927;101-303.

Ward W.S., Coffey D.S. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod 1991;44:569-74.

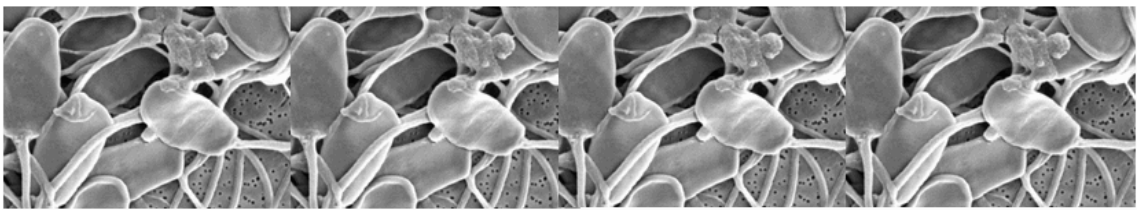
Xu X., Pommier S., Arbov T., Hutchings B., Sotto W., Foxcroft G.R. In vitro maturation and fertilization techniques for assessment of semen quality and boar fertility. J Anim Sci 1998;76:3079-89.

Wolft J. Genetic correlations between production and semen traits in pig. Animal 2009;3:1094-99.

Yilmaz A., Davis M.E., Simmen R.C.M. Estimation of (co)vaciance components for reproductive traits in Angus beef cattle divergently selected for blood serum IGF-I concentration. Journal of Animal Science, 2004;82:2285-92.

Zhang J., Boyle M.S., Smith C.A., Moore H.D.M. Acrosome reaction of stallion spermatozoa evaluated with monoclonal antibody and zona-free hamster eggs. Mol Reprod Dev 1990;27:152-8.

OBJECTIVES



OBJECTIVES

The aim of this thesis was to study the different sources of variation for some of the most important traits related with AI dose production, particularly the genetic ones, in order to explore the consequences of selection for growth rate in these traits and their future inclusion in a genetic selection programme in rabbits. To this end, the specific objectives of the thesis were as follows:

In chapter 1, the objective was to estimate the genetic parameters of sperm production traits (ejaculate volume (V), sperm concentration (CN) and sperm production (PROD) per ejaculate) as well as the genetic relationship with daily gain (DG).

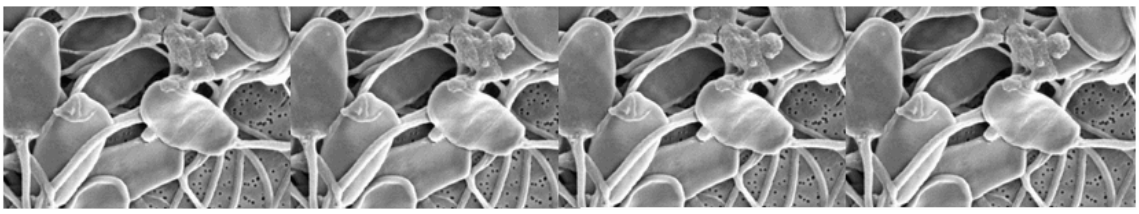
The objective in chapter 2 was to explore the genetic determinism of some quality sperm traits: NAR (%, percentage of sperm with intact acrosome), ANR (%, percentage of sperm abnormalities), MOT (%, percentage of total motile sperm cells) and some motion CASA parameters in fresh semen and their genetic relation with the selection criteria of the paternal rabbit line.

Chapter 3 aimed to determine the repeatability and heritability of sperm head characteristics: width (W, µm), area (A, μm^2), length (L, µm) and perimeter (P, µm), and explore the relationships between them and with the selection objective.

Some environmental and male effects that could have an influence on sperm freezability were studied in Chapter 4.

The last chapter of this thesis focused on estimating the heritability of semen freezability traits and the genetic correlation between frozen-thawed sperm traits and the growth rate in the paternal rabbit line.

CHAPTER ONE



CHAPTER ONE

Genetic parameter estimates for semen production traits and growth rate of a paternal rabbit line

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ABSTRACT

Variance components of sperm production traits (volume in ml, V; concentration in $x10^6$ sperm/ml, CN; sperm production in $x10^6$ sperm, PROD), were estimated in a paternal line of rabbit selected for 25 generations on the basis of daily weight gain (DG, g/day) between 28 and 63 days of age. Features of the marginal posterior distributions for ratios of genetic variance, variance due to non-additive plus environmental permanent male effects, and variance due to common litter of birth effects with respect to phenotypic variance are reported. The correlations between sperm production traits and the selection criteria were also estimated.

Three sets of two-trait analyses were performed, involving 12908 records of DG, 2329 ejaculates corresponding to 412 bucks, and 14700 animals in pedigree file. The heritabilities (h^2) of the semen traits were 0.13±0.05, 0.08±0.04 and 0.07±0.03 for V, CN and PROD respectively. The permanent environmental effects were lower than the corresponding values of h^2 and varied between 0.06 and 0.11. A favorable and moderate genetic correlation was observed between V and DG (0.36±0.34; P>0: 0.83), together with a non-favorable and moderate correlation between permanent environmental effects due to common litter of birth for both traits (-0.35±0.35; P<0: 0.85). On the other hand, the correlation between male permanent environmental effects for semen traits and DG, were moderate and non-favorable (-0.51±0.29 with P<0: 0.95 for DG-CN, and -0.31±0.37 with P<0: 0.79 for DG-PROD).

INTRODUCTION

Commercial production of rabbits for meat is based commonly on crossbred dams derived from a cross between two maternal lines. Those females are mated to bucks from a paternal line (Baselga 2004). Currently, the crossbred females are inseminated (instead of mated) with semen doses from bucks housed in artificial insemination stations. The relative importance of the male in the cost of the artificial insemination dose is around 2–6% (García *et al.,* 1998; García *et al.,* 2004), but this value could be increased if the percentage of males that show adequate reproductive behaviour at six months is low (58%, Pascual *et al.,* 2004) or if the males have a high rate of culling (30–40% of global culling) caused by their low output with respect to semen quality and/or production (Rosell and De la Fuente, 2009).

In rabbits, the genetic parameters, such as heritability, repeatability, genetic correlation or heterosis, for traits related to litter size and growth have been estimated in many populations, and they are well documented (Moura *et al.,* 2001; Piles *et al.,* 2006). However, the corresponding parameters for semen traits have never or rarely been estimated. Only a few studies on the repeatability and crossbreeding parameters can be found in the literature (Bencheikh, 1995; Brun *et al.,* 2002; Castellini *et al.,* 2006; García-Tomás *et al.,* 2006a,b).

The effect of selection of paternal rabbit lines for growth rate on sperm quality and quantity has been reported by some authors (Brun *et al.,* 2006), but there is no information about genetic parameters between semen and growth traits. In other species, selection on the basis of growth traits shows genetic responses that are positively correlated with the weights of reproductive organs but negatively correlated with the efficiency of sperm production and parameters

of semen quality (Eisen and Johnson, 1981 in mice; Johnson *et al.,* 1994 in pigs; Rege *et al.,* 2000 in sheep; Kealey *et al.,* 2006 in cattle).

Knowledge of the genetic parameters of semen traits and their genetic correlation with the selection criteria in paternal rabbit lines is necessary to predict the correlated response on semen traits and to determine a possible strategy of selection for semen production traits. Therefore, the aims of this study were to estimate the genetic parameters of traits related to semen in a paternal rabbit line selected for post-weaning daily weight gain, and to estimate the genetic correlation between these semen traits and the growth rate.

MATERIALS AND METHODS

Animals and data

Animals that belonged to a paternal rabbit line (Line R) were used for the experiment. Line R was selected for daily weight gain (DG) between 28 and 63 days of age (Estany *et al.* 1992) by individual selection.

After weaning, the animals were housed in collective cages (eight rabbits per cage) and subjected to a temperature that ranged from 15 to 25ºC. At the age of 63 days, male rabbits were moved to three AI stations. The males were placed in individual cages, subjected to a photopheriod of 16 h light/day, and fed *ad libitum* with a commercial rabbit diet (on a dry matter basis: 17.5% crude protein, 3.5% ether extract, 16.7% crude fiber, 2938 kcal/kg). In all stations, the environmental conditions were controlled with the aim of maintaining the temperature between 17 and 24ºC.

Males started the training period at 150-170 days of age. The training was performed for 2 weeks. After the training, the males entered in the production period. For the training and production period, each week, two ejaculates per male were collected on a single day using an artificial vagina, with a minimum of 30 min between collections. Collection of semen from each male during the experiment was performed on the same day of the week.

The semen samples for the experiment were collected during two different periods:

- Period A: 1 week after finishing the training period.

- Period B: more than 3 months after period A.

Only ejaculates that exhibited a white color were used in the experiment; samples containing urine and cell debris were discarded whereas gel plugs were removed. The volume of the ejaculate (V, ml) was recorded. For determination of concentration (CN, 10^6 spz/ml), an aliquot from each ejaculate (20 µl) was fixed with 180 µl of 2% glutaraldehyde solution in DPBS. The sperm concentration was determined using a Thoma-Zeiss counting cell chamber (Marienfield, Germany). Sperm production per ejaculate (PROD, 10^6 spz) was determined as V*CN.

A total of 12908 records for DG were used in the experiment. DG data used belonged to animals from twelve generation before. In addition to DG, semen traits PROD, V and CN were recorded from 2006 to 2007. This involved 2329 ejaculates from 412 males. The pedigree file included 14700 animals.

In order to reduce bias in the estimation of the genetic parameters of the semen traits resulting from the selection for DG, each of the semen traits was analyzed jointly with DG (Sorensen and Johansson, 1992). Therefore a set of two-trait analyses were performed.

Statistical analyses

The mixed model used for the semen traits was:

$$y_{sijokl} = \mu_s + S_{si} + O_{sj} + P_{so} + a_{sk} + p_{sk} + c_{sl} + e_{sijokl}$$

where y_{sijokl} is a record of the semen trait, μ_s is the overall mean, S_{si} is the systematic effect of station–year–season in which the ejaculate was collected with 64 levels, O_{sj} is the systematic effect of ejaculate order with two levels (first and second ejaculate on the same day), P_{so} is the systematic effect of age of the male with 3 levels (≤ 6 months, 6–8 months, more than 8 months), a_{sk} is the animal additive genetic effect, p_{sk} is the permanent environmental effect over all the ejaculates of the male *k*, c_{sl} is the random effect of the litter in which the male *k* was born, and e_{sijokl} is the residual. It was assumed that the different random effects (additive, permanent, litter of birth and residual) followed normal distributions and were independent among and within the effects, excepting the additive values of the animals, which were correlated through the numerator relationship matrix.

The mixed model used for DG was:

$$y_{dijkl} = \mu_d + b*LS_{dl} + YS_{di} + OP_{dj} + a_{dk} + p_{dk} + c_{dl} + e_{dijkl}$$

where y_{dijkl} is the daily gain of animal *k*, μ_d is the overall mean, LS_{dl} is the covariate litter size at birth, YS_{di} is the systematic effect of year–season in which

the animal was weaned, with 30 levels, OP_{dj} is the systematic effect of parity order in which the animal was born, with three levels (first, second, and higher), a_{dk} is the animal additive genetic effect, c_{dl} is the random effect of the litter in which the animal *k* was born; the residual of the model was split into two components: p_{dk}, which corresponds to the part of the residual correlated with the permanent environmental effect for semen traits, and e_{dijkl}, which corresponds to the part of the residual uncorrelated with any other random effect, within and among traits.

The assumptions for the random effects for DG are the same as those indicated above for the semen traits.

Further assumptions, concerning correlations between random effects of DG (a_d, p_d, c_d, e_d) and random effects of one semen trait (a_s, p_s, c_s, e_s), are summarized in the following matrices:

$$\mathbf{G}=\begin{bmatrix} \sigma^2_{a_d} & \sigma_{a_d,a_s} \\ \sigma_{a_s,a_d} & \sigma^2_{a_s} \end{bmatrix};$$

$$\mathbf{P}=\begin{bmatrix} \sigma^2_{p_d} & \sigma_{p_d,p_s} \\ \sigma_{p_s,p_d} & \sigma^2_{p_s} \end{bmatrix};$$

$$\mathbf{C}=\begin{bmatrix} \sigma^2_{c_d} & \sigma_{c_d,c_s} \\ \sigma_{c_s,c_d} & \sigma^2_{c_s} \end{bmatrix};$$

$$\mathbf{R}=\begin{bmatrix} \sigma^2_{e_d} & 0 \\ 0 & \sigma^2_{e_s} \end{bmatrix}$$

where the components **G**, **P**, **C** and **R** are the additive, permanent, common litter and residual variances for the daily gain and the semen trait in the diagonal, and the corresponding covariances between both traits, out of the diagonal.

The variance–covariance components were estimated using a Bayesian approach implemented in the TM program developed by Legarra *et al.* (2008). Flat priors were used for systematic effects and variance components.

The following prior distributions for random effects were assumed:

$$p\left(\begin{bmatrix} a_d \\ a_s \end{bmatrix} \middle| \mathbf{G}\right) \sim N(\mathbf{0}, \mathbf{A} \otimes \mathbf{G}),\ p\left(\begin{bmatrix} p_d \\ p_s \end{bmatrix} \middle| \mathbf{P}\right) \sim N(\mathbf{0}, \mathbf{I} \otimes \mathbf{P}),\ p\left(\begin{bmatrix} c_d \\ c_s \end{bmatrix} \middle| \mathbf{C}\right) \sim N(\mathbf{0}, \mathbf{I} \otimes \mathbf{C})$$

where **A** is the additive genetic relationship matrix, **0** is a vector of zeroes, **I** is an identity matrix, and **G**, **P** and **C** are the (co)variance matrices summarized above.

After some exploratory analysis, chains of 1000000 samples were used, with a burning period of 100000. Only one sample of each 100 was saved. The convergence was checked on each chain by the Z Geweke criterion (Geweke, 1992).

RESULTS

The number of records, means and standard deviations of the traits studied are presented in **Table 1.1**, where the high variability of the semen traits can be observed.

The results for the study of systematic effects proved the importance of the order of ejaculate to V and CN, but not to PROD (**Table 1.2**). The second ejaculate had a lower volume and was more concentrated than the first ejaculate. On the other hand, the age of the male also showed an important effect on seminal traits: males of ≤ 6 months had the worst seminal production traits, and males with ages between 6 and 8 months, and more than 8 months, did not show any relevant difference.

Table 1.1: Crude means and standard deviations for semen traits and daily weight gain

	n	Mean	Standard deviation
DG (g/d)	12908	46.2	6.9
V (ml)	2329	0.59	0.36
CN (10^6spz/ml)	2297	190.9	154.9
PROD (10^6spz)	2297	107	110

n: number of data. DG: daily gain between days 28 and 63. V: volume of ejaculate. CN: concentration. PROD: number of sperm per ejaculate.

Table 1.2: Descriptive statistics of the posterior marginal distributions of the estimable functions between ejaculate order (E1, E2) and age of the male (A1, A2, A3) for volume of ejaculate (V), sperm concentration (CN) and number of sperm per ejaculate (PROD)

	V (ml)		CN (10^6spz/ml)		PROD (10^6spz)	
	PM	$HPD_{95\%}$	PM	$HPD_{95\%}$	PM	$HPD_{95\%}$
A1-A2	-0.13	[-0.20 -0.07]	-37.38	[-65.87 -9.18]	-38.83	[-59.95 -18.08]
A1-A3	-0.12	[-0.18 -0.05]	-27.05	[-55.05 0.58]	-32.40	[-52.59 -11.95]
A2-A3	0.02	[-0.03 0.07]	10.34	[-12.29 33.05]	6.43	[-10.34 22.86]
E1-E2	0.11	[0.08 0.14]	-31.44	[-45.13 -17.68]	4.87	[-5.49 15.51]

E1: first ejaculate; E2: second ejaculate; A1: ≤6 months; A2: 6-8 months; A3: > 8 months; PM: posterior mean. $HPD_{95\%}$: interval of highest density of 95%.

Features of the estimated marginal posterior distributions (PM: posterior mean. $HPD_{95\%}$: interval of highest density of 95%) of heritabilities (h^2), and proportions of phenotypic variance due to genetic non-additive plus permanent environmental effects (p^2), and common litter of birth environmental effects (c^2) are presented in **Table 1.3**. The same information corresponding to the genetic (r_g), permanent environmental (r_p), and common litter (r_c) correlations among traits is shown in **Table 1.4**.

Table 1.3: Descriptive statistics of the posterior marginal distributions of heritability (h^2), ratio of permanent effects variance to phenotypic variance (p^2) and ratio of litter of birth effects variance to phenotypic variance (c^2), for daily gain (DG), volume of ejaculate (V), sperm concentration (CN), and number of sperm per ejaculate (PROD)

	h^2			p^2			c^2		
	PM	**$HPD_{95\%}$**	**$Min_{95\%}$**	**PM**	**$HPD_{95\%}$**	**$Min_{95\%}$**	**PM**	**$HPD_{95\%}$**	**$Min_{95\%}$**
DG	0.18	[0.12 0.23]	0.13	0.27	[0.06 0.48]	0.07	0.27	[0.25 0.30]	0.25
V	0.13	[0.04 0.23]	0.05	0.09	[0.02 0.17]	0.03	0.05	[0.01 0.10]	0.01
CN	0.08	[0.01 0.17]	0.03	0.11	[0.04 0.20]	0.05	0.07	[0.01 0.14]	0.02
PROD	0.07	[0.01 0.12]	0.02	0.06	[0.02 0.13]	0.03	0.04	[0.01 0.08]	0.01

PM: posterior mean. $HPD_{95\%}$: interval of highest density of 95%. $Min_{95\%}$: value for which the probability of higher values is 95%.

The highest heritability of the semen traits was for the volume of the ejaculate (PM: 0.13, posterior standard deviation (PSD): 0.05).

The posterior means of p^2 were lower than the corresponding values of h^2 and varied between 0.06 and 0.11.

The litter of birth effect explained a high proportion of the phenotypic variance for DG (0.27, **Table 1.3**), but not for the semen traits. The posterior mean of c^2 for the semen traits ranged between 0.04 and 0.07.

A favorable and moderate genetic correlation was observed between the volume of the ejaculate and DG (0.36; P>0: 0.83), together with an unfavorable and moderate litter of birth correlation between these variables (-0.35; P<0: 0.85). On the other hand, the environmental correlations between semen traits (sperm concentration and sperm production) and DG were moderate and non-favorable (-0.51 with P<0: 0.95 for DG-CN, -0.31 with P<0: 0.79 for DG-PROD).

Table 1.4: Descriptive statistics of the posterior marginal distributions of the genetic effects (r_g), permanent effects (r_p) and litter of birth effects (r_c) correlations with daily gain (DG) and volume of ejaculate (V), sperm concentration (CN), and number of sperm per ejaculate (PROD)

	r_g		r_p		r_c	
	PM	**$HPD_{95\%}$**	**PM**	**$HPD_{95\%}$**	**PM**	**$HPD_{95\%}$**
V-DG	0.36	[-0.26 0.96]	0.10	[-0.72 0.86]	-0.35	[-0.99 0.24]
CN-DG	-0.09	[-0.85 0.65]	-0.51	[-0.99 0.01]	0.13	[-0.43 0.69]
PROD-DG	0.17	[-0.51 0.98]	-0.31	[-0.99 0.36]	-0.02	[-0.70 0.69]

PM: posterior mean. $HPD_{95\%}$: interval of highest density of 95%.

DISCUSSION

Artificial insemination (AI) is used widely in rabbits. In Spain, 81.5% of the rabbits produced are derived from the use of artificial insemination (MARM, 2009).

Given that most of the semen used on commercial farms comes from males of paternal lines, which are commonly selected for growth traits such as DG, it is very important to know how the selection of these lines can affect the reproductive capacity of the males. In AI stations, the selection of the males is related to their libido and the number of useful doses of semen that they can produce. These doses also need to have a level of quality that guarantees no reduction in fertility and prolificacy.

The semen traits considered here are those involved in sperm production (volume, concentration and production per ejaculate). The average ejaculate volume observed in this experiment is similar to the values reported for males selected on the same objective (Brun *et al.,* 2006; Lavara *et al.,* 2008; Safaa *et al.,* 2008).

However, the concentration and, therefore, production of sperm were similar to those observed in previous work with males of line R (145–206x10^6spz/mL, Lavara *et al.,* 2008; Safaa *et al.,* 2008), but lower than those observed in other males selected for a similar objective (245–738 x10^6spz/mL, Brun *et al*., 2006; García-Tomás *et al.,* 2006a). The differences observed in ejaculate order and the age of the male rabbit are in agreement with previous results found in the literature.

No previous studies have estimated the genetic parameters for semen traits in rabbits, but estimates of repeatability for some semen traits have been reported by some authors.

The heritability values estimated in the current study for the semen traits were similar to those reported for bulls (0.09 for volume and 0.16 for concentration; Kealey *et al.,* 2006), and for pigs (0.14–0.18 for volume, 0.14–0.26 for

concentration and 0.17–0.25 for sperm production; Brandt and Grandjot, 1998; and Smital *et al.,* 2005).

If the repeatability is computed as the sum of h^2, p^2 and c^2, the values obtained would be 0.27 for the volume, 0.26 for concentration and 0.17 for sperm production per ejaculate. These values are of a similar magnitude to the values of repeatability estimated by Castellini *et al.* (2006) which were estimated to be 0.17 and 0.22 for concentration and sperm production, respectively, but lower than the values given by Bencheik (1995) and Garcia-Tomas *et al.* (2006b), which were around 0.3–0.4.

The genetic correlations of DG with CN and PROD showed a wide $HPD_{95\%}$, and the probabilities of an absolute value lower than 0.2 were 0.63 and 0.68, respectively; for this reason the results should be viewed with caution.

The estimated genetic correlation between V and DG was positive, as previous estimates published in pigs (Brandt and Grandjot, 1998) and bulls. With regard to the latter, some authors have predicted that bulls genetically superior for growth would be expected to sire sons with testicles of larger circumference and with the ability to produce larger quantities of semen (Knights *et al.,* 1984, Smith *et al.,* 1989). This finding is due to the fact that large testicles produce more testosterone that implies higher accessory glands which in turn could produce more seminal plasma.

The same general pattern was observed by Johnson and Eisen (1975) for the genetic correlation between semen production and DG in mice. They found a lower efficiency of sperm production in a line of mice selected for growth after weaning than in the unselected control line. Owing to the inaccuracy of the

estimates, no comment can be made about the other genetic and environmental correlations between DG and the semen traits.

In most farmed species, the ejaculate is diluted in an extender before AI and the number of spermatozoa per AI dose is standardized. The number of spermatozoa per dose and the type of the semen used (fresh, refrigerated or frozen) is species specific. The dilution of the ejaculate could reduce the direct advantage of high sperm output in the ejaculate on potential fertility, because when the ejaculate is diluted to standardize the cell number, the seminal plasma is diluted as well. Some authors relate the seminal plasma composition with sperm viability (Kohsaka *et al*., 2003), and it has been suggested that the composition of the seminal plasma may influence fertility in ewes (Maxwell *et al*., 1999). In rabbits, Castellini *et al*. (2000) showed that dilution of seminal plasma more than 20-fold caused a decline in motility, and accelerated the peroxidation of rabbit spermatozoa. In poultry, the seminal plasma seems to have an important role in fertility because dilution of fresh fowl semen has been shown to have harmful effects on spermatozoa (Blesbois and de Reviers, 1992). Bratte and Ibe (1989) worked with undiluted chicken semen and semen diluted one-, two- or three-fold, showed a downward linear trend in fertility.

Several authors have found that the composition of seminal plasma varies among species, but also among males, and among ejaculates from the same male in rams (Maxwell *et al.,* 1999; Pérez-Pe *et al.,* 2001) and in boars: (Zhu *et al.,* 2000). Garner *et al*. (2001) and Haugan *et al.* (2007), working with bulls, found that the effect of dilution on sperm viability was specific to the male. The male-dependent effect of the dilution ratio of seminal plasma to extender may

be related to proteins in the seminal plasma that are associated with fertility (Killian *et al.,* 1999).

With regard to rabbits, several authors have studied the relationship between ejaculate characteristics (volume and concentration) and fertility. Brun *et al.* (2002) found a significant association between sperm production and kindling rate. However, in rabbits AI is performed with heterospermic doses instead of homospermic doses, and the optimum number of spermatozoa in the seminal dose varies between studies, Viudes-de-Castro and Vicente (1997), Castellini and Lattaioli (1999). These results could be due to differences in the male line (different composition of the seminal plasma (Viudes de Castro *et al.,* 2004)) and/or conditions of sperm storage (male-specific responses to sperm conservation, Piles *et al.,* 2008).

Previous studies in the genetic parameters of semen have reported a negative genetic correlation between volume and sperm concentration, and a positive genetic correlation between sperm concentration and the total number of sperm per ejaculate. The importance of these correlations depends on species: they are medium–low in poultry (-0.27 and 0.52), as reported by Barbato (1999), and high in boars. In boars, Smital *et al.* (2005) and Oh *et al.* (2006) obtained a positive correlation between semen volume and the total number of spermatozoa (range between 0.6 and 0.7). These results, combined with knowledge of the relationship between ejaculate characteristics and fertility, indicate that, if some seminal trait is included as a selection objective, the useful trait could be sperm production in which both volume and concentration are included.

Finally, there is currently no evidence to suggest that selection for DG in rabbits will affect sperm production adversely. More data will be needed to improve the accuracy of the genetic correlations. If in future studies the low correlation between PROD and DG were confirmed, it would be possible to select paternal lines of rabbits for DG and PROD jointly.

REFERENCES

Barbato G.F. Genetic relationships between selection for growth and reproductive effectiveness. Poult Sci 1999;78:444-52.

Baselga M. Genetic improvement of meat rabbits.Programmes and diffusion. In: Proc 8th World Rabbit Congress 2004;1:1-13.

Bencheikh N. Effet de la fréquence de collecte de la semence sur les caractéristiques du semence et des spermatozoïdes récoltés chez le lapin. Ann Zootech 1995 ;44:263-79.

Blesbois E., de Reviers M. Effect of different fractions of seminal plasma on the fertilizing ability of fowl spermatozoa stored in vitro. Reprod Fert 1992;95:263-8.

Brandt H., Grandjot G. Genetic and environmental effects of male fertility of AI-boar. In: Proc 6th World Congress on Genetics Applied to Livestock Production 1998

Bratte L., Ibe S.N. Optimal insemination interval, semen dose and dilution for optimum fertility in chickens under humid tropical conditions *Anim Reprod Sci* 1989;20:179-85.

Brun J.M., Theau-Clément M., Bolet G. Evidence for heterosis and maternal effects on rabbit semen characteristics. Anim Res 2002;51:433-42.

Brun J.M., Theau-Clément M., Esparbié J., Falières J., Saleil G., Larzul C. Semen production in two rabbit lines divergently selected for 63d body weight. Theriogenolgy 2006;66:2165-72.

Castellini C., Lattaioli P. Effect of number of motile sperms inseminated on reproductive performance of rabbit does. Anim Reprod Sci 1999;57:111-20.

Castellini C., Lattaioli P., Minelli A., Moroni M. Effect of seminal plasma on the characteristics and fertility of rabbit spermatozoa. Anim Reprod Sci 2000;63:275-82.

Castellini C., Lattaioli P., Cardinali R., Dal Bosco A. Effect of collection rhythm on spermatozoa and droplet concentration of rabbit semen. World Rabbit Sci 2006;14:101-6.

Eisen E.J., Johnson B.H. Correlated responses in male reproductive traits in mice selected for litter size and body weight. Genetics 1981;99:513-24.

Estany J., Camacho J., Baselga M., Blasco A. Selection response of growth rate in rabbits for meat production. Genet Sel Evol 1992;24:527-37.

García M.L., Cifre J., Viudes de Castro M.P. Comparación entre la inseminación artificial y la monta natural en España. Estudio económico teórico. ITEA 1998;94(A): 81-91.

García M.L., Andrés I., Caselles P., Lavara, R. Estudio de la edad de los machos de conejo en la inseminación artificial. Boletín de cunicultura 2004;135:17-25.

García-Tomás M., Sánchez J., Rafel O., Ramón J., Piles M. Heterosis, direct and maternal genetic effects on semen quality traits of rabbits. Livest Sci 2006a;100:111-20.

García-Tomás M., Sánchez J., Rafel O., Ramón J., Piles M. Variability, repeatability and phenotypic relationships of several characteristics of production and semen quality in rabbit. Anim Reprod Sci 2006b;93:88-100.

Garner D.L., Thomas C.A., Gravance C.G., Marshall C.E., De Jarnette J.M., Allen C.H. Seminal plasma addition attenuates the dilution effect in bovine sperm. Theriogenology 2001;56:31-40.

Geweke J. Evaluating the accuracy of sampling-based approaches to the calculation of posterior moments (with discussion). In J. M. Bernardo, J. O. Berger, A. P. Dawid, & A. F. Smith (Eds.). Bayesian statistics 1992;4:169–93.

Haugan T., Gröhn Y.T., Kommisrud E., Ropstad E., Reksen O. Effects of sperm concentration at semen collection and storage period of frozen semen on dairy cow conception. Anim Reprod Sci 2007;97:1-11.

Johnson B.H., Eisen E.J. Effects of selection for postweaning gain on testicular function in mice. Can J Genet Cytol 1975;17: 93-8.

Johnson R.K., Eckardt G.R., Rathje T.A., Drudik D.K. Ten generations of selection for predicted weight of testes in swine: direct response and correlated response in body weight, backfat, age at puberty, and ovulation rate. J Anim Sci 1994;72:1978-88.

Kealey C.G., MacNeil M.D., Tess M.W., Geary T.W., Bellows R.A. Genetic parameter estimates for scrotal circumference and semen characteristics of Line 1 Hereford bulls. J Anim Sci 2006;84:283-90.

Killian G.J., Chapman D.A., Rogowski L.A. Fertility-associated proteins in Holstein bull seminal plasma. Biol Reprod 1993;49:1202-07.

Knights S.A., Baker R.L., Gianola D., Gibb J.B. Estimates of heritabilities and of genetic and phenotypic correlations among growth and reproductive traits in yearling angus bulls. J Anim Sci 1984;58:887-93.

Kohsaka T., Hamano K., Sasada H., Watanabe S., Ogine T., Suzuki E., Nishida S., Takahara H., Sato E. Seminal immunoreactive relaxin in domestic animals and its relationship to sperm motility as a possible index for predicting the fertilizing ability of sires. Int J Androl 2003;26:115-20.

Lavara R., Vicente J.S., Marco-Jiménez F., Baselga M. Correlation among CASA and ASMA parameters in rabbit semen. In: Proc 9th World Rabbit Congress 2008

Legarra A., Varona L., López de Maturana E. 2008. TM: threshold models. http://cat.toulouse.inra.fr/~alegarra.

MARM 2009. Encuesta nacional de cunicultura 2008. Memoria 2009. Secretaría General del Ministerio de Medio Ambiente, Medio Rural y Marino. Secretaría general técnica. http://www.mapa.es/estadistica/pag/cunicultura/2008cuniculturamemoria.pdf

Maxwell W.M.C., Evans G., Mortimer S.T., Gillan L., Gellatly E.S., McPhie C.A. Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. Reprod Fertil Dev 1999;11: 123-6.

Moura A.S.A.M.T., Costa A.R.C., Polastre R. Variance components and response to selection for reproductive litter and growth traits through a multi-purpose index. World Rabbit Sci 2001;9: 77-86.

Oh S.H., See M.T., Long T.E., Galvin J.M. Estimates of genetic correlations between production and semen traits in boar. *Asian-Australasian* J Anim Sci 2006;19:160-4.

Pascual J.J., García C., Martínez E., Mocé E., Vicente J.S. Rearing management of rabbit males selected by high growth rate: the effect of diet and season on semen characteristics. Reprod Nutr Dev 2004;44:49-63.

Pérez-Pe R., Barrios B., Muiño-Blanco T., Cebriá, J.A. Seasonal differences in ram seminal plasma revealed by partition in an aqueous two-phase system. J Chromatogr B Biomed Sci Appl (2001;760:113-21.

Piles M., García M. L., Rafel O., Ramón J., Baselga M. Genetics of litter size in three maternal lines of rabbits: Repeatability versus multiple-trait models. J Anim Sci 2006;84: 2309-15.

Piles M., Tusell LL., García-Tomás M., López-Bejar M., García-Ispierto I., Ramón J., Rafel O., Baselga M. Interacción genotipo x tipo de dosis de inseminación artificial para la fertilidad del macho de conejo. ITEA 2008;104(2):160-8.

Rosell J.M., De la Fuente L.F. Culling and mortality in breeding rabbits. Preventive Veterinary Medicine 2009;88:120-7.

Safaa H.M., Vicente J.S., Lavara R., Viudes de Castro M.P. Semen evaluation of two selected lines of rabbit bucks. World Rabbit Sci 2008;16:141-8.

Smital J., Wolf J., De Sousa L.L. Estimation of genetic parameters of semen characteristics and reproductive traits in AI boars. Anim Repr Sci 2005;86: 119-30.

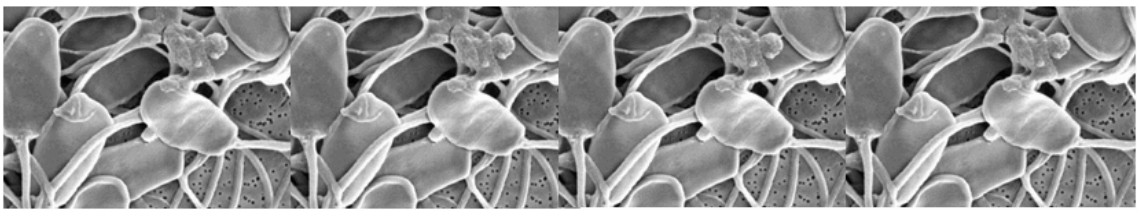
Sorensen D.A., Johansson K. Estimation of direct and correlated responses to selection using univariate animal models. J Anim Sci 1992;*70:*2038-44.

Viudes de Castro M.P., Marco-Jiménez F., Vicente J.S., Navarro E., Lavara R., Mocé E. Sperm kinetic parameters and differences in seminal plasma composition among two rabbit lines. Reprod Domest Anim 2004: poster 276.

Viudes de Castro M.P., Vicente J.S. Effect of sperm count on the fertility and prolificity rates of meat rabbits. Anim Repr Sci 1997;46:313-9.

Zhu J., Zu X., Cosgrove J.R., Foxcroft G.R. Effects of semen plasma from different fractions of individual ejaculates on IVF in pigs. *Theriogenology* 2000;54:1443-5.

CHAPTER TWO



CHAPTER TWO

Estimation of genetic parameters for semen quality traits and growth rate in a paternal rabbit line

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ABSTRACT

Variance components of sperm quality traits were estimated in a paternal line of rabbits selected for the basis of daily weight gain (DG, g/day) between 28 and 63 days of age. Features of the marginal posterior distributions for the ratios of genetic variance, variance due to non-additive plus environmental permanent male effects, and variance due to litter of birth effects with respect to phenotypic variance are reported. The correlation between sperm quality traits and the selection criteria were also estimated. Nine sets of two-trait analyses were performed involving 12908 records of DG, 2231 ejaculates corresponding to 412 males, and 14700 animals in the pedrigree file. Heritabilities (h^2) of quality semen traits commonly evaluated in a classic spermiogram were 0.18, 0.19 and 0.12 for NAR (%, percentage of sperm with intact acrosome), ANR (%, percentage of sperm abnormalities) and MOT (%, percentage of total motile sperm cells) respectively. We also estimate the h^2 of some motion CASA parameters 0.09, 0.11, 0.10, 0.11, 0.11 and 0.11 for VAP (µm/s; average path velocity), VSL (µm/s; straight-line velocity), VCL (µm/s; curvilinear velocity), LIN (%, linearity index), ALH (µm; amplitude of the lateral head displacement), STR (%, straightness). The permanent environmental effects were lower than the corresponding values of h^2 and varied between 0.04 and 0.14. Genetic correlations between DG and semen traits showed a high $HPD_{95\%}$ (interval of highest density of 95%). However there is some consistent evidence of the negativity of the genetic correlations of DG with NAR and MOT (-0.40 and -0.53, respectively). Permanent correlations were low, including the zero in the $HPD_{95\%}$. Litter birth correlations between DG with LIN and STR showed that a favorable effect for growth could be detrimental for them (-0.47 and -0.53).

Therefore, because the magnitude of the genetic correlations do not seem very high it could be possible to define an index of selection including some quality sperm traits that allows to continuing the improvement of DG without diminishing the semen quality.

INTRODUCTION

Intensive meat rabbit production is based on the use of crossbred female, taken from the cross of two maternal lines selected for reproductive traits. These does should be mated to males from a paternal line that is selected only for growth traits. This three-way crossbreeding scheme aims to produce a large number of young rabbits with fast growth and high feed efficiency (Baselga, 2004).

Approximately 80% of the commercial Spanish rabbit production uses artificial insemination (AI) (MARM, 2009). This change in the reproductive management (AI instead of natural mating) is due to the increase in the number of does per farm. Managing high numbers of does calls for the synchronization of large numbers of does in the same physiological status. Consequently, AI stations have appeared in recent decades to satisfy demand for semen doses from paternal rabbit lines. The economic efficiency of these stations depends on the number of fertile seminal doses produced by each male. As in pigs, basic semen traits that affect AI center profitability are volume, sperm concentration and sperm quality (Robinson and Buhr, 2005). The increase of the number of AI doses could be achieved in two different ways; the first is by optimizing the protocols for preparation, conservation and insemination procedure, and the second way is by increasing the quantity and/or quality of sperm produced by each male.

To achieve normal fertility and prolificacy outcomes from the inseminated rabbit sperm doses, apart from the right number of sperm in the insemination doses and the absence of contaminants or residues in the ejaculate as urine, blood and some other residues, the ejaculates used for preparing the seminal

doses need to show a minimum score for quality traits, such as total motile sperm (more than 70%), abnormal sperm (less than 20%) and normal acrosome status (more than 80% of non reacted acrosome). If the ejaculate does not show the minimum score it is discarded (Foxcroft *et al*., 2008). Currently the ejaculate discarded rate in paternal rabbit lines can be as high as 40%, which implies a need to increase the ejaculate quality in these rabbit lines.

It must be taken into account, however, that seminal traits are influenced by genetic, management and environmental factors, as reported in several species (Everett and Bean, 1986; Mathevon *et al*., 1998; Brun *et al*., 2006; David *et al*., 2007). In fact, to optimize management and selection for both growth and sperm dose production, genetic parameters need to be estimated between all the involved traits for efficiently selection of the males to test the possibility to combine the selection for both objectives, i.e., growth and male sperm output. Genetic parameters for quantity sperm traits (such as volume and sperm concentration per ejaculate) in rabbits were previously reported by different authors (García-Tomás *et al*., 2006a; Brun *et al*., 2002; Lavara *et al*., 2011), but no estimation about heritability of quality sperm traits as abnormal forms or acrosome status have been reported.

The objectives of this study were to estimate, in a paternal rabbit line, genetic parameters for sperm quality traits (motility, movement characteristics, acrosome status and presence of abnormal sperms in the ejaculate) and their genetic correlations with the selection objective of the line (daily gain between 28 and 63 days of age).

MATERIALS AND METHODS

Animals and data

Animals of a paternal rabbit line (Line R) were used in the experiment. Line R was selected for daily weight gain (DG) between 28 and 63 days of age (Estany *et al*., 1992) by individual selection.

After weaning, animals were housed in collective cages (8 rabbits per cage) at temperatures ranging from 15 to 25ºC. At age of 63 days, males were moved to three AI stations. Bucks were placed in individual cages subjected to a photopheriod of 16h light/day and fed *ad libitum* with a similar commercial rabbit diet (on dry matter basis: 17.5% crude protein, 3.5% ether extract, 16.7% crude fiber, 2938 kcal/kg). In all stations, environmental conditions were controlled in an attempt to maintain the temperature between 17 and 24ºC.

Males started the training period at 150-170 days of age. Training was carried out for 2 wks, whereupon the bucks entered the production period. For the training and production period, each week, two ejaculates per male were collected on a single day using an artificial vagina, with a minimum of 30 min between collections. Collections from each male during the experiment were performed on the same day of the week.

Semen samples for the experiment were collected during two different periods:

- Period A: 1 week after finishing the training period.

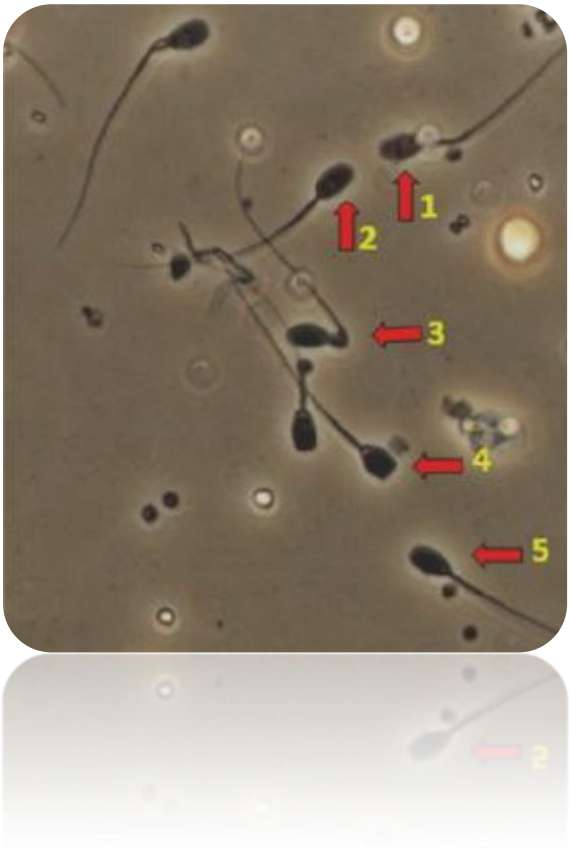
- Period B: more than 3 months after Period A.

Only ejaculates that exhibited a white color were used in the experiment; samples containing urine and cell debris were discarded, whereas gel plugs were removed. Aliquots from each ejaculate (10 µL) were diluted 1:20 in an extender (Tris-citrate acid-glucose) containing bovine serum albumin 0.3% (BSA) to prevent the spermatozoa from sticking to the glassware during motility analysis. 10µL of the diluted sample was placed into a 10 µm deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) for motility analysis using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, S.C.A., Microptic, Barcelona, Spain). Sperm motility was assessed at 37ºC with 10X negative phase contrast objective. For each sample four microscopic fields were analyzed. The CASA system was used to evaluate simultaneously, in addition to the percentage of total motile sperm cells (sperm motility (MOT), %), different sperm motility parameters. All semen samples were recorded at the same frame rate (30 Hz) for 1 second. The following six parameters were measured by the system: average path velocity (VAP, µm/s; the average velocity of the smoothed cell path), curvilinear velocity (VCL, µm/s; the average velocity calculated over the actual point to point track followed by the cell), straight-line velocity (VSL, µm/s; the average velocity calculated in a straight line from the beginning to the end of the track), linearity index (LIN, %; the average value of the ratio VSL/VCL), amplitude of lateral head displacement (ALH, µm; the mean width of the head oscillation as the sperm cells swim), and the straightness (STR, %; the average value of the ratio VSL/VAP).

For morphologic analyses, an aliquot from each ejaculate (20µL) was fixed with 180 µL of a solution of glutaraldehyde 0.2% (Electron Microscopy Science, Washington) in Dubbelco's Phosphate Buffered Saline (DPBS). A minimum of

100 spermatozoa were evaluated at a magnification of 400X by positive phase contrast microscopy. Sperm abnormalities (AS) and acrosome status of normal sperm [intact (AI) or reacted (AD)] were evaluated (**Figure 2.1**). Sperm with morphologic defects in head, tail or neck-midpiece was classified as abnormal (AS). The percentage of abnormal sperm (ANR) was calculated as the ratio: [AS/(AI + AD + AS)] x 100. The percentage of sperm with normal acrosome status (NAR) was calculated as the ratio: [AI/(AI + AD)] x 100. Sperm concentration was determined using a Thoma-Zeiss counting cell chamber (Marienfield, Germany).

Figure 2.1: Sperm morphology.



Abnormal sperm (AS): 3,4. Acrosome status of normal sperm: intact (AI): 5,2; reacted (AD): 1.

To reduce bias in the estimation of the genetic parameters of semen traits resulting from the selection for DG, each of the semen traits was analyzed jointly with DG (Sorensen and Johansson, 1992). A set of two-trait analyses was thus performed.

A total of 12908 records for DG were used in the experiment. DG data used belonged to animals from twelve generation before. In addition to DG, the semen traits (percentage of sperm motility (MOT, %), percentage of sperm with normal acrosome status (NAR, %), percentage of abnormal sperm (ANR, %) and CASA traits were recorded, involving 2231 ejaculates from 412 males. The pedigree file included 14700 animals.

Estimation of the genetic correlation between the sperm traits was not attempted, because the number of males with semen records was deemed not enough to obtain accurate estimates of those correlations.

Statistical analyses

The mixed model used for the spem traits was:

$$y_{sijokl} = \mu_s + S_{si} + O_{sj} + P_{so} + a_{sk} + p_{sk} + c_{sl} + e_{sijokl}$$

where y_{sijokl} is a record of the semen trait, μ_s is the overall mean, S_{si} is the systematic effect station–year–season in which the ejaculate was collected, with 64 levels, O_{sj} is the systematic effect of ejaculate order with two levels (first and second ejaculate on the same day), P_{so} is the systematic effect of age of the male with 3 levels (≤ 6 months, 6–8 months, more than 8 months), a_{sk} is the animal additive genetic effect, p_{sk} is the permanent environmental effect over all the ejaculates of the male *k*, c_{sl} is the random effect of the litter in which the

male *k* was born, and e_{sijokl} is the residual. It was assumed that the different random effects (additive, permanent, litter of birth and residual) followed normal distributions and were independent among and within the effects, except for the additive values of the animals, which were correlated though the numerator relationship matrix.

The mixed model used for DG was:

$$y_{dijkl} = \mu_d + b*LS_{dl} + YS_{di} + OP_{dj} + a_{dk} + p_{dk} + c_{dl} + e_{dijkl}$$

where y_{dijkl} is the daily gain of animal *k*, μ_d is the overall mean, LS_{dl} is the covariate litter size at birth and b the corresponding regression coefficient, YS_{di} is the systematic effect of year–season in which the animal was weaned, with 30 levels, OP_{dj} is the systematic effect of parity order in which the animal was born, with three levels (first, second, and higher), a_{dk} is the animal additive genetic effect, c_{dl} is the random effect of the litter in which the animal *k* was born; the residual of the model was split into two components: p_{dk}, which corresponds to the part of the residual correlated with the permanent environmental effect for semen traits and e_{dijkl} that corresponds to the part of the residual uncorrelated with any other random effect, within and among traits.

The assumptions for the random effects for DG are the same as those indicated above for the sperm traits.

Further assumptions, concerning correlations between random effects of DG (a_d, p_d, c_d, e_d) and random effects of one semen trait (a_s, p_s, c_s, e_s), are summarized in the following matrices:

$$\mathbf{G}=\begin{bmatrix}\sigma^2_{a_d} & \sigma_{a_d,a_s}\\ \sigma_{a_s,a_d} & \sigma^2_{a_s}\end{bmatrix};$$

$$\mathbf{P}=\begin{bmatrix}\sigma^2_{p_d} & \sigma_{p_d,p_s}\\ \sigma_{p_s,p_d} & \sigma^2_{p_s}\end{bmatrix};$$

$$\mathbf{C}=\begin{bmatrix}\sigma^2_{c_d} & \sigma_{c_d,c_s}\\ \sigma_{c_s,c_d} & \sigma^2_{c_s}\end{bmatrix};$$

$$\mathbf{R}=\begin{bmatrix}\sigma^2_{e_d} & 0\\ 0 & \sigma^2_{e_s}\end{bmatrix}$$

where the components of **G**, **P**, **C** and **R** are the additive, permanent, birth litter and residual variances for the daily gain and the sperm trait in the diagonal, and the corresponding covariances between both traits, out of the diagonal.

Variance–covariance components were estimated using a Bayesian approach implemented in the TM program developed by Legarra *et al*. (2008). Flat priors were used for systematic effects and variance components.

The following prior distributions for random effects were assumed:

$$p\left(\begin{bmatrix}a_d\\ a_s\end{bmatrix}\middle|\mathbf{G}\right)\sim N(\mathbf{0},\mathbf{A}\otimes\mathbf{G}),\ p\left(\begin{bmatrix}p_d\\ p_s\end{bmatrix}\middle|\mathbf{P}\right)\sim N(\mathbf{0},\mathbf{I}\otimes\mathbf{P}),\ p\left(\begin{bmatrix}c_d\\ c_s\end{bmatrix}\middle|\mathbf{C}\right)\sim N(\mathbf{0},\mathbf{I}\otimes\mathbf{C})$$

Where **A** is the numeratior relationship matrix, **0** is a vector of zeroes, **I** is an identity matrix, and **G**, **P** and **C** are the (co)variance matrices summarized above.

After some exploratory analysis, chains of 1000000 samples were used, with a burning period of 100000. Only one sample of each 100 was saved. The

convergence was checked on each chain by the Z Geweke criterion (Geweke, 1992).

RESULTS

The number of records, means and standard deviations of the traits studied are presented in **Table 2.1**. Sperm traits were highly variable. All traits had a large coefficient of variation, with the highest being 78% for ANR and 42% for MOT.

Features of the estimated marginal posterior distributions (PM: posterior mean. $HPD_{95\%}$: interval of highest density of 95%) of heritabilities (h^2), and ratios to the phenotypic variance of the permanent (p^2), and birth litter variances (c^2) are presented in **Table 2.2**. Similar information that relates to the genetic (r_g), permanent (r_p) and birth litter (r_c) correlations is shown in **Table 2.3**

The heritability of DG had a PM of 0.18 and $HPD_{95\%}$ of [0.12; 0.23]. This $HPD_{95\%}$ is narrower than the ones showed in **Table 2.2** for the semen traits because the very high number of records for DG.

The highest heritability of sperm traits was for the percentage of sperm abnormalities in the ejaculate [PM: 0.19, PSD (posterior standard deviation): 0.05]. The posterior means of p^2 were lower than the corresponding values of h^2 and varied between 0.04 and 0.10.

Litter of birth effect is an environmental effect that includes the environment provided by the mother on the development of her offspring during the uterine and lactation period and also the specific environment associated with each litter. This effect is common to all young rabbits born in the same litter. The c^2 estimates were, in general lower than the corresponding values of p^2 and

ranged between 0.03 and 0.08, but in the case of sperm velocity traits were higher than p^2 estimates.

Table 2.1: Crude mean and standard deviation for semen traits and daily weight gain

	n	Mean	Standard deviation
DG (g/d)	12908	46.2	6.9
NAR (%)	2225	87.6	15.1
ANR (%)	2225	17.0	13.2
MOT (%)	2231	65.0	27.4
VAP(µm/s)	2083	58.1	16.1
VSL (µm/s)	2083	48.5	16.1
VCL (µm/s)	2083	84.3	19.6
LIN (%)	2083	59.5	15.8
ALH (µm)	2083	3.2	1.1
STR (%)	2083	80.7	11.2

n, number of data; DG, daily gain between days 28 and 63; NAR, normal acrosome status; ANR, sperm abnormalities; MOT, sperm motility; VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; LIN, linearity index; ALH, amplitude of lateral head displacement; STR, straightness.

Table 2.2: Descriptive statistics of the posterior marginal distributions of heritability (h^2), ratio of permanent variance to phenotypic variance (p^2) and ratio of litter of birth variance to phenotypic variance (c^2), for semen traits

	h^2			p^2			c^2		
	PM	**$HPD_{95\%}$**	**$Min_{95\%}$**	**PM**	**$HPD_{95\%}$**	**$Min_{95\%}$**	**PM**	**$HPD_{95\%}$**	**$Min_{95\%}$**
NAR	0.18	[0.09 0.27]	0.10	0.10	[0.02 0.18]	0.04	0.05	[0.01 0.10]	0.01
ANR	0.19	[0.08 0.29]	0.10	0.14	[0.05 0.23]	0.07	0.08	[0.01 0.15]	0.02
MOT	0.12	[0.04 0.20]	0.05	0.10	[0.03 0.17]	0.04	0.05	[0.01 0.11]	0.01
VAP	0.09	[0.01 0.17]	0.03	0.04	[0.01 0.09]	0.01	0.08	[0.01 0.14]	0.03
VSL	0.11	[0.02 0.20]	0.03	0.06	[0.01 0.11]	0.01	0.07	[0.01 0.13]	0.02
VCL	0.10	[0.02 0.18]	0.04	0.04	[0.01 0.08]	0.01	0.07	[0.01 0.13]	0.02
LIN	0.11	[0.03 0.20]	0.04	0.08	[0.01 0.15]	0.02	0.05	[0.01 0.11]	0.02
ALH	0.11	[0.03 0.19]	0.04	0.11	[0.03 0.18]	0.05	0.03	[0.01 0.08]	0.01
STR	0.11	[0.03 0.19]	0.04	0.07	[0.01 0.13]	0.02	0.03	[0.01 0.07]	0.01

PM, posterior mean; $HPD_{95\%}$, interval of highest density of 95%, $Min_{95\%}$, value for which the probability of higher value is 95%; NAR, normal acrosome status; ANR, sperm abnormalities; MOT, sperm motility; VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; LIN, linearity index; ALH, amplitude of lateral head displacement; STR,

Table 2.3: Descriptive statistics of the posterior marginal distributions of the genetic (r_g), permanent (rp) and litter of birth (r_c) correlations of semen traits with daily gain (DG)

	r_c		r_p		r_c	
	PM	**HPD95%**	**PM**	**HPD95%**	**PM**	**HPD95%**
NAR-DG	-0.40	[-0.78 -0.02]	0.12	[-0.53 0.94]	-0.02	[-0.62 0.61]
ANR-DG	0.25	[-0.18 0.66]	-0.06	[-0.77 0.54]	-0.19	[-0.69 0.33]
MOT-DG	-0.53	[-0.95 0.02]	-0.34	[-0.88 0.22]	-0.06	[-0.85 0.70]
VAP-DG	0.03	[-0.55 0.57]	-0.12	[-0.96 0.62]	-0.02	[-0.44 0.38]
VSL-DG	0.01	[-0.53 0.56]	-0.13	[-0.93 0.59]	-0.13	[-0.58 0.35]
VCL-DG	-0.11	[-0.61 0.41]	-0.33	[-0.98 0.38]	0.17	[-0.30 0.63]
LIN-DG	-0.02	[-0.56 0.50]	0.14	[-0.51 0.85]	-0.47	[-0.99 -0.02]
ALH-DG	-0.02	[-0.49 0.48]	-0.25	[-0.83 0.25]	0.36	[-0.24 0.99]
STR-DG	-0.14	[-0.68 0.38]	-0.20	[-0.94 0.41]	-0.53	[-0.99 0.01]

PM, posterior mean; $HPD_{95\%}$, interval of highest density of 95%; DG, daily weight gain; NAR, normal acrosome status; ANR, sperm abnormalities; MOT, sperm motility; VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; LIN, linearity index; ALH, amplitude of lateral head displacement; STR, straightness.

Concerning correlations, the first question that arises is the very high $HPD_{95\%}$ showing that a very high number of males are needed for their accurate estimation due to the low values of the corresponding ratios of variance (h^2, p^2 and c^2). However there is some consistent evidence of the negativity of the genetic correlations of DG with NAR and MOT and, also, of the litter birth correlations between DG with LIN and STR.

DISCUSSION

Artificial insemination has been widely used in rabbit production over the last 15 years. Commercial sperm doses currently come from males from paternal lines, which are commonly selected for growth traits, such as DG and feed efficiency. In AI stations, the relative importance of the males is determined not only by the growth performance they are able to transmit to their progeny, but also by the number of useful sperm doses that they can produce for use on commercial farms, with a quality that ensures the fertility and prolificacy of the females will not be reduced.

The sperm traits considered in this work are involved in sperm quality (motility, motion characteristics, sperm abnormalities and acrosome status). The literature on estimation of genetic parameters for sperm quality traits in rabbits is scarce. Brun *et al*. (2009) reported heritabilities from motility and some motion characteristics. To our knowledge, no existing research addresses the heritability for sperm abnormalities and acrosome status in rabbits, as only estimates of repeatability have been reported by some authors (Brun *et al*., 2002; García-Tomás *et al*., 2006a).

Sperm motility and CASA motion parameters

Sperm motility is essential to achieve normal fertility and prolificacy after AI. Although CASA systems in recent decades have improved the accuracy and repeatability records for sperm motility (Gravance and Davis, 1995; Holt and Palomo, 1996; Verstegen *et al*., 2002), correlations between the percentages of motile sperm in one semen sample and fertility are still not consistent among different studies (Holt, 1996). Flowers (1997) concluded that motility could give a quantitative estimation about fertility in sows when the ejaculates had a low motility score (less than 60%). The same pattern was found by Tardif *et al.* (1999), when the AI was performed with a limited number of spermatozoa, the motility could be related with the fertility at birth. Motility is a compensable trait, and the concomitant problems could be solved by increasing the number of spermatozoa in the AI dose.

It must be taken into account that sperm fertility evaluation depends on several factor (analysis strength, sperm AI technique used, type of fertility recorded), including the fact that the ejaculate is composed of different sperm population. Spermatozoa heterogeneity is reflected not only in differences in motility or morphology, but also in the capacity to remain fertile over time, to sustain sperm selection and transport in the female tract, all of which account for variation in the fertilizing ability among doses, ejaculates and males (Rodríguez-Martínez, 2007).

The sperm motility and movement characteristics obtained in the present study were similar to those reported in rabbit without ejaculate selection (Lavara *et al*., 2008; Safaa *et al*., 2008). However, results were different from those in which

a previous selection of the ejaculates took place before AI dose preparation (Lavara *et al*., 2005; Quintero-Moreno *et al*., 2007).

The heritability value estimated for motility was of the same magnitude as DG. If the repeatability is computed as the sum of h^2, p^2 and c^2, the value obtained would be 0.27. This value is similar to the repeatability values estimated by Brun *et al*. (2002 and 2009); García-Tomás *et al*. (2006a) and Tusell *et al*. (2010).

Visual estimation of the percentage of motile sperm suffers from human bias. This human bias can be curtailed by evaluating sperm motion using a CASA system. The motility evaluation at present work was measured with the aid of a CASA system, which allowed the motility to be gauged in a continuous scale (0-100%) instead of a discrete scale (from 0 to 5). Motility estimates reported in rabbits by Brun *et al*. (2009) and Tusell *et al*. (2010) were lower than those obtained in this study. The possible reason for this difference could be due to the subjective manner in which this trait was measured, since it is dependent on technician skills. Alternatively, Brun *et al*. (2009) reported estimates of heritability for individual motility similar than those obtained at present work, corroborating this hypothesis.

The heritability values estimated for motility in different species are usually based on data from subjective evaluation. The literature shows a huge range of estimates from 0.07 to 0.72, as these values depend on species and population studied. In pig, Smital *et al*. (2005) demonstrated that a possible reason for differences in sperm trait heritability estimations could be the age of the males, so it can be assumed that in young males the expression of the sperm traits is not stabilized yet. Alternatively, in ram, David *et al*. (2007) studied the motility at young and adult age as two different traits, obtaining different estimations. This

last point could be a determining factor to our heritability estimations, as the ejaculates used in this work were from males at the onset of sexual maturity and adult males. Future estimations could be due in both types, young and adult males.

Heritability and repeatability estimations for motion CASA traits were similar as for motility (between 0.09 and 0.11 for heritability and between 0.21 and 0.25 for repeatability). Very little information was available from the literature on the motion genetic parameters, in rabbits only Brun *et al*. (2009) reported similar information about VAP and LIN. However, there are discrepancies between different studies over the biological meaning of some CASA traits and their relationship with fertility (reviewed by Verstegen *et al*., 2002). Many studies have shown that motility integrates the biochemical events occurring in the spermatozoa. The sperm samples are heterogeneous because spermatozoa with different motility patterns coexist in the same ejaculate (Katz and Davis, 1987), and the analysis of the different motility patterns associated with different sperm subpopulations may help assess the status of the sperm sample and its fertility potential instead of using the raw datasets (Davis *et al*., 1995). So, in the future, as the relationship between sperm subpopulation and fertility was found previously by Quintero-Moreno *et al*. (2007), genetic parameters of sperm subpopulations should be studied in detail.

Sperm abnormalities and acrosome status

In rabbits, sperm fertility must be demonstrated in terms of fertility rate and prolificacy of does. It is well known that there are many factors which have an impact on results obtained after AI. Some of the factors that influence the results are the sperm abnormalities and acrosome status. Several studies in

different species corroborate the relationship between abnormal sperm rate and fertility reduction (Xu *et al*., 1998; Farrell *et al*., 1993; Lavara *et al*., 2005). Nevertheless, fertility of ejaculates with abnormal sperm rate is compensated by an increased number of normal sperm in the doses (Braundmeier and Miller, 2001) but this is not a good practice. In addition, if we could obtain ejaculates with less abnormal spermatozoa, we could increase the number of AI doses produced by each male.

Unfortunately, an increase in acrosome-reacted sperm rate in an ejaculate might not be compensated by increasing the number of sperm with an intact acrosome. The inclusion of high numbers of acrosome-reacted spermatozoa in a seminal dose, reduce the conservation time and compromise the future results of the AI doses.

The mean values for ANR and NAR obtained in the present study were similar to those observed in previous studies with the same rabbit line (Lavara *et al*., 2008; Safaa *et al*., 2008; García-Tomás *et al*., 2006b).

The heritability estimates for ANR and NAR presented in this study are medium (0.19 and 0.18). To our knowledge there is no information in the literature concerning heritability estimates for these traits, and only a few authors have reported repeatability estimates for sperm traits in rabbits (Bencheikh, 1995; García-Tomás *et al*., 2006a). Repeatability estimates for ANR and NAR in these papers agree with our results (0.40 and 0.33) indicating that an important part of the phenotypic variance was due to male-related sources of variation.

Correlations between DG and semen traits

It is important to have good knowledge about the genetic correlations between the criteria of selection and sperm traits to prevent the possible effect of selection on semen quality and finally on sperm doses fertility.

We know that sperm traits, such as motility and acrosome status strongly depend on the collection and manage conditions before and during sperm assessment. Therefore, we tried to include all important factors with an effect on semen traits in the models for the (co) variance estimation. A further problem in parameter estimation is that we have enough data for accurate estimates of h^2 for DG and for less accurate but acceptable estimates of h^2 for the sperm traits. The problem increases with the estimates of the correlations between DG and the sperm traits that have very wide $HPD_{95\%}$, despite the high number of records of DG. These intervals should be higher for the estimates of the correlations between sperm traits and therefore we have discarded their estimation. In future, large data sets should be used to achieve more accurate results, but it is necessary bear in mind the extreme difficulty and expense of obtaining these data in rabbits, where AI stations are small and sometimes the relationship between the males is lacking.

The genetic correlation of DG with MOT was medium and negative and showed a wide $HPD_{95\%}$. In another paternal rabbit line also selected for growth rate, the estimated r_g between MOT and DG was low (Tusell *et al*., 2010). However in that work, motility was measured subjectively, so it could not be considered as the same trait.

The value and sign of the correlation found at present study were similar to those previously found in pigs by Smith *et al*. (1989). However, as in other previous research, genetic correlations in which seminal traits are involved showed high standard errors due to the low heritability estimates of those traits and the relatively small number of records. Thus the lower limit of the corresponding $HPD_{95\%}$ is always negative and relatively far from zero and the upper limit is always positive and far from zero, excepted for NAR and MOT that are very close to zero, negative for NAR and positive for MOT. Consequently, findings of no effects or small effects of the selection for DG on motion traits cannot be discarded.

The permanent correlations between DG and the sperm traits were very low, including the zero in the $HPD_{95\%}$ and the limits of these intervals were far from zero, an indication of the low information of the data related to these correlations. Consequently, conclusions cannot be drawn for them. For birth litter correlations between DG and the sperm traits the trend was the same, including zero in the $HPD_{95\%}$ except for LIN. For the last trait, the PM was -0.47, suggesting that a favorable effect for growth could be detrimental to the linearity of spermatozoa trajectory. However, it necessary to know more about this trait to better understand the nature of this relationship, as there are discrepancies between different studies about the biological meaning of this CASA trait (reviewed by Martínez-Pastor *et al*., 2010).

CONCLUSIONS

Paternal lines are selected mainly on the basis of the individual records for growth traits, whereby no information on the breeding values for sperm traits is taken into account. Selection on sperm quality traits should be effective given

the magnitude of heritability estimates in the present study and the high coefficient of variation of the traits. Nevertheless, it must be taken into account that a rigorous study of the economic values of these sperm traits should be done previously to determine their economic weights. AS the magnitude fo the genetic correlations does not seem very high, it may be possible to define a selection index, including some sperm quality traits (as motility and acrosome status) that allows ongoing the improvement in daily gain without diminishing the sperm quality.

REFERENCES

Baselga M. Genetic improvement of meat rabbits. Programmes and diffusion. In: Proc. 8th Word Rabbit Congress, Puebla, Mexico 2004;1:1-13.

Bencheikh N. Effet de la fréquence de collecte de la semence sur les caractéristiques du sperme et des spermatozoïdes récoltés chez le lapin. *Ann Zootech* 1995;44:263-79.

Braundmeier A.G., Miller D.J. The search is on: finding accurate molecular markers of male fertility. J Dairy Sci 2001;84:1915-25.

Brun J.M., Theau-Clément M., Bolet G. Evidence for heterosis and maternal effects on rabbit semen characteristics. Anim Res 2002;51:433-42.

Brun J.M., Theau-Clément M., Esparbié J., Faliéres J., Saleil G., Larzul C. Semen production in two rabbit lines divergently selected for 63d body weight. Theriogenology 2006;66:2165-72.

Brun J.M., Sanchez A., Duzert R., Saleil G., Theau-Clément M. Paramètres génètiques des caractéristiques de la semence de lapin. In: *13èmes* Journ Rech Cunicole 2009;11:17-8.

David I., Druart X., Lagriffoul G., Manfredi E., Robert-Granié C., Bodin L. Genetic and environmental effects on semen traits in Lacaune and Manech tête rousse Al rams. Genet Sel Evol 2007;39:405-19.

Davis R., Drobnis E., Overstreet J. Application of multivariate cluster, discriminate function, and stepwise regression analyses to variable selection and predictive modeling of sperm cryosurvival. Fertil Steril 1995;63:1051-7.

Estany J., Camacho J., Baselga M., Blasco A. Selection response of growth rate in rabbits for meat production. Genet Sel Evol 1992;24:527-37.

Everett R.W., Bean B. Semen fertility: An evaluation system for artificial insemination sires, technicians, herdsm and systematic fixed effects. J Dairy Sci 1986;69:1630-41.

Farrell P.B., Foote R.H., Simkin M.E., Clegg E.D., Wall R.J. Relationship of semen quality, number of sperm inseminated, and fertility in rabbits. J Androl 1993;14:464-71.

Flowers W.L. Management of boars for efficient semen production. J Reprod Fertil 1997;52:67-8.

Foxcroft G.R., Dyck M.K., Ruiz-Sanchez A., Novak S., Dixon W.T. Identifying useable semen. Theriogenology 2008;70:1324-36.

García-Tomás M., Sanchez J., Rafel O., Ramon J., Piles M. Heterosis, direct and maternal genetic effects on semen quality traits of rabbits. Livest Sci 2006a;100:111-20.

García-Tomás M., Sanchez J., Rafel O., Ramón J., Piles M. Variability, repeatability and phenotypic relationships of several characteristics of production and semen quality in rabbit. Anim Reprod Sci 2006b;93:88-100.

Geweke J. Evaluating the accuracy of sampling-based approaches to the calculation of posterior moments (with discussion). In: Bernardo JM., Berger J O., Dawid AP., & Smith AF. (Eds.). Bayesian statistics 1992; 4:169–93.

Gravance C.G., Davis R.O. Automated sperm morphometry analysis (ASMA) in the rabbit. J Androl 1995;16:88-93.

Holt W.V., Palomo M.J. Optimization of a continuous real-time computerized semen analysis system for ram sperm motility assessment and evaluation of four methods of semen preparation. Reprod Fertil Dev 1996;8:219-30.

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

Katz D.F., Davis R.O. Automatic analysis of human sperm motion. J Androl, 1987;8:170-81.

Lavara R., Mocé E., Lavara F., Viudes de Castro M.P., Vicente J.S. Do parameters of seminal quality correlate with the results of on-farm inseminations in rabbits? Theriogenology 2005;64:1130–41.

Lavara R., Vicente J.S., Marco-Jiménez F., Baselga M. Correlation among CASA and ASMA parameters in rabbit semen. In: Proc. 9th World Rabbit Congress 2008; 381-6.

Lavara R., Vicente J.S., Baselga M. Genetic parameter estimates for semen production traits and growth rate of a paternal rabbit line. J Anim Breed Genet 2011;12:44-51.

Legarra A, Varona L, López de Maturana E. (2008) TM: threshold models. http://cat.toulouse.inra.fr/~alegarra.

MARM (2009). Encuesta nacional de cunicultura 2008. Memoria 2009. Secretaría General del Ministerio de Medio Ambiente, Medio Rural y Marino. Secretaría general técnica.http://www.mapa.es/estadistica/pag/cunicultura/2008_cunicultura_memoria.pdf

Martínez-Pastor F., Tizado E.J., Garde J.J., Anel L., de Paz P.. Statistical series: Opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 2010;75:783-95.

Mathevon M., Buhr M.M., Dekkers J.C.M. Environmental, management, and genetic factors affecting semen production in Holstein bulls. J Dairy Sci 1998;81:3321-30.

Quintero-Moreno A., Rigau T., Rodríguez-Gil J.E. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. Reprod Domest Anim 2007;42:312-9.

Rodríguez-Martínez H. State of the art in farm animal sperm evaluation. Reprod Fertil Dev 2007; 19: 91-101.

Robinson J.A.B., Buhr M.M. Impact of genetic selection on management of boar replacement. Theriogenology 2005;63:668-78.

Safaa H.M., Vicente J.S., Lavara R., Viudes de Castro M.P. Semen evaluation of two selected lines of rabbit bucks. World Rabbit Sci 2008;16:141-8.

Smital J., Wolf J., De Sousa L.L. Estimation of genetic parameters of semen characteristics and reproductive traits in AI boars. Anim Repr Sci 2005;86:119-30.

Smith B.A., Brinks J.S., Richardson G.V. Estimation of genetic parameters among breeding soundness examination components and growth traits in yearing bulls. J Anim Sci 1989;67:2892-6.

Sorensen D.A., Johansson K. Estimation of direct and correlated responses to selection using univariate animal models. J Anim Sci 1992;70,2038-44.

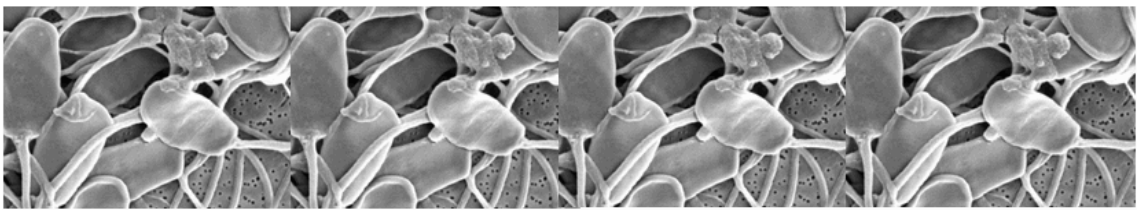
Tardif S., Laforest J.P., Cormier N., Bailey J.L. The importance of porcine sperm parameters on fertility in vivo. Therigenology 1999;52:447-59.

Tusell LL., Garcia-Tomas M., Rafel O., Ramon J., Piles M. Genetics of semen production and its relationship with growth rate. In: XV Reunión Nacional de Mejora Genética Animal. Vigo Junio 2010.

Verstegen J., Iguer-Ouda M., Onclin K. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 2002;57:149–79.

Xu X., Pommier S., Arbov T., Hutchings B., Sotto W., Foxcroft G.R. In vitro maturation and fertilization techniques for assessment of semen quality and boar fertility. J Anim Sci 1998;76:3079-89.

CHAPTER THREE



CHAPTER THREE

Genetic variation in head morphometry of rabbit sperm

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ABSTRACT

The aim of this study was to determine the repeatability and heritability of sperm head characteristics: width (W, µm), area (A, µm2),length (L, µm) and perimeter (P, µm), and explore the relationships between them and with the selection objective in a rabbit paternal line selected for daily weight gain (DG, g/day) between 28 and 63 days of age.

Six sets of three-trait analyses were performed involving 12908 DG records, with 1795 ejaculates corresponding to 283 males and 14700 animals in pedigree file. The results obtained showed that sperm head dimensions are heritable (ranged between 0.2 and 0.29). The genetic correlations between sperm traits were always high and positive (between 0.72 and 0.90), with the exception of L-W genetic correlation, which was moderate. Regarding the genetic correlations between DG and sperm head characteristics, the resulting means ranged from -0.09 for L-DG to -0.43 for W-DG, showing a consistent evidence of the negativity of the genetic correlations. Further studies should be focused on the relationship between sperm head morphometry and different semen or ejaculate characteristics and also between sperm head morphometry and field fertility and prolificacy.

INTRODUCTION

One of the principal functions of sperm structure is to protect and transport the paternal haploid genotype to the oocyte. Genetic information is located inside the sperm head (Ward and Coffey, 1991) and comprises around 65% of the total sperm head volume (Barth and Oko, 1989). Previous reports indicate that a reduction in sperm head surface area is related to decreased fertility (Barth *et al.*, 1992).

Traditional methods for evaluating sperm morphology adopt a subjective approach, classifying the sperm as normal or abnormal depending on its morphological appearance. However, when employing this methodology only sperm exhibiting clearly smaller or larger than average heads may be classified as abnormal. Morphometry analysis is thus more convenient, as it reveals smaller differences in size and shape than routine evaluation (Graham, 1996). In this sense, morphometry analysis has been used to describe the morphology of ejaculated sperm from different domestic species including rabbit (Gravance and Davis, 1995; Marco-Jiménez *et al.*, 2005 and 2010) and is recommended as part of the spermiogram for domestic animals (Rodríguez-Martínez, 2007).

Early studies on sperm head dimensions observed that bull fertility was correlated with the uniformity of sperm head length, indicating that the high coefficient of variation was related to low fertility in bulls (Williams and Savage (1927) cited by Barth and Oko (1989)). Further research applied multiple regression methodology intended to predict the fertilising capacity of seminal doses in different species with contradictory results (Casey *et al*. (1997) in goats;

Gravance *et al*. (2008) in bulls and Marco-Jiménez *et al*. (2005 and 2010) in rabbits).

In addition to the well-known between-species differences (Gage, 1998), there is significant variation in sperm morphometry between individual males within a species (reviewed by Ward (1998)), and some authors have postulated that sperm morphometry could be male-specific (Morrow and Gage, 2001).

How much of the variation in sperm characteristics such as motility, sperm abnormality and acrosome integrity can be attributed to genetic causes has been already studied in rabbits (Brun *et al*., 2009; Lavara *et al*., 2011 and 2012; Tusell *et al*., 2012). However, knowledge of the genetic basis for male differences in sperm head measurements is scarce and no studies concerning genetic correlations between sperm head dimensions and selection criteria have been conducted in commercial rabbit lines.

The present study aims to determine the repeatability and heritability of sperm head characteristics and to explore the relationships with post-weaning daily gain a common selection objective in rabbit paternal lines.

MATERIAL AND METHODS

Animals and data

Animals from a paternal rabbit line (Line R) were used in the experiment. Line R was selected for daily weight gain (DG) between 28 and 63 days of age (Estany *et al*., 1992) by individual selection. After weaning, animals were housed in collective cages (8 rabbits per cage) at temperature ranging from 15 to 25ºC. At 63 days of age, males were moved to three commercial AI stations

belonged to the Red UPV-IRTA (Spain), the first one located in Zarzadilla de Totana (Murcia, Spain) with 250 males capacity, the second one in Aras de los Olmos (Valencia, Spain) with 500 males capacity, and the third one in Fabara (Zaragoza, Spain) with 500 males capacity. Bucks were placed in individual cages, subjected to a photopheriod of 16h light/day and fed *ad libitum* with a similar commercial rabbit diet (on dry matter basis: 17.5% crude protein, 3.5% ether extract, 16.7% crude fibre, 2938 kcal/kg). In all stations, the males were housed in special rooms with temperature control systems to ensure optimal moisture, ventilation and temperature (between 17 and 24ºC).

Males started the training period at 150-170 days of age. Training was carried out for 2 weeks, whereupon the bucks entered the production period. For the training and production period, each week two ejaculates per male were collected on a single day using an artificial vagina, with a minimum of 30 min between collections. Collections from each male during the experiment were performed on the same day of the week.

Semen samples for the experiment were collected during two different periods:

- Period A: 1 week after finishing the training period.

- Period B: more than 3 months after period A.

Only ejaculates that exhibited a white colour were used in the experiment; samples containing urine and cell debris were discarded when gel plugs were removed.

For morphological analyses, one sample per ejaculate (20µl) was fixed with 180 µl of a glutaraldehyde solution at 0.2% (Electron Microscopy Science, Washington) in Dubbelco's Phosphate Buffered Saline (DPBS).

One slide per ejaculate was prepared and spermatozoa with normal head morphology were evaluated with the aid of software (see Marco-Jiménez *et al.* (2010) for details). The morphometric dimensions of length (L), width (W), area (A) and perimeter (P) of a minimum of 100 normal sperm heads were randomly analysed from each slide.

A total of 12908 records for DG were used in the experiment. DG data used belonged to animals from twelve generation before. In addition to DG, the sperm traits length (L), width (W), area (A) and perimeter (P), were recorded involving 1795 ejaculates from 283 males. The pedigree file included 14700 animals.

Statistical analyses

To reduce bias in the estimation of the genetic parameters of sperm traits resulting from the selection for DG, the sperm traits were analysed jointly with DG (Sorensen and Johansson, 1992). A set of three-trait analyses were thus performed to estimate the correlations among traits.

The mixed model used for the sperm traits was:

$$y_{sijokl} = \mu_s + S_{si} + O_{sj} + P_{so} + a_{sk} + p_{sk} + c_{sl} + e_{sijokl}$$

where y_{sijokl} is a record of the sperm trait, μ_s is the overall mean, S_{si} is the systematic effect station–year–season in which the ejaculate was collected, with 64 levels, O_{sj} is the systematic effect of ejaculate order with two levels (first

and second ejaculate on the same day), P_{so} is the systematic effect of age of the male with 3 levels (≤ 6 months, 6–8 months, more than 8 months), a_{sk} is the animal additive genetic effect, p_{sk} is the permanent environmental effect over all the ejaculates of the male *k*, c_{sl} is the random effect of the litter in which the male *k* was born, and e_{sijokl} is the residual. It was assumed that the different random effects (additive, permanent, litter of birth and residual) followed normal distributions and were independent among and within the effects, except for the additive values of the animals, which were correlated through the numerator relationship matrix.

The mixed model used for DG was:

$$y_{dijkl} = \mu_d + b*LS_{dl} + YS_{di} + OP_{dj} + a_{dk} + p_{dk} + c_{dl} + e_{dijkl}$$

where y_{dijkl} is the daily gain of animal *k*, μ_d is the overall mean, LS_{dl} is the covariate litter size at birth and b the corresponding regression coefficient, YS_{di} is the systematic effect of year–season in which the animal was weaned, with 30 levels, OP_{dj} is the systematic effect of parity order in which the animal was born, with three levels (first, second, and higher), a_{dk} is the animal additive genetic effect, c_{dl} is the random effect of the litter in which the animal *k* was born; the residual of the model was split into two components: p_{dk}, which corresponds to the part of the residual correlated with the permanent environmental effect for sperm traits and e_{dijkl} which corresponds to the part of the residual uncorrelated with any other random effect, within and among traits.

The assumptions for the random effects for DG are the same as those indicated above for the sperm traits.

Further assumptions, concerning correlations between random effects of DG (a_d, p_d, c_d, e_d) and random effects of first sperm trait (a_{s1}, p_{s1}, c_{s1}, e_{s1}) and second sperm trait (a_{s2}, p_{s2}, c_{s2}, e_{s2}) are summarised in the following matrices:

$$\mathbf{G}=\begin{bmatrix} \sigma^2_{a_d} & \sigma_{a_d,a_{s1}} & \sigma_{a_d,a_{s2}} \\ \sigma_{a_{s1},a_d} & \sigma^2_{a_{s1}} & \sigma_{a_{s1},a_{s2}} \\ \sigma_{a_{s2},a_d} & \sigma_{a_{s2},a_{s1}} & \sigma^2_{a_{s2}} \end{bmatrix};$$

$$\mathbf{P}=\begin{bmatrix} \sigma^2_{p_d} & \sigma_{p_d,p_{s1}} & \sigma_{p_d,p_{s2}} \\ \sigma_{p_{s1},p_d} & \sigma^2_{p_{s1}} & \sigma_{p_{s1},p_{s2}} \\ \sigma_{p_{s2},p_d} & \sigma_{p_{s2},p_{s1}} & \sigma^2_{p_{s2}} \end{bmatrix};$$

$$\mathbf{C}=\begin{bmatrix} \sigma^2_{c_d} & \sigma_{c_d,c_{s1}} & \sigma_{c_d,c_{s2}} \\ \sigma_{c_{s1},c_d} & \sigma^2_{c_{s1}} & \sigma_{c_{s1},c_{s2}} \\ \sigma_{c_{s2},c_d} & \sigma_{c_{s2},c_{s1}} & \sigma^2_{c_{s2}} \end{bmatrix};$$

$$\mathbf{R}=\begin{bmatrix} \sigma^2_{e_d} & 0 & 0 \\ 0 & \sigma^2_{e_{s1}} & \sigma_{e_{s1},e_{s2}} \\ 0 & \sigma_{e_{s2},e_{s1}} & \sigma^2_{e_{s2}} \end{bmatrix}$$

where the components of **G**, **P**, **C** and **R** are the additive, permanent, birth litter and residual variances for the daily gain and the sperm traits in the diagonal, and the corresponding covariances between traits, out of the diagonal.

Variance–covariance components were estimated using a Bayesian approach implemented in the TM program developed by Legarra *et al.* (2008). Flat priors were used for systematic effects and variance components.

The following prior distributions for random effects were assumed:

$$p\left(\begin{bmatrix} a_d \\ a_{s1} \\ a_{s2} \end{bmatrix}\middle|\mathbf{G}\right)\sim N(\mathbf{0},\mathbf{A}\otimes\mathbf{G}),\ p\left(\begin{bmatrix} p_d \\ p_{s1} \\ p_{s2} \end{bmatrix}\middle|\mathbf{P}\right)\sim N(\mathbf{0},\mathbf{I}\otimes\mathbf{P}),\ p\left(\begin{bmatrix} c_d \\ c_{s1} \\ c_{s2} \end{bmatrix}\middle|\mathbf{C}\right)\sim N(\mathbf{0},\mathbf{I}\otimes\mathbf{C})$$

Where **A** is the numerator relationship matrix, **0** is a vector of zeroes, **Is** are identity matrices and **G**, **P** and **C** are the (co)variance matrices summarised above.

After some exploratory analysis, chains of 2000000 samples were used, with a burning period of 500000. Only one sample from each 100 was saved. The convergence was checked on each chain by the Z Geweke criterion (Geweke, 1992). Summary statistics from the marginal posterior distributions were calculated directly from the samples saved.

RESULTS AND DISCUSSION

In this study we explore the source of variation in sperm morphometry in rabbits using a huge data set involving more than 150,000 sperm measurements from 1795 ejaculates. Summary statistics for the sperm traits are shown in **Table 3.1**. The mean values obtained showed similar values to the ones obtained in previous studies (Marco-Jiménez *et al*. (2005 and 2010), Lavara *et al*., 2008). The recorded DG values of the different males used in the present study varied between 39.1 g/day and 65.43 g/day (data not shown in tables). The source of variation of sperm morphometry could be related to environmental effects, genetic effects (additive or non additive) or a combination of both, as in abnormal sperm forms (Lavara *et al*., 2012). Morphometry variables had smaller variation coefficients (between 2 to 5%, **Table 3.1**) than variables describing ejaculate features such as volume or sperm production (Lavara *et al*., 2011; Tusell *et al*., 2012).

As we commented above differences in sperm head morphometry may derive from environmental influences. Possible environmental influences include

semen processing (semen handling and cryoconservation), season of the year, age of the male or ejaculate order, as have been previously reported on several rabbit semen characteristics (Roca *et al.,* 2005; Lavara *et al.,* 2001, 2013).

Table 3.1: Descriptive statistics for sperm head dimension traits

	n	Mean	Standard deviation
W(µm)	1795	4.7	0.2
A(µm^2)	1795	31.9	1.7
L(µm)	1795	8.5	0.2
P(µm)	1795	23.9	1.0

n: number of ejaculates; W: width of sperm head; A: area of sperm head; L: length of sperm head; P: perimeter of sperm head.

Results from the study of environmental systematic effects proved that neither the order of ejaculate nor the age of the male has any relevant effect on sperm morphometric traits (**Table 3.2**), due to the low magnitude of the change (around 0.04% and 0.7%), in agreement with Beatty and Napier (1961) and Gage and Cook (1994), who reported the limited effect of environmental factors on these traits. Most of the literature on sperm head morphometry among different species is focused on semen processing effects, including the effects of cryopreservation and smear preparation techniques previous to morphometric analyses (Gravance and Davis (1995); Esteso *et al.,* 2003; Gravance *et al.,* 2008). Only a few studies conducted in rams, however, have examined the effects of other environmental influences as the season effect, associated with photoperiod (Martí *et al.,* 2012), the herd of origin (Maroto-

Morales *et al.,* 2012), or the age of the male, related with the sexual maturity (Martí *et al.,* 2011), suggesting that these effects could explain an important part of the observed variation in sperm head morphometry, both in fresh (Maroto-Morales *et al.,* 2012) and after cryopreservation process (Martí *et al.,* 2011 and 2012). Regarding to the age of the male, Martí *et al.* (2011) showed that except for L all morphometric dimensions (A, P, W) values decreased with the increase of male age. This finding is partially in agreement with results from our study, where the highest values of W, A and L belonged to males with 6-8 months of age that could be considered as mature rabbit males (**Table 3.2**).

Results from the study of environmental systematic effects proved that neither the order of ejaculate nor the age of the male has any relevant effect on sperm traits (**Table 3.2**), due to the low magnitude of the change (around 0.04% and 0.7%), in agreement with Beatty and Napier (1961) and Gage and Cook (1994), who reported the limiting effect of environmental factors on these traits.

Repeatability, heritability, permanent and common litter effects

Features of the estimated marginal posterior distributions (PM: posterior mean. $HPD_{95\%}$: interval of highest density of 95%) of heritability (h^2), permanent environment (p^2), common litter of birth (c^2) and male effects for each trait are presented in **Table 3.3**. We computed total male effect (or repeatability) as the sum of h^2, p^2 and c^2 effects. Male effect represents the implication of male to each sperm trait, both from the male's genotype and from the non genetic factors that affect sperm head dimensions of a particular male throughout its life. Male effects of morphometric sperm traits (length, width, perimeter and area) were high, ranging from 0.35 to 0.5, whereas the repeatabilities of traits routinely measured in artificial insemination centres (motility, sperm

concentration, volume, abnormal forms, acrosome) were a little low (Lavara *et al*., 2011 and 2012; Tusell *et al*., 2012).

Regarding the proportion of variance due to the common litter effect being lower than h^2 estimates, these results are in agreement with those published previously in related seminal traits (Lavara *et al*., 2011 and 2012; Tusell *et al*., 2012).

Resulted heritabilities showed that sperm head dimensions are heritable (ranged between 0.20 and 0.29, **Table 3.3**). Although the genetic determination of sperm size traits is a relatively unexplored area, an early study conducted by Napier (1961) found very high heritability estimates for several sperm morphometry traits in rabbits (sperm head length 0.72±0.18; width 0.71±0.13 and area 0.74±0.5). All of these estimates are unusually high, probably due to incorrect experimental design. The main point was that the males used belonged to two different breeds (Edinburgh AS and Netherland Dwarfs) which exhibited high differences in sperm morphometry and the author did not include the breed effect in the father-son regression model, resulting in a biased estimation of heritability. Moreover, no other permanent effects were included in the model. In our study complete pedigree information was employed, in addition as we used males from a selected line information regarding selection procedure was included in order to had an unbiased estimation of different variance components.

Table 3.2: Descriptive statistics of the posterior marginal distributions of the estimable functions between ejaculate order (E1, E2) and age of the male (A1, A2, A3) for width (W), area (A), long (L) and perimeter (P) of the sperm head

	W(µm)		A(µm²)		L(µm)		P(µm)	
	PM	$HPD_{95\%}$	PM	$HPD_{95\%}$	PM	$HPD_{95\%}$	PM	$HPD_{95\%}$
A1-A2	-0.01	[-0.04; 0.02]	-0.21	[-0.5; 0.07]	-0.07	[-0.12;-0.03]	0.08	[-0.06; 0.22]
A1-A3	0.01	[-0.02; 0.04]	-0.11	[-0.41; 0.19]	-0.06	[-0.11; -0.02]	0.13	[-0.01; 0.28]
A2-A3	0.02	[-0.01; 0.04]	0.11	[-0.12; 0.33]	0.01	[-0.02; 0.05]	0.05	[-0.06; 0.16]
E1-E2	0.002	[-0.01; 0.01]	-0.06	[-0.2; 0.07]	-0.02	[-0.04; 0.01]	0.02	[-0.05; 0.08]

E1, first ejaculate; E2, second ejaculate; A1≤6 months; A2, 6-8 months; A3, >8 months; PM, posterior mean; $HPD_{95\%}$, interval of highest density of 95%

Table 3.3: Descriptive statistics of the posterior marginal distributions of heritability (h^2), ratio of permanent effects variance to phenotypic variance (p^2), ratio of common litter of birth effects variance to phenotypic variance (c^2), and male effects (or repeatability) for width (W, µm), area (A, µm^2), length (L, µm) and perimeter (P, µm) of sperm head

	h^2			p^2			c^2			Male effects		
	PM	**HPD$_{95\%}$**	**Min$_{95\%}$**	**PM**	**HPD$_{95\%}$**	**Min$_{95\%}$**	**PM**	**HPD$_{95\%}$**	**Min$_{95\%}$**	**PM**	**HPD$_{95\%}$**	**Min$_{95\%}$**
W(µm)	0.28	[0.14, 0.43]	0.16	0.09	[0.01, 0.17]	0.03	0.12	[0.02, 0.23]	0.04	0.48	[0.41, 0.53]	0.42
A(µm^2)	0.20	[0.09, 0.31]	0.11	0.11	[0.02, 0.19]	0.04	0.06	[0.01, 0.13]	0.01	0.35	[0.29, 0.42]	0.30
L(µm)	0.25	[0.11, 0.39]	0.14	0.18	[0.06, 0.30]	0.08	0.06	[0.01, 0.13]	0.01	0.47	[0.40, 0.53]	0.41
P(µm)	0.29	[0.18, 0.41]	0.19	0.10	[0.02, 0.17]	0.03	0.06	[0.01, 0.13]	0.01	0.43	[0.36, 0.49]	0.38

PM: posterior mean. HPD$_{95\%}$: interval of highest density of 95%. Min$_{95\%}$: value for which the probability of higher values is 95%.

Table 3.4: Descriptive statistics of the posterior marginal distribution of the genetic effects (r_g), permanent effects (r_p) and common litter of birth effects (r_c) correlations between sperm head morphometry traits, width (W, µm), area (A, µm^2), length (L, µm) and perimeter (P, µm)

	r_g			r_p			r_c		
	PM	**HPD$_{95\%}$**	**P**	**PM**	**HPD$_{95\%}$**	**P**	**PM**	**HPD$_{95\%}$**	**P**
W-A	0.72	[0.42, 0.97]	1.00	-0.26	[-1.00, 0.69]	0.67	0.45	[-0.63, 1.00]	0.84
W-L	0.48	[0.05, 0.94]	0.97	0.19	[-0.86, 1.00]	0.68	-0.30	[-1.00, 0.66]	0.70
W-P	0.87	[0.75, 0.97]	1.00	0.59	[-0.46, 1.00]	0.90	0.86	[0.56, 1.00]	0.99
A-L	0.85	[0.67, 0.97]	1.00	0.95	[0.81, 1.00]	1.00	0.85	[0.47, 1.00]	0.98
A-P	0.90	[0.75, 0.99]	1.00	0.55	[-0.43, 1.00]	0.89	0.72	[-0.10, 1.00]	0.94
L-P	0.80	[0.62, 0.98]	1.00	0.90	[0.65, 1.00]	0.99	0.17	[-1.00, 0.87]	0.68

PM: posterior mean. HPD$_{95\%}$: interval of highest density of 95%.P: probability of r_i being >0 (<0) when PM of r_i is >0 (<0), for i=g, p, c.

Table 3.5: Descriptive statistics of the posterior marginal distributions of the genetic effects (r_g), permanent effects (r_p) and common litter of birth effects (r_c) correlations between daily gain (DG) and width (W, µm), area (A, µm²), length (L, µm) and perimeter (P, µm) of sperm head

	r_g			r_p			r_c		
	PM	**HPD95%**	**P**	**PM**	**HPD95%**	**P**	**PM**	**HPD95%**	**P**
W-DG	-0.43	[-0.84, 0.01]	0.97	-0.51	[-0.99, 0.11]	0.93	-0.09	[-0.58, 0.43]	0.65
A-DG	-0.32	[-0.86, 0.14]	0.88	-0.10	[-0.99, 0.68]	0.61	0.10	[-0.48, 0.99]	0.60
L-DG	-0.09	[-0.60, 0.42]	0.63	-0.35	[-0.99, 0.25]	0.86	0.09	[-0.56, 0.99]	0.57
P-DG	-0.34	[-0.74, 0.06]	0.94	-0.65	[-0.99, -0.14]	0.97	-0.01	[-0.76, 0.81]	0.53

PM: posterior mean. $HPD_{95\%}$: interval of highest density of 95%.P: probability of r_i being >0 (<0) when PM of r_i is >0 (<0), for i=g, p, c.

Correlations between sperm traits, and between DG and sperm traits

The estimated marginal posterior distributions of the genetic, the permanent environmental and the common litter effects correlations between sperm traits are presented in **Table 3.4**, whereas the correlations between DG and sperm traits are shown in **Table 3.5**.

The genetic correlations (rg) between sperm traits, with the exception of L-W, where the correlation was moderate, were always high and positive (between 0.72 and 0.90).

To our knowledge, no previous studies on genetic relationship between male growth traits and morphometry sperm traits have been reported. Estimates of genetic correlations between DG and sperm traits showed larger $HPD_{95\%}$ than estimates of genetic correlations between sperm traits, as a more accurate estimation of them needs a higher number of males. Means of the genetic correlations between DG and sperm traits studied were negative and small with L, while other traits showed larger estimated correlations with DG, ranged from -0.32 for A to -0.34 for P and it was -0.43 with W. However, there is consistent evidence of the negativity of the genetic correlation (probability of being lower than 0 is higher than 0.88) for W, A and P with DG (**Table 3.5**).

Concerning common litter effect correlations, the estimates were in general lower than for genetic correlations and again the estimates in general showed a great uncertainty associated with them (**Table 3.5**). Our results show some evidence of the existence of a genetic relationship between sperm morphometry and DG, and the consequence of direct selection for increased

daily gain during the fattening period could produce a decrease in sperm head dimensions due to the negative magnitude of the genetic correlations.

Our estimates are in line with the phenotypic results obtained in an experiment conducted with bulls from two breeds selected for different purposes (meat and dairy) in which the meat breed had smaller sperm than the dairy breed (Morrow and Gage, 2001). The antagonism between growth rate and seminal traits has been observed in previous studies where DG selection seems to have a slightly detrimental effect on ejaculate volume (Tusell *et al*., 2012) and sperm motility (Lavara *et al*., 2012). In contrast, the sperm production was not affected by selection (Lavara *et al*., 2011; Tusell *et al*., 2012). In addition Lavara *et al*. (2012) concluded that there is an apparent tendency for genes favouring increased daily weight gain to slightly decrease normal acrosome status and increase abnormal sperm forms. Regarding genetic correlation between growth and male contribution to fertility in rabbit, Piles and Tusell (2012) found that both traits seemed not to be genetically correlated. Further studies should be focused on the relationship between sperm head morphometry and different semen or ejaculate characteristics and also between sperm head morphometry and field fertility and prolificacy.

REFERENCES

Barth A.D., Oko R.J. Abnormal morphology of bovine spermatozoa, Iowa State University Press, 1989, Ames, IA, USA.

Barth A.D., Bowman P., Bo B., Mapletoff R.J. Effect of narrow sperm head shape on fertility in cattle. Cn Vet J 1992;33:31-9.

Beatty R.A., Napier R.A.N. Genetics of gametes. I. A quantitative analysis of five characteristics of rabbit spermatozoa. Proc Roy Soc Edinb. B, 1961;68:1-16.

Brun J.M., Sanchez A., Duzert R., Saleil G., Theau-Clément M. Paramètres génètiques des caractéristiques de la semence de lapin. In: *13èmes Journ. Rech. Cunicole,* Le Mans, France. 2009;11:17-8.

Casey P.J., Gravance C.G., Davis R.O., Chabot D.D., Liu I.K. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology 1997;47:575-82.

Estany J., Camacho J., Baselga M., Blasco A. Selection response of growth rate in rabbits for meat production. *Genet Sel Evol* 1992;24:527-37.

Esteso M.C., Fernández-Santos M.R., Soler A.J., Garde J.J. Head dimensions of cryopreerved red deer spermatozoa are affected by thawing procedure. Cryo Lett 2003;24:261-8.

Gage M.J.G., Cook .PA. Sperm size or number? Effects of nutritional stress upon eupyrene and apyrene sperm production strategies in the moth *Plodia interpunctella* (Lepidoptera: Pyralidea). Funct Ecol 1994;8:594-9.

Gage M.J.G. Mammalian sperm morphometry. Proc R Soc Lond B 1998;265:97-103.

Geweke J. Evaluating the accuracy of sampling-based approaches to the calculation of posterior moments (with discussion). In J. M. Bernardo, J. O. Berger, A. P. Dawid, & A. F. Smith (Eds.). Bayesian statistics 1992;4:169–93.

Graham J.K. Analysis of stallion semen and its relation to fertility. Veterinary clinics of North America. Equine practice1996;12:119-30.

Gravance C.G., Davis R.O. Automated sperm morphometry analysis (ASMA) in the rabbit. J Androl 1995;16:88-93.

Gravance C.G., Casey M.E., Casey P.J. Pre-freeze bull sperm head morphometry related to post-thaw fertility. Anim Reprod Sci 2008;114:81-8.

Lavara R., Vicente J.S., Marco-Jiménez F., Baselga M. Correlation among CASA and ASMA parameters in rabbit semen. In: Proc. 9th World Rabbit Congress, 2008, Verona, Italy.

Lavara R., Vicente J.S., Baselga M. Genetic parameter estimates for semen production traits and growth rate of a paternal rabbit line. *J Anim Breed Genet* 2011;12:44-51.

Lavara R., Vicente J.S., Baselga M. Estimation of genetic parameters for semen quality traits and growth rate in paternal rabbit line. Theriogenology 2012;78:567-75.

Lavara R., David I., Mocé E., Baselga M., Vicente J.S. Environmental and male variation factors of freezability in rabbit semen. Theriogenology 2013;79:582-89.

Legarra A., Varona L., López de Maturana E. TM: threshold models. 2008. http://cat.toulouse.inra.fr/~alegarra.

Marco-Jiménez F., Balasch S., Vicente J.S., Lavara R., Viudes de Castro M.P., Mocé E. Detection of sub-fertile rabbits by means of sperm head morphometric analysis. In: American Society of Andrology (ed.), Proceeding of the 30th Annual Meeting of Journal of Andrology. EEUU, Seattle 2005, pp.124.

Marco-Jiménez F., Vicente J.S., Lavara R., Balasch S., Viudes de Castro M.P. Poor prediction value of sperm head morphometry for fertility and litter size in rabbit. Reprod Dom Anim 2010;45:e118-e23.

Martí J.I., Aparicio I.M., García-Herreros M. Sperm morphometric subpopulations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. Theriogenology 2011;76:97-109.

Martí J.I., Aparicio I.M., Leal C.L.V., Garía-Herreros M. Seasonal dynamics of sperm morphometric subpopulations and its association with sperm quality parameters in ram ejaculates. Theriogenology 2012;78:528-41.

Maroto-Morales A., Ramón M., García-Álvarez O., Soler A.J., Fernández-Santos M.R.; Roldán E.R.S., Gomendio M., Pérez-Guzmán M.D., Garde J.J. Morphometrically-distinct sperm subpopulations defined by a multistep statistical procedure in ram ejaculates: Intra- and interindividual variation. Theriogenology 2012;77:1529-39.

Morrow E.H., Gage M.J.G. Consistent significant variation between individual males in spermatozoa morphometry. J Zool Lond 2001;254:147-53.

Napier R.A.N. Fertility in the male rabbit. III. Estimation of spermatozoan quality by mixed insemination, and the inheritance of spermatozoan characters. J Reprod Fert 1961;2:273-89.

Piles M., Tusell L. Genetic correlation between growth and female and male contributions to fertility in rabbit. J Anim Breed Genet 2012;129:298-305.

Roca J., Martínez S., Orengo J., Parrilla I., Vázquez J., Martínez E.A. Influence of constant long days on ejaculate parameters of rabbits reared under natural environment conditions of Mediterranean area. Livest Prod Sci 2005;94:169-74.

Rodríguez-Martínez H. State of the art in farm animal sperm evaluation. Reprod Fertil Dev 2007; 19:91-101.

Sorensen D.A., Johansson K. Estimation of direct and correlated responses to selection using univariate animal models. *J Anim Sci1992; 70:* 2038-44.

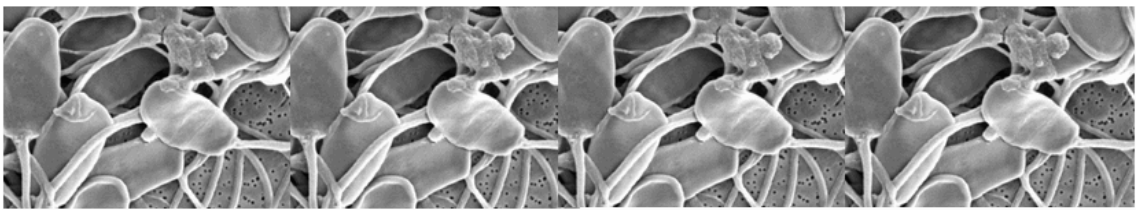
Tusell L., Legarra A., García-Tomás M., Rafel O., Ramon J., Piles M. Genetic basis of semen traits and their relationship with growth rate in rabbits. J Anim Sci 2012;90:1385-97.

Ward W.S., Coffey D.S. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod 1991; 44: 569-74.

Ward P.I. Intraespecific variation in sperm size characters. Heredity 1998;80:655-659.

Williams W.W., Savage A. Observations on the seminal micropathology of bulls. Cornell Vet 1927;15:353-75.

CHAPTER FOUR



CHAPTER FOUR

Environmental and male variation factors of freezability in rabbit semen

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ABSTRACT

The aim of this study was to analyze the environmental and male effects that could have an influence on sperm freezability using a recursive model. A total of 853 ejaculates from 217 males belonged to a paternal rabbit line were collected and frozen. Six different traits were evaluated: the sperm concentration (CONC, 10^6spermatozoa/mL), the acrosome integrity on fresh (NAR, %) and frozen-thawed semen (Nar-FT, %), the sperm motility on fresh (MOT, %) and frozen-thawed semen (Mot-FT, %), and the percentage of viable sperm on frozen-thawed semen (Live-FT, %). In addition, two synthetic traits were computed, the relative reduction of acrosome integrity (Rnar, %) and relative reduction of motility (Rmot, %) after the freezing-thawing process. A multiple-trait recursive model was used to analyze the relationships between the semen traits considered. For the fixed effects studied, the season had the highest impact on post-thaw semen characteristics. Results of the analysis of recursive coefficients showed that fresh semen concentration and motility influence the future freezability of the semen. All traits studied presented moderate repeatabilities ranging from 0.11 to 0.38. These results provide conclusive evidence that sperm freezability in rabbits could be heritable. Regarding male correlations, there were large positive male correlations between fresh traits (r_m=0.77-0.57), as well as between direct frozen-thawed traits (r_m=0.72-1). Male effects on fresh and direct frozen-thawed traits were generally positively correlated. This correlation was moderate to high for MOT with all frozen-thawed traits (r_m=0.41-0.74) and for Mot-FT and all fresh traits (r_m=0.5-0.74), these results suggest that these traits could be genetically related.

Further studies involving more males and ejaculates should be conducted in the future in order to estimate the heritabilities and genetic correlations of post-thaw semen traits in rabbits.

INTRODUCTION

Rabbit artificial insemination is usually performed with cooled semen stored for short periods of time (under 36 h) with good results in fertility and prolificacy (Viudes de Castro *et al*., 1999; Roca *et al*., 2000; Lavara *et al*., 2005). There is a need to develop this technique to dissociate the days of collection and insemination for bio-security reasons and due to the interest of commercial rabbit AI stations in establishing sperm cryobanks. To date, in rabbits, there are some freezing-thawing protocols with acceptable results in fertility, such as those reviewed by Mocé and Vicente (2009). These procedures can be used for experimental purposes and in genetic conservation programmes where reproductive performances lower than those achieved with fresh semen are admissible.

Rabbit ejaculates have shown variability in their survival after freezing-thawing process related to differences between lines (Mocé *et al*., 2003) and between males within lines (Castellini *et al*., 1992; Polgár *et al*., 2004). These results point out that a genetic component is involved in rabbit sperm freezability that deserves to be studied in association with the currently most acceptable procedure of cryopreserving rabbit semen.

None of the studies found in the literature concerning sperm cryopreservation consider the fact that there is a complex relationship between fresh and frozen-thawed sperm traits. Obviously, post-thawing traits are affected by the fresh semen traits in addition to environmental and male effects. When fresh semen traits are only considered as fixed effects for studying cryoresistance (Roca *et al*., 2006), the fact that both fresh and post-thawing traits are determined genetically and that they might be genetically correlated is ignored. On the other hand, when only the genetic correlation is taken into account (Safranski *et al*., 2011), the cause-and-

effect relationships between both types of trait is ignored. Gianola and Sorensen (2004) outlined a solution for this kind of biological system, describing the use of recursive multiple-trait models in a quantitative genetic context.

This approach is able to handle properly the relationship between fresh and post-thawing semen and provides estimates for genetic parameters and estimates of the effect of initial traits on post-thawing traits.

To address the question of whether genetic selection for freezability in rabbit could be possible, we need to know which part of the observed variation is due to a genetic additive component (heritability) or at least which part is due to variation within males (upper limit of heritability). In addition, better knowledge about the relationship between fresh and frozen-thawed traits could be interesting to facilitate an eventual selection programme. Because of the absence of such estimates in the literature, we aim to analyse the environmental and male effects that could have an influence on sperm freezability using a recursive model and linked to the currently acceptable procedure for freezing-thawing rabbit semen.

MATERIALS AND METHODS

Animals and experimental design

Data were collected from 217 males belonging to a paternal rabbit line (Line R). Line R was selected for daily weight gain (DG) between 28 and 63 days of age by individual selection (Estany *et al*., 1992). After birth, number of total born (LB) was recorded. At weaning (28 days of age), number of weaned (LW) and individual weight (W28d) were recorded. After weaning, animals were housed in collective cages (8 rabbits per cage) subjected to a temperature ranging from 15 to 25ºC. At 63 days of age, the weight was recorded (W63d) and males were moved to two AI

stations. Males were placed in individual cages, subjected to a photoperiod of 16 h light/day and fed *ad libitum* with a commercial rabbit diet (on dry matter basis: 17.5% crude protein, 3.5% ether extract, 16.7% crude fibre, 2938 kcal/kg). In both stations, environmental conditions were controlled maintaining the temperature between 17 and 24ºC.

Males began the training period at 150-170 days of age. The training was performed for 2 weeks. After training, the males started the production period. For the training and production period, two ejaculates were collected per male and week on a single day using an artificial vagina, with a minimum of 30 min between collections. Collections from each male during the experiment were performed on the same day of the week. The mean number of collections per male was 5. Only ejaculates that exhibited a white colour were used in the experiment (n=853). Samples containing urine and cell debris were discarded, whereas gel plugs were removed and the ejaculates processed separately.

Freezing-thawing protocols

All the chemicals used were purchased from Sigma-Aldrich (Madrid-Spain). Sperm were cryopreserved by diluting the ejaculates 1:1 (v:v) with the freezing extender. The freezing extender was composed of Tris-citric acid-glucose (0.25 M of Tris(hydroxymethyl)aminomethane (Sigma, cat. no. T-1503), 88mM of anhydrous citric acid (Sigma, cat. no. C-0759), and 47mM of D(+)glucose (Sigma, cat. no. G-8270) as base media, and 3.5 M of dimethyl sulfoxide (DMSO, Sigma, cat. no. D-5879) and 0.1 M of sucrose (Sigma, cat. no. S-8501), added as cryoprotectants (Vicente and Viudes de Castro, 1996). All sperm manipulations were performed at 22ºC. The sperm were packaged in 0.25 mL plastic straws (IMV® Technologies, L'Aigle, France) and sealed with modelling paste (JOVI, S.A. Barcelona, Spain, NRI 8-6650). Sperm

were cooled at 5ºC for 30 min. To freeze sperm, straws were suspended horizontally in liquid nitrogen vapour 5cm above the liquid nitrogen level for 10 min before plunging into the liquid nitrogen (LN_2). The straws were kept in an LN_2 bank until use. After storage in LN_2, thawing was performed submerging the straws in a water bath at 44ºC for 12s.

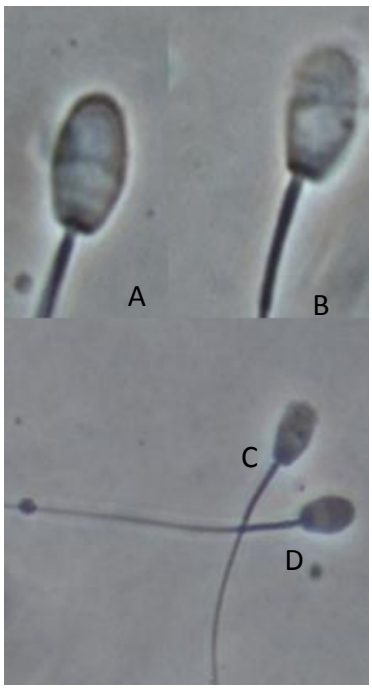
Semen evaluation and traits

Fresh semen traits

Three different variables were assessed in fresh semen: the sperm concentration, the acrosome status and the motility.

Sperm concentration (CONC, 10^6spermatozoa/mL) was determined using a Thoma-Zeiss counting cell chamber (Marienfield, Germany).

Figure 4.1: Acrosome in rabbit spermatozoa



A,D: non reacted acrosome; B,C: reacted acrosome;
A and B (100x objective). C and D (40x objective)

For the acrosome status evaluation, an aliquot from each ejaculate (20 µL) was fixed with 180 µL of a 0.2% solution of glutaraldehyde (Electron Microscopy Science, Washington) in Dulbecco's Phosphate Buffered Saline (DPBS). A minimum of 100 spermatozoa were evaluated at a magnification of 400X by phase positive contrast microscopy. Acrosome status of normal sperm was classified as intact (AI) or reacted (AD), for details see **Figure 4.1**. The percentage of sperm with normal acrosome status (NAR, %) was calculated as the ratio: [AI/(AI + AD)] x 100. For motility analyses, an aliquot from each ejaculate (10 µL) was diluted 1:20 in an extender (Tris-citric acid-glucose) containing bovine serum albumin 0.3% (BSA) to prevent the spermatozoa from sticking to the glassware during the image capture analysis. Then, 10 µL of the diluted sample were placed into a 10 µm deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) for motility analysis using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, S.C.A., Microptic, Barcelona, Spain). Sperm motility was assessed at 37ºC with 10X negative phase contrast objective. Four microscopic fields were captured for each sample. Individual sperm tracks were visually assessed to eliminate possible debris and misdiagnosed tracks. The percentage of total motile sperm cells (MOT, %) was recorded.

Frozen-thawed semen traits

Three traits were measured in frozen-thawed semen: the percentage of viable sperm, the sperm motility and the acrosome integrity.

The percentage of viable (plasma membrane intact) sperm (Live-FT, %) in each frozen-thawed sample was determined using flow cytometry, as described by Purdy and Graham (2004). Briefly, a sample from each thawed straw was diluted with Tris-BSA to 30 x 10^6 sperm/ mL. Then, each sample was stained for flow cytometric

analysis by transferring a 0.1 mL aliquot into a tube containing 0.45 mL Tris-BSA diluent, 2.5 µL SYBR-14 (stock solution: 10 µM in DMSO) and 2.5 µL PI (stock solution: 1.5 mM in distilled water). The samples were incubated for 10 min at room temperature and filtered through a 40 µm nylon mesh before being analysed using an Epics XL-MCL flow cytometer (Beckman Coulter, IZASA, Barcelona, Spain) equipped with an argon laser tuned to 488 nm at 15 mW power. Fluorescence from 10,000 cells was measured using a 550 long pass filter (LP) combined with a 525 nm band pass filter (BP) to detect SYBR-14 and a 645 nm LP combined with a 620Nm BP filter to detect PI. Using this protocol, all cells stain with SYBR-14, but only non-viable cells stain with PI. In the frozen-thawed semen, sperm motility (Mot-FT, %) and acrosome integrity (Nar-FT, %) were determined in the same way as for fresh semen.

In addition, two synthetic traits were computed, the relative reduction of acrosome integrity (Rnar, %) and relative reduction of motility (Rmot, %) after the freezing-thawing process. The two variables were defined as the reduction of the trait between fresh and frozen-thawed sperm divided by the value of the trait in fresh semen.

Model

A multiple-trait recursive model was used to analyse the relationships between the semen traits considered. A graphical description of the model is illustrated in Figure 1. Let y_{ijk} and $z_{i'jk}$ be the kth measurements for individual j on fresh trait $i(i=1,2,3)$ and frozen trait $i'(i'=1,2,3,4,5)$, respectively. We note $R_{ii'}=1$ if there is a hypothetical recursive relation ($\lambda_{ii'}$) between fresh trait i and frozen trait i', $R_{ii'}=0$, otherwise (**Figure 4.2**) and we note $B_{i'}=\{i \text{ such that } R_{ii'}=1\}$. The recursive model assumed for fresh traits i and frozen-thawed semen traits i' is the following:

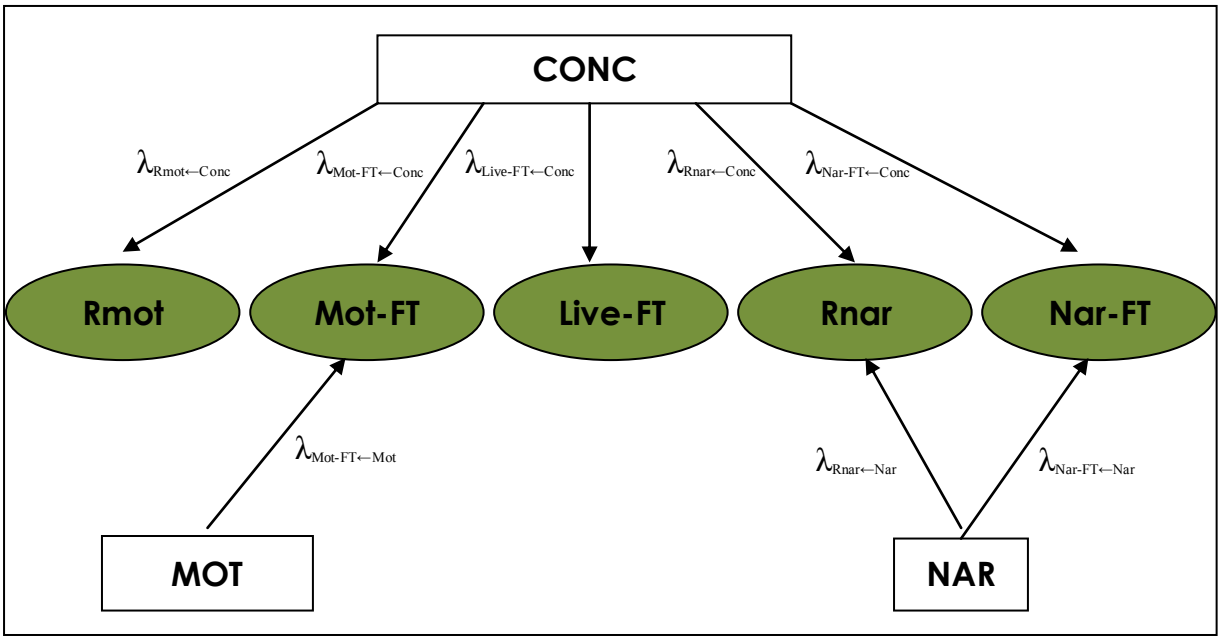
$$y_{ijk} = \boldsymbol{x}_{ijk}^{'}\boldsymbol{\beta}_i + p_{ij} + e_{ijk}$$

$$z_{i'jk} = \sum_{l \in B_{i'}} \lambda_{li'} y_{ljk} + \boldsymbol{h}_{ijk}^{'}\boldsymbol{\alpha}_{i'} + m_{i'j} + t_{i'j} + e_{i'jk}^{'}$$

Where $\boldsymbol{\beta}_i$ and $\boldsymbol{\alpha}_{i'}$ are the vectors of fixed effects related to each trait, which will be determined as explained below, with corresponding known incidence vectors $\boldsymbol{x}_{ijk}^{'}$ and $\boldsymbol{h}_{i'jk}^{'}$; p_{ij} and $m_{i'j}$ are the random male effects on fresh trait i and frozen trait i' with $\begin{bmatrix} \boldsymbol{p} \\ \boldsymbol{m} \end{bmatrix} \sim N(0, \boldsymbol{H} \otimes \boldsymbol{I}_N)$, where $\boldsymbol{H}$ is the variance covariance matrix between male effects of the different traits and $\boldsymbol{I}_N$ an identity matrix of size N, the number of males; $t_{i'j}$ is the random effect of thaw session, $[\boldsymbol{t}] \sim N(0, \boldsymbol{Q} \otimes \boldsymbol{I}_n)$, where $\boldsymbol{Q}$ is a diagonal matrix of variances of thaw session effect for the different frozen traits, $\boldsymbol{I}_n$ is an identity matrix of size n, the number of thaw sessions; $e_{i'jk}$ and e'_{ijk} are the residuals, $\begin{bmatrix} \boldsymbol{e} \\ \boldsymbol{e'} \end{bmatrix} \sim N(0, \boldsymbol{K} \otimes \boldsymbol{I}_L)$, where $\boldsymbol{K}$ is a diagonal matrix of variances of residual effect for the different traits, and $\boldsymbol{I}_L$ is an identity matrix of size L, the number of records. Random effects are assumed to be independent of each other.

Fixed effects, included in the model for fresh semen traits, were previously reported by Lavara *et al*. (2011) on the same dataset (i.e. year, age of the male, day of recovery and order of ejaculate). Fixed effects included in the model for frozen semen traits (**Table 4.1**) were selected in two main steps. The first main step consists of selecting effects that can be included in the model. All factors that were significantly related to the trait in a single-trait univariate model with a p_value lower than 0.2 (Mickey and Greeland, 1989) and factors that are known to affect frozen traits were selected. All the selected effects and all one-way interactions with biological meaning were then included in a saturated model and selected in the second main

Figure 4.2: Scheme of the hypothetical recursive relationships between fresh and frozen-thawed semen traits. λ's are recursive coefficients



Fresh semen traits: CONC: sperm concentration, 10^6 spermatozoa/mL, NAR: percentage of spermatozoa with non-reacted acrosome, %. MOT: percentage of motile spermatozoa, %; Frozen semen traits: Rmot: relative reduction of motile spermatozoa,%; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen, %; Live-FT: percentage of viable spermatozoa in frozen-thawed semen, %; Rnar: relative reduction of spermatozoa with non-reacted acrosome, %; Nar-FT: percentage of spermatozoa with non-reacted acrosome in frozen-thawed semen, %.

step by descending procedure. Models were compared using the likelihood ratio test. For these selections, models were fitted using the mixed procedure of SAS version 9.0 (SAS, 2000) and the maximum likelihood method. Once the effects and interactions for each trait were selected, the estimations of fixed effects and variance components were obtained using Asreml software (Gilmour *et al*., 2002) and the multi-trait recursive model. Repeatability was computed as Gianola and Sorensen (2004) indicated for recursive models.

RESULTS

A summary of the data statistics presented in **Table 4.2** confirms that the freezing-thawing process drastically reduces the quality of sperm. On average, only 17% of the motile spermatozoa in fresh semen are still motile in frozen-thawed semen, while

less than 30% of the spermatozoa keep their acrosome intact during the freezing-thawing process.

Table 4.1: List of fixed effects (and its levels) tested in the analyses

	Significant effects retained in the final model				
Tested effects	**Rmot**	**Rnar**	**Mot-FT**	**Nar-FT**	**Live-FT**
DG (g/day); [5 levels] [30-44; 44,1-47; 47.1-49; 49.1-52; 52.1-60.3]					
Litter size at birth (LB, n); [3 levels] [<7; 7-10; >10]					
Litter size at weaning (LW, n); [3 levels] [<6; 7-9; >9]	X		X		
Weight at 28d (W28d, g); [4 levels] [<650; 650-749; 750-900; >900]			X		
Weight at 63d (W63d, g); [4 levels] [<2200; 2200-2399; 2400-2700; >2700]					X
Day of ejaculate collection; [38 levels] [each day of collection represent one level]					
Age of male at collection; [2 levels] [<6 months; >6 months]					
Ejaculate order (order); [2 levels] [1stejaculate; 2nd ejaculate]		X		X	X
AI station; [2 levels] [centre 1; centre 2]					
Year; [2 levels] [2006; 2007]					X
Season; [4 levels] [fall; winter; spring; summer]	X	X	X	X	X
Season*order interaction		X		X	X
Season*year interaction					X
Time in LN_2; [3 levels] [<1.2 years; 1.2-1.4 years; >1.4 years]					

DG: daily gain between 28 and 63 days of age; AI: artificial insemination; Rmot: relative reduction of motile spermatozoa ,%; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen, %; Live-FT: percentage of viable spermatozoa in frozen-thawed semen, %; Rnar: relative reduction of spermatozoa with non-reacted acrosome, %; Nar-FT: percentage of spermatozoa with non-reacted acrosome in frozen-thawed semen, %.

Table 4.2: Summary statistics for semen traits

Trait	n	Mean	SD
CONC	853	186.4	155.9
MOT	853	60.5	26.7
NAR	853	86.5	15.7
Mot-FT	853	10.4	13.0
Nar-FT	853	23.9	17.6
Live-FT	853	30.2	22.0
Rmot	853	83.1	20.0
Rnar	853	71.8	20.2

CONC: sperm concentration, 10^6 spermatozoa/mL; MOT: percentage of motile spermatozoa in fresh semen, %; NAR: percentage of spermatozoa with non-reacted acrosome in fresh semen, %; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen, %; Nar-FT: percentage of spermatozoa with non-reacted acrosome in frozen-thawed semen, %; Live-FT: percentage of viable spermatozoa in frozen-thawed semen, %; Rmot: relative reduction of motile spermatozoa ,%; Rnar: relative reduction of spermatozoa with non-reacted acrosome, %. N: number of ejaculates; SD: standard deviation.

Analysis of fixed effects

Changes due to the fixed effects reaching significance for at least one trait are presented in **Table 4.3**. For frozen-thawed motility trait (Mot-FT), most of the variation was due to litter size at weaning and weight at 28d, while the importance of season and litter size effects was quite similar for the reduction of motile spermatozoa. Changes in acrosome traits (Nar-FT, Rnar) were due to the combination season*ejaculate order. The main effects affecting the percentage of viable spermatozoa were the combinations season*order and season*year.

Table 4.3: Importance of significant fixed effects expressed by their maximal effect (i.e. maximal difference between estimates of the levels of the factor) for each frozen trait

	Rmot	Rnar	Mot-FT	Nar-FT	Live-FT
Order					1.8
W28d			10.4		
W63d					0.9
Lw	7.0		8.5		
Year					1.2
Season	6.6		4.0		
Season*order combination		10.9		5.1	5.9
Season*year combination					7.2

Rmot: relative reduction of motile spermatozoa ,%; Rnar: relative reduction of spermatozoa with non-reacted acrosome, %; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen, %; Nar-FT: percentage of spermatozoa with non-reacted acrosome in frozen-thawed semen, %; Live-FT: percentage of viable spermatozoa in frozen-thawed semen, %; Order: Ejaculate order; W28d: weight at 28 days of age; W63d: weight at 63 days of age; LW: litter size at weaning; Year: year of ejaculate recover; Season: season in which the ejaculate was recovered

Analysis of recursive coefficients

Estimated changes in frozen semen traits due to change in fresh semen traits are presented in **Table 4.4**. Recursive effect estimate of concentration on frozen-thawed semen traits shows that an increment in the number of spermatozoa in one ejaculate would decrease the traits Rmot and Rnar, and would increase the traits Mot-FT, Nar-FT and Live-FT. Concerning the recursive effect of Mot and Nar on frozen-thawed semen traits, an increase in Mot increases the expectation of Mot-FT and an increase

in Nar also increases the expectation of RNar and Nar-FT, although the effect is very small.

Repeatabilities, correlations and thaw session variance

The repeatabilities, phenotypic and male correlations of the different traits are reported in **Table 4.5**. We divided the traits into three groups: fresh, direct and computed frozen-thawed traits. All traits presented moderate repeatabilities ranging from 0.11 to 0.38. There were large positive male correlations between fresh traits (r_m=0.77-0.57), as well as between direct frozen-thawed traits (r_m=0.72-1). In the case of computed traits, the observed male correlation was intermediate (r_m=0.54).

Male effects on fresh and direct frozen-thawed traits were generally positively correlated. This correlation was moderate to high for MOT with all frozen-thawed traits (r_m=0.41-0.74) and for Mot-FT and all fresh traits (r_m=0.5-0.74). The other male correlations were very low. Male effect on the relative reduction of the percentage of motile spermatozoa was negatively correlated with all fresh and frozen-thawed semen traits (r_m=-0.33 to -0.77), indicating that a male with a positive effect on fresh traits or frozen traits tended to have a lower reduction of motile spermatozoa. In contrast, male effect on Rnar seems to be negatively correlated with frozen-thawed traits but slightly positively correlated with the fresh trait NAR.

Phenotypic correlations were in the same direction as male correlations but with smaller values.

The variance due to the thaw session for each direct frozen-thawed trait accounted for around 1-5% of the total variance (**Table 4.6**).

Table 4.4: Estimated recursive coefficients of fresh traits on frozen-thawed semen traits (Means ±SD)

	Rmot	Rnar	Mot-FT	Nar-FT	Live-FT
λ_{CONC}	-0.016 ± 0.026	-0.09E-03 ± 0.31E-03	0.54E-01 ±0.11E-02	0.81E-03 ± 0.28E-03	0.84E-03 ± 0.37E-03
λ_{MOT}			0.89E-02 ± 0.62-E03		
λ_{NAR}		0.17E-02 ±0.32E-03		0.53E-03 ± 0.28E-03	

Rmot: relative reduction of motile spermatozoa ,%; Rnar: relative reduction of spermatozoa with non-reacted acrosome, %; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen, %; Nar-FT: percentage of spermatozoa with non-reacted acrosome in frozen-thawed semen, %; Live-FT: percentage of viable spermatozoa in frozen-thawed semen, %; CONC: sperm concentration, 10^6 spermatozoa/mL; MOT: percentage of motile spermatozoa in fresh semen, %; NAR: percentage of spermatozoa with non-reacted acrosome in fresh semen, %. SD: standard deviation.

Table 4.5: Repeatabilities on diagonal, phenotypic correlations (above diagonal) and male correlations (below diagonal) for fresh and frozen-thawed semen traits, mean (SE)

	CONC	MOT	NAR	Rmot	Rnar	Mot-FT	Nar-FT	Live-FT
CONC	**0.24 (0.04)**	*0.28 (0.05)*	*0.09 (0.01)*	*-0.27 (0.11)*	*-0.09 (0.01)*	*0.32 (0.08)*	*0.12 (0.46)*	*0.14 (0.09)*
MOT	0.63 (0.10)	**0.30 (0.04)**	*0.52 (0.05)*	*-0.03 (0.04)*	*0.01 (0.08)*	*0.51 (0.05)*	*0.04 (0.07)*	*0.21 (0.05)*
NAR	0.57 (0.09)	0.77 (0.06)	**0.38 (0.04)**	*-0.10 (0.02)*	*0.16 (0.06)*	*0.42 (0.08)*	*0.09 (0.02)*	*0.19 (0.04)*
Rmot	-0.41 (0.14)	-0.38 (0.14)	-0.33 (0.13)	**0.26 (0.05)**	*0.31 (0.07)*	*-0.48 (0.06)*	*-0.19 (0.04)*	*-0.21 (0.10)*
Rnar	-0.06 (0.15)	0.07 (0.14)	0.35 (0.12)	0.54 (0.13)	**0.24 (0.04)**	*-0.09 (0.06)*	*-0.39 (0.06)*	*-0.21 (0.11)*
Mot-FT	0.50 (0.11)	0.74 (0.08)	0.71 (0.08)	-0.77 (0.09)	-0.14 (0.14)	**0.27 (0.04)**	*0.18 (0.03)*	*0.36 (0.04)*
Nar-FT	0.09 (0.20)	0.41 (0.19)	0.10 (0.17)	-0.59 (0.13)	-0.81 (0.16)	0.74 (0.18)	**0.11 (0.03)**	*0.34 (0.04)*
Live-FT	0.29 (0.14)	0.52 (0.12)	0.29 (0.12)	-0.52 (0.14)	-0.38 (0.14)	0.72 (0.10)	1.0 (0.15)	**0.23 (0.04)**

CONC: sperm concentration, 10^6 spermatozoa/mL; MOT: percentage of motile spermatozoa in fresh semen, %; NAR: percentage of spermatozoa with non-reacted acrosome in fresh semen, %; Rmot: relative reduction of motile spermatozoa ,%; Rnar: relative reduction of spermatozoa with non-reacted acrosome, %; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen, %; Nar-FT: percentage of spermatozoa with non-reacted acrosome in frozen-thawed semen, %; Live-FT: percentage of viable spermatozoa in frozen-thawed semen, %. SE: standard error.

Table 4.6: Thaw-session effect. Ratio of explained variance for direct frozen-thawed traits, mean (SE)

Trait	$\frac{\sigma^2_{thaw}}{\sigma^2_p}$ (SE)
Mot-FT	0.01 (0.01)
Nar-FT	0.03 (0.02)
Live-FT	0.05 (0.02)

Mot-FT: percentage of motile spermatozoa in frozen-thawed semen, %; Nar-FT: percentage of spermatozoa with non-reacted acrosome in frozen-thawed semen, %; Live-FT: percentage of viable spermatozoa in frozen-thawed semen, %. σ^2_{thaw}: thaw session variance; σ^2_p: phenotypic variance. SE: standard error.

DISCUSSION

It is known that cryoconservation protocols drastically reduce the number of motile and viable spermatozoa in rabbits (see review by Mocé and Vicente (2009)). Freezing and thawing damage sperm membranes and the membrane becomes less functional after thawing as a result of these injuries (Peña *et al*., 2004). Furthermore, the surviving spermatozoa have a shorter lifespan and suffer alterations in their functionality that lead to failure in the fertilisation process (Roca *et al*., 2006). This fertilisation reduction could be attributed to changes in the motility and altered plasma membrane structure and acrosome integrity during cryopreservation, which makes the spermatozoa more susceptible to capacitating factors (Siqueira *et al*., 2011). Plasma and outer acrosome membranes are the most cryosensitive structures in domestic species (Salomon and Maxwell, 2000). In fact, the reduction of non-reacted acrosome after

freezing-thawing process observed in the present study is comparable with the damage detected in acrosome membranes in mammal species such as rams, bulls (Watson, 2000), pigs (Roca *et al*., 2006) and monkeys (Okada *et al*., 2001), among others. When AI with frozen-thawed sperm was performed, the possible losses of fertility were compensated by increasing the number of spermatozoa in the insemination doses (Salomon and Maxwell, 2000). For instance, despite the reduction in the number of motile and non-reacted acrosome sperm, the protocol used in the present study reported successful fertility rates, as shown in previous studies (Vicente and Viudes de Castro, 1996; Mocé *et al*., 2003; Mocé *et al*., 2010).

Artificial insemination in rabbits is performed with fresh or cooled, diluted semen rather than frozen, due to the poor fertility obtained with cryopreserved sperm (Castellini *et al*., 1992 and 2006) (for a review see: Mocé and Vicente (2009)). In rabbit, frozen semen is occasionally used for genetic resources conservation, international export and research. To increase the use of cryopreserved rabbit sperm in commercial farms, it is necessary to establish reliable sperm cryobanks. To achieve this objective, it is mandatory to determine the main environmental and genetic effects affecting the sperm survival during cryopreservation. This study considered and jointly analysed eight different traits; the models were built to estimate the male and thawing session effect free of environmental variation effects, while taking into account the fresh sperm characteristics.

Environmental effects

Season effect was one of the principal causes of variation in nearly all frozen-thawed sperm traits. The influence of heat stress on fresh sperm traits is well known (see review by Marai *et al*., 2002), and the variation in sperm quality and

quantity in summer-autumn period is a classic effect in rabbits (Nizza *et al*., 2003; Pascual *et al*., 2004; Safaa *et al*., 2008). This effect is mainly determined by changes in temperature, humidity and photoperiod, and affects rabbit reproductive performance (Theau-Clèment *et al*., 1998).

However, in our models post-thaw semen traits are corrected by sperm concentration and initial sperm quality, and due to this premise we can speculate that this variation could be due to differences in seminal plasma or sperm membrane composition in different seasons. These differences could explain part of the variation in sperm freezability. In fact, in many species, peroxidation of lipids of the plasma membrane has been cited as a major factor involved in sperm quality after thawing. Moreover, alterations in the sperm membrane fluidity could alter the activation of signal transduction pathways, critical for sperm function (Macías-García *et al*., 2011). Some research has demonstrated that the membrane can exchange lipid components with the extracellular environment such as seminal plasma or, particularly in rabbits, with prostatic secretory granules (Mourvaki *et al*., 2010; Castellini *et al*., 2012), and seminal plasma composition could in fact be altered by the diet (Surai *et al*., 2000). In addition, biochemical properties of spermatozoa changed with season (Lovercamp *et al*., 2007).

One interesting finding of the present study was the influence of litter size and weight at weaning on variation of motility traits after thawing. This particular effect could be related with Sertoli and Leydig cell proliferation. In prepubertal rabbit, Leydig cell proliferation occurs over a time interval between 5-10 weeks of age (Gondos *et al*., 1977). Sertoli cell proliferation occurs during the foetal and neonatal period and is representative of the adult Sertoli cell population

(Gondos *et al*., 1993). We must keep in mind that weaning in males occurs at 4 weeks of age, and their weight at that point could influence the proliferation of both types of cells. Regarding litter size at weaning, males used in this study came from litters composed of 2 to 13 young rabbits. In this way, Flowers (2006) suggested that management very early in life could influence the future sperm quality of the boars. Boar males from smaller litters have higher sperm output compared with boars from larger litters. Perhaps for this reason litter size at weaning had great influence on the variation of motility traits.

Recursive parameters

Commonly, only ejaculates exceeding certain quality limits are selected for cryopreservation in all species. However, to our knowledge, there have been no specific studies in which initial semen traits were correlated with sperm quality post-thawing in rabbit, when ejaculates had not been selected prior to cryopreservation. However, the experiments in which that relationship was studied did not take into account that fresh and post-thaw traits could be genetically correlated. With the use of a recursive multiple-trait model, we assumed that there is a phenotypic and genetic connexion between both types of traits. In our results, the estimated recursive coefficients indicate that an increase in the concentration of the ejaculate leads to an increase in sperm freezability; an increase in initial motility leads to an increase in post-thaw motility and the effect of an increase in acrosome status is low on sperm freezability. These obtained relationships between initial and post-thaw traits are in accordance with several studies in different species that evidenced a positive correlation between fresh and post-thaw variables (in boars: Roca *et al*., (2006); in stallions: Loomis *et al*., (2008); among others).

Genetic background

The inter male variability in post-thaw sperm quality in different species was found previously (in rabbits: Castellini *et al*., 1992; in boars: Holt *et al*., (2005)). The reason for male variability in sperm cryosurvival is unknown at present, although some authors suggested it might have a genetic origin (Thurston *et al*., 2002; Fraser *et al*., 2008). In our work we studied the repeatability of sperm variables. Repeatability indicates the rate of total variation associated with a semen trait that is due to genes controlling that trait and the non genetic common factors to all the observations relating to the same male. This is the upper limit of heritability.

The values obtained for fresh traits in this experiment were generally in agreement with those previously reported by other authors (Brun *et al*., 2002; García-Tomás *et al*., 2006). However, there are no previous studies concerning the repeatability of post-thaw semen traits in rabbits. Repeatability of the post-thaw semen traits was moderate for most of them, indicating that almost one third of the observed phenotypic variance was due to male-related sources of variation. As expected, the repeatabilities of fresh traits were higher.

Correlations

Very little information is available in the literature regarding the correlation between male effects for most of the semen quality traits measured in this study, and only some genetic correlations between fresh semen traits have been reported (Brun *et al*., 2009; Tusell *et al*., 2010). In contrast, to our knowledge no estimates of genetic parameters are reported in the literature regarding frozen-thawed sperm. Correlation between male effects indicates

the correlation between genes controlling traits and the environmental factors common to all the records relating to the same male.

High male correlations could be due to the fact that traits are genetically related and/or common environmental male effects are related between them. In this study, quality traits measured in fresh sperm or in frozen-thawed sperm showed high correlations among them, suggesting that great parts of the common environment and/or genetic control are similar. We expected that correlation between the same trait measured in fresh and frozen-thaw samples would be high and positive. This hypothesis was corroborated for motility but not for acrosome status of spermatozoa. Thus, these two traits seem quite different and seem to not have the same meaning. Genes and common environment that affect the capacity of the spermatozoa to suffer no acrosome damage due to the freezing-thawing process are different from those which control the acrosome status in fresh semen.

The magnitude of correlations between different kind of traits measured in fresh and frozen-thawed semen samples are moderate and favourable (for instance motility in fresh and live sperm after thawing, or normal acrosome status in fresh and motility after thawing). These results are in agreement with those reported in previous studies where genetic correlations between fresh and frozen-thawed semen traits in bulls were studied (Druet *et al*., 2009).

Moderate favourable male correlations were found between concentration and motilities (in fresh and frozen-thawed semen) and normal acrosome status in fresh semen. However, no relationship was obtained between concentration and normal acrosome status in frozen-thawed semen. In contrast, negative

moderate to high correlations were found between computed variables and frozen-thawed traits.

Thaw session variance

Apart from inter male variability, other sources of variability in sperm cryosurvival were observed in different species and studies (Roca *et al*., 2006, among others). The reasons provided by various authors for this variation are not consistent, but it may be related to poor sustainability of the cryopreservation process (Thurston *et al*., 2001). Post-thaw session variability was observed in the current study, although it was less important than male variation. To date, experiments conducted to study this effect in rabbits are lacking. The variation associated with post-thawing session is an estimation of the differences in the sample handling and specific environmental changes associated with the thawing process. In this study, a higher proportion is associated with percentage of live sperm and percentage of spermatozoa with normal acrosome status than with motility. This could be due to the fact that live sperm and acrosome status were traits more sensitive to handling and environmental changes, as stated previously.

CONCLUSION

For the fixed effects studied, the season had the highest impact on post-thaw semen characteristics. Fresh semen concentration and motility influence the future freezability of the semen. Male effect estimation with a recursive multivariate model that took into account fresh sperm characteristics provides conclusive evidence that sperm freezability in rabbits could be heritable. The

high male correlations found between different groups of traits (fresh and frozen-thawed) suggested that these traits could be also genetically related.

Further studies involving more males and ejaculates should be conducted in the future in order to estimate the heritabilities and genetic correlations of post-thaw semen traits in rabbits.

REFERENCES

Brun J.M., Theau-Clément M., Bolet G. Evidence for heterosis and maternal effects on rabbit semen characteristics. Anim Res 2002;51:433-42.

Brun J.M., Sánchez A., Duzert R., Saleil G., Theau-Clément M. Paramètres génètiques des caractéristiques de la semence de lapin. In : Proceedings of the 13èmes Journ. Rech. Cunicole, Le Mans, France 2009; 11:17-18.

Castellini C., Battaglini M., Lattaioli P. Effects of cryoprotectants and freezing on rabbit semen quality. J Appl Rabbit Res 1992;15:431-8.

Castellini C., Pizzi F., Theau-Clément M., Lattaioli P. Effect of different number of frozen spermatozoa inseminated on the reproductive performance of rabbit does. Theriogenology 2006;66:2182-7.

Castellini C., Mourvaki E., Cardinali R., Collodel G., Lasagna E., Del Vecchio M.T., Dal Bosco A. Secretion patterns and effect of prostate-derived granules on the sperm acrosome reaction of rabbit buck. Theriogenology 2012;78:715-23.

Druet T., Fritz S., Sellem E., Basso B., Gérard O., Salas-Cortés L., Humblot P., Druart X., Eggen A. Estimation of genetic parameters and genome scan for 15 semen characteristics traits of Holstein bulls. J Anim Breed Genet 2009;126:269-77.

Estany J., Camacho J., Baselga M., Blasco A. Selection response of growth rate in rabbits for meat production. Genet Sel Evol 1992;24:527-37.

Flowers W.L. Increasing sperm production in mature boars via manipulation of their neonatal environment. In: Proceedings of the National Swine Improvement Federation. December 7-8, 2006, Nashville, TN, USA pp. 23-25.

Fraser L., Pareek C.S., Strzezek J. Identification of amplified fragment length polymorphism markers associated with freezability of boar semen: a preliminary study. Med Vet 2008;64:646-9.

García-Tomás M., Sánchez J., Rafel O., Ramon J., Piles M. Variability, repeatability and phenotypic relationships of several characteristics of production and semen quality in rabbit. Anim Reprod Sci 2006;93:88-100.

Gianola D., Sorensen D. Quantitative genetic models for describing simultaneous and recursive relationships between phenotypes. Genetics 2004;167:1407-24.

Gilmour A.R., Gogel B.J., Cullis B.R., Welham S.J., Thompson R. ASReml User Guide Release 1.0, Ltd V.I., Editor, 2002.

Gondos B., Morrison K.P., Renston R.H. Leydig cell differentiation in the prepubertal rabbit testis. Bio Reproduction 1977;17:745-8.

Gondos B., Berndston W.E. Postnatal and pubertal development. In: The Sertoli Cell, Russell LD, Griswold MD (Eds.). Cache River Press, 1993, pp. 115-54.

Holt W.V., Medrano A., Thurston L.M., Watson P.F. The significance of cooling rates and animal variability for boar sperm cryopreservaton: insights from the cryomicroscope. Theriogenology 2005;63:370-82.

Lavara R., Mocé E., Lavara F., Viudes de Castro M.P., Vicente J.S. Do parameters of seminal quality correlate with the results of on-farm inseminations in rabbits? *Theriogenology* 2005;64:1130–41.

Lavara R., Vicente J.S., Baselga M. Genetic parameter estimates for semen production traits and growth rate of a paternal rabbit line. J Anim Breed Genet 2011;128:44-51.

Loomis P.R., Graham J.K. Commercial semen freezing: Individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Reprod Sci 2008;105:119-28.

Lovercamp K.W., Safranski T.J., Fischer K.A., Manandhar G., Sutovsky M., Herring W. Arachidonate 15-lipoxygenase and ubiquitin as fertility markers in boars. Theriogenology 2007;67:704-18.

Macías-García B., González-Fernández L., Ortega-Ferrusola C., Morillo-Rodríguez A., Gallardo-Bolaños J.M., Rodríguez-Martínez H. Fatty acids and plasmalogens of the phospholipids of the sperm membranas and their relation with the post-thaw quality of stallion spermatozoa. Theriogenology 2011;75:811-8.

Marai I.F.M., Habeeb A.A.M., Gad A.E. Rabbit´s productive, reproductive and physiological performance traits as affected by heat stress: A review. Livest Prod Sci 2002;78:71-90.

Mickey R.M., Greeland S. The impact of confounder selection criteria on effect estimation. Am J Epidemiol 1989;129:125-37.

Mocé E., Vicente J.S., Lavara R. Effect of freezing-thawing protocols on the performance of semen from three rabbit lines after artificial insemination. Theriogenology 2003;60:115-23.

Mocé E., Vicente J.S. Rabbit sperm cryopreservation: A review. Anim Reprod Sci 2009;110:1-24.

Mocé E., Lavara R., Vicente J.S. Effect of cooling rate to 5ºC, straw size and farm on fertilizing ability of cryopreserved rabbit sperm. Reprod Domest Anim 2010;45:e1-e7;doi:10.1111/j.1439-2009.01.507.x

Mourvaki E., Cardinali R., Roberti R., Dal Bosco A., Castellini C. Desmosterol, the main sterol in rabbit semen: distribution among semen subfractions and its role in the in vitro spermatozoa acrosome reaction and motility. Asian Journal of Andrology 2010;12:862-70.

Nizza A., Di Meo C., Taranto S. Effect of collection rhythms and season on rabbit semen production. Reprod Domest Anim 2003;38:436-9.

Okada A., Igarashi H., Kuroda M., Terao K., Yoshikawa Y., Sankai T. Cryopreservation-induced acrosomal vesiculation in live spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). Human Reproduction 2001;16:2139-47.

Pascual J.J., García C., Martínez E., Mocé E., Vicente J.S. Rearing Management of rabbit males selected by high growth rate: the effect of diet and season on semen characteristics. Reprod Nutr Dev 2004;44:49-63.

Peña F.J., Johannisson A., Wallgren M., Rodríguez-Martínez H. Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lípido architecture. Zygote 2004;12:117-24.

Polgár Z.S., Virag G.Y., Baranyai B., Bodó S.Z., Kovács A., Gózca E. Evaluation of effects of cryopreservation on rabbit spermatozoa membranes with trypan blue-Giemsa staining. Proceedings of the 8th World Rabbit Congress, Septembre 7-10, 2004, Puebla Mexico pp. 322-29.

Purdy P.H., Graham J.K. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology 2004;48:36-45.

Roca J., Martínez S., Vázquez J.M., Lucas X., Parrilla I., Martínez E.A. Viability and fertility of rabbit spermatozoa diluted in Tris-buffer extenderds and stored at 15 C. Anim Reprod Sci 2000;64:103-12.

Roca J., Hernández M., Carvajal G., Vázquez J.M., Martínez E.A. Factors influencing boar sperm cryosurvival. J Anim Sci 2006;84:2692-9.

Safaa H.M., Vicente J.S., Lavara R., Viudes de Castro M.P. Semen evaluation of two selected lines of rabbit bucks. World Rabbit Sci 2008;16:141-8.

Safranski T.J., Ford J.J., Rohrer G.A., Guthrie H.D. Genetic selection for Freezability and its controversy with selection for performance. Reprod Domest Anim 2011;46:31-4.

Salomon S., Maxwell W.M. Storage of ram semen. Anim Reprod Sci 2000;62:77-111.

SAS 2000, SAS/STAT TM Guide for Personal Computers, Version 9, SAS Institute Inc., Cary, NC, 1029 pp.

Siqueira A.P., Wallgren M., Hossain M.S., Johannisson A., Sanz L., Calvete J.J. Quality of boar spermatozoa from the sperm-peak portion of the ejaculate after simplified freezing in MiniFlatpacks compared to the remaining spermatozoa of the sperm-rich fraction. Theriogenology 2011;75:1175-84.

Surai P.F., Brillard J.P., Speake B.K., Blesbois E., Seigneurin F., Sparks N.H. Phospholipid fatty acid composition, vitamin E content and susceptibility to lipid peroxidation of duck spermatozoa. Theriogenology 2000;53:1025-39.

Theau-Clèment M., Castellini C., Maertens L., Boiti C. Biostimulations applied to Rabbit reproduction: Theory and practice. World Rabbit Sci 1998;6:179-84.

Thurston L.M., Watson P.F., Mileham A.J., Holt W.V. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculate correlate with variation in boar semen quality following cryopreservation. J Androl 2001;22:382-94.

Thurston L.M., Siggins K., Mileham A.J., Watson P.F., Holt W.V. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol Reprod 2002;66:545-54.

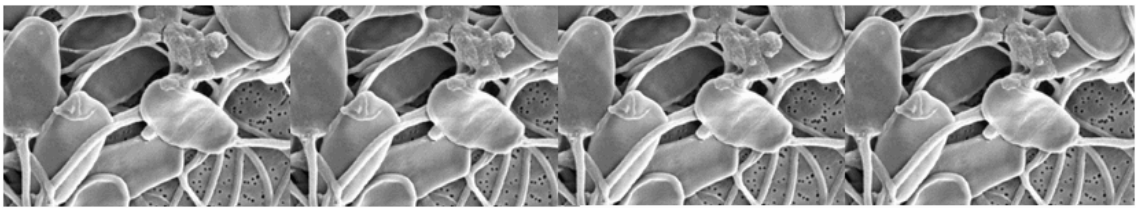
Tusell L., García-Tomás M., Rafel O., Ramon J., Piles M. Genetics of semen production and its relationship with growth rate. In: Proocedings of 15th Reunión Nacional de Mejora Genética Animal 16-18 Junio 2010, Vigo Spain. pp. 1-4.

Vicente J.S., Viudes de Castro M.P. A sucrose-DMSO extender for freezing rabbit semen. Reprod Nutr Dev 1996;36:485-92.

Viudes de Castro M.P., Vicente J.S., Lavara R. Effet du nombre de spermatozoïdes sur la fertilité de la semence conservée 24 heures chez le lapin. Ann Zootech 1999; 48: 407-12

Watson P.F. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;61:481-92.

CHAPTER FIVE



CHAPTER FIVE

Genetics of freezability in rabbit semen

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ABSTRACT

The aim of this study was to estimate the heritability of semen freezability and to estimate the genetic correlation between frozen-thawed sperm traits and the growth rate in a paternal rabbit line. Estimated heritabilities showed that frozen-thawed semen traits are heritable (ranged between 0.08 and 0.15). In the case of Live-FT the estimated heritability is the highest one and suggests the possibility of effective selection. After the study of genetic correlations seems that DG was negatively correlated with sperm freezability, but due to the high HPD95% no further conclusions could be done. More data should be included in order to obtain better accuracy for the estimates of these genetic correlations. If the results obtained at present study were confirmed, it would implied that selection for DG could alter sperm cell membranes or seminal plasma composition, both components related to sperm cryoresistance.

INTRODUCTION

Artificial insemination (AI) is used in rabbit industry, as in other species, to improve breeding management. In rabbit farms AI is performed with fresh or cooled semen rather than frozen because of the poor fertility resulting after thawing (Mocé and Vicente, 2009). However, frozen-thawed rabbit semen is used for conservation of banking resources (endangered breeds or high-value males); international export (semen from selected lines) and research. The inter-animal, within species variation in the ability of spermatozoa to survive cryopreservation is evident in many publications (Froman and Bernier, 1987, Willoughby *et al.,* 1996, Blesbois *et al.,* 2007; Long *et al.,* 2010), suggesting that sperm freezability would have a genetic component. In fact, selection experiments conducted on avian species showed that sperm freezability has a favourable selection response (Ansah and Buckland, 1983).

Recently in rabbits, Lavara *et al.* (2013) provide estimates of repeatability for some frozen-thawed sperm traits, indicating that sperm freezability in rabbits could be heritable. Previously, Mocé *et al.* (2003), showed differences in fertility and prolificacy after AI with frozen-thawed semen from different selected rabbit lines. The line selected on the basis of growth rate during the fattening period, showed the lowest fertility and prolificacy, despite the fact that fresh semen from this line yielded high fertility and prolificacy rates with fresh semen. In this sense, knowledge of the genetic correlation between frozen-thawed sperm traits and the selection criteria would allow us to predict the future correlated response on semen freezability on this selected rabbit line.

Therefore, the aims of this study were to estimate the heritability of semen freezability traits and to estimate the genetic correlation between frozen-thawed sperm traits and the growth rate in a paternal rabbit line.

MATERIALS AND METHODS

Animals and experimental design

Data were collected from 255 males belonging to a paternal rabbit line (Line R). Line R was selected for daily weight gain (DG) between 28 and 63 days of age by individual selection (Estany *et al.,* 1992). After weaning, animals were housed in collective cages (8 rabbits per cage) subjected to a temperature ranging from 15 to 25ºC. At 63 days of age, the weight was recorded and males were moved to two AI stations. Males were placed in individual cages, subjected to a photoperiod of 16 h light/day and fed *ad libitum* with a commercial rabbit diet (on dry matter basis: 17.5% crude protein, 3.5% ether extract, 16.7% crude fibre, 2938 kcal/kg). In both stations, environmental conditions were controlled maintaining the temperature between 17 and 24ºC.

Males began the training period at 150-170 days of age. The training was performed for 2 weeks. After training, the males started the production period. For the training and production period, two ejaculates were collected per male and week on a single day using an artificial vagina, with a minimum of 30 min between collections. Collections from each male during the experiment were performed on the same day of the week. Only ejaculates that exhibited a white colour were used in the experiment. Samples containing urine and cell debris were discarded, whereas gel plugs were removed and the ejaculates processed separately.

Freezing-thawing protocols

All the chemicals used were purchased from Sigma-Aldrich (Madrid-Spain). Sperm were cryopreserved by diluting the ejaculates 1:1 (v:v) with the freezing extender. The freezing extender was composed of Tris-citric acid-glucose (0.25 M of Tris(hydroxymethyl)aminomethane (Sigma, cat. no. T-1503), 88mM of anhydrous citric acid (Sigma, cat. no. C-0759), and 47mM of D(+)glucose (Sigma, cat. no. G-8270) as base media, and 3.5 M of dimethyl sulfoxide (DMSO, Sigma, cat. no. D-5879) and 0.1 M of sucrose (Sigma, cat. no. S-8501), added as cryoprotectants (Vicente and Viudes de Castro, 1996). All sperm manipulations were performed at 22ºC. The sperm were packaged in 0.25 mL plastic straws (IMV® Technologies, L'Aigle, France) and sealed with modelling paste (JOVI, S.A. Barcelona, Spain, NRI 8-6650). Sperm were cooled at 5ºC for 30 min. To freeze sperm, straws were suspended horizontally in liquid nitrogen vapour 5cm above the liquid nitrogen level for 10 min before plunging into the liquid nitrogen (LN_2). The straws were kept in an LN_2 bank until use. After storage in LN_2, thawing was performed submerging the straws in a water bath at 44ºC for 12s.

Semen evaluation and traits

Three traits were measured directly in frozen-thawed semen: the percentage of viable sperm, the acrosome integrity and the sperm motility .

The percentage of viable (plasma membrane intact) sperm (Live-FT, %) in each frozen-thawed sample was determined using flow cytometry, as described by Purdy and Graham (2004). Briefly, a sample from each thawed straw was diluted with Tris-BSA to 30 x 10^6 sperm/ mL. Then, each sample was stained for flow cytometric analysis by transferring a 0.1 mL aliquot into a tube containing 0.45 mL Tris-BSA

diluent, 2.5 µL SYBR-14 (stock solution: 10 µM in DMSO) and 2.5 µL PI (stock solution: 1.5 mM in distilled water). The samples were incubated for 10 min at room temperature and filtered through a 40 µm nylon mesh before being analysed using an Epics XL-MCL flow cytometer (Beckman Coulter, IZASA, Barcelona, Spain) equipped with an argon laser tuned to 488 nm at 15 mW power. Fluorescence from 10,000 cells was measured using a 550 long pass filter (LP) combined with a 525 nm band pass filter (BP) to detect SYBR-14 and a 645 nm LP combined with a 620Nm BP filter to detect PI. Using this protocol, all cells stain with SYBR-14, but only non-viable cells stain with PI.

For the acrosome status evaluation, an aliquot from each frozen-thawed straw (20 µL) was fixed with 180 µL of a 0.2% solution of glutaraldehyde (Electron Microscopy Science, Washington) in Dulbecco's Phosphate Buffered Saline (DPBS). A minimum of 100 spermatozoa were evaluated at a magnification of 400X by phase positive contrast microscopy. Acrosome status of normal sperm was classified as intact (AI) or reacted (AD). The percentage of sperm with normal acrosome status (Nar-FT, %) was calculated as the ratio: [AI/(AI + AD)] x 100. For motility analyses, an aliquot from each frozen-thawed straw (10 µL) was diluted 1:20 in an extender (Tris-citric acid-glucose) containing bovine serum albumin 0.3% (BSA) to prevent the spermatozoa from sticking to the glassware during the image capture analysis. Then, 10 µL of the diluted sample were placed into a 10 µm deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) for motility analysis using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, S.C.A., Microptic, Barcelona, Spain). Sperm motility was assessed at 37ºC with 10X negative phase contrast objective. Four microscopic fields were captured for each sample. The percentage of total motile sperm cells (Mot-FT, %) was recorded.

In addition, two synthetic traits were computed, the relative reduction of acrosome integrity (Rnar, %) and relative reduction of motility (Rmot, %) after the freezing-thawing process. The two variables were defined as the reduction of the trait between fresh and frozen-thawed semen divided by the value of the trait in fresh semen.

A total of 12908 records for DG were used in the experiment. DG data used belonged to animals from twelve generations before. In addition to DG, the sperm traits described above were recorded involving 1292 ejaculates from 255 males. The pedigree file included 14700 animals.

Statistical analyses

To reduce bias in the estimation of the genetic parameters of sperm traits resulting from the selection for DG, the sperm traits were analysed jointly with DG (Sorensen and Johansson, 1992). A set of two-trait analyses were thus performed to estimate the correlations among traits.

The mixed model used for the semen traits was:

$$y_{sbcijkl} = \mu_s + S_{sb} + O_{sc} + T_{si} + P_{sj} + a_{sk} + p_{sk} + c_{sl} + e_{sijokl}$$

where y_{sijokl} is the frozen-thawed semen trait recorded, μ_s is the overall mean, S_{sb} is the systematic effect station–year–season in which the ejaculate was collected, with 47 levels (two AI station with 28 and 19 weeks of collection for each one, where each week of collection on each station represents one different level), O_{sc} is the systematic effect of ejaculate order with two levels (first and second ejaculate on the same day), T_{si} is the systematic effect of thawing session with 19 levels, P_{sj} is the systematic effect of age of the male with 3 levels (≤ 6 months, 6–8 months, more

than 8 months), a_{sk} is the animal additive genetic effect, p_{sk} is the permanent environmental effect over all the ejaculates of the male *k*, c_{sl} is the random effect of the litter in which the male *k* was born, and e_{sijokl} is the residual. It was assumed that the different random effects (additive, permanent, litter of birth and residual) followed normal distributions and were independent among and within the effects, excepting the additive values of the animals, which were correlated though the numerator relationship matrix.

The mixed model used for DG was:

$$y_{dijkl} = \mu_d + b*LS_{dl} + YS_{di} + OP_{dj} + a_{dk} + p_{dk} + c_{dl} + e_{dijkl}$$

where y_{dijkl} is the daily gain of animal *k*, μ_d is the overall mean, LS_{dl} is the covariate litter size at birth and b the corresponding regression coefficient, YS_{di} is the systematic effect of year–season in which the animal was weaned, with 30 levels, OP_{dj} is the systematic effect of parity order in which the animal was born, with three levels (first, second, and higher), a_{dk} is the animal additive genetic effect, c_{dl} is the random effect of the litter in which the animal *k* was born; the residual of the model was split into two components: p_{dk}, which corresponds to the part of the residual correlated with the permanent environmental effect for semen traits and e_{dijkl} that corresponds to the part of the residual uncorrelated with any other random effect, within and among traits.

The assumptions for the random effects for DG are the same as those indicated above for the semen traits.

Further assumptions, concerning correlations between random effects of DG (a_d, p_d, c_d, e_d) and random effects of one semen trait (a_s, p_s, c_s, e_s), are summarized in the following matrices:

$$\mathbf{G}=\begin{bmatrix}\sigma^2_{a_d} & \sigma_{a_d,a_s}\\ \sigma_{a_s,a_d} & \sigma^2_{a_s}\end{bmatrix};$$

$$\mathbf{P}=\begin{bmatrix}\sigma^2_{p_d} & \sigma_{p_d,p_s}\\ \sigma_{p_s,p_d} & \sigma^2_{p_s}\end{bmatrix};$$

$$\mathbf{C}=\begin{bmatrix}\sigma^2_{c_d} & \sigma_{c_d,c_s}\\ \sigma_{c_s,c_d} & \sigma^2_{c_s}\end{bmatrix};$$

$$\mathbf{R}=\begin{bmatrix}\sigma^2_{e_d} & 0\\ 0 & \sigma^2_{e_s}\end{bmatrix}$$

where the components of **G**, **P**, **C** and **R** are the additive, permanent, litter of birth and residual variances for the daily gain and the semen trait in the diagonal, and the corresponding covariances between both traits, out of the diagonal.

The variance–covariance components were estimated using a Bayesian approach implemented in the TM program developed by Legarra *et al.* (2008). Flat priors were used for systematic effects and variance components.

The following prior distributions for random effects were assumed:

$$p\left(\begin{bmatrix}a_d\\ a_s\end{bmatrix}\middle|\mathbf{G}\right)\sim N(\mathbf{0},\mathbf{A}\otimes\mathbf{G}),\ p\left(\begin{bmatrix}p_d\\ p_s\end{bmatrix}\middle|\mathbf{P}\right)\sim N(\mathbf{0},\mathbf{I}\otimes\mathbf{P}),\ p\left(\begin{bmatrix}c_d\\ c_s\end{bmatrix}\middle|\mathbf{C}\right)\sim N(\mathbf{0},\mathbf{I}\otimes\mathbf{C})$$

Where **A** is the numeratior relationship matrix, **0** is a vector of zeroes, **I** is an identity matrix, and **G**, **P** and **C** are the (co)variance matrices summarized above. The ⊗ symbol stays for the direct product.

After some exploratory analysis, chains of 3000000 samples were used, with a burning period of 750000. Only one sample of each 100 was saved. The convergence was checked on each chain by the Z Geweke criterion (Geweke, 1992).

RESULTS AND DISCUSSION

Semen characteristics after the frozen-thawed procedure are summarized in **Table5.1**, where it can be observed the dramatic reduction of sperm motility (Rmot=83%) and normal acrosome status (Rnar=74%). For Mot-FT, Nar-FT and Live-FT, the means obtained are lower than the values reported for the same line in studies in which the ejaculates are preselected for cryopreservation (Mocé *et al.,* 2003). One important difference of this study was the assessment of individual, rather than pooled ejaculates, and the no pre-selection of the ejaculates before freezing. The standard deviations obtained showed the high variability of these traits. In addition, some of them have an effect on male reproductive performance after AI (Mocé and Vicente, 2009).

Table 5.1: Crude mean and standard deviation for semen traits

	n	Mean	SD
Mot-FT	1292	11.2	12.8
Nar-FT	1227	22.4	16.6
Rmot	1292	83.2	17.8
Rnar	1227	74.5	18.3
Live-FT	1199	30.0	19.5

Mot-FT: percentage of motile spermatozoa in frozen-thawed semen; Nar-FT: percentage of spermatozoa with no reacted acrosome in frozen-thawed semen; Rnar: relative reduction of spermatozoa with no reacted acrosome,%, Rmot: relative reduction of motile spermatozoa, %; Live-FT: percentage of live spermatozoa in frozen-thawed semen, %.

Repeatability, heritability, permanent and common litter effects

Table 5.2 shows features of the estimated marginal posterior distributions (PM: posterior mean. HPD95%: interval of highest density of 95%) of heritability (h^2); ratio of permanent variance to phenotypic variance (p^2) and ratio of litter of birth variance to phenotypic variance (c^2) for frozen-thawed semen. We computed the ratio of the phenotypic variance due to the male effects (or repeatability) as the sum of h^2, p^2 and c^2 values. The estimates were moderate, ranging from 0.20 to 0.3, being slightly lower than the repeatabilities of fresh semen traits (Lavara *et al.,* 2011 and 2012; Tusell *et al.,* 2012), indicating the existence of important individual variation for frozen-thawed semen traits in rabbits. Little differences were reported by Lavara *et al.* (2013) using a subset sample of the present database, due probably to differences in the model used and in the number of data. The main difference between studies is the use or not of the information related to the selection criteria. In the first case, Lavara *et al.* (2013) did not use it and in the present study we included the information related to the selection process in order to had an unbiased estimation of the variance components due to the fact that the DG and the frozen-thawed traits could be correlated.

Estimated heritabilities showed that frozen-thawed semen traits are heritable (ranged between 0.08 and 0.15, **Table 5.2**). To our knowledge no previous heritability estimates for frozen-thawed semen traits in rabbits have been reported. The literature estimates of heritabilities for corresponding traits is fresh semen were similar in the case of motility measured with CASA system (0.12-0.14 for Mot,%; Brun *et al.,* 2009; Lavara *et al.,* 2012;) and slightly higher in the case of normal acrosome status (0.18 for Nar,%; Lavara *et al.,* 2012).

Table 5.2: Descriptive statistics of the posterior marginal distributions of heritability (h^2), ratio of permanent variance to phenotypic variance (p^2) and ratio of litter of birth variance to phenotypic variance (c^2), for frozen-thawed semen traits

	h^2		p^2		c^2	
	PM	**$HPD_{95\%}$**	**PM**	**$HPD_{95\%}$**	**PM**	**$HPD_{95\%}$**
Mot-FT	0.13	[0.02 0.25]	0.13	[0.02 0.22]	0.03	[0.00 0.09]
Nar-FT	0.09	[0.01 0.20]	0.11	[0.02 0.21]	0.07	[0.00 0.15]
Rmot	0.08	[0.01 0.18]	0.11	[0.02 0.19]	0.03	[0.00 0.08]
Rnar	0.11	[0.01 0.21]	0.08	[0.02 0.14]	0.05	[0.00 0.13]
Live-FT	0.15	[0.04 0.26]	0.15	[0.05 0.25]	0.02	[0.00 0.06]

PM: posterior mean. $HPD_{95\%}$: interval of highest density of 95%; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen; Nar-FT: percentage of spermatozoa with no-reacted acrosome in frozen-thawed semen; Rnar: relative reduction of spermatozoa with no-reacted acrosome, %, Rmot: relative reduction of motile spermatozoa, %; Live-FT: percentage of live spermatozoa in frozen-thawed semen, %.

In the case of Live-FT the estimated heritability is the highest one and suggests the possibility of effective selection. In this sense a divergent selection experiment would be interesting in order to have better knowledge about the freezability process in rabbits, and could be used as a valuable way for assessing cryoresistance biological basis in rabbit semen. In chicken after 8 generations of selection, physiological changes and biochemical differences were reported between the selected line for frozen-thawed semen fertility and control line. Sperm from the selected line had lower cholesterol and lower cholesterol:phospholipid ratio compared with control line, in addition seminal plasma cholesterol and phospholipid levels also were lower in the selected line (Ansah and Buckland, 1983).

Regarding the proportions of variance due to the common litter effect, they are lower than the corresponding h^2 estimates. This result is in agreement with those published previously in related fresh semen traits (Lavara *et al.,* 2012).

Correlations between sperm traits and DG

Estimates of genetic, permanent and litter correlations between DG and traits of frozen-thawed semen are presented in **Table 5.3**.

Concerning permanent and litter correlations, the estimates were in general lower than the genetic correlation and showed a great uncertainty associated with them.

Regarding genetic correlations, the estimates published previously show antagonistic correlations between fresh semen traits as Nar (%) and Mot (%, objectively measured) and DG (Lavara *et al.,* 2012). In concordance, these traits after the frozen-thawed process must maintain a similar genetic correlation pattern. In our case after the study of genetic correlations seems that DG was negatively correlated with sperm freezability, but due to the high HPD95% no further conclusions could be done. More data should be included in order to obtain better accuracy for the estimates of these genetic correlations. If the results obtained at present study were confirmed, it would implied that selection for DG could alter sperm cell membranes or seminal plasma composition, both components related to sperm cryoresistance. In fact, selection for DG in this rabbit line changed carcass fat levels at the same age compared with lines selected for litter size, and this would affect indirectly lipid membranes in sperm, or cholesterol: phospholipid ratio (Hernández *et al.,* 2006).

Table 5.3: Descriptive statistics of the posterior marginal distributions of the genetic (r_g), permanent (r_p) and litter of birth (r_c) correlations of daily gain (DG) with frozen-thawed sperm traits

	r_g		r_p		r_c	
	PM	**HPD$_{95\%}$**	**PM**	**HPD$_{95\%}$**	**PM**	**HPD$_{95\%}$**
Mot-FT&DG	-0.59	[-1 -0.12]	-0.18	[-0.86 0.50]	-0.24	[-0.99 0.61]
Nar-FT&DG	-0.48	[-0.98 0.24]	-0.36	[-0.96 0.24]	0.11	[-0.48 0.79]
Rmot&DG	0.31	[-0.49 0.94]	0.15	[-0.61 0.86]	0.33	[-0.45 1.00]
Rnar&DG	0.52	[-0.07 0.98]	0.24	[-0.50 1.00]	-0.22	[-1.00 0.40]
Live-FT&DG	-0.44	[-0.96 0.11]	-0.52	[-0.99 0.06]	0.133	[-0.58 1.00]

PM: posterior mean. HPD95%: interval of highest density of 95%; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen; DG: daily gain; Nar-FT: percentage of spermatozoa with no-reacted acrosome in frozen-thawed semen; Rnar: relative reduction of spermatozoa with no-reacted acrosome,%; Rmot: relative reduction of motile spermatozoa, %; Live-FT: percentage of live spermatozoa in frozen-thawed semen, %.

Estimates of genetic correlations between different semen traits and selection criteria in rabbits is scarce (for a review see Piles *et al*., 2012), and estimates are generally imprecise making difficult to draw reliable conclusion, so in the future more efforts should be done in order to better assess the genetic correlations.

From our study, it can be concluded that selection on semen freezability should be effective given the magnitude of heritability estimates in the present study. In addition there are apparently negative effects of selection for increased growth

rate on semen freezability. However, the uncertaintity of obtained estimates, make difficult to predict the correlated effect of selection with enough accuracy.

REFERENCES

Ansah G.A., Buckland R.B. Eight generations of selection for duration of fertility of frozen-thawed semen in the chicken. Poultry Sci 1983; 62:1529–38.

Blesbois E., Seigneurin F., Grasseau I., Limouzin C., Besnard J., Gourichon D., Coquerelle G., Rault P., Tixier-Boichard M. Semen cryoconservation for ex situ management of genetic diversity in chicken: Creation of the French avian cryobank. Poult Sci 2007;86:555-64.

Brun J.M., Sanchez A., Duzert R., Saleil G., Theau-Clément M. Paramètres génètiques des caractéristiques de la semence de lapin. In: *13èmes Journ. Rech. Cunicole,* Le Mans, France. 2009;11:17-8.

Estany J., Camacho J., Baselga M., Blasco A. Selection response of growth rate in rabbits for meat production. Genet Sel Evol 1992;24:527-37.

Froman D.P., Bernier P.E. Identification of heritable spermatozoa degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 1987;37:969-77.

Geweke J. Evaluating the accuracy of sampling-based approaches to the calculation of posterior moments (with discussion). In: Bernardo JM., Berger J O., Dawid AP., & Smith AF. (Eds.). Bayesian statistics 1992;4:169–93.

Hernández P., Ariñó B., Grimal A., Blasco A. Comparison of carcass and meat characteristics of three rabbit lines selected for litter size or growth rate. Meat Science 2006;73:645-50.

Lavara R., Vicente J.S., Baselga M. Genetic parameter estimates for semen production traits and growth rate of a paternal rabbit line. J Anim Breed Genet 2011;12:44-51.

Lavara R., Vicente J.S., Baselga M. Estimation of genetic parameters for semen quality traits and growth rate in paternal rabbit line. Theriogenology 2012;78:567-75.

Lavara R., David I., Mocé E., Baselga M., Vicente J.S. Environmental and male variation factors of freezability in rabbit semen. Theriogenology 2013;79:582-89.

Legarra A., Varona L., López de Maturana E. TM: threshold models. http://cat.toulouse.inra.fr/~alegarra. 2008

Long J.A., Bongalhardo D.C., Pelaéz J., Saxena S., Settar P., O'Sullivan N.P., Fulton J.E. Rooster semen cryopreservation: Effect of pedigree line and male age on postthaw sperm function. Poult Sci 2010;89:966-73.

Mocé E., Vicente J.S., Lavara R. Effect of freezing-thawing protocols on the performance of semen from three rabbit lines after artificial insemination. Theriogenology 2003;60:115-23.

Mocé E., Vicente J.S. Rabbit sperm cryopreservation: A review. Anim Reprod Sci 2009;110:1-24.

Piles M., Tusell L., Lavara R., Baselga M. Breeding programs for improving male reproductive performance and efficiency of AI dose production in paternal lines: feasibility and limitations. In: Proceedings 10th World Rabbit Congress; September 2012, Sharm El-Sheikh-Egypt:1-17.

Purdy P.H., Graham J.K. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology 2004;48:36-45.

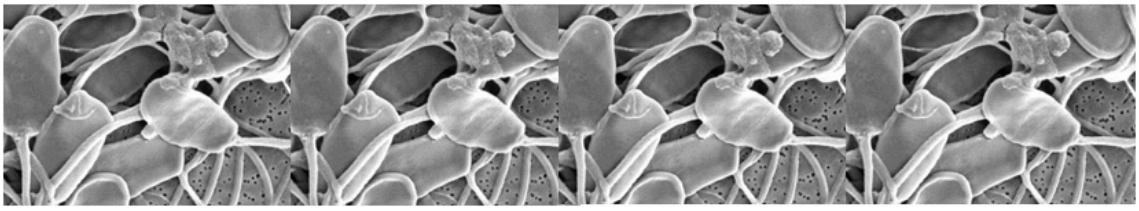
Sorensen D.A., Johansson K. Estimation of direct and correlated responses to selection using univariate animal models. J Anim Sci1992;70:2038-44.

Tusell L., Legarra A., García-Tomás M., Rafel O., Ramon J., Piles M. Genetic basis of semen traits and their relationship with growth rate in rabbits. J Anim Sci 2012;90:1385-97.

Vicente J.S., Viudes de Castro M.P. A sucrose-DMSO extender for freezing rabbit semen. Reprod Nutr Dev 1996;36:485-92.

Willoughby C.E., Mazur P., Peter A.T., Critser J.K. Osmotic tolerance limits and properties of murine spermatozoa. Biol Reprod 1996;55:715–27.

GENERAL DISCUSSION



GENERAL DISCUSSION

AI centres rely on the ability of their males to produce a sufficient amount of good quality semen in order to achieve high field fertility. The relative importance of the male in the cost of the artificial insemination dose is around 2-6% (García *et al.,* 1998, 2004), but this value could be increased to 15-20% due to the low percentage of males that show adequate reproductive behaviour at 6 months (58%, Pascual *et al.,* 2004) or if the males have a high rate of culling (30-40% of global culling) caused by their low output with respect to semen quality and/or production (Rosell and De la Fuente, 2009). Culling of high value males due to impaired semen production may result in important economic losses. Nowadays, the criterion to determine the use or not of the ejaculate for AI is based on a set of macroscopic and microscopic evaluations. Macroscopic evaluation involves the volume and aspect of the ejaculate, whereas microscopic evaluation takes into account the sperm concentration, motility and the percentages of abnormal sperms and intact acrosomes. If the ejaculate does not fit the minimum requirements, it is rejected. Finally, the ejaculates classified as optimum are pooled (heterospermia) to avoid the negative effects of unknown subfertile ejaculates.

Most of the semen used in commercial rabbit farms belongs to males from paternal lines. Breeding objectives of the improvement programmes in these lines have generally been focused on production traits such as growth, feed efficiency and carcass traits (Rochambeau *et al.,* 1989; Estany *et al.,* 1992; Larzul and Rochambeau, 2005; Nagy *et al.,* 2006), and no selection has been

performed for male reproductive traits such as semen quantity or quality in fresh or after the frozen-thawing process.

The genetic determinism of some semen traits, important to the AI centres, and their relationship with the selection criteria, were investigated in this thesis in order to analyse their eventual inclusion in future selection indices and the expected correlated responses on these traits of the current selection programmes.

This thesis was developed in two different scenarios, the current scenario of the AI centres where the inseminations must be performed with fresh or cooled semen, and the future scenario in which the insemination will be performed with frozen-thawed semen. With these objectives, the first three studies were conducted in fresh semen and the two last studies were carried out to gain better knowledge of the genetic determinism concerning frozen-thawed semen.

Genetic determinism of seminal traits

The sperm traits studied in the first study of this thesis are those involved in sperm production (ejaculate volume, concentration and sperm production per ejaculate). The traits presented moderate values of repeatability according to the literature. The estimated h^2 for these ranged from 0.07 to 0.12 for single ejaculates; these h^2 are lower than those recently reported by Tusell *et al.* (2012), for the pool of two consecutive ejaculates in rabbits (from 0.23 and 0.27), but similar to those previously reported for bulls (Kealey *et al.,* 2006) and pigs (Brandt and Grandjot 1998; Smital *et al.,* 2005).

In rabbits, the ejaculate is diluted in an extender before AI. Dilution of the ejaculate could reduce the direct advantage of high sperm output in the ejaculate on potential fertility, because when the ejaculate is diluted to standardise the number of spermatozoa, the seminal plasma is diluted as well, and this dilution of seminal plasma caused an increase in the peroxidation of rabbit spermatozoa and a decline in motility (Castellini *et al.,* 2000). Thus, selection to increase semen production could be effective, and due to the inverse relationship between the dilution of seminal plasma and fertility in rabbits (when dilution rate is more than 20-fold; Castellini *et al.,* 2000), the most useful trait could be sperm production, because concentration and also volume are included and indirectly the seminal plasma quantity could be improved.

Production traits are directly responsible for the number of seminal doses that we can obtain from one ejaculate, but to achieve good fertility and prolificacy, the ejaculate must have enough sperm with ability to progress through the female tract, complete the capacitation process and fertilise the oocyte. We try to assess these characteristics through some traits such as motility (%), normal acrosome status (%), abnormal forms (%) and morphometric sperm dimensions or sperm motion characteristics.

For this reason, we conducted the second and third study to determine the genetic parameters of traits involved in sperm quality. The second study is focused on some traits routinely measured in AI centres, i.e. motility (%), normal acrosome status (%) and abnormal forms (%). The sperm quality characteristic most checked in AI centres is sperm motility, as an indirect measure of sperm viability. Although the evaluation technique is simple, it is highly dependent on

the experience of the operator. Nowadays, the objectivity of motility measurements has also been improved by the use of CASA, which measures a number of characteristics of sperm motility and could be applied in the future to routine assessment of rabbit semen.

However, considering the complexity of the fertilisation process, measurements of any of these single sperm quality attributes cannot reflect the real fertilising ability of a semen sample. They could, however, be useful in eliminating samples of very poor quality (Graham *et al.,* 1989; Hirano *et al.,* 2001). For this reason the range in fertility in commercial rabbit farms is usually narrow and high, due to the high pre-selection of the ejaculates

The h^2 estimates for motility and sperm motion characteristics reported in this study ranged between 0.09 and 0.11. Recently, Tusell *et al.* (2012) reported lower h^2 for individual motility in rabbits. The possible reason for this difference could be due to the subjective manner in which this trait was measured, since it is dependent on technician skills. Regarding the sperm motion characteristics, very little information was available from the literature on its genetic parameters; in rabbits only Brun *et al.* (2009) have reported similar information about some traits. As mentioned previously, sperm abnormalities and acrosome status are related with fertility and also with prolificacy of rabbit does after AI. The h^2 estimates for abnormal forms (%) and normal acrosome status (%) presented in this thesis are medium. Only a few authors have reported repeatability estimates for these traits (0.40 and 0.33) in rabbits, supporting our findings. Thus, selection on semen quality traits should be effective, given the magnitude of heritability estimates and the high coefficient of variation reported for these traits.

Traditional methods for evaluating sperm morphology adopt a subjective approach, classifying the sperm as normal or abnormal depending on its morphological appearance (Barth and Oko, 1998). However, when employing this methodology only sperm exhibiting clearly smaller or larger than average heads may be classified as abnormal. Although it is not routinely carried out in AI centres, morphometry analysis could be beneficial, as it reveals smaller differences in size and shape than routine evaluation (Graham, 1996) and provides information relative to sperm physiology status that could possibly be related with its storage resistance (Rodríguez-Martínez, 2007). With this premise, we conducted the third study to estimate the variance components of the sperm head morphometry in our population. Results obtained reported that morphometric sperm traits are more repeatable and heritable than traits routinely measured in AI centres. If in the future the relationship between sperm head morphometry and storage resistance is confirmed, considering the h^2 estimates of these traits, selection for one of these traits could be recommended.

Relationship between seminal traits and DG

In order to select males for sperm production and/or sperm quality jointly with DG it is necessary to know the h^2 of seminal traits, but also their genetic correlations with the selection criteria of paternal lines. Those parameters are also needed to predict the expected responses in sperm production and quality when selection is performed on DG.

In this thesis, all the studies were conducted using males from the R line, selected for growth rate during the fattening period. Thus, the genetic relationship between seminal traits and growth rate was estimated. Our results

show that there is no evidence to suggest that selection for DG will affect sperm production adversely, but there is an apparent tendency for genes favouring DG to slightly decrease functional sperm per ejaculate (less motility and normal acrosome status and more abnormal forms) and also to decrease the sperm head dimensions. Our findings are in accordance with the results reported in pigs, where the genetic correlations between growth and sperm production traits always had low magnitude (Oh et *al.*, 2006; Wolft, 2009), and also in rabbits where Tusell *et al.* (2012) concluded that selection for increasing DG in paternal lines is not expected to have detrimental correlated effects on seminal traits involved in sperm production.

Estimates of genetic correlations between DG and sperm traits reported in this thesis showed large $HPD_{95\%}$, perhaps in part because the magnitude of the correlations is not high (accuracy of estimates tended to be inversely related to the magnitude of the correlation). We know that with more data the accuracy could be increased, but we need to take into account the special difficulties in achieving the size needed to do so. However, there is consistent evidence of the negativity of the genetic correlation for quality traits such as normal acrosome status, motility and sperm head dimensions, and the unfavourable genetic relationship between DG and abnormal forms.

Freezability in rabbit semen

As mentioned above, the last two studies in this thesis are focused on the genetic determinism of frozen-thawed rabbit ejaculates. Nowadays semen cryopreservation in rabbits is limited to gene banking for conservation of genetic diversity and insurance for losses of genotypes (in cases, for instance, of compulsory slaughter, or the elimination of certain selected lines that eventually

result in losses of valuable genetics), but in the future could be used due to commercial advantages. As commercial advantages we can cite the introduction of superior genetics to the selection nucleus, or the commercialisation of genetics in the international market. In the latter case, sales of frozen semen, instead of the fresh or cooled semen usually employed, entail a better sanitary safeguard of the ejaculate (Purdy, 2008). Doses of semen can be kept frozen until the semen, or the males that provided those samples, are tested for biological agents that could be transmitted to the inseminated females, thus providing a better guarantee for the semen. But before doing so, we need to have better knowledge about freezability in rabbit semen, so one possible experimental design could be to obtain two lines divergently selected for sperm freezability in order to assess the biological basis of cryoresistance in rabbit semen

In the fourth study, we analyse the environmental and male effects that could have an influence on sperm freezability. For this purpose, we used a recursive model to take into account the relationships between fresh and frozen-thawed sperm traits. This model is able to handle the relationship properly between initial and post-thawing semen traits in order to obtain unbiased variance components (Gianola and Sorensen, 2004). The results obtained pointed out that the ejaculate concentration and the initial motility influence the future sperm freezability. It was also shown that, within the environmental effects studied, the season had the highest impact on post-thaw semen characteristics. In addition, the repeatability values of frozen-thawed semen traits were moderate for most of them, indicating that almost one third of the observed phenotypic variance was due to male-related sources of variation. Very little information is available in the literature regarding the correlation

between male effects for semen quality traits in rabbits, and only some genetic correlations between fresh semen traits have been reported (Brun *et al.*, 2009; Tusell *et al*., 2012). In contrast, no estimates of genetic parameters are reported regarding frozen-thawed sperm. In this study, motility measured in fresh sperm and in frozen-thawed sperm showed a high favourable correlation between them, suggesting that great parts of common environment and/or genetic control are similar. This finding is in agreement with those previously reported in bulls by Druet *et al.,* 2009 and Karoui *et al.,* 2011. For some fresh and frozen-thawed traits, the magnitude of male correlations between them was moderate and favourable (such as motility in fresh and live sperm after thawing, or normal acrosome status in fresh and motility after thawing) suggesting that these traits could also be genetically related.

Finally, the last study in this thesis attempted to determine the heritability of frozen-thawed sperm traits and its genetic correlation with the selection criteria. We observed that frozen-thawed semen traits could be improved through selection due to their estimated heritability (h^2 range between 0.08 and 0.15) and its high coefficient of variation. Regarding the study of genetic correlations, it seems that DG was negatively correlated with sperm freezability but due to the high HPD95% no further conclusions could be drawn. In the future, if this genetic relationship is confirmed, it would imply that selection for DG could alter sperm cell membranes or seminal plasma composition, both components related to sperm cryoresistance.

LITERATURE CITED

Barth A. D., Oko R.J. Abnormal morphology of bovine spermatozoa. Ames 1989; ia: Iowa state university press.

Brandt H., Grandjot G. Genetic and environmental effects of male fertility of AI-boar. In: Proc 6th World Congress on Genetics Applied to Livestock Production 1998, Armidale, Australia.

Brun J.M., Sanchez A., Duzert R., Saleil G., Theau-Clément M. Paramètres génètiques des caractéristiques de la semence de lapin. In Proc: 13èmes Journ Rech Cunicole 2009;11: 17-18.

Castellini C., Lattaioli P., Minelli A., Moroni M. Effect of seminal plasma on the characteristics and fertility of rabbit spermatozoa. Anim Reprod Sci 2000;63:275-82.

Druet T, Fritz S, Sellem E, Basso B, Gérard O, Salas-Cortés L, Humblot P, Druart X, Eggen A. Estimation of genetic parameters and genome scan for 15 semen characteristics traits of Holstein bulls. J Anim Breed Genet 2009;126:269-77.

Estany J., Camacho J., Baselga M., Blasco A. Selection response of growth rate in rabbits for meat production. Genet Sel Evol 1992;24:527-37.

García M.L., Cifre J., Viudes de Castro M.P. Comparación entre la inseminación artificial y la monta natural en España. Estudio económico teórico. ITEA 1998;94(A): 81-91.

García M.L., Andrés I., Caselles P., Lavara, R. Estudio de la edad de los machos de conejo en la inseminación artificial. Boletín de cunicultura 2004;135:17-25.

Gianola D., Sorensen D. Quantitative genetic models for describing simultaneous and recursive relationships between phenotypes. Genetics 2004;167:1407-24.

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

Graham J.K. Analysis of stallion semen and its relation to fertility. Vet Clin N AM Equine Pract 1996;12:119-30.

Hirano Y., Shibahara H., Obara H., Suzuki T., Takamizawa S., Yamaguchi C., Tsunoda H., Sato I. Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates in vitro. Journal of Assisted Reproduction and Genetics 2001;18:213-18.

Karoui S., Díaz C., Serrano M., Cue R., Celorrio I., Carabaño M.J. Time trends, environmental factors and genetic basis of semen traits collected in holstein bulls under commercial conditions. Anim Reprod Sci 2011;124:28-38.

Kealey C.G., MacNeil M.D., Tess M.W., Geary T.W., Bellows R.A. Genetic parameter estimates for scrotal circumference and semen characteristics of Line 1 Hereford bulls. J Anim Sci 2006;84:283-90.

Larzul C., De Rochambeau H. Selection for residual feed consumption in the rabbit. Livest Prod Sci 2005;95: 67-72.

Nagy I., Ibañez N., Romvári R., Mekkawy W., Metzger Sz., Horn P., Szendro Zs. Genetic parameters of growth and in vivo computerized tomography based carcass traits in Pannon White Rabbits. Livest Sci 2006;104:46-52.

Oh S.H., See M.T., Long T.E., Galvin J.M. Estimates of genetic correlations between production and semen traits in boar. Asian-Australasian Journal of Animal Science 2006;19:160-4.

Pascual J.J., García C., Martínez E., Mocé E., Vicente J.S. Rearing management of rabbit males selected by high growth rate: the effect of diet and season on semen characteristics. Reprod Nutr Dev 2004;44:49-63.

Purdy P.H. Swine gene banking: A quality control perspective on collection, and analysis of samples for a national repository. Theriogenology 2008;70:1304-9.

Rochambeau H. de, Fuente L.F., Rouvier R., Ouhayoun J. Selection sur la vitesse de croissance post-sevrage chez le lapin. Genet Sel Evol 1989;21:527-46.

Rodríguez-Martínez H. State of the art in farm animal sperm evaluation. Reprod Fertil Dev 2007;19:91-101.

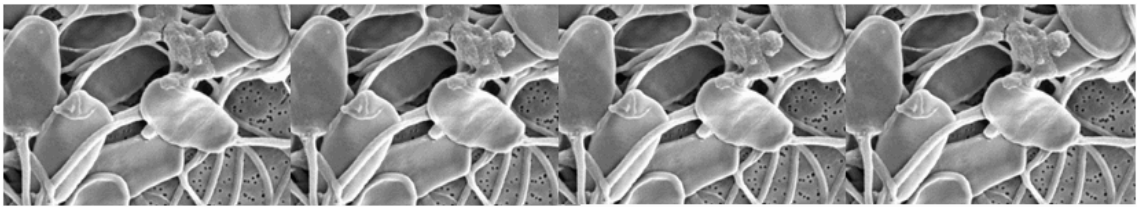
Rosell J.M., De la Fuente L.F. Culling and mortality in breeding rabbits. Preventive Veterinary Medicine 2009;88:120-7.

Smital J., Wolf J., De Sousa L.L. Estimation of genetic parameters of semen characteristics and reproductive traits in AI boars. Anim Repr Sci 2005;86: 119-30.

Tusell L., Legarra A., García-Tomás M., Rafel O., Ramon J., Piles M. Genetic basis of semen traits and their relationship with growth rate in rabbits. J Anim Sci 2012;90:1385-97.

Wolft J. Genetic correlations between production and semen traits in pig. Animal 2009;3:1094-99.

CONCLUSIONS



CONCLUSIONS

The traits related with sperm production and motility in fresh semen showed low heritability, whereas traits related with sperm morphology exhibited moderate heritability.

Regarding the relation between fresh and post-thawed semen, initial ejaculate characteristics such as concentration and motility influence the future freezability of the semen. Season also had the highest impact on post-thaw semen characteristics. Moreover, selection on semen freezability should be effective given the magnitude of heritability estimates.

In addition, there is an apparent tendency for genes favouring increased daily weight gain to slightly decrease sperm quality traits both in fresh and in frozen-thawed semen. However, the uncertainty of the estimates obtained makes it difficult to predict the correlated effect of selection with enough accuracy.