Freezability genetics 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 (percentage of viable sperm after freezing), the estimated heritability is the highest one, and suggests the possibility of effective selection. After the study of genetic correlations it seems that daily weight gain (DG) was negatively correlated with sperm freezability, but no further conclusions could be drawn due to the high HPD95%. 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 imply that selection for DG could alter sperm cell membranes or seminal plasma composition, both components related to sperm cryoresistance.

Keywords: Rabbit-semen; Heritability; Genetic-correlation; Frozen-semen

1.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 [1]. 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 [2, 3, 4, 5], suggesting that sperm freezability has a genetic component. In fact, selection experiments conducted on avian species showed that sperm freezability has a favourable selection response [6].

Recently in rabbits, Lavara *et al.* [7] provide estimates of repeatability for some frozen-thawed sperm traits, indicating that sperm freezability in rabbits could be heritable. Previously, Mocé *et al.* [8], 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. 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.

2. MATERIALS AND METHODS

2.1 Animals and experimental design

Data were collected from 255 males belonging to a paternal rabbit line (Line R), born between 2006 and 2007. Line R is a synthetic line that has been selected since 1980 for daily weight gain (DG) between 28 and 63 days of age by individual selection [9]. This line was formed by crossing a California line with a synthetic line created by mating commercial crossbred rabbits [10]. 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 to 170 days of age. The training was performed for two 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.

2.2 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 [11]. All sperm manipulations were performed at 22°C. The sperm were packaged in 0.25 mL plastic straws (IMV® Technologies, L'Aigle, France), sealed with modelling paste (JOVI, S.A. Barcelona, Spain, NRI 8-6650)and then cooled at 5ºC for 30 min. Cooled temperature was provided storing straws in a refrigerator set at 5ºC. To freeze sperm, straws were suspended horizontally in liquid nitrogen vapour 5 cm 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 12 s.

2.3 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 Mocé et al. [12]. 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 propidium iodide (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 X400 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 X10 negative phase contrast objective. Four microscopic fields were captured for each sample, and then revised and correct manually in order to avoid the possible problems due to sperm granules present in the rabbit semen plasma [13]. 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.

2.4 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 [14]. A set of two-trait analyses were thus performed to estimate the correlations among traits.

The mixed model used for the semen traits was:

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

where $y_{sijopkl}$ is the frozen-thawed semen trait recorded, μ_s is the overall mean, S_{si} 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_{sj} is the systematic effect of ejaculate order with two levels (first and second ejaculate on the same day), T_{so} is the systematic effect of thawing session with 19 levels, P_{sp} 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 $e_{sijopkl}$ 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.

A Bayesian framework was adopted for inference. Denote Ω as the vector including all the unknown parameters in the model. The joint posterior distribution of all parameters for the joint analyses of two traits was:

$$p(\Omega \mid y_{sijopkl}, y_{dijkl}) \propto p(y_{sijopkl}, y_{dijkl} \mid \Omega) \times p(\Omega)$$

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 numerator relationship matrix off all the individuals, **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.

The marginal posterior distributions of the parameters of interest were derived from the joint posterior density of all the unknowns. The Gibbs sampler algorithm was used to estimate the marginal posterior distributions of the systematic effects and the variance-covariance components implemented in the TM software developed by Legarra *et al.* [15]. Details of the fully conditional distributions of the model parameters, can be found in Sorensen and Gianola [16].

After some exploratory analysis, chains of 3000000 iterations 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 [17].

3. RESULTS AND DISCUSSION

Semen characteristics after the freezing-thawing procedures are summarized in Table1, where the dramatic reduction of sperm motility (Rmot=83%) and normal acrosome status (Rnar=74%) can be observed. 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 [8, 12]. Important differences of the present study were the assessment of individual, rather than pooled ejaculates, and the lack of ejaculates pre-selection 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 [1].

3.1 Repeatability, heritability, permanent and common litter effects

Table 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

212 to phenotypic variance (c^2) for frozen-thawed semen. We computed the ratio of the
213 phenotypic variance due to the male effects (or repeatability) as the sum of h^2, p^2
214 and c^2 values. The estimates were moderate, ranging from 0.20 to 0.3, being slightly
215 lower than the repeatabilities of fresh semen traits [18, 19, 20], indicating the existence
216 of important individual variation for frozen-thawed semen traits in rabbits. Little
217 differences in frozen-thawed semen repeatabilities estimates were observed using a
218 subset sample of the present database [7], probably due to differences in the model
219 used and in the number of data. The main difference between studies is the decision
220 of using the information related to the selection criteria, at the present study we
221 included the information related to the selection process in order to have an unbiased
222 estimation of the variance components due to the fact that the DG and the frozen-
223 thawed traits could be correlated.
224 Estimated heritabilities showed that frozen-thawed semen traits are heritable (they
225 ranged between 0.08 and 0.15, Table 3). To our knowledge no previous heritability
226 estimates for frozen-thawed semen traits in rabbits have been reported. The literature
227 estimates of heritabilities for corresponding traits in fresh semen were similar in the case
228 of motility measured with CASA system (0.12-0.18 for Mot,%; [19, 21, 22]) and slightly
229 higher in the case of normal acrosome status (0.18 for Nar,%; [7]). The estimated
230 heritability of Live-FT is the highest one and suggests the possibility of effective
231 selection. To test this hypothesis, a divergent selection experiment should be
232 conducted in order to gain knowledge about the sperm freezabilty in rabbits, as well
233 as to assess cryoresistance biological basis in rabbit semen. After 8 generations of
234 selection in chicken, physiological changes and biochemical differences were
235 reported between the selected line for frozen-thawed semen fertility and control line.
236 Sperm from the selected line had lower cholesterol and lower
237 cholesterol:phospholipid ratio compared with control line, in addition seminal plasma

238 cholesterol and phospholipid levels were lower in the selected line [6]. Regarding the
239 proportions of variance due to the common litter effect, they are lower than the
240 corresponding h^2 estimates. This result is in agreement with those published previously
241 in related fresh semen traits [19].

242 **3.2 Correlations between sperm traits and DG**

243 Estimates of genetic, permanent and litter correlations between DG and traits of
244 frozen-thawed semen are presented in Table 3.

245 Concerning permanent and litter correlations, the estimates were in general lower
246 than the genetic correlation and showed a great uncertainty associated with them.
247 Regarding genetic correlations, the estimates published previously show antagonistic
248 correlations between fresh semen traits as Nar (%) and Mot (%, objectively measured)
249 and DG [19]. In concordance, these traits after the freezing-thawing process must
250 maintain a similar genetic correlation pattern. After the study of genetic correlations
251 it seems that DG was negatively correlated with sperm freezability, but no further
252 conclusions could be drawn due to the high HPD95%. More data should be included
253 in order to obtain better accuracy for the estimates of these genetic correlations. If
254 the results obtained at present study were confirmed, it would imply DG selection
255 could alter sperm cell membranes or seminal plasma composition, both components
256 related to sperm cryoresistance. Therefore, the future knowledge of plasma
257 biochemistry characteristics [23] and mitochondrial activity of sperm cells [24] in this
258 selected line could be of great value.

259 In fact, selection for DG in this rabbit line changed carcass fat levels at the same age
260 compared with lines selected for litter size, and this would affect indirectly lipid
261 membranes in sperm, or cholesterol: phospholipid ratio [25]. Estimates of genetic
262 correlations between different semen traits and selection criteria in rabbits are scarce
263 (for a review see Piles *et al*. [26]), and estimates are generally imprecise making it

difficult to draw reliable conclusions, so in the future more efforts should be done in order to better assess the genetic correlations.

4. CONCLUSIONS

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 uncertainty of obtained estimates difficults to make a prediction about the correlated effect of selection on sperm freezabilty with enough accuracy.

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Table 1: Crude mean and standard deviation for semen traits

	n	Mean	Standard deviation
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 an intact acrosome in frozen-thawed semen; Rnar: relative reduction of spermatozoa with an intact acrosome,%, Rmot: relative reduction of motile spermatozoa, %; Live-FT: percentage of live spermatozoa in frozen-thawed semen, %.

370 **Table 2:** Descriptive statistics of the posterior marginal distributions of heritability (h^2), ratio of permanent variance to phenotypic

371 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	**HPD95%**	**PM**	**HPD95%**	**PM**	**HPD95%**
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]

372 PM: posterior mean. HPD95%: interval of highest density of 95%; Mot-FT: percentage of motile spermatozoa in frozen-thawed semen; Nar-FT:

373 percentage of spermatozoa with an intact acrosome in frozen-thawed semen; Rnar: relative reduction of spermatozoa with an intact acrosome,%,

374 Rmot: relative reduction of motile spermatozoa, %; Live-FT: percentage of live spermatozoa in frozen-thawed semen, %.

375 **Table 3:** Descriptive statistics of the posterior marginal distributions of the genetic
376 (r_g), permanent (r_p) and litter of birth (r_c) correlations of daily gain (DG) with
377 frozen-thawed sperm traits.
378

	r_g		r_p		r_c	
	PM	**HPD95%**	**PM**	**HPD95%**	**PM**	**HPD95%**
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]

379 PM: posterior mean. HPD95%: interval of highest density of 95%; Mot-FT: percentage of
380 motile spermatozoa in frozen-thawed semen; DG: daily gain; Nar-FT: percentage of
381 spermatozoa with an intact acrosome in frozen-thawed semen; Rnar: relative reduction
382 of spermatozoa with an intact acrosome,%; Rmot: relative reduction of motile
383 spermatozoa, %; Live-FT: percentage of live spermatozoa in frozen-thawed semen, %.
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385