

GUIDELINES FOR THE HANDLING OF RABBIT BUCKS AND SEMEN

INTERNATIONAL RABBIT REPRODUCTION GROUP¹

ABSTRACT: This paper contains recommendations for procedures to be used in applied reproduction trials performed with rabbit bucks and/or semen. The factors influencing sperm production (environment, age, health status and feeding strategies) are described as well as the effects of semen handling (dilution, storage conditions, etc) on spermatozoa characteristics. Basic tools for analysing spermatozoa characteristics are given. Procedures, material and methods to evaluate sperm metabolism are precisely defined in an appendix. The present guidelines have been discussed and approved by a panel of experts (see footnote) in the course of various meetings in the context of COST action 848 sponsored by EU.

Key words: rabbit buck, semen, spermatozoa, guidelines.

INTRODUCTION

Semen is a mixture of spermatozoa suspended in a liquid medium, secreted at different locations by the epididymus and various glands, which, at the time of ejaculation, are combined.

Semen evaluation must provide information on the fertilizing capability of spermatozoa. The most important parameters pertaining to fertility are the number of spermatozoa inseminated and their motility (CASTELLINI and LATTAIOLI, 1999; BRUN *et al.*, 2002).

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These seminal characteristics are affected by many factors (breed, feeding, health status, rearing condition, season and collection frequency, etc.) and there is thus a wide variety in semen traits (ALVARIÑO, 2000).

Additionally, semen evaluation is a very difficult topic and differences in laboratory methodologies can introduce substantial variations in the evaluation of sperm parameters (sperm counts, motility and morphology) (WHO, 1999).

The purpose of this paper is to furnish simple and standard procedures for rearing rabbit bucks and for processing semen samples. Only standard procedures will be presented, but obviously many other traits could be investigated for their specific experimental features.

FACTORS INFLUENCING SEMEN PRODUCTION

Different factors such as the collection frequency, lighting programmes, buck age and health as well as feeding strategies, can influence qualitative and quantitative sperm production.

Frequency of collection.

The effect of collection frequency on semen characteristics is considerable and must be recorded in detail. Two ejaculates collected once a week (in a period of at least 15 min) give good semen production results (BENCHEIKH, 1995, MOCE *et al.*, 2000). Since the duration of spermatogenesis is about 7-8 weeks, a minimum period of 10 weeks is recommended to determine the effect of certain exogenous factors.

Light

The length of time exposed to light affects the hypothalamus-pituitary axis and consequently hormonal release and spermatozoa production. A daily constant 16L:8D light program increases sperm production (qualitative and quantitative aspects)

compared with a shorter light duration (8L:16D, THEAU-CLÉMENT *et al.*, 1994).

Age

Sexual maturity occurs approximately at 5 months (depending on the strain) and semen quality generally decreases in older rabbit bucks (> 2 years).

Health status

It has been widely verified that inflammation of male reproductive apparatus (sub-clinical or manifest; O'BRYAN *et al.*, 2000) impairs testicle functions and seminal characteristics by affecting biosynthesis of proinflammatory eicosanoids (prostaglandins and leukotriens) and the release of cytokines (KNAPP, 1990). The health of rabbit bucks must be closely controlled, especially in aging animals.

Feeding strategies

Specific dietary recommendations for rabbit bucks are not available (DE BLAS and WISEMAN 1998), and thus it is quite impossible to indicate guidelines on this topic. However, some suggestions can be given.

Feed quantity. Males are preferentially fed *ad libitum* (LUZI *et al.*, 1996). A commercial diet with low energy content is helpful in order to avoid excessive fattening.

Feed quality.

- *Crude protein.* Diets with more than 15% of crude protein are recommended to ensure adequate sperm production (NIZZA *et al.*, 2000).
- *Fat.* The balanced fatty acid composition seems to be more important than the total amount (WESLEY, 1998). Since a very high amount of spermatozoa lipids are PUFA of n-3/n-6 series and these fatty acids modify membrane fluidity and its competence, it is important that diets provide a sufficient amount (at least 1 %, precursor or elongated).
- *Antioxidant.* The high unsaturation of spermatozoa membrane renders these

cells very susceptible to peroxidation, which degrades membrane structure, physiology and DNA integrity. Antioxidant protection is assured by seminal plasma, which is strongly affected by dietary supplementation. Bucks fed a high level of antioxidants (200 mg/kg vitamin E and 0.5 g/L vitamin C, CASTELLINI *et al.* 2000), have reduced lipoperoxidation in the semen. These trends are more pronounced after semen storage or when diets contain an adequate amount of PUFA.

SEMEN COLLECTION

Rabbit semen is collected by means of an artificial vagina filled with a warm liquid (about 45°C). A doe is fitted with this device and presented to the buck. Some authors reported that previous stimulation of the buck increases sperm concentration, and it may help to previously leave a doe on top of the buck's cage for several minutes.

The type of artificial vagina influences the adaptation of the buck to the collection. A vagina with a wider collection orifice facilitates adaptation. A different artificial vagina should be used for each collection. There is generally a lot of bacterial contamination from the environment and it is important to collect the semen sample under hygienic conditions (MERCIER and RIDEAUD, 1990).

Libido is generally evaluated as the lapse of time from when the doe is placed in the buck's cage to the moment of ejaculation.

SEMEN HANDLING

As a general rule, conditions should be carefully controlled to avoid contamination of the sample (temperature, chemical) during semen handling.

Within 5 min of collection, the semen is diluted (1/2-1/5) with a buffer medium at the same temperature to avoid heat or cold shocks. The transport to the laboratory and the semen handling should be scheduled to avoid comparing samples not collected at a uniform time.

Storage conditions

Temperature. Regarding storage temperature, 15-18 °C is generally a good range (up to 48 h) for rabbit semen. Nevertheless, optimal temperature could depend on the extender used.

Extenders. Two important factors in the control of variations are the medium used to dilute semen and the dilution rate. A comparison of different media showed that any physiologic buffered saline solution is adequate for a very short storage period (≤ 1 h). However, for longer storage times there are differences in the survival capacity of the sperm (SEED *et al.*, 1996). A Tris-buffer is adequate for a storage time of 24-48 hours (see Appendix).

A high dilution rate (more than 1/100) has a detrimental effect on motility and causes excessive dilution of the seminal plasma, which plays an important role (MINELLI *et al.*, 2001) and reduces the kinetic characteristics of spermatozoa.

For analyses that require simulation of the uterine environment, the incubation of semen could be useful.

Incubation. In response to specific demands for semen for further laboratory use or for artificial insemination, a suitable procedure should be chosen to maintain vitality and physiological fertilisation capacity over a defined time interval. Semen should thus be diluted or the spermatozoa separated and placed in the final medium. For both semen dilution and for spermatozoa containing medium, optimal conditions can be ensured by selecting the appropriate buffer systems, adapted to reproductive organs. Carbon dioxide (5%) incubators will provide long term semen/spermatozoa availability.

MACROSCOPICAL EVALUATION

A normal sperm sample has a homogeneous white opalescent appearance. The presence of red blood cells (reddish) or urine (yellow) is easily detected. Dark, yellow or other abnormal semen samples are generally discarded. If present, gel plug should be removed immediately after collection.

Quantity

Volume may be measured. The weight of the semen can be evaluated by weighing the tube before and after collection.

pH

The pH should be measured immediately after collection since it is modified by metabolism.

MICROSCOPICAL EVALUATION

Microscopical evaluation allows an estimate to be made of concentration, motility and the presence of elements other than spermatozoa (other cells, droplets, or particles). A phase contrast microscope is recommended for examination of unstained preparations. Rabbit semen presents a variety of particle quantities, which interferes with the cell count when using a spectrophotometer.

Spermatozoa count

Semen is diluted to a final dilution of 1/100 with a (10 ml 35% v/v formalin in 1 L of 0.9% NaCl) solution to allow counting by haemocytometer (Thoma, Burker or Neubauer chamber).

Live/dead cells

Different protocols of staining (normal or fluorescent) can be used. The percentage of live spermatozoa can be determined by using stains that penetrate cells with damaged membranes.

One of the more useful methods is assessment by fluorescent microscopy with Propidium Iodide and Carboxyfluorescein diacetate, counting at least 200 cells per sample.

Capacitation

Capacitation is a natural process that takes place when spermatozoa reach uterine fluids. It should be measured either by direct or indirect staining techniques, or by measuring the hyperactivated spermatozoa. Hyperactivation of spermatozoa occurs after capacitation and implies several changes to the kinetic parameters of cells. Generally, VCL, ALH, BCF increase, whereas LIN and STR decrease (definitions are given below). The physiological reason for such behaviour is related to the necessity of enhancing movement strength for crossing the zona pellucida of the oocyte.

Acrosome reaction (AR)

The acrosome reaction is an exocytotic process occurring after the binding of spermatozoa to the zona pellucida. To fertilise the oocyte, both capacitation and the AR of the spermatozoa are necessary, but the location and the correct timing of the occurrence are also very important. Premature capacitation or AR causes the loss of the fertilising ability of spermatozoa, thus a low Spontaneous AR (SAR) and a high Inducible AR (IAR) are highly likely (see appendix for further details).

Assessment of sperm motility

Immediately after collection, sperm motility can be assessed visually by the operator, but such evaluation is subjective. Computer Assisted Semen Analysis (C.A.S.A.) systems have been developed for an objective evaluation of the motility pattern. These systems include a phase contrast microscope, equipped with heating stage, connected to a high-resolution videocamera and a computer. Several commercial manufacturers provide C.A.S.A. systems (e.g. Hamilton Thorne, Hobson Sperm Tracking and others). The following kinetic parameters are generally evaluated:

- % motile spermatozoa = $\text{Number of motile sperms} / \text{total} \times 100$;
- VCL = Velocity of the sperm head along its actual curvilinear path or

curvilinear velocity ($\mu\text{m/s}$);

- VSL = Velocity of the sperm head along a straight line or straight line velocity ($\mu\text{m/s}$);
- VAP = Velocity of the sperm head along its average path or average path velocity ($\mu\text{m/s}$);
- LIN = The linearity of the curvilinear path (VSL/VCL),
- STR = The straightness of the path velocity (VSL/VAP);
- % progressive spermatozoa = percentage of spermatozoa with a $\text{VAP} > 40 \mu\text{m/s}$ and straightness $> 80\%$;
- ALH = Amplitude of Lateral Head displacement about its average path (μm);
- BCF = Beat Cross Frequency. The average rate at which the track speed crosses its average path (beat/s);

For better understanding some of them are represented in the Figure 1.

Reliable estimations can be obtained only after the system has been correctly set up and when operational procedures are strictly followed. For each system, a specific set-up must be defined for rabbit semen. The most important operational procedures are: the number of fields analysed, the number of drops of each sample

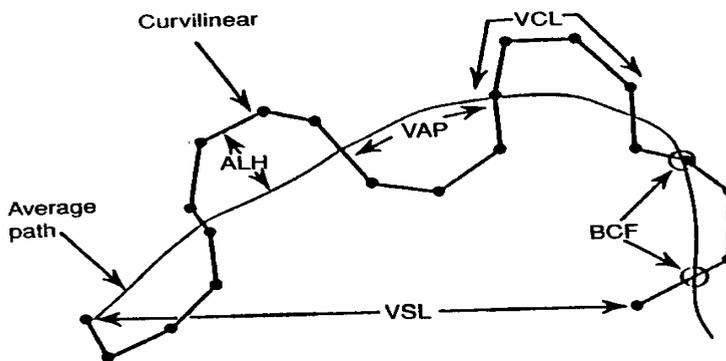


Figure 1: Graphic of the motility of a spermatozoa.

analysed, the temperature of the sample during the analysis and the concentration of the sample. A high concentration of cells per mL and the presence of particles of various sizes, as in rabbit semen, would bias C.A.S.A. estimates. When particles are erroneously counted as dead spermatozoa, the concentration of spermatozoa is overestimated and the percentage of motility is underestimated. An accurate set-up of the C.A.S.A system screens out the particles on the basis of size, shape and brightness (THEAU-CLÉMENT *et al.*, 1996).

MORPHOLOGICAL AND ULTRA-STRUCTURAL EVALUATION

Morphological analysis of the rabbit spermatozoa is essential in order to establish the physiological parameters of the semen; it can be performed by optical microscopic procedures using different staining techniques. The Papanicolaou staining method, suitably modified for sperm, is the most appropriate system for distinguishing different cellular structures and their anomalies (WHO, 1999).

Some other aspects related to the ultra-structure of the sperm, such as the beginning of the acrosome reaction, the maturity-immaturity of the whole cell, the plasma-membrane and the mitochondrial-helix integrity can be precisely determined only by electron microscopy techniques such as Scansion Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

The shape of rabbit sperm is similar to that of other mammals (Figure 2). The dimensions of the ovoid head are about $7 \times 4 \times 0.5 \mu\text{m}$. The length of the tail is $45 \mu\text{m}$. The acrosomal cap is situated on the top of the head for three-quarters of its extent and presents an increase along its edge (Figure 3).

The longitudinal section of the rabbit sperm head shows a very compact nuclear chromatin surrounded by the acrosomal complex. The axonemal complex shows a typical mammalian model composed of 2 central microtubules and 9 peripheral microtubules doublets surrounded by 9 accessory fibres. (Figure 4).

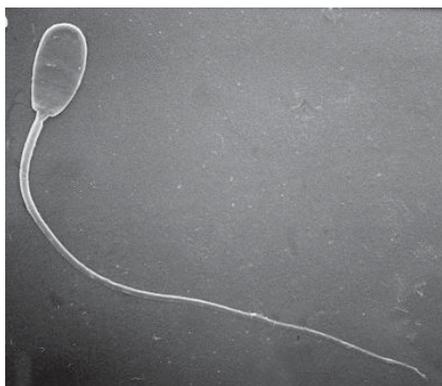


Figure 2: SEM view of a complete rabbit sperm
× 2.600.

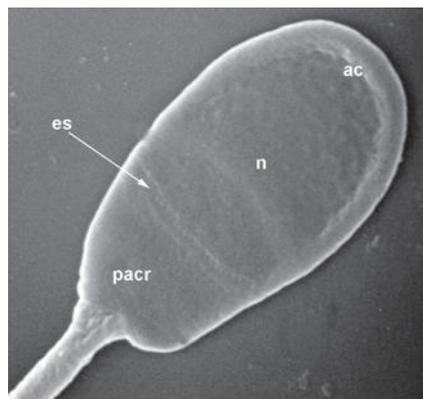


Figure 3: SEM view of Rabbit sperm head:
ac- acrosome, n- nucleus, es- equatorial
segment, pacr- post-acrosomal region.
×10.000.

FUNCTIONAL PROPERTY OF MEMBRANE

The functional property of plasma membrane is an important aspect of sperm biology. It is involved in exchanges with the surrounding medium and plays an important role in the events that take place during fertilisation (capacitation, acrosome reaction, sperm-oocyte fusion). This aspect offers reliable information on the potential fertility of semen and, in association with routine semen analyses (concentration, motility and morphology), enlarges the power of discrimination between different ejaculates on fertility. Other assays evaluating different functional parameters of the spermatozoa (e.g. cervical mucus and oocyte penetration, acrosin activity) are time-consuming and expensive. The Hypo-Osmotic Swelling test (HOS), measures the response of a sperm membrane to a hypo-osmotic medium. It seems that the HOS-test gives information mainly regarding the sperm tail membrane (Ducci *et al.*, 2002). In the HOS-test viable sperms with a functional tail membrane show a curved flagellum, whereas non-viable sperms maintain tail linearity.

CONCLUSION

Although more accurate and sophisticated analyses have recently been developed, to date the relations between the different semen characteristics have not been studied

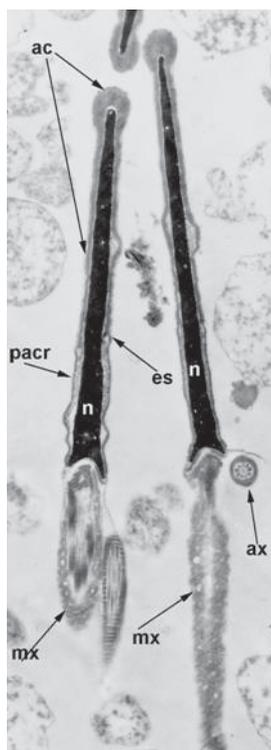


Figure 4: Longitudinal section of the rabbit sperm head ac- acrosome, es- equatorial segment, pacr - post-acrosomal region, mx- mitochondrial helix, ax- axoneme, n- nucleus. $\times 10.500$.

in detail. Nevertheless, the insemination of a sufficient quantity of spermatozoa with the appropriate functional properties (e.g., motility, HOS-test, SAR and IAR) would normally prevent conception failures due to defective semen.

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REFERENCES

- ALVARIÑO J.M.R. 2000. Reproductive performance of male rabbits. *In: Proc. 7th World Rabbit Congr., Valencia Jul., 2000, vol. A, 13-35.*
- BENCHEIKH N. 1995. Effet de la fréquence de collecte de la semence sur les caractéristiques du sperme. *Ann. Zoot. 44, 263-279.*
- BRUN J.M., THEAU-CLÉMENT, M., BOLET, G. 2002. The relationship between rabbit semen characteristics and reproductive performance after artificial insemination. *Anim. Reprod. Sci., 70, 139-149.*

- CASTELLINI C. and LATTAIOLI P. 1999. Effect of motile sperms inseminated on reproductive performance of rabbit does. *Anim. Sci.*, 57, 111-120.
- CASTELLINI C., LATTAIOLI P., BERNARDINI M., DAL BOSCO A. 2000. Effect of dietary α -tocopheryl acetate and ascorbic acid on rabbit semen storage at 5 °C. *Theriogenology*, 54, 523-533.
- DUCCI M., GAZZANO A., VILLANI C., CELA V., ARTINI P.G., MARTELLI F., GENAZZANI A.R. 2002. Membrane integrity evaluation in rabbit spermatozoa. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, 102, 53-56.
- DE BLAS C. and WISEMAN J. 1998 - The nutrition of the rabbit. *CABI Oxon, UK*.
- GLIOZZI T.M., LUZI F., CEROLINI S. 2003. Assessment of sperm viability in boar, rabbit and rooster: a modification of the fluorometric ethidium bromide exclusion procedure. *Theriogenology*, 60, 635-645.
- KNAPP H.R. 1990. Prostaglandins in human semen during fish oil ingestion: evidence for in vivo cyclooxygenase inhibition and appearance of novel trienoic compounds. *Prostaglandins*, 39: 407-423.
- LUZI F., MAERTENS L., MIJEN P., PIZZI F. 1996. Effect of feeding level and dietary protein on libido and semen characteristics of bucks. In: *Proc. 6th World Rabbit Congr., Toulouse, vol. 2, 87-92*.
- MENDOZA C, CORRERAS A, MOOS J, TESARIK J. 1992. Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. *J. Reprod. Fertil.*, 95, 755-763.
- MERCIER P. et RIDEAUD P. Bactériologie du sperme frais de lapin. *Prod. Anim.*, 3(3), 215-221.
- MINELLI A., MORONI M., CASTELLINI C. 2001. Isolation of the IGF-I protein complex from rabbit seminal plasma: effects on sperm mobility and viability. *J. Exp. Zoology*, 290: 279-290.
- MOCE, E.; LAVARA, R; LAVARA, F; VICENTE, J.S. 2000 . Effect of reproductive rhythm on seminal parameters from a rabbit line selected with high growth rate. In: *Proc. 7th World Rabbit Congress. Valencia. July 2000. Vol A: 197-201*.
- NIZZA A., DI MEIO C., TARANTO S. 2000. Influence of dietary protein content on libido and semen characteristics bucks. in: *Proc. 7th World Rabbit Congr. Valencia jul.2000, 217-224*.
- O'BRYAN M.K., SCHLATT S., PHILLIPS D.J., DE KRETZER D.M., HEDGER M.P. 2000. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo. *Endocrinology*, 141: 238-246.
- SEED J., CHAPIN R.E., CLEGG E.D., DOSTAL L.A., FOOTE R.H., HURTT M.E., KLINEFELTER G.R., MAKRIS S.L., PERREAULTYS S.D., SCHRADER S., SEYLER D., SPARANDO R., TREINE K.A., VEERAMACHENENI D.N.R., WISE L.D. 1996. Method for the assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. *Reprod. Toxicol.* 10: 237-244.
- THEAU-CLÉMENT M., MICHEL N., ESPARBIÉ J., BOLET G. 1994. Effect of artificial photoperiods on sexual behaviour and sperm output in the rabbit. *Anim. Sci.* 60,

143-149.

- THEAU-CLÉMENT M., LATTAIOLI P., ROUSTAN A., CASTELLINI C. 1996. Reliability and accuracy of a computerized semen image analyses to evaluate various biological parameters in rabbit semen. *in: Proc. 6th World Rabbit Congr., Toulouse, Jul., vol. 2, 139-143.*
- WESLEY A.J. (1998). Immunonutrition: the role of w-3 fatty acids. *Nutrition, 14: 627-633.*
- WHO (1999). Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. *4th Ed. Cambridge University Press.*

APPENDIX

METHODS TO EVALUATE SPERM METABOLISM
AND PRODUCTION**Composition of more used media**

Components and adequate properties of Tris Buffer.		Components and adequate properties of Tris Buffer.	
Tris	3.029 g	NaCl	5.690 g
Citric acid H ₂ O	1.676 g	KCl	0.231 g
Dehydrate D-Glucose	1.250 g	CaCl ₂ 2H ₂ O	0.294 g
Streptomycin	75.000 IU	MgCl ₂ 6H ₂ O	0.081 g
G-Penicillin	166.200 IU	Na ₂ HPO ₄	0.040 g
Distilled water	To 100 mL	NaHCO ₃	2.090 g
pH	7.14	Na pyruvate	0.022 g
Osmolarity	299 mOsm/kg	Dehydrate D-Glucose	0.900 g
		Lactic acid	3.680 mL
		HEPES	2.380 g
		Streptomycin	75.000 IU
		G-Penicillin	166.200 IU
		Distilled water	To 1 L
		pH	7.40
		Osmolarity	296 mOsm/kg

Alternatively HEPES (biological buffer available in the sigma H7523 or other catalogues) or MOPS (*idem* - M5162, 30-150mM) can be used.

Sperm preparation techniques

The separation of spermatozoa from seminal fluid and from debris is sometimes important for several purposes. Many procedures may be used and the two main methods of separation are:

a) Swim Up - Spermatozoa may be selected on the basis of their vigour in swimming. This procedure permits the selection of the more motile spermatozoa. To date, this procedure is mainly developed for other species; however, it could be useful to gain further information on rabbit semen.

Basically, the procedure is adapted to the use of frozen/thawed semen. One mL capacitation medium is poured into Falcons (conical tubes). Semen is thawed (38-39 °C, 12 s) and 100 µL are carefully transferred to the bottom of the Falcon so that the capacitation medium and the semen extender do not become mixed.

The Falcon tube is placed in an incubator for 45 min (37 °C). Any movement should be avoided. After the swim up technique, 900 µL of the clear capacitation medium are introduced into a pipette and transferred to a tube for centrifugation at 200 g for 10 to 15 min. The liquid phase is removed and the remainder is re-suspended for estimation of spermatozoa concentration.

Centrifugation: For the separation of seminal plasma a gentle centrifugation at 700xg × 10 min at 4 °C or at room temperature is performed. Centrifugations at different density gradients Percoll (35%/70%) are used to separate sperm from lipid droplets (GLIOZZI *et al.*, 2003).

b) HOS-test - The HOS-test is performed by mixing (1:10) 100 mL of sample with 900 ml of 60 mOsmol fructose solution, since the standard sodium-citrate medium showed slight toxicity. After 5' of incubation at 37 °C a drop of 30 mL is smeared on a microscope slide and observed in a phase contrast microscope. A minimum of 200 cells should be observed.

CASA analysis - Operational procedure

The analysis of fresh semen samples should start as soon as possible (never more than 1 hour after collection). If the analysis cannot be carried out within this time limit, the samples must be protected against cold shock and are usually kept at room temperature. Whatever the storage conditions before starting the C.A.S.A analysis, the samples must be maintained for 10 min at 37°C in a water bath.

Motility can be assessed in the undiluted semen, but when the concentration is higher than 50×10^6 sperm/ml, as is the case in rabbit semen, samples must be diluted. A high spermatozoa concentration in the field being processed and the consequent possible cell collisions are likely to induce errors. According to the initial cell concentration a further dilution is performed, to avoid a severe “dilution effect” the final concentration of sample should not be less than 10-15 million cells/mL. For a reliable CASA analysis 40-50 cells per field are processed. A Tris or TALP medium could be used for the dilution of fresh and frozen-thawed samples, respectively.

For fresh and frozen-thawed semen samples the following standard procedure is usually adopted: 10 μ L of diluted material is placed in a preheated chamber (Makler or similar type) at 37 °C. After a 1-min interval, to allow the sperms to settle, the analysis is started. Four different fields per chamber containing at least 200 cells are analysed. As replicates within samples, two successive drops are analysed.

During the time lapse of the analysis a deterioration in the motility pattern can be expected; a compromise between the number of cells to be analysed and the time of the analysis should thus be found.

Lipid oxidation

The most frequently used method to estimate lipid oxidation is that of Thiobarbituric Acid Reactive Substances (TBARS). Lipid peroxidation is induced by incubating 1 mL of semen containing 50×10^6 cells/mL with 0.25 mL ferrous sulphate (0.8 mM) and sodium ascorbate (1 mM) at 37 °C for 1 h. The TBARS value of spermatozoa is evaluated by mixing 1 mL of this reaction mixture with 2 mL of a stock solution containing 15% w/v trichloroacetic acid, 0.375% w/v TBA and 0.25N HCl. The solution is incubated at 90 °C for 15 min and after cooling to room temperature the precipitate is removed by centrifugation at $2,500 \times g$ for 10 min. The samples are read on a spectrofluorimeter using excitation and emission wavelengths of 510 nm and 553 nm, respectively.

Reference values of fresh rabbit semen.

Trait	Standard value
Spermatozoa/ml (nx10 ⁶)	250-600
Progressive motility %	30-90
Volume (ml)	0.3-0.9
pH	7.1
VCL	80-100
VSL	30-50
VAP	50-70
LIN	35-80
STR	40-80
ALH	2.0-6.0

Reference values of fresh rabbit semen

(THEAU-CLÉMENT *et al.*, 1996; CASTELLINI *et al.*, 2000).

Capacitation and acrosomal status

To assess capacitation and acrosomal status, the most frequently used methods are:

Positive staining (detection of sperm with total AR)

- *Concanavalin Ensiformis* Agglutinin - The lectin binds specifically to glycoproteins of the inner membrane.
- GB24 or CD46 antibody - Specific monoclonal antibodies associated with a fluorochrome. They recognise two proteins of the inner membrane.

Negative staining (detection of both partial and total AR):

- *Pisum Sativum* Agglutinin (PSA) - The lectin has specificity toward a-linked mannose-containing oligosaccharides, with a N-acetylchitobiose-linked a-fucose residue included in the receptor sequence. PSA has been used to fractionate cells, to isolate glycoproteins and glycopeptides, to distinguish

between normal and virally transformed cells.

- *Arachis Hypogea* Agglutinin - The lectin specifically binds to glycoproteins of the outer membrane.
- Chlortetracycline (CTC) - CTC may bind the membrane-associated calcium or a plasma membrane protein but the exact mechanism of CTC labelling is still unclear.

Since PSA is one of the more widely used and easily executable methods (MENDOZA *et al.*, 1992) more details on the procedure are reported:

- Pool ejaculate samples and centrifuge at $600 \times g$ for 5 min at 4 °C;
- Suspend the pellet in modified Tyrode's albumin-lactate-pyruvate (TALP) previously equilibrated overnight at 37°C, 5% CO₂, without BSA at final concentration of 10⁶ sperm/mL;
- Drop 5 µL of suspension on a slide to assess the rate of Spontaneous AR (SAR);
- Add 0.3% BSA to induce capacitation and incubate for 30 min at 37°C, 5% CO₂;
- Add to the suspension 100 µg/mL of a-LPC (lysophosphatidilcholine) to induce AR (IAR) on BSA capacitated sperms (15 min at 37°C, 5% CO₂);
- Drop 5 µL of suspension on a slide.

Assessment of slides

- Make a chamber with a silicon pen. Wash rapidly with PBS buffer and permeabilize with cold methanol (-20 °C) for 3 min.
- Wash with PBS for 1 min and add the blocking solution (PBS + BSA1%) for 20 min RT. Add PSA solution (25 µg/mL) in PBS for 45 min in the dark. Wash 3 times with PBS for 5 min. Add a drop of mounting medium and a coverslip. The slides are stored at 4 °C in a box in darkness and analysed with an epifluorescence microscope (excitation filter 335-425 nm). A minimum of 200 cells should be counted.

Samples staining.

ethanol 80%	10 plunges
ethanol 70%	10 plunges
ethanol 50%	10 plunges
Distilled H ₂ O	10 plunges
Harris Haematoxylin	3 min
Running H ₂ O	3-5 min
ethanol 100%	2 plunges
Running H ₂ O	3-5 min
Scott solution	4 min
Distilled H ₂ O	1 plunge
ethanol 50%	10 plunges
ethanol 70%	10 plunges
ethanol 80%	10 plunges
ethanol 90%	10 plunges
Orange G6	2 min
ethanol 95%	10 plunges
ethanol 95%	10 plunges
EA-50	5 min
ethanol 95%	5 plunges
ethanol 95%	5 plunges
ethanol 95%	5 plunges
ethanol 99.5%	2 min
xilene	3 time

Optical microscopy procedures – preparation of samples

The spermatozoa samples must be creped on the microscopy glass, air dried and then fixed in a 50% alcohol 95°/ ether solution for 5-15 min.

After dehydration, samples must be immediately protected with a glass cover using Depex medium as adhesive.

Scansion and transmission electron microscopy (SEM and TEM)

Rabbit spermatozoa should be collected directly into the Karnovsky fixative in a volume ratio of 1/10 semen/fixative. The suspension should be gently stirred with a plastic pipette in order to prevent possible agglomerates due to fixative action on the protein components of the seminal plasma. The time of fixation is 2 hours at 5°C.

After fixation, the sperm-pellet is recovered by centrifugation at $380 \times g$ for 10 min. at room temperature. Excess liquid is discarded. The sperm- pellet is washed in 10 ml of the cacodilate buffer 0.1 M pH 7.2 for 12-24 h. This long washing-time is necessary in order to remove excess fixative from the cells. The sperm sample is recovered by centrifugation at $500 \times g$ at room temperature for 10 min. The sperm is post-fixed with 2-4 ml of OsO_4 1% in cacodilate buffer 0.1 M pH 7.2 for 1 h. Then the sample is washed in 10 ml of the cacodilate buffer 0.1 M pH 7.2 for at least 1 h. The sperm sample can be stored in this solution for 2-4 days at a temperature of 2-5 °C without sustaining damage. The sperm suspension is then divided into two parts: one for TEM and the other for SEM.

TEM. The sperm pellet is recovered by centrifugation at $380 \times g$ at room temperature for 10 min. Excess liquid is discarded. The sperm sample is dehydrated in a series of alcohol (50, 75, 95, absolute) and in a 1,2- Propylene oxide. After each alcohol step, recovery of the sample and propylene oxide is performed by centrifugation at $100 \times g$ for 10 min. After dehydration, the sample is set in a 50% solution of 1.2 propylene-oxide/epon-araldite resin for 12 hours and then centrifuged at $840 \times g$ for 15 min. The sperm sample is placed in pure epon–araldite resin for 12 hours at 2 - 4 °C and then centrifuged at $4000\text{-}2340 \times g$ for 15 min. The sample is embedded in pure epon-araldite resin and polymerized at 45 °C for 48 hours.

SEM. A drop of the sperm sample is placed on a glass slide (1x1 cm square) previously treated with polylysine 1% and dehydrated in a series of alcohol (50, 75, 95, absolute) 10 min for each step. The action of the polylysine prevents the

detachment of the sperm from the glass surface during the dehydration procedures. The sample is placed in a critical point drier in order to remove the residue of alcohol. The dried samples are stuck to the SEM mounting base using conductive carbon cement and gold coated by means of a sputter coater.
