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ALVARIÑO J.M.R.

REPRODUCTIVE PERFORMANCE OF MALE RABBITS

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ALVARIÑO, J.M.R.

Departamento de Producción Animal, E.T.S.I.Agrónomos,
Universidad Politécnica de Madrid, Ciudad Universitaria s/n 28040 Madrid, Spain.

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INTRODUCTION

The control of rabbit reproduction has experienced a great change in the last decade, mainly as a consequence of the development of new techniques such as artificial insemination (A.I.) on a commercial level.

Undoubtedly male rabbits are the basis of the reproductive success, but they have not received the attention they should have, mainly if we consider that one single male is affecting the fertility and prolificacy of about one hundred females when A.I. is performed as a routine in rabbit farms.

This review has been undertaken with the objective of gathering together the most relevant information available in the last decade, although in some cases classic works have been rescued to offer a global vision of knowledge in the physiology of reproduction in rabbit males. Some of the areas reviewed are of practical interest, while others could provide the basis for new developments in the near future.

At the time of the 5th Congress of the World Rabbit Science Association (McNITT, 1992), there was rather sparse literature relating to the male. Since then, a lot of work has been done, so that the information available is much more important and the intention of this review is more to include salient work rather than to provide an exhaustive information.

SEMEN CHARACTERISTICS

2.1) The composition of semen

The ejaculated semen in rabbits comprises the spermatozoa suspended in the seminal plasma. Seminal plasma contains a number of substances secreted by epididymis and accessory glands. This is a liquid containing high concentrations of fructose, citric acid, and also includes inositol, glycerol, ergothioniene, glutamic acid, certain enzymes, proteins, electrolytes and small lipid drops.

The volume of semen varies between 0.3 and 6.0 ml depending on the secretion of accessory glands (gel fraction). Sperm concentration range from 50 to 500 x 10^6/ ml. The pH measured just after semen collection ranges between 6.8 - 8.4 and is a good index to estimate semen quality.

Table 1. Composition of semen in rabbits (HOLTZ and FOOTE, 1978; SETCHELL, 1989; BATTAGLINI *et al.,* 1992).

Parameters	*First ejaculate*	*Second ejaculate*
Volume (without gel fraction) (ml)	0.1 - 1.1	0.2 - 0.4
Volume of the gel fraction (ml)	0.32 - 0.50	0.1 - 0.18
Ejaculates with gel fraction (%)	54	15
Spermatozoa/ml semen (x 10^6)	280 - 1,050	420 - 800
Sperm motility (%)	58 - 90	57 - 87
Motility rate (0 - 5)	2.3 - 3.3	2.0 - 4.8
Distal cytoplasmatic droplet (0 - 5)	0.6 - 1.0	0.4 - 0.8
Sperm agglutination (0 - 5)	1.2 - 2.0	0.8 - 1.6
pH	7.7 - 8.4	
Seminal plasma		
Fructose (mg/ 100 ml)	40 – 150	
Sorbitol (mg/100ml)	80	
Citric acid (mg/ 100 ml)	70 – 200	
Protein (mg/ 100 ml)	4 – 15	
Glycerylphosphorylcholine (mg/100ml)	215 – 370*	
Sodium (mmoles/l)	80 – 140	
Potassium (mmoles/l)	23 – 120	
Phosphorus (mmoles/l)	1 – 3	
Magnesium (mmoles/l)	2 – 4	
Calcium (mmoles/l)	2 – 8	

* Whole semen

2.2) Factors affecting semen composition

2.2.1) Breed and age.

Several parameters, such as the volume of the semen and gel fraction, sperm motility, sperm concentration, morphological alterations, or fructose concentration show high variations among the different breeds (DUBIEL *et al.,* 1985; EL-EZZ *et al.,* 1985). Such differences should be relatively considered because of the high individual variability observed in each breed (Table 2).

The mean volume of semen, mean sperm concentrations, as well as the fertility and litter size at birth are influenced by the age of the bucks. Globally, these parameters increase over time and higher values are observed in bucks of 5 month to 24 months versus older bucks (MIROS and MIKHNO, 1982). A significative effect of the age on sperm concentration, libido, sperm volume, motility and pH was also reported by LUZI *et al.,* (1996) and MINELLI *et al.,* (1999), confirming previous data.

Table 2. Average characteristics of semen in different rabbit breeds (DUBIEL *et al.,* 1985).

Breed	Semen volume (ml)	Gel volume (ml)	Motility (%)	Sperm concentration x10^6	Morphology alterations (%)
Black and Tan	0,68	0.32	54	97.6	19
New Zealand White	0,97	0.13	66	309.6	11
New Zealand Red	0.83	0.79	49	221.7	27
German Pied Giant	1.51	0.27	71	502.5	14

2.2.2) Seasonality of breeding and spermatogenesis.

In some domestic species such as the boar, horse, ram, and rabbit spermatogenesis shows seasonal variations related chiefly to photoperiod and temperature. Wild bucks show a seasonal breeding pattern with the peak activity occurring in the spring. Several studies found bucks with testes exhibiting active spermatogenesis during every month of the year with a peak of fecundity occurring during April, May and June (BOYD and MYHILL, 1987). Changes in the scrotal testis length provide a good indicator of the reproductive status of the male (BOYD, 1985). This parameter may be influenced by changes in photoperiod (BOYD, 1985; BOYD, 1986). GnRH release is influenced by the photoperiod with a higher level of GnRH release in the evening than in the afternoon hours (LIN and RAMIREZ ,1988). Seasonal variations in GnRH release occurred even in bucks maintained in a fixed 12L:12D photoperiod (LIN and RAMIREZ, 1991). There were seasonal changes in the secretion of GnRH with the lowest values occurring just before the winter solstice. The frequency of GnRH pulses increased after the winter solstice, and GnRH release increased during and within one month after the summer solstice (LIN and RAMIREZ, 1991). Testis weight was highest in August and lowest during the winter.

These strong seasonal breeding patterns are not observed in the domestic bucks, while the highest sperm volume and concentrations were found from March to June and the lowest at the beginning of the Autumn. These parameters are also influenced by the local climate (DUBIEL *et al.,* 1985; YAN *et al.,* 1985). Changes in the pH of semen and morphological alterations of the sperm increases during the summer (AMIN *et al.,* 1987). High temperatures (higher than 27 ºC) can affect fertility due to increasing semen pH values and morphological alterations, as well as a decrease in sperm motility and libido (BROCKHAUSEN *et al.,* 79; BAGLIACCI *et al.,* 1987).

2.2.3) Feeding

The libido and sperm output/ejaculate are influenced by the feeding level, but semen quality seemed to be unaffected (LUZI *et al.,* 1996).

Males fed ad libitum showed increased semen volume, spermatozoa/ ejaculate and better libido. However, their sperm concentration (spermatozoa/ ml) was comparable with the males fed with a restricted diet. Excepting the pH, semen quality was not affected by the diet. Only a slight effect on the initial pH was observed.

Severe feed restriction can affect the sperm volume and number of spermatozoa/ ejaculate. This management is not recommended for young males. Males fed ad libitum or with low or high protein levels showed no acrosome defects nor alterations of the live:dead spermatozoa ratio (LUZI *et al.,* 1996).

Recently, MINELLI *et al.* (1999) reported that semen quality was not affected by a dietary supplementation of vitamins C and E. ABD-ELGAWAD *et al.* (1999) described a negative effect on sperm parameters and blood concentrations of LH and FSH of lead and cadmium given in drinking water. Similar results were found by MOORMAN *et al* (1998) after subcutaneous injections of lead acetate. Exposure of rabbit semen in vitro to lead, reduced the fertilizing ability of treated sperm, lowering the fertility rate from 82 to 68% (FOOTE, 1999).

2.3) New quality measurement methods.

2.3.1) Electrical properties of rabbit semen in different dilution media

The electrical parameters for the raw sperm or diluted sperm are correlated with the metabolic activity. Results reported by MICU *et al.* (1996) indicate that for living spermatozoa, the current-voltage characteristics have an accentuated hysteresis and an exponential increase of the current intensity with the applied voltage. That behaviour may be due to the semiconducting properties of the lipid membranes (BHATTACHARYYA *et al*., 1993) and to the negative charge that interacts with the electric field and stimulates the sperm motility (ROFFEY, 1994). For dead spermatozoa, the current intensity may decrease because the spermatozoa lose their negative charge, or some of them may change their type of conductivity.

2.3.2) Water content estimated by electron paramagnetic resonance (EPR).

CASTELLINI *et al.* (1996) proposed EPR to directly estimate the cell water content at different sub-zero temperatures (-5 ºC, -10 ºC). This method is based on the use of a spin label tampon and the broadening agent potassium-chromium oxalate. The determination of the intracellular water showed that a maximum of about 44% of the cellular water is involved in cell dehydration during freezing. These results differ substantially from values obtained with osmotic procedures.

2.3.3) Computerised semen image analyses (HTMA-IVOS Hamilton-Thorn).

THEAU-CLEMENT *et al.* (1996, b, c) proposed a computer-assisted semen

analysis (CASA) to evaluate biological parameters in rabbit semen. First results confirm the accuracy of this method for motility analysis. HTMA concentration correlated to a great extent with the exact total number of cells ($r = 0.999$; $p < 0.001$), determined after a 1:40 dilution rate. HTMA allows access to invisible parameters like the percentage of progressive cells, significantly correlated to individual motility. The interest for HTMA-IVOS will be completely established when high correlation with fertility is obtained.

The CASA has been compared with conventional methods and other new techniques by WABERSKI *et al.* (1999), who concluded that a combination of semen tests gives a better indication of fertilizing ability than a single test. Particularly, the amount of specific plasma proteins is related to fertility, and provides an additional method of semen evaluation.

PHYSIOLOGY OF SPERM PRODUCTION

3.1) Sexual maturity

The differentiation of male reproductive organs takes place during foetal life. Formation of the albuginea occurs between days 14 - 15 of pregnancy. A few days later, the seminiferous tubules appear surrounded by germinal cells. Androgen production starts on day 19 of gestation. The Müllerian ducts regress on day 20, and on day 21 the formation of the prostate begins.

Castration of male embryos on day 19 inhibits the Wolf ducts and induces the development of Müllerian ducts with absence of prostate (JOST, 1965). On day 24 the development of Wolf ducts is well established, and Müllerian ducts regress. The regression of the Müllerian ducts depends on the presence of a testis, and the active principle is apparently testosterone or probably several others testis secretions. The plasma testosterone/ dihydrotestosterone rate increases with age, ranging from 1.7 ± 0.4 ng/ml at birth to 2.6 ± 0.7 in the adult male. Testosterone appeared always as the predominant hormone. Nevertheless, in the rete testis fluid of adult bucks the predominant hormones are testosterone, dehydroepiandrosterone and dihydrotestosterone. These hormones probably play a complementary role in the differentiation of the male reproductive tract (LAU and SAKSENA, 1979). In other species, a glucoprotein with Pm 132,000 secreted by Sertoli cells has been identified. This substance is a Müllerian inhibitory hormone that provokes regression of the foetal oviduct, uterus, and vagina (JOHNSON and EVERITT, 1988).

The impuberal testis is pituitary controled. The multiplication and differentiation of Sertoli cells are gonadotrophin dependant. The testis weight and the number of Sertoli cells which decrease after hypophysectomy is maintained after injection of LH. FSH has a synergistic action with LH. Testosterone is ineffective. The mitotic activity of the gonocytes is more pituitary independent but differentiation into spermatogonia, giving rise to spermatogenetic activity with appearance of the primary spermatocytes, is controlled by gonadotrophins (BYSKOV, 1986).

At birth the testis are situated in an abdominal position descending into the scrotum during puberty. In the wild buck the testis return to the abdominal cavity during sexual inactivity.

Plasma testosterone and FSH reach basal concentrations between birth and 40

days of age, and LH decrease from day 20 (BERGER *et al.,* 1982). The prepuberal stage starts around day 40 and the Leydig cells appears together with a dramatic increase of FSH and testosterone concentrations and a small increase of LH levels. In the prepuberal testis of the hypophysectomized rat, spermatogenesis is stimulated by FSH which also induces the secretion of a specific androgen-binding protein (ABP) by the Sertoli cells (SETCHELL, 1989).

The size of the testis increases steadily until the 8th month of age. A rapid growth of seminal vesicles takes place during this period (MIROS, 1980). The relation between testis weight and body weight changes from 0.89 on the 5th week of age, to 2.89 afterwards. The development of accessory glands shows a similar growth pattern.

Spermatogenesis is established about 70 days of age (appearance of the first meiotic cleavage) but can vary according to breed, environment, and management (BERGER *et al.,* 1982).

The first spermatozoa appear about day 60 of age, although about day 84 all seminiferous tubules are fully active. Differentiation processes are observed from the 4th week of age increasing from the 8th – 10th week. On the 16th week (112 days of age), the differentiation of the epididymis ends, and spermatozoa are observed in the head and body of epididymis (LEESON and LEESON, 1970).

The fructose secretion by seminal gland and prostate increases from the 6th week of age. The secretor activity of accessory glands continues until 1 year of age decreasing the sperm concentration.

The mating behaviour begins on day 60 – 70 of age, and the first mating about day 100 of age. Sexual maturity is reached in New Zealand breed bucks on day 129 (MACEDO and MIGUEL, 1986). In Fig. 1, the chronological development of sexual differentiation is represented.

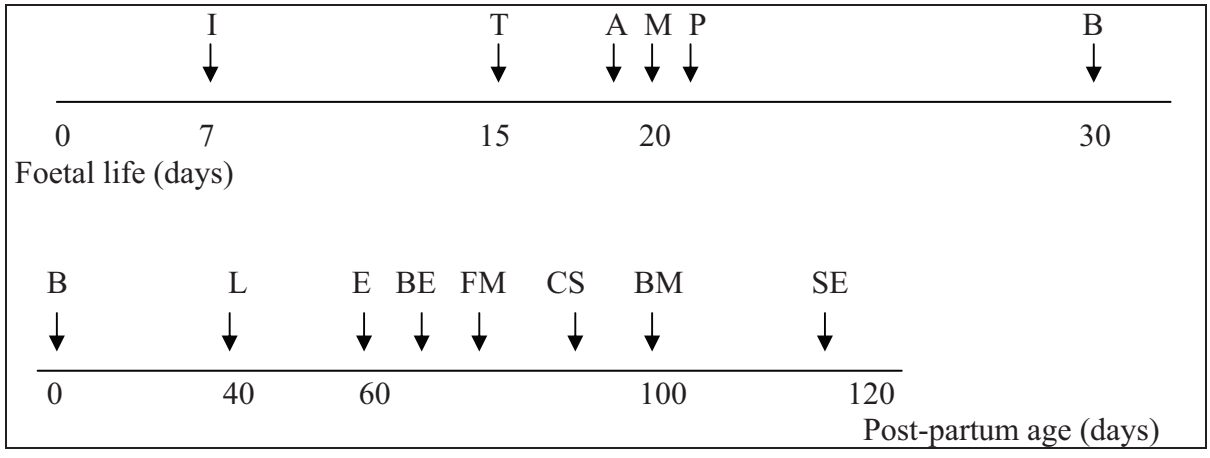

Fig. 1. Chronological development of sexual differentiation in the male rabbit.

I= Implantation; T= Beginning of testis differentiation; A= Beginning of androgen secretion; M= Beginning of Müllerian duct degeneration; P= Beginning of prostate growth; B= Birth; L= Maturation of Leydig cells; E= Appearance of first spermatozoa; BE= Beginning of spermatogenesis; FM= First mating behaviour; CS= Complete development of spermatogenesis; BM= Beginning of mating; SE= Appearance of spermatozoa in epididymis.

3.2) The spermatogenic processes

The total number of spermatozoa produced daily depends upon the rate of development of spermatogenesis. In the adult buck this ranges from 38 to 41 days. The spermatogenic cycle is a biological constant. The time taken for the spermatozoa to pass along the ducts epididymis is about 9 to 10 days in the rabbit (SETCHELL, 1989). The daily spermatozoa production is about 30 to 40 x 10^6/g testis with an average of about 250 x 10^6/day varying among the different breeds (EL-HABBATO *et al.,* 1984).

During the passage through the ducts epididymis, the spermatozoa develop a maturation process (acrosome reduction and loss of cytoplasm droplet). A significant decrease in sperm head size occurs during transit from the seminiferous tubules to the distal kaput of the epididymis, whereas distally the diminution continues at a lower rate and is not relevant during transport and storage within the epididymal cauda and vas deferent (PEREZ-SANCHEZ *et al.,* 1998). The transition from the immobile, infertile spermatozoa which leave the testis, to motile, potentially fertile spermatozoa, occurs at a defined area of the epididymis. The ability of sperm to fertilise is acquired in the distal half of the body of the epididymis, although the proportion of fertile sperm in this zone may still be less than in the tail of the epididymis or the ducts deferent (SETCHELL, 1989).

The capacity for full progressive motility also develops in the corpus epididymis, but in contrast with fertility, motility can develop in rabbit spermatozoa when they are held by ligature in any part of the epididymis. However, spermatozoa held in the caudal retain the capacity for full motility for 30 to 60 days, whereas the motility of those held in the kaput persists for only a few days. Thus, there appears to be a possible separation of the development of motility from that of fertility (SETCHELL, 1989).

The analysis of sperm morphological abnormalities appearing in the male reproductive tract indicates that there must be a mechanism for the disposal of defective spermatozoa, and cells may undergo a form of autolysis within the lumen of the duct (PEREZ-SANCHEZ *et al.,* 1997).

KUZMINSKY *et al.* (1996) produced an illustrated guide for the classification of rabbit sperm abnormalities, which reach a mean value of 18.2%, corresponding 2.9% to the head, 13.6% to the tail, and 1.7% to broken spermatozoa, although variability was very high.

The survival of spermatozoa passing through the epididymis depends on normal androgen production by the testis. Castration or hypophysectomy causes the death of the spermatozoa, but injections of testosterone can keep them motile and fertile.

In the adult testis, LH acts through androgenic secretion. Androgen receptors are present in seminiferous tubules (SETCHELL, 1989). Moreover, ABP transports the androgens to the germ cells. The seminiferous epithelium secretes inhibin, a protein which regulates FSH. This secretion is negatively related to spermatogenic activity (BERGER *et al.,* 1982). In conclusion, although the rate of speed of spermatogenesis cannot be affected by hormones such as gonadotrophins or androgens, the number of germ cells produced during spermatogenesis is influenced by these hormones.

The spermatozoa leave the testis in the rete testis fluid which carries them into the kaput epididymis. There most of the fluid is reabsorbed and their further movement along the duct seems to be unaffected by ligation of the efferent duct. It seems likely that the contractile activity of the muscle surrounding the epididymal duct is responsible for their movement within the epididymis. The transit time of spermatozoa through the epididymis is about 8-10 days in the rabbit. There is a slight decrease in the transit time in animals which are ejaculating frequently but this reduction is usually only about 10 to 20% of the total transit time (BEDFORD, 1975).

The epididymis is a main spermatozoa storage as can be seen in Table 3. The total spermatozoa storage is about 1.000 x 10^6 spermatozoa, distributed as follows: 275 x 10^6 in the kaput and the body, 650 x 10^6 in the tail of the epididymis and 50 x 10^6 in duct deferent. Nevertheless, the storage of spermatozoa in the epididymis tail varies depending on the sexual activity and daylight duration. A total amount of 1,500 x 10^6 spermatozoa in a buck with long sexual inactivity may decrease to 500 x 10^6 spermatozoa after various consecutive ejaculations.

Table 3. Number of spermatozoa in different parts of the male reproductive organ (HOLTZ and FOOTE, 1978)

Organ	*No. of spermatozoa (x 10^6) during*	
	Sexual inactivity	*Sexual activity*
Testis	704 ± 498	581 ± 258
Kaput of epididymis	243 ± 88	218 ± 87
Body of epididymis	31 ± 15	41 ± 18
Tail of epididymis	649 ± 286	740 ± 359
Deferent duct (proximal)	48 ± 34	15 ± 10
Ampulla	8 ± 10	5 ± 6
Accessory glands	0	0

FACTORS AFFECTING REPRODUCTIVE PERFORMANCE

4.1) Buck management

LÓPEZ *et al.* (1996, a) reported improvement of semen production when males were grouped together 3 hours before semen collection. This technique revealed to be a simple and effective method to enhance the volume of ejaculates and sperm motility. This management enhances sexual behaviour and probably stimulates the secretion of accessory glands increasing the volume of ejaculates. Previously, a relationship was observed between activation of sexual behaviour and a higher volume of ejaculates (HOLTZ and FOOTE, 1978). The sperm motility may be enhanced by a modification in the seminal fluid composition. Similarly, higher number of live spermatozoa were found in male rabbits stimulated before semen collection (HOLTZ and FOOTE, 1978).

Since sperm production is highly variable between bucks, as well as, between successive ejaculates from the same male, the frequency of ejaculation revealed to have a great effect on the quality of semen, the reproductive performance of the male and the buck-doe rate in the farm (LÓPEZ *et al.,* 1996, a; BUNACIU *et al.,* 1996; BODNÁRK

et al., 1996).). The sperm volume, sperm concentration and number of seminal doses decreased from the 2nd ejaculate when 4 ejaculates were taken in the same day, but were not affected when semen collection was separated (2 + 2) in two consecutive days (LÓPEZ *et al.,* 1996, a).

Intense mating rhythm causes spermatogenesis alterations with a high proportion of immature spermatozoa and low fertility results. Good sperm parameters and fertility rates were obtained after 3 times/week mating frequency and one rest day after each double mating (BUNACIU *et al.,* 1996; BODNÁRK *et al.,* 1996).

4.2) Hormonal treatments

Male rabbits with low libido and poor sperm production are usually observed among rabbitries. Several authors reported improvement of these animals after treatments with GnRH or HCG (HSU *et al.,* 1987, REBOLLAR *et al.,* 1998), or prostaglandins (EL-GAAFARY *et al.,* 1991). These hormones are also administered to stimulate steroid secretion and spermatogenesis in young bucks. More seminal doses/ ejaculate were obtained in New Zealand x California breed bucks aged 4-5 months after weekly administrations of GnRH during seven weeks, compared to HCG treatment (REBOLLAR *et al.,* 1998).

MARAI *et al* (1998) reported that GnRH administration once a week increased total sperm output of heat-stressed low fertile male rabbits, as well as kindling rate and litter size. The effects of GnRH were greater in the summer than in winter.

4.3) Dilution rate

The attempt to determine the lower limit of sperm concentration when A.I. is performed with 24 hour stored semen hasn't led to a clear conclusion. According to ALVARIÑO *et al.* (1996, a), on a commercial scale, a dose of 20 million negatively affects fertility and a dose of 14 million also negatively affects prolificacy. When fresh semen is employed, it seems clear that the reduction from 60 million to a half of this does not affect either fertility or prolificacy but the reduction to a third, which means 20 million spermatozoa/dose lowers the conception rate by 12%. Thus, the lowest advisable limit should be over 20-million spermatozoa/ dose, which differs greatly from other proposals (HELLEMANN *et al.,* 1979; WEITZE, 1 981; ROUSTAN, 1982; BATTAGLINI *et al.,* 1992; PIZZI *et al.,* 1996) but agrees with Adams results (ADAMS, 1972).

PIZZI *et al.* (1996), consider that l5 x 10^6 motile spermatozoa/ml (7.5 x 10^6 per dose) seem to be enough to reach a high fertility level, the use of doses containing a higher number of motile spermatozoa having little influence on the percentage of pregnancy. Values under 5 x 10^6 motile spermatozoa/doses, are likely to reduce the reproductive performance.

The number of motile spermatozoa/dose needed to obtain a threshold value of 95% of the maximum fertility, depends on the sexual receptivity of females, being 11 million for receptive and 13 million for non receptive does. On the contrary, the

physiological state doesn't affect litter size results, and only 4 million are necessary to reach the threshold value (CASTELLINI and LATTAIDI, 1999).

A dose of 12 million spermatozoa refrigerated at 16-18 ºC for 26-30 hr showed a similar fertility rate to 6 million preserved 0-4 hours (VIUDES de CASTRO *et al.,* 1999).

The reduction from 12 to 4 million spermatozoa/dose did not affect fertility (90%) nor the number of implanted embryos (10.7), although a further reduction to 2 or 1 million provoked a lowering of these parameters (VIUDES de CASTRO *et al.,* 1997).

REMOIS *et al.* (1996) studied the effect of a dilution rate of 1:5 or 1.10. Semen dilution factor 1: 10 induced on the average lower litter size, without affecting birth rate in comparison with dilution 1: 5. Although no general recommendations could be put forward because of variability of results between the weeks.

According to LÓPEZ and ALVARIÑO (1998) the mean litter size at birth is less sensitive to low semen concentrations as only a negative effect was detected at a 14 and 8 million spermatozoa/dose. Although structural damage of spermatozoa linked to cooling was not evaluated, authors assume that the number of intact spermatozoa is well above 0,3 million, reported previously as enough to get satisfactory results in A.I. (WEITZE, 1981).

FARREL *et al.* (1993) reported that the minimum sperm numbers required for normal fertility were 0.05 million, and 0.1 million for normal litter size.

Probably the lowest advisable dose is not the same for different breeds and selected strains of rabbit bucks, so that specific study should be performed in each case. The determination of the optimal spermatic concentration could help to reach a more efficient utilisation of the bucks, and to necessitate a lower number of them in the rabbitry, thus being of clear economical and practical utility.

4.4) Addition of sperm stimulating substances

The motility and metabolic activity of sperm stored for large periods has been increased in several species by adding cAMP, calcium, prostaglandin, oxytocin, adrenaline, or caffeine (SIMPSON and WHITE, 1987; EL-GAAFARY, 1987; DAADER *et al.,* 1989; EL-GAAFARY, 1994; ADB EL-KARIM *et al.,* 1998). Several studies have indicated that cyclic AMP and calcium either separately or synergistically regulate the motility of mammalian sperm (GABERS *et al.,* 1971; PETERSON *et al.,* 1979). Although addition of C-AMP or phosphodiesterase inhibitors (e.g. caffeine, theophylline, papaverine) appears to stimulate sperm motility, added calcium can have positive or negative effects depending on the concentration and species (DAVIS, 1978).

Intratesticular injections of oxytocin or adrenaline given to low fertility males increased libido, ejaculate volume, sperm motility, sperm concentration and litter size (ADB EL-KARIM *et al.,* 1998).

Increased sperm motility was observed after adding 1 ml of caffeine (10 mM/l)

to a pool of semen stored for 24 hour at 18 ºC, although this increment was not associated to improved fertility or prolificacy rates in farm conditions, when performing AI. (LÓPEZ and ALVARIÑO, 2000). Semen stored for longer periods (72 –96 hr) was unable to react to added caffeine. The number of live kits /litter, and the litter size at birth, was reduced when using higher caffeine concentrations (100 mM/l) (LÓPEZ and ALVARIÑO, 2000). These results could not confirm the positive effect of caffeine reported previously by BODNAK (1998) on a low number of females. According to this author, caffeine salicylate improved conception rate and increased the rate of new-born male rabbits by 15%.

Prostaglandins added to the semen can affect fertility. Addition of prostaglandin $F_2 \alpha$ depressed sperm motility and kindling rates probably due to changes in the acrosome affecting the release of the enzymes (EL-GAAFARY, 1994; ALVARIÑO and REBOLLAR, 1991). The addition of high doses of prostaglandin $F_2\alpha$ to the semen resulted in a rise in the number of sperm with damaged acrosomes, and an increased release of several acrosomal enzymes (EL-GAAFARY *et al.,* 1991). Addition of prostaglandin E_2 caused an increase of fertility, probably due to the activation of uterine motility and a faster sperm transport to the ampullas (ALVARIÑO and REBOLLAR, 1991).

4.5) Sex preselection

Intact, viable X and Y chromosome-bearing rabbit spermatozoa can be separated according to DNA content with a flow cytometer/cell sorter, attaining purities of 86% for X-bearing, and 81% for Y-bearing sperm populations. After insemination with X-bearing sperm, 94% of offspring born were females (JOHNSON *et al.,* 1989).

According to McNUTT and JOHNSON (1996), flow-sorted sperm are capable of fertilizing mature oocytes under in vitro conditions, although flow sorting seems to interfere during early embryonic and foetal development, reducing the speed of early division and the number of foetus over time.

High-speed cell sorting technology has improved the yielding by a new sperm orienting nozzle, producing up to 6 million sperm per hour (90% purity) or 20 million (75-80% purity) (JOHNSON and WELCH, 1999; RENS *et al.,* 1999).

SPERM PRESERVATION

Rabbit semen conservation is to date, one of the main problems for a wide utilisation of artificial insemination. Rabbit semen is more sensible to hypertonic solutions compared to other species (CASTELLINI *et al.,* 1992), and to cryoprotective agents containing glycerol (MAURER *et al.,* 1976; ARRIOLA, 1982). To date, several steps towards an effective cryoprotective substance have been given, so freezing could soon be a method to preserve valuable semen from selected bucks. Several techniques have been developed to keep diluted semen during short periods of time, usually up to 48 hour at 5 to 25 ºC. Most extenders are based on Tris-citric acid combined with egg yolk, which acts as a protective agent (SINKOVICKS *et al.,* 1983; MERCIER and

RIDEAUD, 1992). Dimethylsulphoxide, ethylene glycol or acetamide showed low toxicity (HANADA and NAGASE, 1980; CHEN *et al.,* 1989, b) showing good sperm motility at 20 ºC (HANADA and NAGASE, 1980). Glycerol revealed no toxic effects on diluted semen at least under 5% concentration (CASTELLINI *et al.,* 1992).

5.1) Short term storage

5.1.1) Effects of short term storage on spermatozoa morphology and motility

LÓPEZ *et al.,* (1996, b) showed that it is possible to preserve rabbit spermatozoa during 24 hours at a wide range of cooling temperatures (6 to 25 ºC), which is in agreement with previous works (SINKOVICS *et al.,* 1983; BONANNO and COSTANZO, 1985; BATTAGLINI *et al.,* 1988; FREYCHAT *et al.,* 1989; THEAU-CLEMENT and ROUSTAN, 1991; FACCHIN, 1992; ALVARIÑO, 1993).

Whereas GOTTARDI, (1993), and BERGONZONI and ZAMBELLI, (1994) showed better motility and acrosome integrity at 15 ºC. LÓPEZ *et al.* (1996, b) obtained best A.I. results at 18 and 19 ºC, being 18 ºC the preservation temperature recommended.

Motility at 24 hours is greatly reduced in relation to the observation made immediately after semen dilution. It seems clear that relatively high cooling temperatures (25 ºC) show higher motility than low ones (6 an 11 ºC), but it has no practical consequences as no correlation has been found between motility after 24 hours and fertility (LÓPEZ *et al.,* 1996, b).

Only pooled ejaculates showing a motility score under 1 (in a scale 0 to 5), provoked a lower conception rate, although results were not disastrous and litter size was normal. That means that apparently dead spermatozoa are only in a sleeping state and are reactivated once inside the female genital tract (LÓPEZ *et al.,* 1996, b).

No differences were observed in the seminal parameters between fresh and refrigerated semen at 16-18 ºC for 26-30 hours (VIUDES de CASTRO *et al.,* 1999).

5.1.2) Effects of storage on fertility and litter size

The extender MA24 (Spanish patent no. 2106686) based on glucose, fructose, EDTA and sodium citrate, has been tested at the commercial level.

Globally, the performance of the A.I. with 24 hour stored diluted semen using the MA 24 extender has been satisfactory, without any decrease in fertility or prolificity. The fertility reached an average rate of 80% and litter size was 8.5 in lactating does (ALVARIÑO *et al.,* 1996).

Fertility was significantly affected by cooling temperature, reaching the highest values in a range of 17 to 19 ºC, and decreasing at lower (15, 11 and 6), or higher (21 and 5 ºC) cooling temperatures (LÓPEZ *et al.,* 1996, b). This could be explained by the effect of temperature on motility, acrosomal damages and depletion of endogenous energetic reserves. Although metabolic activity slows down, temperatures under 17 ºC

are not beneficial to rabbit spermatozoa preservation because acrosomal damage increases (GOTTARDI, 1993). Temperatures over 19 ºC would have a negative effect because of a lower acrosome integrity (GOTTARDI, 1993) probably associated to a partial alteration of the ability to employ exogenous energetic resources.

Litter size was affected by cooling temperature. The highest value was obtained at 18 ºC. Mortality at birth was increased at 6 and 11 ºC compared to higher semen preservation temperatures (LÓPEZ *et al.,* 1996, b). A similar increase in birth mortality was reported in PMSG stimulated does (MAERTENS and LUZI, 1995), which suggest that some mechanisms could exist relating gamete quality and birth mortality.

VIUDES de CASTRO *et al.* (1999) did not find differences in fertility and prolificacy between fresh and refrigerated semen at 16-18 ºC for 26-30 hours.

LÓPEZ and ALVARIÑO (1998) reported three marked periods of performance of the MA 24 extender. In the first period, between 2 and 48 hours, means of fertility are near to or higher than to 80%. The second interval, between 48 and 72 hours of conservation, reflects an important decrease of fertility, with a reduction of 12 points. Periods of conservation longer than 72 hours led to a decrease of fertility (around 40 %) unacceptable from an economic point of view. These results agree with previous data, as LAZZARONI *et al.* (1992) reported for periods of conservation of 50, 62 and 72 hours, values of fertility of 51, 60 and 45 % respectively, using the same extender and identical conservation temperature.

PERRIER *et al.* (1998) have obtained similar fertility and litter size after preserving semen diluted in the commercial diluent GALAP, for 72 hours at 18 ºC.

In a work carried out by ANSELMINO and TOMATIS (1989), using an extender of non published composition, acceptable values of fertility are presented for the period between 24 and 72 hours (71.1%), or between 72 and 108 hours (61.1 %), although the number of inseminations carried out with each group was relatively low (52 and 18 respectively).

According to LÓPEZ and ALVARIÑO (1998), the number of total born kits was higher than 8 and similar for periods of conservation up to 48 hours, but at 72 and 96 hours a decrease of 1 kit or 3 kits per litter was observed, respectively. ANSELMINO and TOMATIS (1989), reached 7.5 with a conservation interval between 24 and 72 hours, and 6.9 kits after 72-108 hours.

The readiness of an extender able to maintain rabbit semen during periods of time longer than 72 hours would be of great utility, allowing the integration of farms with breeding females and fattening rabbits on one hand, and semen production centres on the another. With this objective at commercial level, the search for new products has been intensified, with launchings of new extenders (MARTINEZ, 1996). At the moment, the extenders for rabbit semen are working well between 1 and 3 days, although diverse centres are working to increase the time of conservation. Thus, a new organisation system, based on transportation of rabbit semen from specialised centres to the all female rabbit farms, could be set up (LÓPEZ and ALVARIÑO, 1998).

5.2) Freezing techniques

Freeze preservation requires extenders capable of maintaining the vitality and fecundant capacity of spermatozoa submitted to several types of management and very low temperatures. Semen extenders must provide an osmotic pressure isotonic with spermatozoa, a proper balance of minerals essential to the life of sperm cells, energy source for sperm metabolism, buffering capacity against metabolic products, be free of, and protect sperm from bacterial or infectious organisms, and finally, provide protection for sperm cells against cold shock. In addition, semen extenders must contain cryoprotective agents to protect the sperm cells during freezing. These agents include: DMSO (dimetylsulfoxide), glycerol, etilenglycol, acetamide, or lactamide.

Generally, dilution rates range between 1:5 and 1:10. Semen is packaged in straws, frozen and stored in liquid nitrogen at –196 ºC.

One of the more common semen extenders was described by STRAZINGER *et al.* (1971). The general method includes the following steps:

1) Dilution rate 1:4
2) Progressive cooling until 5 ºC during about 3 hours.
3) Addition of glycerol 6% 30 minutes before freezing.
4) Packaging of diluted and cooled semen in: a) glass ampoules containing 0.5 – 2.5 ml of semen, b) polyvinyl chloride straws, c) plastic straws.
5) Freezing in nitrogen steam during 6 – 10 minutes
6) Storing in liquid nitrogen.

Thawing is performed during 20 seconds using a thawing solution at 37 ºC (ANDRIEU, 1974).

A combined dilution was proposed by ANDRIEU (1974) consisting in predilution at 35 ºC with Strazinger extender, followed by cooling at 5 ºC and dilution with the NAGASE and GRAHAM (1964) extender containing lactose, glycerol and egg yolk. This method could permit the initial addition of an extender containing DMSO and egg yolk without glycerol, which at 32 ºC is toxic for spermatozoa, although it is basic as a cryoprotective agent.

Amides have been employed as cryoprotective agents, as the damage of spermatozoa decreases during freezing and storing, also sperm motility is not so high when using DMSO combined with glycerol (CHEN *et al.,* 1989; HANADA and NAGASE, 1980).

COURTENS and THEAU-CLEMENT (1996) analysed the ultrastructural morphology of rabbit spermatozoa during the steps of freezing and thawing, using either the techniques devised by ANDRIEU and COUROT (1976) or CHEN *et al.,* (1989). The main cellular alterations resulted from osmotic problems. Andrieu and Courot diluents are hyperosmotic and induce cell dehydration both before and during freezing. When thawed, spermatozoa are more sensitive to osmotic shock and many plasma membranes and acrosomes are damaged. CHEN *et al.* (1989) diluent is physically hyperosmotic, but biologically hypoosmotic. It induces membrane swelling and

breakage before thawing. The cells are dehydrated during the freezing step and are rehydrated at thawing. Due to the higher hydration in the frozen stage, many ice crystals damages are present. According to the technique used, cellular damage appears respectively before and during freezing (CHEN *et al.,* technique), or during thawing (ANDRIEU and COUROT, technique). The thawing temperatures (37 ºC or 45 ºC) are equivalent for main morphology but more plasma membranes are intact after thawing at 45 ºC.

A simple freezing extender for freezing rabbit semen from selected males, based on sucrose 0.5M and DMSO 1.75M, has been proposed by VICENTE and VIUDES de CASTRO (1996), obtaining a post-thawing motility of 42% and normal acrosoma rates of 66%.

5.2.2) Reproductive performance with frozen semen

Generally, experiments concerning the comparison between fresh and deep frozen semen, show that freezing significantly affects fertility and prolificacy (BATTAGLINI *et al,* 1988; FARGEAS, 1995). Nevertheless, some authors do not agree but generally register reproductive performances on a quite low size sample (GRASER, 1978; CHEN *et al.,* 1990). Satisfactory storage of rabbit semen has not yet been reported on a large scale.

Acetamide cryopreservant, used along with the rapid thawing procedure, was more effective in preserving the proportion of progressively motile spermatozoa than the combination of glycerol and DMSO (CHEN *et al.,* 1989, a). This supports the observation by HANADA and NAGASE (1980) that acetamide was more protective than glycerol for rabbit spermatozoa during freezing. Rabbit spermatozoa frozen in an egg-yolk-acetamide extender were as fertile as unfrozen spermatozoa extended in the same medium.

According to THEAU-CLEMENT *et al.* (1996, a) on receptive does, deep frozen semen preserved five years in liquid nitrogen, can lead to good reproduction performance. Fertility in this study was slightly lower than that obtained by ANDRIEU and COUROT (1976) (84 Vs 66%), but litter size at birth was higher (6.9 Vs 9.1).

It seems that 5 years storage does not affect the fertility ability of the semen as concluded by MAURER *et al.* (1976), GRASER (1978) and WEITZE *et al.* (1982) on shorter periods (12 to 15 months). Nevertheless, freezing causes cellular damage respectively before, and during freezing, or during thawing (COURTENS and THEAU-CLEMENT, 1996), leading as suggested by MAURER *et al.* (1976), to fertilisation failures and important embryo losses before implantation. Effectively, after "in vitro" culture, these authors showed that embryos coming from frozen semen were smaller than others.

Freezing increased the number of lost or damaged acrosomes, but insemination at different times, in relation to ovulation time, did not indicate that the time required to capacitation of sperm was reduced by freezing and thawing. Fertility declines similarly in does inseminated at the expected time of ovulation, compared with inseminations performed 10 hr before ovulation (CHEN *et al.,* 1989, b). This contrasts with the results of PARRISH and FOOTE (1986) who found, with competitive fertilisation, that frozen

sperm inseminated 5 hr before expected ovulation, produces more embryos than frozen sperm inseminated 10 hr before expected ovulation, whereas the highest fertilisation rate was achieved with fresh semen placed in the female tract 10 hr before ovulation.

It has been suggested that freezing sperm may affect the capacitation time, but in rabbits the work of CHEN *et al*. (1989, a) could not be conclusive about that, although it was clear that there are differences among bucks.

VICENTE and VIUDES de CASTRO (1996) tested the morphological normality and viability of embryos recovered after insemination with frozen semen in a DMSO sucrose extender. It was found that normal embryos obtained for donor does was similar to data from fresh semen (8.9), as well as survival after vitrification (52% foetuses at 29 days of gestation).

A work studying the effect of dilution rate with frozen-thawed semen, showed that the conception rate and litter size at birth was maximum for a dilution rate of 1: 10, but decreased for a dilution rate of 1:25, 1:50 or 1:100, the litter size being especially affected (THEAU-CLEMENT and ROUSTAN, 1982).

The number of motile inseminated cells greatly vary between authors: from 1.6 (CHEN *et al.,* 1989, b; CHEN *et al.,* 1990) to 7 to 26 10^6 (ANDRIEU and COUROT, 1976). In this experiment, the minimum and the amplitude were higher (9 to 65 10^6), and this can perhaps explain the lack of significant correlation between the fertility or the prolificacy and the number of inseminated cells alive. Consequently, this suggests that decreasing the number of inseminated cells is possible without affecting the reproductive performance of the does, but further studies are necessary to precise the optimal number.

SUMMARY

The above mentioned data are the output of much effort and research, and indicate that there exists a strong possibility of improving the efficiency of rabbit industries through application of the recent knowledge in the collection, analyses, dilution and storage techniques of sperm manipulation. At the same time special attention should be paid to the individual influence of males, not only on the characteristics of semen, but also on the reproductive performance after AI. In the future, the availability of selected bucks on the basis of prolificacy or growth rate, linked to improved freezing techniques will surely offer new prospects in the control of rabbit reproduction.

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