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The application of reproductive technologies to natural populations of red deer

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Contents

Over the past decade, there has been increasing interest in the application of reproductive technology to the conservation and management of natural populations of deer. The application of ART within natural population of deer is in its infancy. However, its future potential is enormous, particularly in relation to genetic management or conservation. This paper reviews the present state of such technologies for a wild subspecies of red deer, the Iberian red deer, by discussing the major components of oestrous synchronization, semen collection/cryopreservation and insemination techniques. In addition, findings made during the course of natural populations studies have enormous potential for the understanding of novel reproductive mechanism that may not explain during livestock animal or human studies. Finally, the results of these studies are also reviewed here.

Introduction

Rapid expansion of the red deer (*Cervus elaphus*) farming industry around the world within the last 35 years has been accompanied by equally rapid development and adoption of a number of assisted reproduction technologies (Asher et al. 2000). These assisted reproduction technologies (ART) have not only facilitated increased rates of genetic improvement on individual farms, but have also allowed widespread movement of genetic material around the world, have been implicated in genetic rescue of rare genotypes/individuals, and have allowed farmers and researches to cross species boundaries in the production of potentially useful hybrids (Asher 1998; Asher et al. 2000). The principle tools that have been used, within the red deer farming, include artificial insemination (AI), multiple ovulation embryo-transfer (MOET) and in vitro embryo production (IVP).

If one excludes reindeer, red deer and their hybrids with wapiti/elk are by far the most widely farmed species. In New Zealand, there are now around 2.6 million farmed deer, of which 85% are red deer and the balance hybrid red deer (Fletcher 2001); there are also small

numbers of farmed fallow deer. During 2000, this industry exported deer products worth $81 million US, of which venison contributed $60 million and velvet $11 million; the balance was made up of hides and other co-products (Fletcher 2001). Demand for New Zealand farmed venison was exceptionally high in Europe in 2001 and the New Zealand deer farmer experienced prices 35% higher than at the same time in 2000, and 63% higher than in 1999 (Fletcher 2001). These figures are especially impressive given that this industry has been developed entirely in the last 35 years in a nation of only 3.5 million people, and receiving no agricultural support. Other national farmed deer herds are much smaller than New Zealand's.

With such a rapid build up of farmed deer herds, there has been economic pressure for deer breeders to purchase specific bloodlines based on heavily promoted, phenotypically high-performing individual animals. By the mid 1980s, artificially insemination with cryopreserved semen was commercially available to New Zealand deer farmers (Asher et al. 2000). The efficiency of laparoscopic intrauterine insemination in red deer, assessed as conception rate (pregnancies established per 100 hinds inseminated), generally ranges from 55% to 70% for fresh and frozen-thawed semen, respectively (Asher et al. 2000). Conservative estimates of AI practice in New Zealand indicates insemination of 10000 red deer hinds annually, representing only 1–2% of breeding hinds (Asher 1998). This percentage has not changed significantly, although the recent development of transcervical insemination (Willard et al. 2002), and improved genetic evaluation, especially through the development of sire-referencing, could lead to more widespread use.

In recent years, there has been increasing interest in the application of reproductive technology to the conservation and management of natural populations of deer (Jabbour and Bainbridge 1997). The application of ART within natural population of deer is in its infancy. However, its future potential is enormous, particularly in relation to genetic management or conservation, as in the case of the Iberian red deer (*Cervus elaphus hispanicus*). It is the largest free-living ruminant of the Iberian peninsula, which lives remarkably southern than the

much more studied Scottish red deer (*C. elaphus scoticus*). This subspecies differs from *C. elaphus sp.* mainly because it is smaller in size. ART may play an important role for the purpose of ensuring genetic preservation and/or genetic progress, which are becoming increasingly important as a result of the genetic isolation of wild populations within fenced games estates (Martinez et al. 2002). Deleterious effects of inbreeding have been found on some components of the fitness of hinds (Coulson et al. 1998) and also on male reproductive function in others ungulate species (Roldán et al. 1998; Gomendio et al. 2000). In this situation, germplasm conservation of Iberian red deer offers the possibility of genetic variability preservation via biotechnological reproduction programmes. In addition, the only commercial benefit obtained from this subspecies is that of hunting. The genetic value of the Iberian red deer population is well known worldwide, and the conservation of this genetic potential is an important incentive for the Spanish game breeding and hunting industry. Besides, there is also a remarkable interest in the use of ART for genetic salvage, particularly from harvested trophy males (Asher et al. 2000). Unfortunately, Iberian red deer is the carrier of tuberculosis (Gortazar et al. 2005) and theileriosis (Höfle et al. 2004)). This subspecies might therefore not be translocated from one part of Spain to any other area, due to the potential of spreading these diseases (Fernandez de Mera et al. 2003). ART thus represent methods to breed disease-free Iberian deer and may have economic potential for game farming in the future.

The reasons stated above highlight the importance that ART may have in managing deer natural populations, as is the case of Iberian red deer. Of the genetic material in cryo-banks, the collection, storage and subsequent use of spermatozoa has found the most wide-spread application (Watson and Holt 2001). According to this, cryopreservation of spermatozoa combined with artificial insemination (AI) has been the method of ART that has been most extensively applied to deer species (Asher et al. 2000). In our context, post-mortem recovery of spermatozoa from the epididymides of hunted stags has been proposed as a

widely used source (Platz et al. 1982), since collection by other methods is often very difficult or unaffordable. Also, if the species are hunted, a considerable number of samples may be available each year, which can eventually be used to restore populations challenged by inbreeding and to reinvest the valuable genetic material in the population (Platz et al. 1982).

Thus, this paper reviews the present state of such technologies for a wild subspecies of red deer, the Iberian red deer, by discussing the major components of oestrous synchronization, semen collection/cryopreservation and insemination techniques. In addition, findings made during the course of natural populations studies have enormous potential for the understanding of novel reproductive mechanism that may not explain during livestock animal or human studies. Finally, the results of these studies are also reviewed here.

Oestrous synchronization

Iberian red deer hinds are seasonally polyestrous (García et al. 2002), and exhibit an oestrous cycle varying from 19 to 20 days (García et al. 2003a). This subspecies of red deer is a short-day breeder that mates during a rut occurring shortly after the autumn equinox (García et al. 2002). Indeed, it is well known that mammalian species from temperate areas trigger their reproductive physiology in response to environmental cues. It has long been accepted that the photoperiod is the primary environmental cue controlling seasonal breeding in deer. Thus, the photoperiodic signal is transduced by the pineal gland into a pattern of melatonin secretion which, in turn, provides a critical endocrine signal to regulate secretion of other hormones involved in the onset and end of the annual breeding season. We found for first time in red deer that the pineal gland of the adult female is highly responsive to both daily and seasonal changes in natural environmental illumination, although overnight levels lasted longer than the photoperiodic night in all cases, particularly at the winter solstice (García et al. 2003b).

In these species where females exhibit regular cycles of sexual activity, synchronization of oestrous and ovulation is essential, to allow assisted breeding procedures

129 to be applied at a time when the chances of conception are maximal. In most species of deer,
130 recurrent ovulation occurs as a direct consequence of failed conception during the previous
131 cycle (García et al. 2003a). The progesterone-secreting corpus luteum is destroyed by the
132 action of protaglandins secreted by the non-pregnant uterus, and this precipitates a subsequent
133 cycle of oestrous activity and ovulation. There are a number of practical advantages to
134 exerting external control over timing of reproductive events, particularly in terms of
135 improving efficiency of artificial insemination. In addition, natural (spontaneous) oestrous is
136 difficult to detect. Furthermore, natural synchrony is no better than 10-14 days, been
137 necessary successive AI of hinds over this period, a situation which is clearly impractical
138 (Asher 1998). Therefore, oestrous synchronization of red deer hinds is an important facet of
139 all AI programmes of this species.

140 Synchronization of oestrous and ovulation in a group of females can be induced
141 artificially by altering the endogenous endocrine environment of a reproductive active, non-
142 pregnant female through the exogenous administration of prostaglandins or progesterone
143 (Asher et al. 1993). A large number of studies have investigated the use of the controlled
144 internal drug releasing (CIDR) device, containing progesterone, for efficacy control of oestrous
145 in red deer. Besides, the additional administration of equine chorionic gonadotrophin (eCG) at or
146 near of CIDR device withdrawal is generally performed for farmed red deer. For more details
147 see Asher et al. (1993). This procedure has also proven successful for Iberian deer by our group
148 (García et al. 1998). Thus, we have adopted this method for artificial synchronization of
149 oestrous as a more cost-effective alternative to detection of natural oestrous (Soler et al.
150 2003b; Malo et al. 2005a). The use of prostaglandin injections to synchronize hinds has been
151 investigate, but has generally proved to be of lower efficacy than intravaginal CIDR (Asher et
152 al. 1993). It should be borne in mind, however, that the optimum timing of insemination in
153 relation to the time of oestrous synchronization treatment varies between species and is
154 dependent on the subtle interplay between the hormones oestrogen (secreted by the

155 developing follicle) and LH (secreted by the pituitary), which leads to follicle rupture and
156 release of the egg. The aim must be deposit the spermatozoa at a time close to ovulation, so
157 that only one spermatozoon will penetrate the ovum, leading to successful fertilization and
158 subsequent embryonic development.
159
160 **Semen collection and quality**
161 Farming of red deer have allowed to apply conventional artificial reproduction techniques for
162 obtaining semen from stags, using either artificial vagina or electroejaculation. However, in
163 the context of natural populations, the use of techniques based in collection by artificial
164 vagina must be discarded, although it has been successful with tamed animals (Gordon 1997;
165 Gizejewski 2000, 2004). To the contrary, electroejaculation can be used combined with
166 darting, thus it is feasible to apply it to wild animals. Nevertheless, the most practical
167 technique is post-mortem recovery of epididymal spermatozoa, since this species is legally
168 hunted in many countries. These techniques have been tested not only in red deer, but also in
169 many other wild ruminant species (Asher et al. 2000; Holt 2001). Despite of the many
170 published studies dealing with deer semen, few of them give enough detail on the method
171 used for seminal collection, techniques vary and it is difficult to compare different studies.
172 Electroejaculation must be used when the objective is either to obtain a representative
173 number of samples from natural populations not subjected to hunting, or to repeatedly recover
174 samples from selected males. To achieve a viable methodology, not only a suitable
175 electroejaculation technique must be developed, but also effective and secure darting and
176 anesthesia. Reports on electroejaculation of red deer stags are referred from more than 30
177 years ago (Jaczewski and Jasiorowski 1974). The major problems related to this techniques
178 are related to the possible risks of the anesthetic protocol (Krzywinski and Wierzchos 1992),
179 and to the quality and quantity of the semen sample, since dead of valuable individuals
180 because of anesthesia or bad quality or insufficient semen sample can make a collection plan

unworthy. Indeed, electroejaculated semen has a different composition that semen collected by "natural" methods (artificial vagina), because of differential stimulation of seminal glands by the electroejaculation probe and the difficulty of achieve repeatability between electroejaculation sessions. This may negatively affect semen quality, but it is still to be confirmed for red deer (Asher et al. 1993, 2000).

Several authors have reported successful electroejaculation with restrained males (Haigh et al. 1984b; Comizzoli et al. 2001b), which may not be applicable due to animal welfare concerns. Regarding anesthetic protocols, successful collection has been achieved using fentanil citrate, xylacine or azaperone (Fennessy et al. 1990). Sipko et al. (1997) reported the use of diacethylcholine+displacine for Siberian Maral (*C. e. sibiricus*). Our group has reported xylacine+ketamine as an effective and secure preparation for Iberian red deer (*C. e. hispanicus*) anesthesia prior to electroejaculation, with yohimbine as inhibitor (Anel et al. 2000; Garde et al. 2000; Martínez et al. 2004; Martínez-Pastor et al. 2004, 2006). We have carried out 192 electroejaculations on 25 red deer stags with no fatal incidents, recommending intubation and monitoring of the animals during anesthesia (Anel et al., unpublished data).

Regarding the electroejaculation protocol, few articles give details on probe characteristics, electroejaculation process or the applied current. Probe size and characteristics are described in few reports (Haigh et al. 1984b; Fennessy et al. 1990). In our experiments with Iberian red deer, we used a 250×30 mm probe with three longitudinal electrodes (Martínez et al. 2004; Martínez-Pastor et al. 2004, 2006), achieving ejaculation at average voltage and amperage values of 4.5 V and 90 mAmp, respectively. According to Sipko et al. (1997), successful electroejaculation has been achieved in Siberian Maral using 3–4 stimulations of 5 s each, at 10 s intervals, with 8 V as maximum.

Samples obtained by electroejaculation are suitable for preparation of seminal doses and cryopreservation. However, electroejaculation method, individual and season influences the outcome (Fennessy et al. 1990; Sipko et al. 1997). However, electroejaculation can

stimulate urine emission, which may spoil good quality samples and contribute to sample variability. Regarding this issue, we have reported that 22 samples of 79 obtained by electroejaculation from Iberian red deer were contaminated with urine (Martinez-Pastor et al. 2003a).

Seasonality is a major factor conditioning sample collection in red deer, not only quality, but also quantity. Haigh et al. (1984a) could obtain seminal doses during the breeding season of wapiti, but no semen was obtained in June and July. Anel et al. (2000) studied the quality and quantity of semen collected by electroejaculation in autumn, winter and spring (after antler casting), finding that semen concentration and volume reached its highest values in autumn, and decreased in winter to a minimum in spring. However, quality was similar in autumn and winter, only dropping in spring. Other characteristics of the ejaculate vary too (Gizejewski et al. 2003; Gizejewski 2004). For instance, during the rut, semen is accompanied by a sticky and viscous yellow secretion of the vesicular glands, termed “honey”, whose presence may affect spermatozoa quality. Anel et al. (2000) reported presence of this secretion when electroejaculating in autumn, but absence in winter.

Post-mortem seminal recovery is the most practical option to obtain spermatozoa samples from wild populations of red deer. Besides, hunting provides a constant source from harvested animals. The source of post-mortem spermatozoa is the cauda epididymis. The maturation stage and fertility of spermatozoa stored in this part of the epididymis are similar to those of ejaculated spermatozoa and, therefore, they are suitable for germplasm banking (Foote 2000). Sperm recovery is usually carried out either by performing some cuts on the cauda epididymis (Anel et al. 2002; Garde et al. 2000; Gizejewski et al. 1998; Martínez-Pastor et al. 2002, 2006) or by retrograde flushing of the cauda from the vas deferens (Comizzoli et al. 2001a; Esteso et al. 2003; Fernández-Santos et al. 2006a,c; Garde et al. 1998a, 1998b; Soler et al. 2003b, 2005a, 2005b; Zomborszky et al. 1999). Recovery by means of cuts is quicker and more easily performed than retrograde flushing, but it has the important

drawback of contamination, negatively affecting the spermatozoa. We have compared both methods (Martinez-Pastor et al. 2006), finding that the total amount of spermatozoa recovered was equivalent. Sperm quality, both before and after cryopreservation, was slightly better when retrograde flushing was used, and sample contamination was clearly higher for the cuts method. In fact, the median values of the concentration of erythrocytes for cuts and flushing were around 7×10 and 10 mL, respectively. The presence of such contaminants in the sample may be deleterious, thus, in routine recovery for germplasm banking it would be preferable the use of methods which minimize contamination.

As in the case of electroejaculation, many factors affect semen quality, and, apart from the technique of sperm recovery and factors intrinsic to the male, season is the most significant one (Guerra et al. 2002). Our group (Martinez-Pastor et al. 2005d) showed that the number of recovered spermatozoa varied dramatically from a median value of 35.1×10 mL during the rut (September to mid-October) to 13.2×10 mL in the post-rut (mid-October to December) and to 2.1×10 mL in February (non-breeding season). However, in the same study, sperm quality was as good, and even higher (acrosomal status and sperm viability), in the post-rut as in the rut. However, sperm recovered in February was barely motile. In another study (Martinez-Pastor et al. 2005a), we found differences between samples collected during the rut and during the post-rut, regarding motility subpopulations. These differences were attributed to the maturational status of the epididymal spermatozoa, and its impact on sperm fertility has not been established yet.

In the case of post-mortem samples, the time between the death of the animal and sperm collection and processing is an important factor to take into account. Indeed, several studies on Iberian red deer indicate that sperm samples undergo a quick drop in motility after 24 h post-mortem, although other characteristics are not affected but many days post-mortem (Anel et al. 2002; Garde et al. 1998b; Martinez-Pastor et al. 2005a, 2005c; Soler and Garde 2003; Soler et al. 2005a). Therefore, epididymal samples must be recovered and

cryopreserved as soon as possible after the death of the stag. Nevertheless, a few hours of delay (considering transportation from the field to the lab) may not represent a difference.

Another fundamental landmark in a semen recovery and cryopreservation program is the assessment of the quality of the samples. Many conventional and novel techniques have been successfully used for the assessment of red deer spermatozoa, and recently developed techniques have been adapted to analyze red deer spermatozoa, increasing the amount of information available. Thus, CASA systems have been used to analyze sperm motility parameters (Malo et al. 2005b; Martinez-Pastor et al. 2005c, Martínez-Pastor et al. 2006b,c), and it has been possible to identify subpopulations defined by motility patterns, and their changes in different circumstances (Martínez-Pastor et al. 2005a, 2005b). Moreover, fluorescent probes (either by fluorescence microscopy or by flow cytometry) have been used to analyze different physiological parameters of red deer semen, as viability and acrosomal status (Anel et al. 2002; Martínez-Pastor et al. 2006a, b, c), or mitochondrial membrane potential (Martínez-Pastor et al. 2002; Soler et al. 2005a). Flow cytometric techniques for DNA assessment (García-Macias et al. 2006; Soler et al. 2005a) and automated morphometric analysis of sperm heads (Esteso et al. 2003; Soler et al. 2005b) have been adapted for its use in this species.

Cryopreservation of red deer semen

The cryopreservation of the seminal doses is a fundamental step in the management of germplasm banks, and one of the most critical ones. Spermatozoa must endure a series of stresses, and semen samples undergo a more or less marked loss of viability and fertility. Cryopreservation of red deer seminal samples has been reported for many years (Jaczewski et al. 1976, 1978; Krzywinski 1981; Sipko et al. 1997a), and both refrigerated and frozen storage are carried out in farmed red deer (Asher et al. 2000; Gordon 1997). However, there is a lack of optimized protocols, especially for epididymal spermatozoa. Most protocols for

285 cryopreserving red deer sperm come from existing methods for ejaculated semen of domestic
286 ruminants, with little modifications.

287 Jaczewski et al. (1978) used protocols from bull, reindeer and goat for the
288 cryopreservation of red deer semen obtained by electroejaculation, and freezing the semen in
289 pellets on dry-ice. These authors obtained good post-thawing motility after using the goat
290 extender, although they could not determine if it was due to good extender choice or to the
291 different season of collection. Pellet freezing has been used by other authors (Krzywinski
292 1981; Sipko et al. 1997b), with good results. However, health standards and practical reasons
293 make preferable packaging in straws (Holt 2001), being 0.25 mL straws the preferred choice
294 for red deer.

295 Asher et al. (2000) reviewed thoroughly the storage and freezing of semen from many
296 species of deer. For red deer, they indicated that most authors have used protocols from sheep
297 and cattle with no modifications, but few studies had attempted to search new extenders for
298 red deer. Veldhuizen (1994) tried five extenders and three cryoprotectants (glycerol, dimethyl
299 sulfoxide and propan-1,2-diol). The results indicated that the Tris-citrate-egg yolk-glycerol
300 rendered the highest motility post-thawing, whereas lactose-egg yolk-glycerol better protected
301 acrosomes. However, in vivo fertility did not indicated differences. In any case, glycerol is the
302 preferred permeating cryoprotectant in deer. More recently, other authors have reported good
303 semen quality using commercial extenders for cattle (Zomborszky et al. 2005). Garde et al.
304 (2000) tried 4% and 8% glycerol concentrations on electroejaculated semen (Tes-Tris-
305 fructose-egg yolk extender), finding that 4% rendered slightly better results after thawing, but
306 not significant. In a recent report, we found that sperm viability after thawing was
307 significantly higher in samples frozen with 4% glycerol (Martínez-Pastor et al. 2006b). In the
308 same study, we compared three extenders with osmolalities of 320, 380 and 430 mOsm/kg,
309 finding higher post-thawing progressive motility and sperm viability with the 320 mOsm/kg
310 extender.

Nevertheless, epididymal spermatozoa have some peculiarities that make compulsory to adapt or develop new extenders. These spermatozoa are in an environment totally different from ejaculated ones. Epididymal fluid has a much higher osmolality than seminal plasma, and its composition differs considerably regarding proteins and antioxidants. Therefore, epididymal spermatozoa are not submitted to some interactions with seminal plasma components, which may alter their capacity to reach and fertilize the oocyte. Despite these facts, there are reports of successful use of cryopreserved epididymal spermatozoa for AI on red deer after using extenders and protocols for cattle. Garde et al. (1998a) obtained four fawns after vaginal AI of 17 synchronized hinds, using Triladyl® as semen extender. In another experiment, our group tried three extenders: sodium citrate-fructose, Triladyl® and a Tris-lactose extender (Garde et al. 1998c; Ortiz et al. 1997). We found that the lactose extender (slightly hypertonic relative to the other two extenders) was the most suitable for freezing high quality samples, but low quality samples were better preserved by Triladyl®. Besides, Zomborszky et al. (1999) reported AI of superovulated hinds with epididymal spermatozoa frozen in Tris-citric acid-fructose-egg yolk extender (two fractions; 6% final glycerol concentration); uterus were flushed, recovering three embryos, which were cryopreserved and two of them transplanted, given one fawn.

Recently, many studies have approached the development of optimized extenders and protocols for red deer epididymal spermatozoa cryopreservation. Thus, Garde et al. (2000) tried 4% and 8% glycerol for freezing epididymal spermatozoa in a Tes-Tris-fructose-egg yolk extender, being 8% more effective protecting the membrane functionality as assessed post-thawing. In another study, Martínez-Pastor et al. (2006b) confirmed this result, although they only obtained statistically higher results regarding acrosomal status. Furthermore, in the second part of their study on epididymal spermatozoa, Martínez-Pastor et al. (2006b) found that 380 and 430 mOsm/kg extenders were more adequate than a 320 mOsm/kg extender for freezing. These results differ from those corresponding to semen obtained by

337 electroejaculation, as showed above. Our results indicated that the mean osmolalities of
338 ejaculated and epididymal samples were 336 and 387 mOsm/kg, and therefore it would
339 influence the suitability of extender osmolality. This kind of studies point out the differences
340 between ejaculated and epididymal spermatozoa, and the need of treat them differentially
341 when designing cryopreservation protocols.

342 Besides, Fernández-Santos et al. (2005) tested the effect of glycerol, ethylene glycol,
343 propylene glycol and dimethyl sulfoxide, added at 22 °C or 5 °C. They found that dimethyl
344 sulfoxide was toxic to red deer epididymal spermatozoa, whereas glycerol performed slightly
345 better than ethylene glycol and propylene glycol. Regardless of the cryoprotectant used, they
346 found that adding it at 22 °C better protected the acrosomes. In two related studies, these
347 authors reported that 6% glycerol provided better protection than 4% glycerol. In the same
348 study, they studied if egg yolk concentration (0, 5, 10 and 20%) and its processing (clarified
349 by centrifugation or whole egg yolk) affected epididymal spermatozoa cryopreservation,
350 concluding that 20% clarified egg yolk improved sperm motility post-thawing. Other
351 experiment (Fernández-Santos et al. 2006a) agreed that 20% egg yolk is a more suitable
352 concentration for red deer epididymal spermatozoa than 0 or 5%, when cooling the semen
353 sample from 22 °C to 5 °C. In the same studies, we also analyzed the effect of different
354 velocities for cooling extended semen samples from 22 °C to 5° C. We found that a fast
355 velocity (4.2 °C/min vs. 0.23 °C/min) improved sperm motility after cooling and after
356 thawing. In other study (Martinez-Pastor et al., 2006c) we found that adding seminal plasma
357 from electroejaculated samples of red deer improved sperm quality before and after freezing.
358 Besides, Fernandez-Santos et al. (2006b) found that several antioxidants (specially catalase
359 and superoxide dismutase) improved freezing of red deer epidymal spermatozoa.

360 Complementarily, in other studies, we have dealt with the thawing procedures on
361 epididymal sperm cryopreservation. Thus, Soler et al. (2003a,b) studied the effect of different
362 thawing rates after freezing semen straws using liquid nitrogen vapor. We found that slow

363 thawing rates (thawing bath at 37 °C) rendered the best results, both for in vitro quality
364 assessment and for fertility (intrauterine AI; 69.7% for 37 °C/min vs. 42.4% for 70 °C/min).
365 Furthermore, morphometric assessment of sperm head dimensions after thawing showed that
366 the rapid thawing procedure (70 °C/min) caused a more marked head size decrease, and a
367 greater loss of sample heterogeneity, which was related to a higher degree of cryoinjury
368 (Esteso et al. 2003).

369 Finally, the most obvious result of our experiments was the clear demonstration of stag-
370 to-stag differences in response of their spermatozoa to freezing and thawing (Soler et al.
371 2003b; Esteso et al. 2006). In addition, we have previously reported that fertility rates also
372 vary markedly between males when frozen-thawed semen was used (Malo et al. 2005a).
373 Differences in the resistance to thawing of the spermatozoa of different individuals have been
374 observed for spermatozoa of other domestic (see Curry 2000) and wild (see Leibo and
375 Songsasen 2002) species. Within this context, semen donors have routinely been categorized
376 as “good” or “bad freezers”. Although similar experiences have been reported for several
377 species, no explanations for these differences have been substantiated. The mechanisms
378 underlying differences in cryosensitivity between different individuals have yet to be
379 elucidated, but there is some evidence for physiological differences between spermatozoa
380 from individuals of the same species (see Leibo and Bradley 1999). In this sense, we have
381 recently reported that differences in epididymal sperm-head area and shape exist between
382 “good” and “bad” freezers stags before freezing, with the smallest overall sperm head
383 dimensions found in the “good” freezers males (Esteso et al. 2006). Besides, we found that
384 the sperm heads in the fresh samples from the “good” freezers were more elongated and
385 narrow than those from the “bad” group (Esteso et al. 2006). In this sense, the sperm head
386 length in the fresh samples from “good” freezers was approximately two times higher than the
387 width. Taken together our results, we hypothesized that sperm head area and shape cause
388 differences in heat exchange as well as in movements of water and ions. It is, therefore,

389 plausible to think that spermatozoa may vary in their physical properties depending on their
390 area and shape and that these variations are, at least partially, responsible of the inter-
391 individual resistance to the cryopreservation process.

392 The observation that sperm head size and shape are highly indicatives of stags sperm
393 survival after cryopreservation is important for two reasons. First, it is possible that sperm
394 head area and shape influence total sperm volume, thus causing differences in heat exchange
395 as well as in movements of water, ions and cryoprotectants and, in turn, on sperm freezability
396 (Curry 2000). Second, it suggests that sperm survival from individuals considered as "bad
397 freezers" (with large and wide sperm heads) can be optimised by modifying either the
398 cryoprotectants concentration or the cooling rates. Thus, previous works have reported that
399 sperm characteristics as surface area and volume, have important implications for determining
400 optimum cooling and warming procedures for sperm cryopreservation (Curry et al. 1996). In
401 this sense, improvements in boar sperm cryosurvival have been reported when spermatozoa
402 were frozen at faster rates (Fiser and Fairfull 1990). When cell suspensions are frozen, they
403 are cooled at finite rates often referred as slow or fast. A rate that is slow for one cell type may
404 be rapid for a second type. The optimum cooling rate is the one at which maximum survival
405 is observed. Thus, the optimum cooling rates for freezing human, boar and ram spermatozoa
406 are different (see Leibo and Bradley 1999). Besides, sperm area and volume have important
407 implications for determining optimum cooling procedures for cryopreservation (Curry et al.
408 1996). These sperm characteristics influence the rate at which the cell can lose water; and the
409 rate at which a cell can lose water is a principal determinant of its optimum cooling rate. For
410 reasons briefly described above, efforts to improve sperm freezing protocols have increased
411 substantially during the past years. In this sense, we propose that sperm samples from the
412 different stags require different cooling rates for optimal cryosurvival. Thus, the results of the
413 present study suggest that the freezability of the spermatozoa from individuals considered as
414 "bad freezers" (with large and wide sperm heads) could be increased in the future using

different cooling rates than those used by us (20ºC/min). We based this hypothesis in the fact that cellular area and volume have important implications for determining optimum cooling rates for cryopreservation (Curry et al.1996; Leibo and Bradley 1999).

Artificial insemination

Artificial insemination, the process of depositing male gametes of genetic value in the female reproductive tract, has been successfully carried out in red, fallow, Eld´s, axis deer and reindeer (Asher et al. 2000). The principle insemination technique for red deer involves placing the thawed or fresh semen directly into the uterus with the aid of a laparoscope. The laparoscopic technique has been adapted from that developed for sheep (Killeen and Caffrey 1982) and was used for red deer because of the relative difficulty of passing the semen pipette through the cervix. Furthermore, intracervical and intravaginal deposition of semen, while relatively easy to perform, have generally been associated with low conception rates (with the possible exception of the use of large, impractical doses of fresh semen). Laparoscopic intrauterine insemination is usually performed at an average time of 54-58 hours after CIDR device removal/eCG injection (Asher 1998), and the technique has proved to be the most reliable form of AI in red deer. It does, however, require a high degree of skill, chemical sedation of recipient hinds and precautionary aseptic procedures.

Initial studies on intravaginal insemination of Iberian red deer with at least 100×10^6 motile thawed spermatozoa resulted in 23.5% (4/17) fawning rate (Garde et al. 1998a). Other trials have yielded similar results of fertility (Table 1). Taken together, our results show that this technique resulted in low conception rates, specially when frozen-thawed semen is used. Success rates were improved when we used the laparoscopic intrauterine insemination technique (Soler et al. 2003b; Malo et al 2005a). Early studies on small numbers of Iberian hinds clearly indicated the potential of the technique (Soler et al. 2003b). More recent trials on larger numbers of hinds inseminated with 100×10^6 total spermatozoa have confirmed the

441 above results (Table 2). Other trial involved comparison of various insemination times, with
442 frozen-thawed semen, following CIDR device withdrawal/eCG application. Results of this
443 trial showed that no significant difference in conception rate were found following
444 intrauterine insemination at 52, 55, 60 and 62 h after device withdrawal. The overall
445 pregnancy rate for 123 Iberian deer hinds was 56.9%. These results indicate that laparoscopic
446 technology also result in increased flexibility in the timing of insemination following oestrous
447 synchronization, without compromising conception rates. The standard regimen presently
448 applied to commercial laparoscopic inseminations of Iberian deer in Spain includes 12 day
449 CIDR device with administration of 250 UI eCG at CIDR device withdrawal and
450 insemination of 100×10^6 total spermatozoa 54-56 h later (Soler et al. 2003b; Malo et al.
451 2005a). The devices are replaced with a new one in each animal on Day 9 to ensure that
452 progesterone concentration remained high throughout the CIDR device insertion period. Finally,
453 we can conclude that the application of males gametes of genetic value by means of artificial
454 insemination within the Iberian deer hunting industry has resulted in the production of high-
455 quality male trophies (Figure 1). As a consequence, the practical application of these
456 technologies is inching closer everyday.
457
458 **Opportunities for studying novel reproductive mechanisms**
459 In our experience, findings made during the course of natural populations studies have
460 enormous potential for the understanding of novel reproductive mechanism that may not
461 explain during livestock animal or human studies. For example, our investigations on fertility
462 of different stags identified that males from natural populations of Iberian red deer vary
463 markedly in their fertility (20 to 75%) even when sperm number were kept constant (Malo et
464 al. 2005a). In addition, our works also showed that differences in fertility rates between males
465 related strongly to sperm swimming velocities as well as to the percentage of morphologically
466 normal spermatozoa (Malo et al. 2005a).

Given the difficulties found in previous studies, it is surprising that such clear associations between specific semen traits and male fertility have emerged. This likely results from males in natural populations showing a wide degree of variation in semen traits, a situation unlikely to be found in domestic species that have been subjected to artificial selection for improved fertility for many generations and in animals that are kept under uniform conditions. Such variation also may be absent from clinical studies that focus exclusively on patients with fertility problems. These results demonstrate that it is possible to identify specific semen traits that determine fertility in natural populations, despite the lack of success when working with domestic species.

We also have found that relative antler size and complexity was associated with relative testes size and sperm velocity (Malo et al. 2005b). Red deer antlers have been widely used as an extreme example of exaggerated male secondary sexual characters traits but have always been regarded exclusively as weapons. The fact that males often display their antlers has been interpreted as a display of fighting ability and the possibility that antlers may signal other male attributes has not been considered so far. Similarly, the fact that males with large antlers father more calves (Kruuk et al. 2002) has been interpreted as being the result of these males winning more fights and forming larger harems, excluding the possibility that males may also signal their fertility or their competitive ability after copulation, and that female choice may play a role. The evidence showing that sperm swimming velocity influence fertilization success (Malo et al. 2005a), lends support to the possibility that antlers may be signaling precisely those reproductive traits that determine male fertility (Roldán et al. 2005).

These findings reveal a new function for male red deer antlers and suggest that among mammals the degree of elaboration of male secondary sexual characters may signal important aspects of male reproductive quality to females and males. Previous studies demonstrated that antler size is related to the number of calves fathered by males, and it has been assumed that this is exclusively the result of males with large antlers being able to win more fights with

493 other males (Kruuk et al. 2002). Our findings suggest that males with large antlers could also
494 achieve higher reproductive success through their enhanced ability to win fertilizations both
495 in competitive and non-competitive contexts and the possible preferences shown by females
496 to mate with them.

497 Finally, we have also found in natural populations of red deer that the main
498 determinants of sperm swimming velocity are the shape of the head and the proportions
499 between the components of the sperm flagellum (Malo et al. 2006). Thus, the hydrodynamic
500 shape of the head and the forces generated by the relative size of the rest of the flagellum
501 seem to be the key determinants of sperm swimming velocity. The large inter-male variation
502 in sperm design also found among natural populations, underlies differences in sperm
503 swimming speed which, in turn, can determine differences in male fertility rates (Malo et al.
504 2006). Therefore, we conclude that, among natural populations, sperm design will be under
505 strong selective pressure (even in the absence of sperm competition) given its role in
506 determining male fertilization success.

507

508 **Conclusions**

509 Important progress has been made in several reproductive technologies applied to natural
510 populations of red deer. As a consequence, the practical application of these technologies is
511 inching closer everyday. Artificial oestrous synchronization overcomes problems associated with
512 the detection of natural oestrous. Synchronization can be successfully achieved through the
513 administration of progesterone (CIDR) plus eCG. However, future studies need to refine
514 synchronization regimens to increase oestrous synchrony, specially when frozen-thawed semen
515 is used for AI. Post-mortem recovery and cryopreservation of spermatozoa from the
516 epididymides of harvested trophy males have been generally successful. This procedure allows
517 valuable genetic material (of individuals of high genetic merit for trophy production) to be
518 reinvested in the population or to be transferred to other populations. However, it can be said

519 with some justification that there has been little original research on formulation of diluents
520 specially for deer semen, probably because the existing diluents have been given acceptable
521 results. However, when we freeze epididymal spermatozoa, this approach would not seem to be
522 very appropriate since it is well known that there are important differences in the
523 physiological characteristics of epididymal *versus* ejaculated spermatozoa, especially in
524 membranes properties that affect sperm cell survival after cooling and freezing. Transcervical
525 access to the uterine lumen has proven difficult in Iberian red deer. Conception rates to per
526 vaginam inseminations (intravaginal or intracervical) have been highly variable and generally
527 low (less than 30%). Conception rates to laparoscopic intrauterine inseminations at 54-56 h
528 after CIDR device withdrawal have been more consistent and generally higher (57-62%). In
529 summary, the technology is now available to apply oestrous/ovulation synchrony and artificial
530 insemination to Iberian deer. What is required is a concerted effort across Spanish game
531 breeding and hunting industry to identify populations that would benefit from, or are in need
532 of, exchange and introduction of male genetic material.
533
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540
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800 **TABLES**

801

802 Table 1. Conception rates of Iberian red deer hinds following intravaginal insemination with

803 frozen-thawed semen (Garde et al., unpublished data)

Game farm	No. of hinds inseminated	No. of hinds pregnant	Conception rate (%)
A	25	0	0.0
B	17	4	23.5
C	50	14	28.0
D	29	6	20.7
E	55	16	29.0
Total	176	40	22.7

804 Oestrous of hinds was synchronized. Briefly, CIDR devices were inserted intravaginally for a

805 total period of 12 days. The devices were replaced with a new one in each animal on Day 9. At

806 CIDR withdrawal the hinds received 250 IU eCG intramuscularly. The hinds were inseminated

807 twice per vagina on consecutive days around 44 and 68 h after CIDR devices withdrawal/eCG.

808 During insemination, hinds were held in an operated deer crush.

809 Conception rate (proportion of hinds which calved) was calculated from the calving records of

810 individual hinds obtained the following spring.

811

812 Table 2. Conception rates of Iberian red deer hinds following laparoscopic intrauterine

813 insemination with frozen-thawed semen (Garde et al., unpublished data)

Game farm	No. of hinds inseminated	No. of hinds pregnant	Conception rate (%)
A	145	85	58.6
B	250	151	60.4
C	368	229	62.2
D	125	71	56.8
Total	888	536	60.4

814 Oestrous of hinds was synchronized. Briefly, CIDR devices were inserted intravaginally for a
815 total period of 12 days. The devices were replaced with a new one in each animal on Day 9. At
816 CIDR withdrawal the hinds received 250 IU eCG intramuscularly. The hinds were inseminated
817 with 100×10^6 total spermatozoa directly into the uterus at around 54 h after CIDR devices
818 withdrawal using laparoscopy.

819 For laparoscopic inseminations, the hinds were sedated with an intravenous injection of xylazine
820 hydrochloride (0.8 mg/kg live weight) and ketamine hydrochloride (2.0 mg/kg live weight).
821 Following insemination, sedation was reversed with an intravenous injection of yohimbine
822 hydrochloride (0.3 mg/kg live weight). Conception rate (proportion of hinds which calved) was
823 calculated from the calving records of individual hinds obtained the following spring.

824 **Legends to figures**

825

826 Figure 1. Iberian deer stags born in May 2001 (five years old) from artificial insemination
827 using epididymal spermatozoa.

35

828 Figure 1

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