Assisted reproduction in Mediterranean wild ruminants: lessons from the Spanish ibex (*Capra pyrenaica*)

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Summary

Despite apparent progress in reproductive technology as applied to wild ruminants, the success achieved in terms of the number of offspring that become healthy adults has remained low. Difficulties often arise through a lack of knowledge regarding appropriate cryopreservation techniques, and indeed through a lack of detailed information on the reproductive physiology of the species in question. The Spanish ibex (*Capra pyrenaica*) is a wild caprid found exclusively in the mountains of Iberia; only two of the original four subspecies still exist. Great efforts need to be made to preserve this species. The endocrine and environmental mechanisms that control its seasonal reproduction need to be properly understood, reproductive technologies (particularly the cryopreservation of gametes) optimised, and genetic resource banks developed. The experience obtained with the Spanish ibex may be useful in *ex situ* conservation strategies designed to preserve other threatened Mediterranean wild ruminants.

Introduction

Many wild ruminant species of the Mediterranean Basin are under threat from the lack of food available where there are high animal population densities, the loss of heterozygosity derived from habitat fragmentation, and other pressures. For example, the Turkish mouflon (*Ovis gmelini anatolica*), which is endemic to central Turkey, is vulnerable to livestock raising and illegal hunting and is currently represented by a single population of 700 animals in the centre-south of the country (Arihan & Bilgin 2001). The Atlas aoudad or Barbary sheep (*Ammotragus lervia lervia*) lives in the mountains of Morocco, northern Algeria and northern Tunisia - although a small population has been introduced into southeastern Spain - and is listed as vulnerable by the IUCN (IUCN 2009). A decline in its numbers in excess of 10% is expected over the next 15 years, mainly as a result of hunting and habitat loss. The original ecotypes of European mouflon (*Ovis orientalis musimon*) inhabiting the islands of Sardinia and Corsica are classified as vulnerable, and reproductive strategies have been established to help preserve the species (Ptak *et al.* 2002). In the early 1900s the Cypriot mouflon (*Ovis orientalis ophion*) came close

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to extinction. Although its numbers have recently become viable it remains threatened by illegal hunting, the introduction of competitive species, climatic factors (excessively warm and dry conditions), and forest fires (Hadjisterkotis 2001).

The Spanish ibex (*Capra pyrenaica*) is an Iberian mountain ungulate, the populations of which have declined significantly over the last few centuries as a result of hunting pressure, habitat fragmentation and agricultural development. The species had disappeared from the French Pyrenees by the mid-nineteenth century, and by around 1890 the subspecies *C. p. lusitanica* became extinct in Portugal (Pérez *et al.* 2002). The Pyrenean subspecies *C. p. pyrenaica* became extinct in January 2000, when the last female died. Thus, only two of the four original subspecies still exit: *C. p. hispanica* and *C. p. victoriae*. Several ibex populations, mainly from southern Spain, have suffered at the hands of sarcoptic mange; in certain cases the mortality rate has been over 95% (Fandos 1991). This disease continues to affect most populations of ibexes in southern Spain, although now with less virulence. The species is protected under the Bern Convention and the EU Habitats and Species Directive. It is listed as Critically Endangered in Portugal, owing to its very small population in that country (Cabral *et al.* 2005). However, recent measures designed to protect the species have played a crucial role in its ongoing recovery in the Iberian Peninsula. A genetic resource bank (GRB) for the different ecotypes of Spanish ibex has also been established, the first of its kind for a Mediterranean mountain ungulate. This will help guarantee the preservation of the species in the face of possible disasters or outbreaks of further disease. The successful use of assisted reproduction technologies with the Spanish ibex may provide a useful model for the *ex situ* conservation of other threatened wild ruminants of the Mediterranean.

Genetic Resource Banks

The long-term cryopreservation of the germplasm of threatened species offers flexibility in their genetic management (Holt 1994) along with a degree of security with respect to disasters or outbreaks of disease that might seriously affect subpopulations (Kirkwood & Colenbrander 2001). A fundamental requirement of any GRB programme is the ability to successfully cryopreserve cells and tissues. Since spermatozoa are more accessible than oocytes or embryos, so they are currently of greater potential in breeding programmes, and at present are the primary cell types preserved in most emerging GRBs. The optimisation of sperm collection methods and the development of more effective cryopreservation protocols of ibex spermatozoa have been a priority.

Sperm collection

Semen collection using artificial vaginas has been used from time to time in wild ruminants maintained in captivity (Gizejewski 2004), but this requires rearing males in close human contact and training these animals in their use. Internal artificial vaginas and vaginal condoms have also been tried (Bainbridge & Jabbour 1998) but in practice their use is very limited. The difficulties associated with sperm recovery in the wild can be partly solved by post-mortem epididymal spermatozoa retrieval or electroejaculation. Viable epididymal spermatozoa can be retrieved from the dead males of a number of wild species (Soler *et* al. 2003; Perez-Garnelo *et al.* 2004). The time that elapses between death and sperm recovery affects the final sperm quality, and this should be taken into account when sperm doses are prepared for use in assisted reproduction. Frozen-thawed epididymal spermatozoa retrieved from ibexes within eight

hours of death offer the maximum guarantee of success in artificial insemination (Santiago-Moreno *et al.* 2006a). The method of sperm collection used may also influence the quality of the spermatozoa. For example, collections can be made by making several small cuts with a scalpel at the tail of the epididymis (Santiago-Moreno *et al.* 2007a). However, spermatozoa thus collected are commonly contaminated with blood and epididymal cells (Martinez-Pastor *et al.* 2006), which may interfere with the optimal cryopreservation of the gametes. Spanish ibex epididymal spermatozoa may also be successfully collected, and with less contamination, by applying air pressure inside the vas deferens. However, high pressure seems to inflict considerable mechanical stress on sperm cells and to have a detrimental effect on their viability (Santiago-Moreno *et al.* 2007b). Alternatively, a larger number of sperm cells more resistant to freezing-thawing can be obtained by retrograde flushing from the vas deferens to the cauda epididymidis employing a Tris, citric acid, glucose, egg yolk-based medium (Santiago-Moreno *et al.* 2009a).

Electroejaculation in living ibexes allows repetitive sperm collection from captive or semi-captive animals. However, the sperm quality is very often poor (Durrant 2009) due to urine contamination, low semen volumes and low sperm concentrations (Giulini *et al.* 2004). A suitable anaesthetic protocol for electroejaculation must be followed to ensure good immobilization and to prevent pain associated with the procedure. The need to select an appropriate anaesthetic is underscored by the fact that several interfere with the neuromuscular mechanisms that control the erectile and ejaculatory functions, while others favour retrograde ejaculation during electrical stimulation (Tecirlioglu *et al.* 2002). In ibexes, an anaesthetic combination based on detomidine 270 µg/kg plus ketamine 1.4 mg/kg has been successfully used; this allows penis protrusion in the majority of animals and minimum urine contamination (Table 1).

Table 1. Effect of anaesthetic protocols on the average number of electrical pulses required for ejaculation, on the percentage of ibexes showing penis protrusion, and on the percentage of ibexes showing urine contamination of the semen.

	Det-Ket-1	Det-Ket-2	Telazol	Det-Ket-Telazol
Electrical pulses	30	40	26	40
Penis protrusion	50%	89%	78%	60%
Urine contamination	11%	14%	10%	9%

Det-Ket-1: detomidine 190 mg/kg and ketamine 2 mg/kg; Det-Ket-2: detomidine 270 mg/kg and ketamine 1.4 mg/kg; Telazol: tiletamine 3.4 mg/kg and zolazepan 3.4 mg/kg; Det-Ket-Telazol: detomidine 100 mg/kg plus ketamine 1 mg/kg plus tiletamine 0.5 mg/kg and zolazepan 0.5 mg/kg. Except when Telazol is used, anaesthesia reversals occur 1-12 min after administration of atipemazole 0.25 mg/kg. All drugs are administered by intramuscular injection.

Breeding seasonality is a limiting factor for successful sperm retrieval in most wild species. Maximum testicular and accessory sex gland activity occur over just a short period of the year, ensuring that sufficient numbers of normal spermatozoa are produced at the right time (rutting season). The identification of this period is important to ensure maximum semen volume retrieval, higher sperm quality, and the greatest resistance to the freezing-thawing process. In Spanish ibex, maximum testicular size and plasma testosterone concentrations occur in October-December (Fig. 1; Toledano-Díaz *et al.* 2007). Surprisingly, the low plasma testosterone concentrations seen during spring and summer do not prevent spermatogenesis in this species, unlike in most other wild ruminants so far studied (Lincoln 1985; Gosch & Fischer 1989). Sperm abnormalities reach their height, however, at this time (Coloma *et al.* 2010).

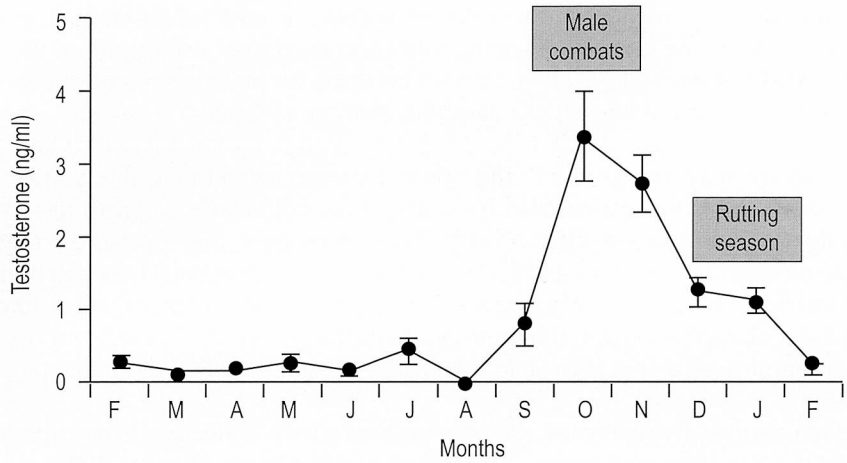

Fig. 1. Changes in plasma concentration of testosterone over the year in the Spanish ibex.

Selective criteria for inclusion of sperm samples in genetic resource banks

The sanitary control of sperm donors avoids the use of contaminated sperm in assisted reproduction (Philpott 1993), reduces the risk of cross-contamination during semen processing (Clarke 1999) and liquid nitrogen storage (Tedder *et al.* 1995; Clarke, 1999), and better maintains values of sperm variables over the freezing-thawing process (Santiago-Moreno *et al.* 2009a). The morphometry of the horns may be a useful criterion for selecting semen samples for GRBs. These secondary sexual characteristics appear to be a sensitive indicator of genetic stress (Parsons 1992) and serve as signals of male vigour that females may use to select mates (Geist 1966, 1991). Certainly, it has been shown that ibexes producing the largest and most symmetrical horns have better sperm quality (Fig. 2; Santiago-Moreno *et al.* 2007b). Reproductive success is therefore related to pre-copulatory strategies such as combat ability associated with horn development, although post-copulatory strategies related to sperm-competition also are involved (Preston *et al.* 2003).

Additives in sperm cryopreservation

The cryoprotection offered by extenders containing different additives has been tested in Ibex sperm samples obtained by electroejaculation and post-mortem collection from the cauda epididymis (Table 2). Egg yolk is beneficial to sperm cryopreservation because it protects against cold shock (Watson 1981); it may also provide certain protection during freezing and thawing (Aboagla & Terada, 2004). Although the replacement of egg yolk with other additives, such as lactose, leads to very low post-thaw motility (Santiago-Moreno *et al.* 2007a), the use of high egg yolk concentrations (12%-20%) can negatively affect the fertilization rate (Santiago-Moreno *et al.* 2006b; 2009b). Thus, the use of extenders containing egg yolk at low concentrations (TCG-6% e.y.) is recommended for the cryopreservation of both epididymal and ejaculated ibex spermatozoa.

The chemical composition of the egg yolks of different avian species varies, particularly in terms of the cholesterol, fatty acid and phospholipid contents (Bair & Marion 1978, Surai *et al.* 1999). This has led to investigations into which type of egg yolk might be more appropriate for use in extenders. Egg yolk from quail has been used successfully in the cryopreservation of

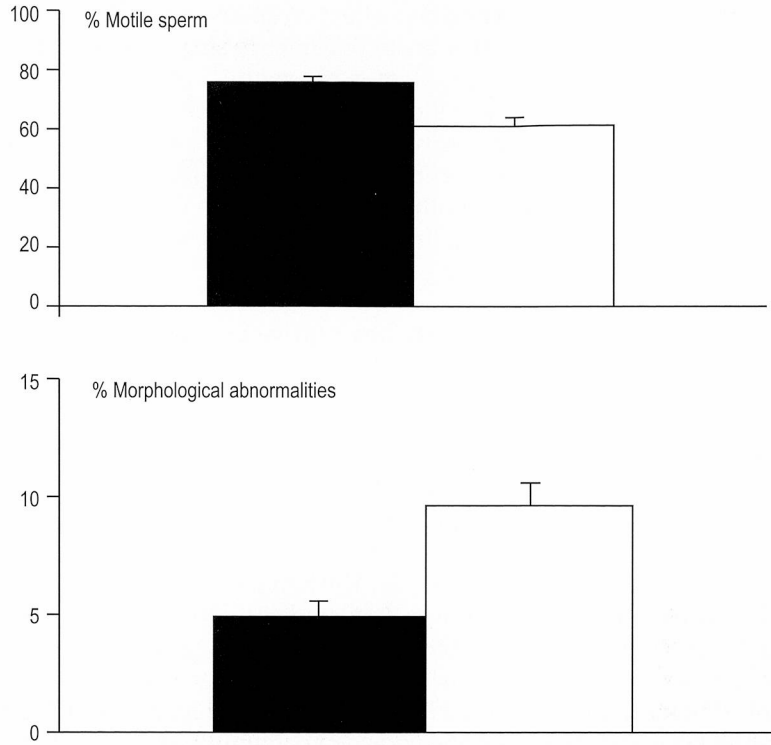

Fig. 2. Percentage of motile spermatozoa and morphological sperm abnormalities from ibexes with good (solid bars) and poor horn condition (open bars). Horn condition is calculated as the sum of the values assigned to the horn length, the horn base perimeter and relative asymmetry between the respective lengths and bases of the left and the right horns, using the method by Luzon *et al.* (2008).

Table 2. Composition of different extenders used to freeze ibex spermatozoa. The best results are usually obtained using TCG-6% egg yolk extender, both with epididymal spermatozoa recovered post mortem and those obtained by electroejaculation.

	Triladyl®	TCG-6% e.y.	TCG-12 % e.y.	TCG-20 % e.y.	TCG-Equex	TTG-6% e.y.	TTG-12% e.y.	TCG-lactose
Glycerol % (v v-1)	6	5	5	5	5	5	5	5
Tris % (w v-1)	?	3.8	3.8	3.8	3.8	1.2	1.2	3.8
Tes % (w v-1)	-	-	-	-	-	4.8	4.8	-
Citric Acid % (w v-1)	?	2.2	2.2	2.2	2.2	-	-	1.7
Glucose % (w v-1)	-	0.6	0.6	0.6	0.6	0.2	0.2	1.3
Fructose % (w v-1)	?	-	-	-	-	-	-	-
Egg yolk % (v v-1)	20	6	12	20	6-20	6	12	-
Lactose % (w v-1)	-	-	-	-	-	-	-	6
Equex Pasta % (v v-1)	-	-	-	-	0.6	-	-	-

sperm in some species, especially equids (Trimeche *et al.* 1997). However, it offers no advantage over chicken egg yolk in the cryopreservation of Spanish ibex epididymal spermatozoa (Santiago-Moreno *et al.* 2008a).

In ibexes, the seminal plasma has a negative effect on sperm survival when egg yolk-based diluents are employed (Coloma *et al.* 2010), an inconvenience also noted with domestic caprine sperm (Iritani & Nishikawa 1963). The problem is caused by a phospholipase secreted from the bulbourethral glands that hydrolyzes the membrane phospholipids of spermatozoa and produces toxic derivates from egg yolk phospholipids (Aamdal *et al.* 1965; Pellicer-Rubio & Combarnous 1998). The removal of the seminal plasma is found to be more beneficial during the time of declining photoperiod than at other times during the year, reflecting the increased activity of the accessory sex glands during the rutting season (Chapman & Chapman 1979).

Artificial insemination in Spanish ibex captive breeding programmes

Factors affecting captive breeding programs

The pregnancy rate in wild ruminant captive breeding programs is usually lower than the natural rate observed in the wild (Asher *et al.* 2000). This is often attributed to the lack of detailed information regarding the reproductive physiology of the species in question. Oestrus and ovulation synchronization protocols should take account the mating period and the characteristics of the sexual cycle defined for each species (Fig. 3). The Spanish ibex is a seasonal, polyoestrous species with alternating periods of oestrous activity and anoestrus. Anoestrus is characterised by low plasma concentrations of progesterone (< 0.5 ng/ml), reflecting complete ovulatory arrest. Ibexes show ovulatory activity with 1 to 3 progesterone cycles (progesterone peaks of 1.4 ± 0.1 ng/ml). The mean duration of the oestrus cycle is 19 days (range: 17–23 days). There is usually one short cycle (10–14 days with average maximum values of 0.8 ± 0.2 ng/ml) prior to or following a cycle of normal duration. The first progesterone cycle takes place between December 3 and 27. The end of seasonal ovulatory activity stretches from January 15 to February 9. Thus, the mean duration of the breeding season is only about 43 days (Santiago-Moreno *et al.* 2003). Furthermore, social interactions may interfere with the use of assisted reproduction techniques in wild species. Indeed, it has been shown that the anovulatory condition in subordinate ibex females (Fig.3) may be related to social status (Santiago-Moreno *et al.* 2007c), with high-ranking females showing a larger number of progesterone cycles. Hence, focusing the use of hormonal treatments on dominant animals might be cost-effective. Stress induced by synchronization treatments can also negatively affect reproduction and, in some cases lead to complete fertilization failure (Morrow *et al.* 2009). Thus, long acclimatization periods are needed under captive conditions, and synchronization protocols requiring minimum handling should be used.

Synchronization of ovulation and artificial insemination

The artificial insemination of ibex females synchronized by administering intravaginal progestagens for 11 days in conjunction with injections of equine chorionic gonadotropin (eCG) and cloprostenol two days before progestagen withdrawal (the standard method used in domestic goats), leads to fertilization rates of just 19-35% (Santiago-Moreno *et al.* 2006a,b). However, protocols based on the injection of luteolytic hormones appear to solve the problems associated with the use of eCG (anti-eCG antibodies are generated in animals repeatedly treated [Baril *et al.* 1996] and premature luteal regression [Saharrea *et al.* 1998]) and intravaginal progestagens (vaginal inflammation). In fact, the synchronization of ovulation in ibex females has been achieved following the IMA-PRO2® method described for dairy goats (Lopez-Sebastian *et al.*

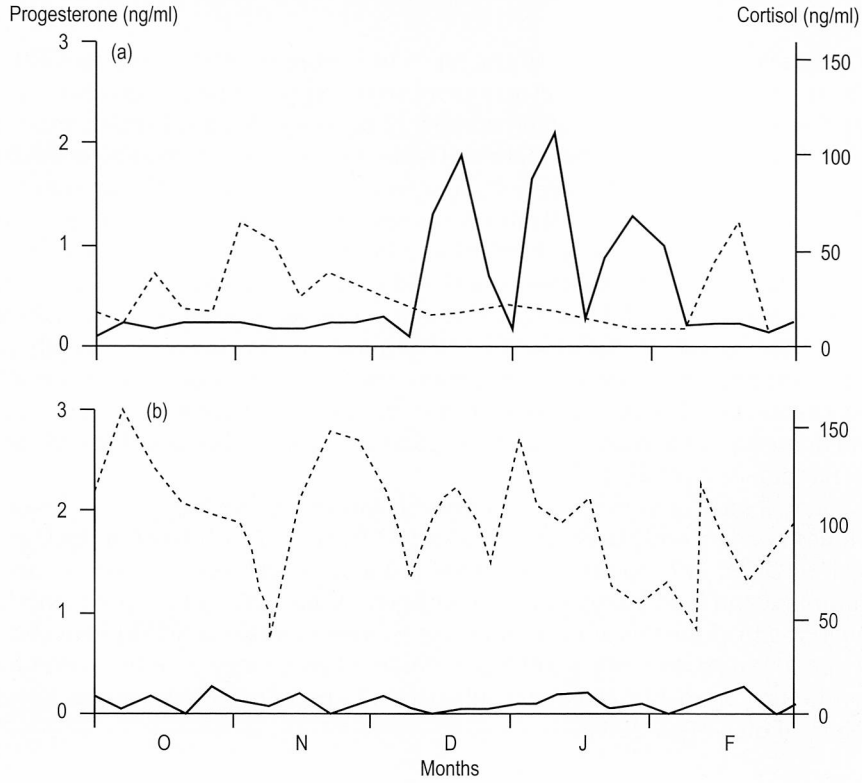

Fig. 3. Progesterone profiles (—) and plasma cortisol concentrations (- - -) in one representative dominant ibex female with cyclic ovulatory activity (a) and in one representative subordinate ibex female with no ovulatory activity (b).

2007). This minimises the number of times an animal is handled and avoids potential vaginal infections. In the breeding season, oestrus and ovulation are synchronised by the intramuscular (i.m.) injection of 25 mg of progesterone (4-pregneno-3,20-dione) in olive oil, plus a 100 μg i.m. injection of cloprostenol on day 0 followed by a single i.m. dose of 100 μg cloprostenol 10 days later. Ibexes are inseminated by laparoscopy with 200 x 10^6 spermatozoa 52 h after the second injection of cloprostenol, allowing fertility rates of 25-63% (Santiago-Moreno *et al.* 2008a,b).

The fertility of the sperm used in artificial insemination must be verified before use. Recent studies have reported the birth of live hybrids (*Capra pyrenaica x Capra hircus*) produced by the insemination of female domestic goats with electroejaculated or epididymal ibex spermatozoa. Along with routine semen analysis, this might be a good way of evaluating fertilising capacity in ibexes (Santiago-Moreno *et al.* 2006a,b). Heterospecific *in vivo* fertilization avoids the use of valuable homologous individuals, and spermatozoa are placed in the domestic female's reproductive tract when conditions are optimal for sperm capacitation, fertilization and embryonic development. The fertilising capacity of frozen-thawed sperm samples of other rare and wild species such as the mouflon (*Ovis gmelini musimon*), the gaur (*Bos gaurus*), Przewlaski's horse (*Equus przewalskii*) or Grant's zebra (*Equus burchelli*) could be tested in domestic sheep (*Ovis aries*), cattle (*Bos taurus*) and horses (*Equus caballus*), respectively.

Future strategies

Although spermatozoa are the primary cell types preserved in most emerging GRBs (because they are far more accessible than oocytes or embryos), embryo cryopreservation and embryo transfer provide ways of maximizing the number of offspring that a valuable female is capable of producing during or even after her lifetime. However, the cryopreservation of the embryos of wild ungulates has not been extensively studied, which may be due to the low rates of embryo recovery following superovulation (Leibo & Songsasen, 2002). Usually, embryo cryopreservation in a wild species involves a standard equilibrium freezing method developed for the embryos of a related domestic species (Leibo 1984; Andrabi & Maxwell, 2007). Interspecific embryo transfer may be a useful strategy in animal conservation programs when there is a lack of suitable female recipients. Interspecific embryo transfer has been successfully performed between the European mouflon (*Ovis orientalis musimon*) and domestic sheep (*Ovis aries*) (Santiago-Moreno *et al.* 2001), urial sheep (*Ovis orientalis*) and domestic sheep, the gaur (*Bos gaurus*) and domestic cattle (Stover *et al.* 1981), and the Spanish ibex and domestic goat (*Capra hircus*) (Fernández-Arias *et al.* 1999).

The Pyrenean ibex (*Capra pyrenaica pyrenaica*) was one of the four subspecies of the Spanish ibex. It has been recently been declared extinct by the Spanish Government, and is listed as such by the IUCN. Prior to the death of the last animal, cells from a skin biopsy were obtained, multiplied and kept frozen in liquid nitrogen. Although recent experiments have been focused on trying to clone this animal, only one recipient female (a hybrid between a Spanish ibex male and a female domestic goat) has maintained pregnancy to term. Unfortunately, the newborn – the first animal of an extinct subspecies to be born - died minutes after birth due to lung defects (Folch *et al.* 2009).

Conclusions

Assisted reproduction technologies may be the only way to guarantee the continued survival of certain species, subspecies or ecotypes at serious risk of extinction. The establishment of conservation plans involving the use of such technologies first requires rigorous studies on the reproductive biology of the taxon in question. Recent advances in our knowledge of seasonal breeding, ovulatory cycles and testicular activity in the Spanish ibex have allowed assisted reproduction technologies to be successfully used. The cryopreservation of sperm is a complex process that involves balancing many factors in order to obtain satisfactory results. Although there are many similarities between ibex and domestic goat sperm, that of the former requires special attention in order to maximise its post-thawing viability. An intricate knowledge of the appropriate methods of sperm collection and the diluents to use is essential to ensure even minimal success. Recent advances in these areas have allowed the establishment of GRBs for the most representative ecotypes of the Spanish ibex. The experience obtained with this species may be useful in *ex situ* conservation strategies designed to preserve other threatened wild ruminants.

Acknowledgements

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References

Aamdal J, Lyngset O & Fossum K 1965 Toxic effect of lysolecithin on sperm. *Nordisk veterinaermedicin (Nor Vet Med)* **17** 633-639.

Aboagla EM & Terada T 2004 Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology* **62** 1160-1172.

Andrabi SMH & Maxwell WMC 2007 A review on reproductive biotechnologies for conservation of endangered mammalian species. *Animal reproduction Science* **99** 223-243.

Arihan O & Bilgin CC 2001 Population biology and conservation of the Turkish mouflon (*Ovis gmelini anatolica*, Valenciennes, 1856). In *Proceedings of the third International Symposium on mouflon*, pp. 31-36. Eds A Náhlik & W Uloth. Sopron, Hungary: Lövér Print.

Asher GW, Berg DK & Evans G 2000 Storage of semen and artificial insemination in deer. *Animal Reproduction Science* **62** 195-211.

Bainbridge DR & Jabbour HN 1998 Potential of assisted breeding techniques for the conservation of mammalian species in captivity: a review. *Veterinary Record* **143** 159-168.

Bair CW & Marion WW 1978 Yolk cholesterol in eggs from various avian species, Poultry Science **57** 1260-1265.

Baril G, Remy B, Leboeuf B, Beckers JF & Saumande J 1996 Synchronization of estrus in goats: The relationship between eCG binding in plasma, time of occurrence of estrus and fertility following artificial insemination. *Theriogenology* **45** 1553-1559.

Cabral MJ, Almeida J, Almeida PR, Dellinger T, Ferrand de Almeida N., Oliveira ME, Palmeirim JM, Queiroz AI, Rogado L & Santos-Reis M (eds) 2005 *Livro Vermelho dos Vertebrados de Portugal*. Instituto da Conservação da Natureza, Lisboa, Portugal.

Chapman NG & Chapman DI 1979 Seasonal changes in the male accessory glands of reproduction in adult fallow deer (*Dama dama*) *Journal of Zoology (London)* **189** 259-274.

Clarke GN 1999 Sperm cryopreservation: is there a significant risk of cross-contamination? Human Reproduction **14** 2941-2943.

Coloma MA, Toledano-Díaz A, López-Sebastián A & Santiago-Moreno J 2010 The influence of washing Spanish ibex (*Capra pyrenaica*) sperm on the effects of cryopreservation in dependency of the photoperiod. *Theriogenology* **73** 900-908.

Durrant BS 2009 The importance and potential of artificial insemination in CANDES (companion animals, non-domestic, endangered species). *Theriogenology* **71** 113-122.

Fandos P 1991 *La cabra montés en el Parque Natural de las Sierras de Cazorla, Segura y las Villas*. Eds ICONA/CSIC. Madrid, Spain.

Fernández-Arias A, Alabart JL, Folch J & Beckers F 1999 Interspecies pregnancy of Spanish ibex (*Capra pyrenaica*) fetus in domestic goat (*Capra hircus*) recipients induces abnormally high plasmatic levels of pregnancy-associated glycoprotein *Theriogenology* **51** 1419-1430.

Folch J, Cocero MJ, Chesné P, Alabart JL, Domínguez V, Cognié Y, Roche A, Fernandez-Arias A, Martí JI, Sánchez P, Echegoyen E, Beckers JF, Sánchez-Bonastre A & Vignon X 2009 First birth of an animal from an extinct subspecies (*Capra pyrenaica pyrenaica*) by cloning. Theriogenology **71** 1026-1034.

Geist V 1966 The evolutionary significance of mountain sheep horns. *Evolution* **20** 558-566.

Geist V 1991 Bones of contention revisited: did antler enlarge with sexual selection as a consequence of neonatal security strategies? *Applied Animal Behaviour Science* **29** 453-469.

Giulini S, Pesce F, Madgar I, Marsella T, Volpe A, Aloysio D & Battaglia C 2004 Influence of multiple transrectal electroejaculation on semen parameters and intracytoplasmic sperm injection outcome. *Fertility and Sterility* **82** 200-204.

Gizejewski Z 2004 Effect of season on characteristics of red deer (*Cervus elaphus L.*) semen collected using modified artificial vagina. *Reproductive Biology* **4** 51-66.

Gosch B & Fischer K 1989 Seasonal changes of testis volume and sperm quality in adult fallow deer (*Dama dama)* and their relationship to antler cycle. Journal of Reproduction and Fertility **85** 7-17.

Hadjisterkotis E 2001 The Cyprus mouflon, a threatened species in a biodiversity "hotspot" area. In *Proceedings of the third International Symposium on mouflon*. pp. 71-81. Eds A. Náhlik & W Uloth. Sopron, Hungary: Lövér Print.

Holt WV 1994 Reproductive technologies. In *Creative Conservation: Interactive Management of Wild and Captive Animals*, pp. 144-166. Eds. PJS Olney, GM Mace & ATC Feistner. London: Chapman and Hall.

Iritani A & Nishikawa V 1963 Studies on the egg yolk coagulating factor in goat semen. III. Release of some acids accompanied by the coagulating phenomena. *Japanese Journal* of *Animal Reproduction* **8** 109-112.

IUCN 2009 *IUCN Red List of Threatened Species*. Version 2009.2. Available from: www.iucnredlist.org

Kirkwood JK & Colenbrander B 2001 Disease control measures fro genetic resource banking. In *Cryobanking the Genetic Resource; wildlife conservation for the future?*, pp. 69-84. Eds PF Watson & WV Holt. London: Taylor and Francis.

Leibo SP 1984. One-step method for direct non-surgical transfer of frozen-thawed bovine embryos. *Theriogenology* **21** 767-790.

Leibo SP & Songsasen N 2002 Cryopreservation of gametes and embryos of non-domestic species. *Theriogenology* **57** 303-306.

Lincoln GA 1985 Seasonal breeding in deer. In *Biology of Deer Production*, pp 165-179. Eds K Drew & P Fennessy. Royal Society of New Zealand, Bull 22, Wellington.

Lopez-Sebastian A, Gonzalez-Bulnes A, Carrizosa JA, Urrutia B, Diaz-Delfa C, Santiago-Moreno J & Gomez-Brunet A 2007 New estrus synchronization and artificial insemination protocol for goats based on male exposure, progesterone and cloprostenol. *Theriogenology* **68** 1081-1087.

Luzón M, Santiago-Moreno J, Meana A, Toledano-Díaz A, Pulido-Pastor A, Gómez-Brunet A & López-Sebastián A 2008. Parasitism and horn quality in male Spanish ibex (*Capra pyrenaica hispanica*) from Andalucía based on coprological analysis and muscle biopsy. *Spanish Journal of Agricultural Research* **6** 353-361.

Martinez-Pastor F, Garcia-Macias V, Alvarez M, Chamorro C, Herraez P, de Paz P & Anel L 2006 Comparison of two methods for obtaining spermatozoa from the cauda epididymides of Iberian red deer. *Theriogenology* **65** 471-485.

Morrow CJ, Penfold LM & Wolfe BA 2009 Artificial insemination in deer and non-domestic bovids. *Theriogenology* **71** 149-165.

Parsons P 1992 Fluctuating asymmetry: a biological monitor of environmental and genomic stress. *Heredity* **68** 361-364.

Pellicer-Rubio MT & Combarnous Y 1998 Deterioration of goat spermatozoa in skimmed milk-based extenders as a result of oleic acid released by the bulbourethral lipase BUSgp60. *Journal of Reproduction and Fertility* **112 95-105.**

Pérez JM, Granados JE, Soriguer RC, Fandos P, Márquez FJ & Crampe JP 2002 Distribution, status and conservation problem of the Spanish Ibex, *Capra pyrenaica* (*Mammalia: Artiodactyla*). *Mammal Review* **32** 26-39.

Perez-Garnelo SS, Garde J, Pintado B, Borque C, Talavera C, Delclaux M, Lopez M & de la Fuente Martinez J 2004 Characteristics and in vitro fertilizing ability of Giant Panda (*Ailuropoda melanoleuca*) frozen-thawed epididymal spermatozoa obtained 4 hours postmortem: a case report. *Zoo Biology* **23** 279-85.

Philpott M 1993 The dangers of disease transmission by artificial insemination and embryo transfer. *British Veterinary Journal* **149** 339-369.

Preston BT, Stevenson IR, Pemberton JM, Coltman DW & Wilson K 2003 Overt and covert competition in a promiscuous mammal: the importance of weaponry and testes size to male reproductive success. *Proceedings of the Royal Society of London* **B 270** 633-640.

Ptak G, Clinton M, Barboni B, Muzzeddu M, Cappai P, Tischner M & Loi P 2002 Preservation of the wild European mouflon: the first example of genetic management using a complete program of reproductive biotechnologies. Biology of Reproduction **66** 796-801.

Santiago-Moreno J, González-Bulnes A, Gómez Brunet A, Cocero MJ, del Campo A, García-García R & López Sebastián A 2001 Procedure for successful interspecific embryo transfer between mouflon (*Ovis gmelini musimon*) and Spanish Merino sheep (*Ovis aries*). *Journal of Zoo and Wildlife Medicine* 32 336-341.

Santiago-Moreno J, Gómez-Brunet A, González-Bulnes A, Malpaux B, Chemineau P, Pulido A & López-Sebastián A 2003 Seasonal ovulatory activity and plasma prolactin concentrations in Spanish ibex (*Capra pyrenaica hispanica*) maintained in captivity. *Reproduction Nutrition and Development* **43** 217-224.

Santiago-Moreno J, Toledano-Díaz A, Pulido-Pastor A, Gómez-Brunet A & López-Sebastián A 2006a Birth of live Spanish ibex (*Capra pyrenaica hispanica*) derived from artificial insemination with epididymal spermatozoa retrieved after death. *Theriogenology* **66** 283-291.

Santiago-Moreno J, Toledano-Díaz A, Pulido-Pastor A, Dorado J, Gómez-Brunet A & López-Sebastián A 2006b Effect of egg yolk concentration on cryopreserving Spanish ibex (*Capra pyrenaica*) epididymal spermatozoa. *Theriogenology* **66** 1219-1226.

Santiago-Moreno J, Toledano-Díaz A, Dorado J, Pulido-Pastor A, Coloma MA & López-Sebastián A 2007a Recovery and cryopreservation of Spanish Ibex epididymal spermatozoa. *Archives of Andrology* **53** 309-316.

Santiago-Moreno J, Toledano-Díaz A, Pulido-Pastor A, Gómez-Brunet A, & López-Sebastián A 2007b Horn quality and postmortem sperm parameters in Spanish ibex (*Capra pyrenaica hispanica*). *Animal Reproduction Science* **99** 354-362.

Santiago-Moreno J, Toledano-Díaz A, Gómez-Brunet A, Pulido-Pastor A & López-Sebastián A 2007c Social dominance and breeding activity in Spanish ibex (*Capra pyrenaica*) maintained in captivity. *Reproduction Fertility and Development* **19** 436-442.

Santiago-Moreno J, Coloma MA, Toledano-Díaz A, Dorado J, Pulido-Pastor A & López-Sebastián A 2008a A comparison of the protective action of chicken and quail egg yolk in the cryopreservation of Spanish ibex epididymal spermatozoa. *Cryobiology* **57** 25-29.

Santiago-Moreno J, Toledano-Díaz A, Gómez-Brunet A, Pulido-Pastor A & López-Sebastián A 2008b Synchronization of ovulation and artificial insemination protocol for Spanish Ibex (*Capra pyrenaica*) based in progesterone and cloprostenol. *Spanish Journal of Agricultural Research* **6** 39-41.

Santiago-Moreno J, Astorga RJ, Luque I, Coloma MA, Toledano-Díaz A, Pulido-Pastor A, Gómez-Guillamon F, Salas-Vega R & López-Sebastián A 2009a Influence of recovery method and microbial contamination on the response to freezing-thawing in ibex (*Capra pyrenaica*) epididymal spermatozoa. *Cryobiology* **59** 357-362.

Santiago-Moreno J, Coloma MA, Dorado J, Pulido-Pastor A, Gómez-Guillamon F, Salas-Vega R, Gómez-Brunet A, & López-Sebastián A 2009b Cryopreservation of Spanish ibex (*Capra pyrenaica*) sperm obtained by electroejaculation outside the rutting season. *Theriogenology* **71** 1253-1260.

Saharrea A, Valencia J, Balcázar A, Mejía O, Cerbón JL, Caballero V, Zarco L 1998 Premature luteal regression in goats superovulated with PMSG: effect of hCG or GnRH administration during early luteal phase. *Theriogenology* **50** 1039-1052.

Soler AJ, Pérez-Guzmán MD & Garde JJ 2003 Storage of red deer epididymides for four days at 5°C: Effects on

sperm motility, viability, and morphological integrity. *Journal of Experimental Zoology* **295A** 188-99.

Stover J, Evans J & Dolensek EP 1981 Interspecies embryo transfer from gaur to domestic Holstein. In *Annual Proceedings of the American Association of Zoo Veterinarians, pp.* 122-124.

Surai PF, Speake BK, Noble RC & Mezes M 1999 Species-specific differences in the fatty acid profiles of the lipids of the yolk and of the liver of the chick. *Journal of the Science of Food and Agriculture* **79** 733-736.

Tecirlioglu RT, Hayes ES & Trounson AO 2002 Semen collection from mice: electroejaculation. *Reproduction Fertility and Development* **14** 363-371.

Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AE, Fielding A, Briggs EM, Irwin D, Blair S, Gorman AM, Patterson KG, Linch DC, Heptonstall J & Brink NS 1995 Hepatitis B transmission from contaminated cryopreservation tank. *Lancet* **346** 137-140.

Toledano-Díaz A, Santiago-Moreno J, Gómez-Brunet A, Pulido-Pastor A & López-Sebastián A 2007 Horn growth related to testosterone secretion in two wild Mediterranean ruminant species Spanish ibex (*Capra pyrenaica hispanica*) and mouflon (*Ovis orientalis musimon*). *Animal Reproduction Science* **102** 300-307.

Trimeche A, Antón M, Renard P, Gandemer G & Tainturier D 1997 Quail egg yolk: a novel cryoprotectant for the freeze preservation of Poitou Jackass sperm. *Cryobiology* **34** 385-393.

Watson PF 1981 The roles of lipid and protein in the protection of ram spermatozoa at 5°C by egg yolk lipoprotein. *Journal Reproduction and Fertility* **62** 483-492.