14 Sperm and conservation

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14.1 The biodiversity crisis

Across the planet, animal populations are becoming smaller and fragmented and, as a result, an increasing number of species faces the risk of extinction (May et al. 1995). In general terms, the major cause of the decline in population sizes is habitat loss, which is the consequence of the exponential growth of the human population and the intensive use of natural resources that it conveys. However, the causes of underlying species loss vary between taxonomic groups, and in some instances (e.g., ungulates) overexploitation is the most frequent cause of threat (Mace & Balmford 2000).

The magnitude of the current biodiversity crisis, coupled with the limited resources available, has led to a situation where the establishment of priorities is essential to maximize the effectiveness of conservation measures. The need to define priorities has generated a heated debate about the role of reproductive studies in conservation. This debate covers two main aspects: (1) is male reproduction impoverished among endangered species thus contributing to further declines? and (2) is the use of reproductive biotechnologies useful in conservation?

14.2 Male reproduction in endangered species: the effect of inbreeding

When population size declines, it may be because mortality rates increase and/or because individuals fail to reproduce. The relative importance of mortality rates versus fecundity rates will vary between species and between populations. In those cases in which fecundity rates decrease, most studies have focused on problems associated with female reproduction, which can be assessed in natural populations. Male reproductive success is more difficult to measure because females often mate with more than one male, so they may reproduce despite having mated with a male of low fertility. Even when females mate with only one male, it is difficult to disentangle male and female effects. Thus, few (if any)

studies have been able to evaluate the role played by male infertility in population declines.

Recent studies on natural populations of red deer have revealed that males differ markedly in fertility rates (measured as the number of females becoming pregnant after being inseminated by one male) (Malo et al. 2005), a finding which challenges the widespread assumption that all males should have uniformly high fertility rates given the strength of selection on this character (see Chapter 6 of this volume). Similarly, a large degree of variation was found between males in several sperm traits. Male fertility rates are determined by specific sperm traits, which in red deer are sperm swimming velocity and the proportion of normal sperm (Malo et al. 2005). The proportion of normal sperm is thought to have a strong genetic component (Smital et al. 2005), whereas sperm swimming velocity seems to be influenced to a greater extent by environmental and social factors (Kilgallon & Simmons 2005; Pizzari et al. 2007).

Given that in natural populations males show varying degrees of fertility, it is worth asking whether male subfertility or infertility are particularly common in endangered species. Semen quality is known to be affected by nutrition, stress and pathogens (Bronson 1989). All these factors are likely to become exacerbated in endangered species, particularly when populations are forced into suboptimal habitats where food resources may be limited, and individuals more susceptible to stress and disease. However, no study has evaluated under these conditions whether population declines are the result of these factors increasing male reproductive failure or mortality rates.

More attention has been given to the effects of inbreeding upon male reproductive physiology and, in particular, male semen quality. Inbreeding is the mating between close relatives, and is likely to become more common as population size declines. Thus, inbreeding will be prevalent in small and isolated populations of endangered species, and in captive breeding programs with small founding populations. The pioneering studies by Wildt and by O'Brien in carnivores drew attention for the first time to the possibility that low levels of genetic variation may be associated with poor semen quality (O'Brien et al. 1983, 1985, 1987; Wildt et al. 1983, 1987a, 1987b; Menotti-Raymond and O'Brien, 1993). Their studies compared populations of cheetahs and lions both in captivity and in the wild. In the case of the cheetah, it was suggested that a recent bottleneck was responsible for the loss of genetic variation that in turn caused a decrease in semen quality and which could be driving the species to extinction. These studies were subsequently criticized on the basis that a comparison between populations cannot control for confounding factors and is thus inappropriate to establish causal links, and that genetic variation was measured at a few allozyme loci which may not be a good indicator of heterozygosity at the genomic level (Caro & Laurenson 1994; Caughely 1994; Merola 1994; May 1995). The debate escalated rapidly and it was suggested that inbreeding depression is rarely expressed in natural populations, and thus of little relevance for conservation (e.g., Lande 1993).

Individual genetic variation is the result of parental relatedness: the offspring of close kin have increased homozygosity and reduced fitness, a phenomenon

known as inbreeding depression (Charlesworth & Charlesworth, 1987). Inbreeding depression has been recognized for a long time in captive and domestic animals (Charlesworth & Charlesworth 1987; Thornhill 1993), the most frequently observed effect being an increase in juvenile mortality (Ralls et al. 1979; Ralls & Ballou 1986). Studies carried out in natural populations in which genealogies have been reconstructed, have revealed that inbreeding decreases female lifetime reproductive success, survival rates among juveniles and adults, and the likelihood of breeding (Keller 1998; Keller et al. 2002). The apparent lack of effects on male reproductive success in some species may be due to the occurrence of extra-pair paternity, which mask any inbreeding depression (e.g., Keller et al. 1994; Keller 1998). The magnitude of inbreeding depression may depend strongly on environmental conditions, since environmental stress exacerbates the effects of inbreeding upon fitness (Keller et al. 1994, 2002). This is true to the extent that in some cases inbreeding depression is only detectable when environmental conditions are poor, meaning that apparently healthy populations may only reveal the impact of inbreeding when conditions deteriorate. These studies show that the levels of inbreeding in island populations can be as high as those found in domestic and captive animals (Keller et al. 2002). In addition, the findings suggest that, even in large populations where inbreeding is rare, when it occurs it has a strong impact (Kruuk et al. 2002). The effects of inbreeding upon fitness components may lead natural populations to extinction, as in the case of a large metapopulation of butterflies (*Melitaea cinxia*) (Saccheri et al. 1998). As animal populations become more and more fragmented worldwide, the incidence of inbreeding is likely to increase, and may be particularly pronounced in endangered species given the low number of individuals.

Given the difficulties associated with constructing pedigrees over several generations in natural populations, an alternative approach has been to exploit the fact that inbreeding reduces heterozygosity. Thus, it has been assumed that inbreeding depression can be detected by correlating multilocus marker heterozygosity of individuals with a trait presumed to be associated with fitness. Studies carried out in natural populations in different species have found associations between heterozygosity and several components of fitness such as birth weight and neonatal survival (Coltman et al. 1998; Coulson et al. 1998; Rossiter et al. 2001), juvenile survival (Coulson et al. 1999), female lifetime breeding success (Slate et al. 2000), male lifetime copulation success (Hoglund et al. 2002), and vulnerability to pathogens (Coltman et al. 1999; Acevedo-Whitehouse et al. 2003; Reid et al. 2003). Thus, there is considerable evidence showing that heterozygosity measured with molecular markers and fitness components are associated. However, the view that individual heterozygosity across a few microsatellite loci reflects inbreeding depression has been challenged (Slate & Pemberton 2002; Coltman & Slate 2003; Pemberton 2004) and its predictive value of known inbreeding coefficients questioned (Slate et al. 2004; Bensch et al. 2006). Individual based simulations show that heterozygosity markers and inbreeding are likely to be correlated under a narrow set of conditions which require frequent and severe inbreeding events, such as under small population

sizes, strong population subdivision and high levels of polygyny (Balloux et al. 2004). The studies in which heterozygosity markers and fitness have been found to be correlated may fulfill these restrictive conditions since they include island populations (Mandarte, Rum, St. Kilda), breeding colonies with high longevity and philopatry (harbor seals, gray seals and albatrosses), or species with strong polygyny (red deer, sea lions and fur seals; reviewed in Balloux et al. 2004). An alternative explanation is that heterozygosity does not reflect genome-wide effects associated with inbreeding, but rather that there is linkage disequilibrium between the neutral markers used and genes experiencing balancing selection (Balloux et al. 2004; Hansson et al. 2004). According to this hypothesis small populations or populations which have suffered bottlenecks, can experience linkage disequilibium, that is, the non-random association of alleles at different loci in gametes, between a marker and linked fitness loci. This phenomenon can arise due to physical linkage or due to demographic processes, being relatively common when the effective population size is small.

These concerns have led to the suggestion that studies using coefficients of inbreeding calculated from genealogies are the most informative (Pemberton 2004). Our research group has studied three species of endangered gazelles for which captive breeding programs have been initiated over 30 years ago and for which detailed breeding records exist (Roldan et al. 2006). Captive populations of these species differ in the levels of inbreeding because the size of the founding populations varies. Males with high inbreeding coefficients suffer a reduction in the proportion of motile spermatozoa, the proportion of morphologically normal spermatozoa and the proportion of spermatozoa with intact acrosomes, traits that are important for fertilization success (Cassinello et al. 1998; Roldan et al. 1998; Gomendio et al. 2000). However, this is only evident in the species with the highest levels of inbreeding, and not in the other two which have intermediate and low levels of inbreeding. This could be because the deleterious effects of inbreeding are only detectable when a threshold of inbreeding is reached, or because of differences in the genetic makeup of the founding populations. A study that manipulated inbreeding experimentally in captive rodents found that inbreeding decreases testes mass and sperm production (Margulis & Walsh 2002). It is unclear if other semen traits were measured.

Because there is evidence that inbreeding depression is milder in captivity than in the wild (Jimenez et al. 1994) it is possible that similar inbreeding levels may have more pronounced effects in natural populations. A recent study has reported that reduced heterozygosity is linked to a high proportion of abnormal sperm and decreased testis size in wild rabbits (Gage et al. 2006). However, the analyses were criticized for not dealing adequately with population stratification. The causal link between heterozygosity and semen quality was questioned because when individuals within populations are compared the relationship did not hold in some populations (Slate & Pemberton 2007). Finally, as mentioned above, heterozygosity is now considered a poor predictor of the inbreeding coefficient.

Given that some of the sperm traits affected by inbreeding influence male fertility, it is likely that inbred males suffer reduced fertility. This is obvious in extreme cases such as the Florida panther where males showed extremely high proportions of abnormal sperm (over 90%) and other reproductive deficiencies such as cryptorchidism (Roelke et al. 1993; Barone et al. 1994). Males of the critically endangered Iberian lynx similarly display a high proportion of abnormal spermatozoa (Figure 14.1). In some experimental studies carried out in captivity, the decline in semen traits associated with inbreeding has not been found to be related to male reproductive success (Margulis & Walsh 2002). However, when inbred and outbred males are mated to the same females, the fertilization success of outbred males is much higher (Konior et al. 2005; Fritzsche et al. 2006). This finding has important implications, since inbred males with low fertility may not fail to reproduce under benign conditions when there is no sperm competition, but may be unable to fertilize when in competition with ejaculates from less inbred males. The poor competitive ability of inbred males leads to high inbreeding depression on sperm competition success, which is equivalent in magnitude to inbreeding depression estimates for traits strongly affecting fitness (Konior et al. 2005).

To date, most studies have measured a few basic semen traits believed to play a role in fertilization success, which typically include sperm numbers, motility, viability, morphology and acrosome integrity. Further studies are required to examine the influence of inbreeding on aspects of sperm function such as survival,

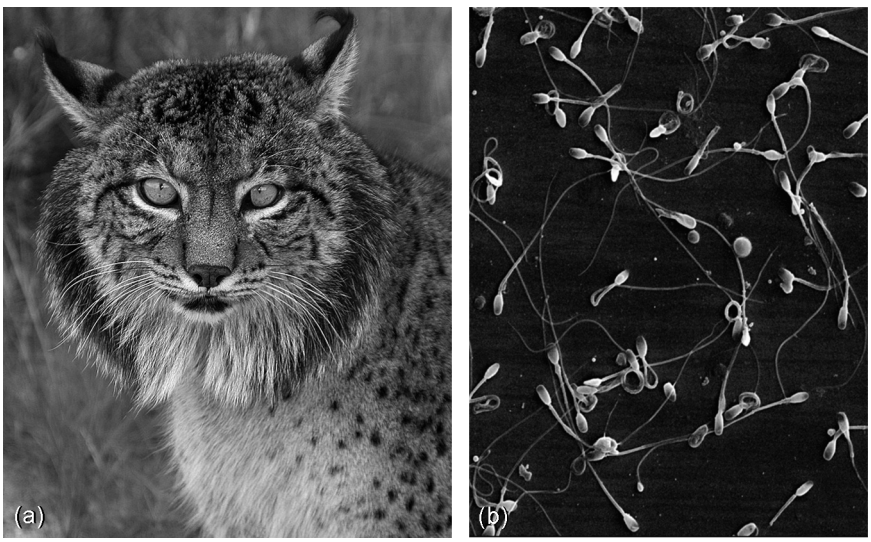

Figure 14.1 Iberian lynx, regarded as the most endangered felid in the world, have a high proportion of abnormal spermatozoa in the ejaculate.
(a) A male Iberian lynx from the captive breeding program. (b) Spermatozoa as seen by scanning electron microscopy.

capacitation, acrosome reaction and sperm–egg interaction. Preliminary results on the three species of endangered gazelles mentioned above suggest that these parameters are also diminished. Current evidence indicates that some basic semen traits, such as the proportion of normal sperm, may reflect hidden aspects of sperm function. For example, normal sperm from males with a high proportion of abnormal sperm are compromised in their ability to undergo capacitation and the acrosome reaction, and to penetrate the zona pellucida (Pukazhenthi et al. 2006b). Of particular interest would be to investigate if inbreeding affects sperm DNA fragmentation, since this would influence embryo viability (Evenson et al. 2007).

Given the costs associated with inbreeding depression, mechanisms of inbreeding avoidance have evolved. These include sex-biased dispersal and female preferences for unrelated males (Pusey & Wolf 1996). It has been suggested that female promiscuity may be a form of inbreeding avoidance if by mating with different males females increase the chances of being fertilized by unrelated males (Hosken & Blanckenhorn 1999; Tregenza & Wedell 2002). Accumulating evidence shows that promiscuity increases offspring survivorship (reviewed in Simmons 2005). However, whether this is the consequence of fertilizations being biased in favor of unrelated males is unclear, because maternal effects may also play a role. Recent work on the Australian *Antechinus* shows that offspring of polyandrous females are three times as likely to survive as offspring of monandrous females and, because most females only mate once in a lifetime, maternal effects are believed to be absent (Fisher et al. 2006). The reason for this huge improvement in offspring survival seems to be that males that are successful at sperm competition have offspring that are more likely to survive. This raises the question as to how such large differences in male 'genetic quality', linked to success in sperm competition, can persist in a population. Theoretical expectations suggest that such differences should be eliminated by strong selection.

One possible explanation that deserves further attention is that males of 'low genetic quality' are in fact inbred males with poor semen quality, which lose out in sperm competition contexts. This would mean that inbreeding in males reduces offspring viability independently of the level of inbreeding in offspring, a possible explanation that has seldom been explored. This idea is supported by the finding that female fur seals mating in crowded breeding sites actively seek males which are not only unrelated (to avoid inbreeding in offspring) but also heterozygous (Hoffman et al. 2007). So what are the costs for females of mating with inbred males? First, females may suffer fecundity costs given that inbred males tend to have poor semen quality and, most likely, lower fertility. Second, inbreeding in males may affect offspring viability if males pass on 'deficient' DNA (for example, fragmented DNA) to their descendants.

The major cause of inbreeding depression is considered to be an increase in homozygosity for deleterious recessive alleles (Charlesworth & Charlesworth 1987), although a decrease in heterozygosity at overdominant loci is another possible explanation which has recently received empirical support (Gemmell & Slate 2006). Inbreeding depression will therefore affect those traits for which there are recessive alleles in the founding population. When the number of

founders is low, as is the case in most captive breeding programs established when species are on the brink of extinction, their genetic architecture will determine that fitness traits decrease under inbreeding. Despite the evidence linking genes on the X and Y chromosomes in mammals to male reproductive traits, such genes should not be affected by an increase in homozygosity since they do not have two alleles in the same loci as happens with autosomal chromosomes. Future work should focus on genes known to influence male semen traits to investigate if homozygous individuals suffer as a consequence of the expression of recessive alleles or due to the loss of heterozygote advantage. Recent work on sheep has demonstrated that there is heterozygote advantage for female fecundity that affects oocyte development and maturation (Gemmell & Slate 2006).

In conclusion, inbreeding in males decreases semen quality, which is likely to reduce their fertility. In addition, indirect evidence suggests that inbreeding in males may also reduce offspring viability. These deleterious effects of inbreeding may have a negative impact upon populations of endangered species since they will contribute to an even further reduction in number of reproductively successful males, and thus promote inbreeding even further. Low male fertility due to inbreeding may also have a negative impact on female reproduction, particularly when females are monandrous; polyandrous females may avoid the costs of mating with low fertility males, since they will be fertilized by sperm from other males. In addition, when females mate with one male and each male controls sexual access to a number of females (polygyny), the number of females that may fail to reproduce as a consequence of low male fertility will be even higher. In natural populations, a significant proportion of females fail to reproduce when sexually receptive, and it has been assumed that this is due to environmental conditions (e.g., limited food resources) or female effects (e.g., poor physical condition). The possibility that low male fertility may also contribute to decrease reproductive rates among females has not been considered. If this is the case, then the effects of low male fertility on population growth may be much larger than expected.

The negative effects of inbreeding may be rapidly reversed by introducing new genes into inbred populations (e.g., Madsen et al. 1999). The exchange of genetic material may be accomplished by moving individuals between populations, or by exchanging gametes through the use of reproductive biotechnologies. This is a novel way in which the study of sperm in endangered species may contribute to their conservation.

14.3 Spermatozoa in assisted reproduction for conservation

14.3.1 Why assisted reproduction?

Reproductive technologies offer possible solutions to the problem of inbreeding depression generated by diminishing numbers of individuals in isolated populations (Wildt 1992; Bainbridge & Jabbour 1998; Wildt & Wemmer 1999). Assisted reproduction may complement efforts of *in situ* conservation because

they can facilitate genetic exchange between populations. Among the various potential uses of assisted reproductive technologies, those involving the collection, manipulation and conservation of sperm cells (or the male germline) are the ones that have received the greatest attention. This is due to the fact that it is relatively easy to gain access to male than to female gametes. In any case, collection of male gametes from wildlife is not easy. It requires a considerable effort to bring together people with different backgrounds, expertise and agendas, to set up conditions that would allow capture, collection and processing of samples (in many cases under field conditions), to develop preliminary research to explore appropriate conditions for sperm processing and storage and, last but not least important, to secure the funding not only for the launching of activities but also for the sustained development of these operations.

When the number of animals in the wild falls below a critical level, the necessity to initiate a captive breeding program is identified and acted upon. This raises the opportunity for the collection and conservation of spermatozoa from individuals under more controlled conditions (including a better health and nutrition program). Under these conditions, it is possible to plan for an organized routine of semen collection and storage. Furthermore, if husbandry conditions are appropriate (and the stress on donor animals limited), the quality and quantity of gametes collected improves. Regular animal examinations, under anesthesia and veterinary care, also generate opportunities for collection of biomaterials (e.g., blood, feces, somatic tissue) that allow studies of the endocrine control of reproduction and the storage of samples.

A captive breeding program can in fact be integrated into a broader approach of *ex situ* conservation and, whenever possible, support *in situ* actions. Thus, health and reproductive expertise gained with a captive breeding program can be used to collect, manipulate and store male germ cells from wild individuals. In some species, road kills are not infrequent. This unwanted fact leads to the opportunity to collect and rescue germ cells and somatic tissues from animals whose reproductive chances would otherwise have ended. Hunting, on the other hand, which is a legally recognized activity in many countries, also generates the opportunity to collect, study and conserve germplasm from natural populations.

Regardless of the source of germplasm, this biological material can be stored in Genome Resource Banks (GRBs) with the purpose of maintaining and securing species genetic diversity almost indefinitely (Loskutoff et al. 1995; Holt et al. 1996; Wildt et al. 1997; Pope & Loskutoff 1999; Wildt & Wemmer 1999; Pukazhenthi & Wildt 2004; Pukazhenthi et al. 2006a; Roldan et al. 2006). GRBs can not only store spermatozoa but they can also potentially store oocytes and embryos (collectively regarded as germplasm) and also somatic tissues that may be used in the future via somatic cell nuclear transfer or, perhaps, via transdifferentiation in culture or after grafting in appropriate host tissue and individuals. Gametes, embryos or somatic tissues conserved in these GRBs can be used across space and time since they can, respectively, support movement of genetic material between populations and allow use many years after the death of an animal.

Moreover, the existence of materials stored in GRBs may help reduce the number of live individuals needed to maintain a viable population thus reducing space for and costs of breeding.

The connection between a captive breeding program (and the overall *ex situ* approach) and *in situ* actions generates the opportunity for *ex situ–in situ* linkage with the ensuing benefit of enlarging the effective population of individuals that contribute to the conservation effort (Swanson et al. 2007). In particular, the collection of sperm samples from wild individuals leads to the opportunity of using this male germplasm, via assisted reproduction, to promote gene flow without actual removal of animals from the wild. This is also true for the potential use of assisted reproduction in natural populations with gametes stored in a GRB, which generates the benefit of reinforcing the animal populations in the wild. The possibility can also be envisaged of promoting 'translocation' of alleles between wild populations without the need to exchange live animals. Translocating animals generates risks due to stress or the spreading of infectious diseases, as well as problems due to a lack of successful integration of individuals in new social groups, not to mention the costs of transportation of large individuals.

14.3.2 Species diversity in sperm parameters

Species vary in their reproductive traits and, with regard to spermatozoa, clear differences exist in shape, dimensions and function, even in closely related species (Figure 14.2a–c). When a species is targeted for conservation, including the development of assisted reproductive techniques, it is sometimes necessary to begin with a basic characterization of sperm parameters and factors affecting male reproductive function (e.g., seasonality). This information will be important to learn about variation in sperm production and quality and to assess potential limits for sperm collection throughout the year. In addition, this information will be valuable when assessing the number of samples to be collected for storage in a GRB, and the time, effort and funds required to collect them.

Differences also exist between individuals within a species. Ejaculate parameters such as sperm concentration, percentage of motile or viable cells, and proportion of morphologically normal spermatozoa vary between individuals, and this variation is also seen in sperm shape, dimensions and velocity. Evidence from natural populations of red deer has shown that these considerable differences in seminal traits between individuals may have substantial effects on male fertility and offspring sex ratio (Malo et al. 2005, 2006; Gomendio et al. 2006). Similarly, spermatozoa from different males vary in their ability to withstand cryopreservation (Loskutoff et al. 1996; Yu et al. 2002). In agriculture, identifying 'good freezers' is an important goal (Thurston et al. 2002), but in wildlife (and more so in endangered species) one may not have the luxury to reject males based on the poor ability of their semen to survive cryopreservation.

A question thus arises in connection with the need to preserve a maximum of genetic diversity. Should one attempt to preserve all this genetic diversity even when

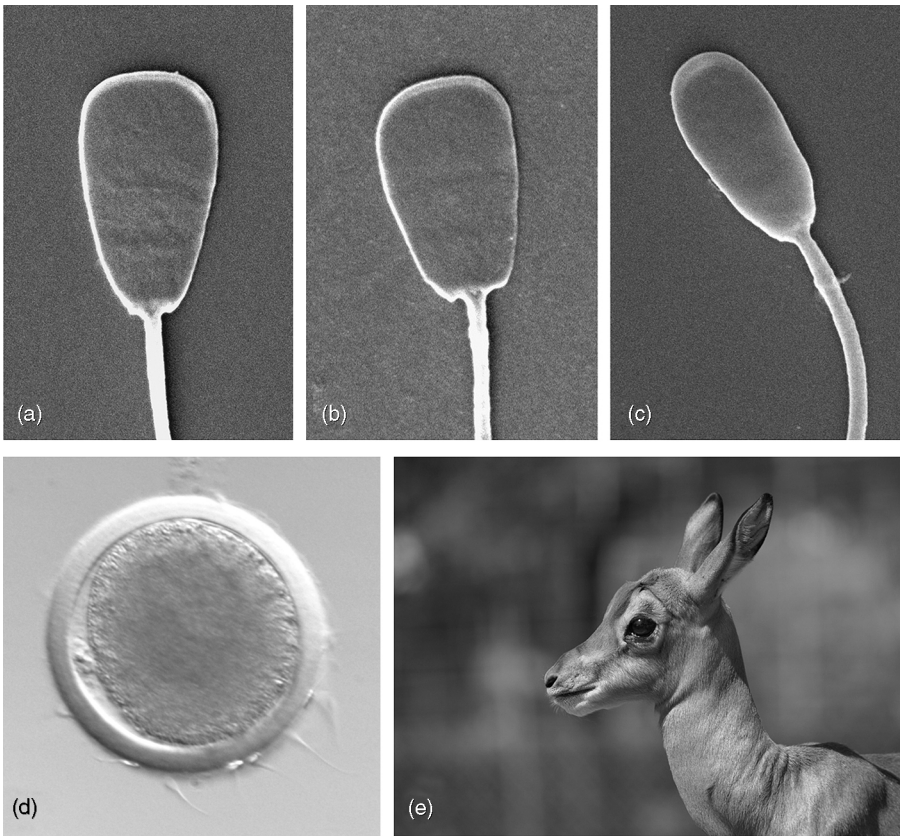

Figure 14.2 Spermatozoa from endangered gazelles.
(a) Spermatozoon from Cuvier's gazelle (*Gazella cuvieri*). (b) Spermatozoon from dama gazelle (*Gazella dama mhorr*). (c) Sperm cell from dorcas gazelle (*Gazella dorcas neglecta*). Note the differences in head shape. (d) *In vitro* fertilization using cryopreserved dama gazelle spermatozoa and *in vitro* matured homologous oocytes. (e) Calf born after artificial insemination using dama gazelle cryopreserved spermatozoa.

considering that certain males may be subfertile or clearly infertile? Or, furthermore, would the effort of preserving sperm samples from most males be possible with the known differences in cryosurvival (including some males who would not freeze well)? What additional difficulties would this create for the subsequent use of this semen samples in artificial insemination or *in vitro* fertilization?

14.3.3 Semen collection, evaluation and preservation

Males from a few mammalian species could be trained in such a way that semen can be collected by using an artificial vagina. In the majority of cases, however,

spermatozoa from wildlife has to be collected by electroejaculation under general anesthesia, or using other forms of artificial stimulation (Pukazhenthi et al. 2006a). The procedure is safe and has no negative consequences for the males as found in many laboratories around the world and on many species. When males die, spermatozoa can be recovered from the epididymides either by flushing or by slicing of tissue and allowing sperm cells to swim out in appropriate culture medium or in cryopreservation diluent.

Sperm evaluation is carried out to estimate concentration, and to assess percentage of motile cells, morphology, organelle integrity and, in certain cases, DNA integrity. In domestic avian and mammalian species and man, additional tests are employed to evaluate the functional capacity of spermatozoa with the aim of estimating their potential fertilizing ability. The objective is to evaluate in the laboratory the capacity of spermatozoa to undergo the series of changes required for fertilization. Tests may measure sperm survival, the capacity to interact *in vitro* with oviductal cells, capacitation (in mammals), the acrosome reaction induced with molecular probes or natural ligands, or sperm–oocyte interactions (see Chapters 7 and 8 of this volume). Work in many laboratories aims to establish relations between the results of these tests and fertility in vivo such as that seen after artificial insemination (Aitken 2006; Rodriguez-Martinez 2006, 2007). In wildlife (and more so in endangered species) these assays are difficult to perform because, among other reasons, sperm samples are scarce. Furthermore, the opportunities for performing artificial inseminations for fertility assessments will be almost nonexistent for these species. Laboratory tests will be required to examine fertility of cryopreserved samples. Sperm–oocyte interactions may be examined by homologous *in vitro* fertilization (Figure 14.2d; Berlinguer et al. 2008) but, due to difficulties in obtaining oocytes from endangered species, assessment is more frequently done by heterospecific *in vitro* fertilization using oocytes from domestic animal models (e.g., cow, sheep and cat; Roth et al. 1999; Berlinguer et al. 2003; Thiangtum et al. 2006).

Sperm evaluation is thus important for the characterization of sperm parameters of individuals from endangered species and for different steps in the process of cryopreservation. Furthermore, periodic evaluation of sperm quality in males from a captive breeding program (e.g., before the breeding season) allows for the identification of possible cases of subfertility or infertility (due to very low sperm counts, poor motility or high proportion of sperm abnormalities) and to evaluate the potential reproductive capacity of males. This information is essential to help managers in the decision-making process regarding breeding schemes.

Cryopreservation of spermatozoa represents a powerful tool for conservation of genetic resources and for an adequate genetic management of both captive and free-living populations. Spermatozoa were first successfully cryopreserved over 50 years ago and used in cattle artificial insemination soon afterwards. The benefits for its use in wildlife relate to the possibility of storing gametes from genetically valuable animals, extend generation times, prevent the possible transmission of infectious diseases, circumvent husbandry factors that prevent

animals from breeding, and facilitate the exchange of genetic material between subpopulations of captive or free-living animals. The latter is particularly important since it will allow the incorporation of founder alleles from the wild without the need to remove animals from natural populations. The value of cryopreservation of spermatozoa from wildlife has been reviewed and many examples have been presented (Figure 14.2e; Wildt et al. 1997; Watson & Holt 2001; Leibo & Songsasen 2002; Pukazhenthi & Wildt 2004; Pukazhenthi et al. 2006a).

There are now many examples of successful cryopreservation of spermatozoa from various vertebrate taxa (Watson & Holt 2001) with the main emphasis being placed on mammals and some birds (Pope & Loskutoff 1999; Leibo & Songsasen 2002; Donoghue et al. 2003), but also with considerable recent advances in fish and shellfish (Tiersch et al. 2007). Despite these interests and advances, considerable efforts are required to improve freezing protocols. Cryopreservation of spermatozoa from wild ungulates has certainly benefited from knowledge developed with cattle sperm cryopreservation (Pukazhenthi & Wildt 2004) leading to success in artificial insemination with frozen semen in antelope, cervids and rhinoceros (Jabbour et al. 1997; Roldan et al. 2006; Hildebrandt et al. 2007). However, translation of protocols used in domestic animal models is not straightforward since differences may exist even between closely related species and cryopreservation protocols will need to be adjusted (Garde et al. 2003, in press). On the other hand, cryopreservation of carnivore spermatozoa present problems and domestic models are of little help because adequate cryopreservation has not been obtained for them (Pukazhenthi & Wildt 2004). Current work in many laboratories tries to understand the many factors that affect successful cryopreservation in various species. Areas that receive attention include the buffer and sugar composition of cryodiluents, refrigeration curves, type and exposure to cryoprotectant agents and velocity of freezing and thawing.

How long do cryopreserved spermatozoa retain fertility? It is speculated that sperm cells could be stored indefinitely. Cryopreserved human spermatozoa can retain motility and good functional ability (as revealed by *in vitro* tests) for over 28 years (Clarke et al. 2006). One factor clearly affecting the results is that the methods used when the first samples were cryopreserved may not be as good as those in use today (see Chapter 15 of this volume). It should be noted that the ability to preserve motility is important only if sperm are to be used in artificial insemination (or *in vitro* fertilization). For intracytoplasmic sperm injection (ICSI), sperm motility is not required. Using ICSI, human spermatozoa stored for 21 years was successfully employed to generate a live baby (Horne et al. 2004). Furthermore, conditions for cryopreservation if spermatozoa are to be used by ICSI may be less strict since it has been found that mouse spermatozoa collected from bodies stored at $-20\,^{\circ}C$ in a freezer can be recovered after 15 years, used for fertilization via ICSI, and produce young (Ogonuki et al. 2006; see Chapter 15 of this volume).

Alternative methods of sperm preservation, such as freeze-drying and desiccation, are also being explored, since cryopreservation may not be adequate for

certain species, or individuals, or for samples collected or stored under certain conditions. Spermatozoa have been successfully freeze-dried, with live births following ICSI, in the mouse (Wakayama & Yanagimachi 1998; Ward et al. 2003), rabbit (Liu et al. 2004) and rat (Hirabayashi et al. 2005). Work is under way with bull and boar spermatozoa (Martins et al. 2007; Nakai et al. 2007), but no live young have been reported so far. Sperm desiccation under nitrogen gas (and storage at 4 or 22 °C), with birth of live young, has been achieved in the mouse (McGinnis et al. 2005). Bull sperm desiccated at temperatures above 50 °C and stored for various times, were capable of fertilizing oocytes by means of ICSI with development up to the blastocyst stage (Lee & Niwa 2006).

Depending on the technique used for sperm preservation, its use via assisted reproduction may vary. Artificial insemination is perhaps the first choice and for its implementation it is important to carry out prior characterization of female estrous cycles, the development of estrous synchronization protocols, and an adequate selection of insemination technique (e.g., cervical or intrauterine via laparoscopy). If the ability of spermatozoa to withstand cryopreservation is poor, if males supply spermatozoa of limited quality, or if immature sperm cells or less differentiated cells from the germline are used, it may be necessary to resort to *in vitro* fertilization or to intracytoplasmic sperm injection. The former requires the development of *in vitro* techniques to allow the sperm cell to undergo capacitation and sperm–oocyte interaction in the laboratory whereas the latter requires the capacity to employ micromanipulation techniques. Both demand the collection of oocytes (usually after synchronization, ovarian stimulation and oocyte *in vitro* maturation) as well as some form of embryo culture *in vitro* and embryo transfer techniques. For ICSI, methods are still in need of improvement for some domestic species (e.g., cattle), in particular with regards to sperm preparation before microinjection in order to achieve proper sperm-triggered activation of oocytes (Roldan 2006). Application of IVF or ICSI to wildlife species poses further challenges due to the stress-induced negative effects on gamete and embryo quality and on subsequent establishment and development of pregnancy to term. In addition, it is important to be aware that techniques such as ICSI remove the need for sperm to overcome a number of barriers present in the female tract, thus allowing fertilization by poor-quality sperm, for example, spermatozoa with poor or no motility which under physiological circumstances may not be able to swim along the female tract. In addition, these techniques may remove potential female barriers aimed at avoiding fertilization by related or genetically incompatible males (see Chapter 7 of this volume). Thus, work with endangered species should try to minimize any negative effects that the absence of such barriers may have on offspring viability and quality.

14.3.4 Sperm sexing

Sex selection by means of sorting X- or Y-chromosome bearing spermatozoa may be a valuable tool in the management of endangered species in captive breeding programs and potentially even if sorted sperm are used in free-living populations.

There is a premium for female calves in the USA and other countries and this is driving the commercial application of sperm sorting. In endangered species, the possibility of selecting the sex of the offspring may have a considerable impact in captive breeding programs facing space restrictions or having to deal with species in which the mating system is such that each male can mate with several females.

Offspring of predetermined sex have been born in cattle, human, sheep, pig, horse, rabbit, elk, buffalo, cat and dolphin (reviewed in Cran 2007). Sperm sorted by flow cytometry, with purities of over 90% of either X- or Y-sperm, can be used for artificial insemination or *in vitro* fertilization with subsequent transfer of the embryos produced. But use will be dictated by the efficiency of sorting and the assisted reproductive techniques available for different species. In cattle relatively high sorting rates, and use for low-dose insemination, are currently achievable, with a minimum of 35% motile sperm upon thawing and pregnancy rates that are about 75% of results obtained with unsorted spermatozoa. Recent studies have shown that sperm can be cryopreserved, thawed, sorted and cryopreserved again for field use (de Graaf et al. 2006) thus opening up new possibilities for collection of spermatozoa from wildlife species and using central facilities for sorting.

14.3.5 Transplantation of germline stem cells and testicular tissue

Sperm samples stored in a GRB can be used in artificial insemination, IVF or ICSI but this also means that the repository for a particular male may become exhausted at some point in time. Also, sperm recovery may not be possible from some males, for example, if animals die either before reaching reproductive maturity, or outside the breeding season, having no opportunity to contributing genetic material. Under these circumstances, collection and storage of germline stem cells (spermatogonia) or testicular tissue represents an option for the rescue, conservation and subsequent use of genetic resources (Dobrinski 2007).

Spermatogonial stem cell transplantation leading to sperm production has been carried out in the mouse, rat, boar, bull, goat and cynomolgus monkey (Pukazhenthi et al. 2006a; Dobrinski 2007). For endangered species, the goal will be to perform transplantation of spermatogonia into a heterologous species, preferably one that is phylogenetically close and readily accessible. Heterologous transplantation of rat, rabbit, dog, boar, bull, stallion, baboon and human spermatogonia into mouse recipients has revealed that successful sperm production occurs when transplantation takes place between closely related species (e.g., rat into mouse, mouse into rat, or hamster into mouse; Clouthier et al. 1996; Ogawa et al. 1999a, 1999b; Zhang et al. 2003). Spermatogonia from more distant species colonize but do not differentiate when transplanted into mouse testes (Dobrinski 2007). Thus, for endangered species, domestic relatives should be examined as potential recipients but bearing in mind that similarities in shape and size of spermatozoa derived from the donor and the host generate the need to develop methods to identify and separate the sperm cells of interest (Pukazhenthi et al. 2006a).

Spermatogonia or a mixture of cells from the germline are placed into the seminiferous tubules, via the efferent ducts, or the rete testis after adequate preparation of the host. Preparation of the spermatogonia or the germ cell mixture to be transplanted involves various steps including enzymatic digestion to first separate seminiferous tubules followed by a second step to isolate and enrich individual cells via sorting. Recipient preparation is also important with the need to deplete the endogenous germline of the host. This can be accomplished using a variety of treatments (Dobrinski 2007).

Since cross-species spermatogonial transplantation is not successful when donor and host are phylogenetically distant, efforts were placed in the development of ectopic grafting of testicular tissue under the back skin of immunodeficient mice as a different approach for the maintenance and propagation of male germ cells that can be more readily applied to different mammalian species (Honaramooz et al. 2002a; reviewed in Dobrinski 2007).

Grafting testis tissue from *immature* individuals to immunodeficient mice has resulted in germ cell differentiation and sperm production in pigs and goats (Honaramooz et al. 2002a), hamsters (Schlatt et al. 2002), rabbits (Shinohara et al. 2002), bulls (Oatley et al. 2004), rhesus monkeys (Honaramooz et al. 2004), cats (Snedaker et al. 2004) and horses (Rathi et al. 2006). Spermatozoa obtained by such means are capable of normal function including fertilization via ICSI (required because spermatozoa are recovered immotile and without the maturation process that takes place in the epididymis). Sperm recovered from allografts (mouse to mouse) and xenografts (monkey to mouse) supported embryo development when injected into oocytes (Schlatt et al. 2003; Honaramooz et al. 2004) and, following embryo transfer, mouse sperm from allografts sired normal progeny (Schlatt et al. 2003).

The onset of spermatogenesis in xenografted pig testis tissue occurred slightly earlier than in the donor species (Honaramooz et al. 2002) and testicular maturation and sperm production in rhesus macaque testis tissue was significantly accelerated (Honaramooz et al. 2004). On the other hand, completion of spermatogenesis of xenografted cat and dog testis tissue is delayed (Snedaker et al. 2004; Pukazhenthi et al. 2006a).

Testis tissue xenografting from mature *adult* individuals of pig, goat, bull, donkey, horse, or rhesus monkey does not support germ cell differentiation after transplantation, although seminiferous tubules with Sertoli cells only survive in some species (Arregui et al. 2008a). Interestingly, testis tissue from subadult donors survives better as xenograft than tissue from mature adult individuals and complete spermatogenesis can occur in some species. These difficulties may perhaps be circumvented in the future by the transplantation of isolated testis cells under the skin of immunodeficient mice, since they seem capable of reorganizing themselves into structures with morphologic and physiologic similarity to normal testis tissue (Honaramooz et al. 2007; Arregui et al. 2008b).

There are some limitations to the use of this technique, since transplantation to immunodeficient mice requires complex and expensive infrastructure. In addition, the lifespan of the mouse host may be shorter than the time required

to obtain proliferation and differentiation of germline from long-lived species. Nevertheless, this technique offers an enormous potential for the production of spermatozoa from endangered species.

For the transplantation of spermatogonia or testicular tissue it will be most useful to have cryopreservation protocols available in order to store cells or tissue and then use them under appropriate conditions. Spermatogonial stem cells from all the species examined can be cryopreserved for long periods with common techniques used for somatic cells (Brinster 2007). With regards to testicular tissue, cryopreservation has been effectively employed in the mouse, hamster, marmoset and humans (Schlatt et al. 2002; Shinohara et al. 2002) whereas cat testes have poor viability after cryopreservation (Pukazhenthi et al. 2006a). Recent studies (Jahnukainen et al. 2007) using rhesus monkey testes xenotransplanted to *nude* mice have explored the possibility of storing testicular tissue by cooling or by cryopreservation using different cryoprotecting agents, and revealed that cooled tissue showed good survival, and that cryopreservation in DMSO allowed grafts to initiate spermatogenesis upon transplantation. These results support the possibility of transport and centralization for xenotransplation at research facilities, and are encouraging for the future collection and storage of testicular tissue from wildlife species.

14.3.6 Spermatozoa derived from embryonic stem cells or somatic cells

Embryonic stem cells (ESCs) are defined as pluripotent stem cell lines derived from early embryos before formation of the tissue germ layers. They are derived usually from the pre-implantation blastocyst and exhibit indefinite proliferative capacity under appropriate conditions *in vitro*. Several investigations have demonstrated that murine ESCs can differentiate to primordial germ cell (PGCs) and subsequently to early gametes (oocytes: Hubner et al. 2003; sperm: Geijsen et al. 2004). Immature sperm cells derived from mouse ESCs in culture have generated live offspring (Nayernia et al. 2006a). Preliminary data indicate that human ESCs most likely display a similar developmental capacity to generate PGCs and, subsequently, gametes (Clark et al. 2004; Aflatoonian et al. 2005; Moore & Aflatoonian 2007). The findings indicate that human ESCs have the potential to differentiate to PGCs (as determined with surface markers and gene expression profiles) although these phenotypes represented a very small proportion of the total cell population. Germ cells will enter meiosis autonomously and develop as oocytes unless meiosis is blocked and cells are induced into a spermatogenic pathway. Interestingly, during human ESC differentiation both pathways seem to occur, regardless of the sex karyotype. Mechanisms underlying the process through which gamete-like cells are generated during stem cell culture remain unclear (Moore & Aflatoonian 2007). This ESC technology offers great potential for new types of reproductive investigations including a readily accessible system to investigate the very earliest stages of gametogenesis including epigenetic modifications of the germline.

These possibilities certainly depend on the availability of ESCs. However, ESCs may not be easily obtained from wildlife embryos produced *in vitro* (due, essentially, to the scarcity of material and technical difficulties) and it may be necessary to consider the use of somatic cell nuclear transfer with heterologous oocytes. Reproductive cloning (by nuclear transfer) has been presented as a possibility for endangered species (e.g., Loi et al. 2001) but success rate will remain low for some time. Conversely, somatic cell nuclear transfer may be useful to generate blastocysts of the desired genotype from endangered species and, from such blastocysts, obtain ESCs that could be used to produce the required PGCs and subsequently, either spermatozoa or oocytes. In this context, somatic cell nuclear transfer may be much more efficient than when it is used for reproductive purposes.

While the evidence is becoming more compelling that in culture ESCs can generate PGCs and germ cells, there remains the question as to whether germ cells can be derived (or transdifferentiated) from adult stem cells residing outside the gonad. There are now reports showing that mouse germ cells can be derived from bone marrow (Nayernia et al. 2006b; Lue et al. 2007). Similarly, human bone marrow have been found to give rise to spermatozoa (Drusenheimer et al. 2007).

These results have exciting implications since they open up many possibilities for the development of studies of mechanisms underlying primordial germ line differentiation and the production of 'synthetic' gametes (Surani 2004). Spermatozoa generated in this way could perhaps one day be used in assisted reproduction or to understand factors determining fertility, and provide an unlimited supply of gametes for endangered species.

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