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Endangered Species Spermatozoa: Diversity, Research, and Conservation

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The global explosion in human population has placed extraordinary pressures on the earth's resources. The result has been a rate of species extinction rivaling that seen during the demise of the dinosaurs. It is a dual-tiered disaster. Not only is the sheer number of species on the planet (biodiversity) being drastically reduced, but the well-being of extant populations is being compromised by relentless exploitation, fragmentation, and pollution of natural ecosystems. The crisis is not going unchallenged, and there are a host of treatises arguing for a wake-up call—that much more attention be given to preserving nature. Perhaps the most eloquent (and alarming) is E.O. Wilson's recent book, *The Diversity of Life*, that documents the impact of global ecosystem destruction, predicting extinction for perhaps 20% of all existing species within the next 30 years (1). In addition to enriching the soil and providing the very air we breathe, ecosystems conceal a wealth of new information on still undeveloped pharmaceuticals, crops, alternative food sources, timber, fibers, petroleum substitutes, and other potential amenities. For these reasons, he convincingly declares that "biological diversity is the key to the maintenance of the world as we know it." Wilson, like many others, makes a plea for developing systematic action plans that will provide stewardship for the earth's resources. To do otherwise or to presume that humankind can thrive in an impoverished biological world is both arrogant and reckless.

The preservation of rare animal and plant species often is entangled in political, economic, and scientific self-interests. Despite these complications, there seems to be real progress on at least two fronts. First, there is a growing realization that preserving native ecosystems and economic development are not necessarily mutually exclusive. Habitat and species can be preserved while fulfilling human needs, a concept known as *sustainable development* (2). Examples include extracting nontimber products

A. Bartke (ed.), *Function of Somatic Cells in the Testis*

from rainforests (i.e., rubber) or highly regulated commercial wildlife hunting in national parks. These types of approaches provide incentive for maintaining high-quality habitat and the species living therein. Second, a growing number of scientists, managers, and politicians are recognizing the real and catastrophic implications of losing biological and genetic diversity. An entire discipline, *conservation biology*, has emerged that seeks to define and understand the many factors that influence the survival of ecosystems, species, subspecies, and even individuals.

In general, there is unanimous consensus that the emphasis should be on maintaining natural ecosystems (in situ conservation), but for some it already is too late. In a sense, many wildlife refuges have become large zoos without fences. Encroachments and destruction have produced small and fragmented populations (similar to that found in captivity) that, if mismanaged further, are vulnerable to loss because of genetic impoverishment. This realization is driving the formation of national and international groups that are cooperating to preserve both habitat and individual species. Zoo scientists play a pivotal role in this new cooperation. Applying computer-based models (developed for managing small captive populations) to predicting wild population viability (3) is one example. Another is the actual reintroduction of zoo-bred offspring into the wild (4–6). In addition to developing new and improved strategies for propagating endangered species (7–10), zoos also contribute through public education/outreach programs that stress conservation themes and even through formal "adoption" of natural reserves (11). But the most important and rapidly evolving function of a zoo is its role as a research center. Of course, all good science explores the unknown, but zoo-oriented research tends to address questions that influence the existence or well-being of *entire species*. Perhaps nothing is more challenging and satisfying in research than understanding and helping to preserve a whole, living thing.

It is not unusual for most scientific professionals to be unaware of the specifics of the biodiversity crisis or recent efforts to address the problems. The issue is real, and for those desiring a "conversion experience," the author recommends studying reference 1. The sequelae involve coming to terms with how science (in this case, reproductive biology) can contribute to conservation. The purpose of this chapter is twofold. First, it is not well understood that high-quality research can be conducted in wildlife species and that findings can be relevant across a range of taxa (including humans). Therefore, one objective is to review some of our laboratory's research on sperm morphology and function in wild felids (cats), virtually all of which are threatened by severe losses in genetic diversity and by extinction. The aim is to demonstrate that novel basic research can be conducted in these unconventional taxa and that findings can be related directly or indirectly to species/population status and, perhaps, to fertility potential. Second, there is an increasing number of organized ex situ

captive breeding programs for endangered animals in which species are propagated strictly on the basis of their genes. Formal *Species Survival Plans* (SSPs) allow management of entire regional populations (many zoos together, rather than individual zoos) while dictating which animals may be mated together (12). The theory is that only genetically vigorous offspring are produced. Assisted reproduction techniques, now used routinely for accelerating livestock production and combating human infertility, have profound potential for managing endangered species. This assertion will be supported by reviewing our recent applied studies.

Genetic Diversity and Reproductive/Health Fitness, Especially Sperm Integrity

Direct human actions exert the most pressure on the extinction process, but habitat quality, demographic variables, and unpredictable catastrophes (e.g., disease and climatic change) also influence species and population survival. One other variable—the level of genetic variation within a species or population—also has attracted attention. In theory, as population size constricts, the incidence of incestuous matings increases, producing a homozygous or inbred population that is less healthy and less reproductively efficient. Producers of livestock have understood for years the sinister effects of monomorphic genotypes, a fact that was long ignored by wildlife managers. Both zoos and natural ecosystems provide ideal venues for studying the cause and impact of inbreeding depression. Due to limited space and because most of their specimens are rare, zoos are destined to deal with small populations. Managers of native ecosystems face a similar dilemma because habitat destruction and fragmentation eliminate natural corridors of genetic exchange. The result is a creeping tendency towards genetic homogenization, with species and populations becoming vulnerable to loss. A vortex is formed in which small population size combined with poor management causes a continued loss of fitness, with an eventual spin-off to extinction (Fig. 1.1).

There is evidence that reduced genetic diversity influences the viability of a wildlife population; specifically, its survivability and reproductive health. The first suggestion of this relationship came in 1979 from Ralls et al. (13) who surveyed survival of captive-bred ungulates with known pedigrees. The results (which in retrospect should not have been surprising) were at the time alarming because juvenile mortality was rampant in inbred compared to outbred offspring. The findings immediately motivated managers throughout North American zoos to begin propagating animals according to genotype, and one eventual result was the formation of the North American SSP program (12). Another outcome was increased interest in systematic, molecular assessments of genetic variation in natural and captive populations, especially in the context of simultaneous

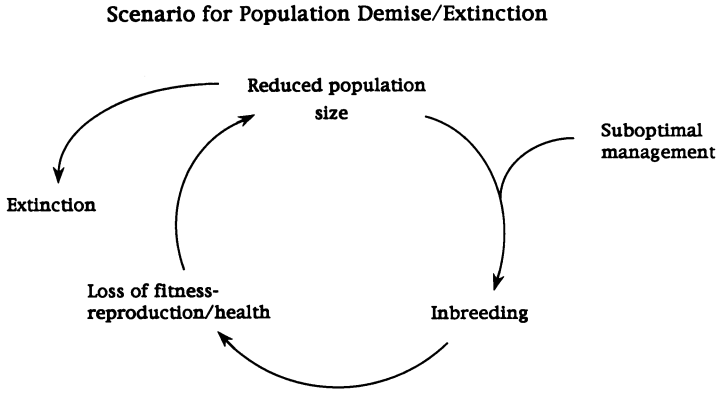

FIGURE 1.1. One scenario for extinction. Small population size, either with or without poor management, results in incestuous matings and inbreeding depression often expressed by decreased reproductive fitness and increased susceptibility to disease and congenital malformations. The cycle continues with further reductions in population size and more inbreeding, eventually leading to extinction.

measurements of reproductive traits. In this context, we have benefited from a long collaboration with the molecular genetics laboratory of O'Brien and associates at the National Cancer Institute. For more than a decade, concurrent studies of genetic variation and reproductive characteristics have produced compelling evidence that genetic impoverishment is related to poor ejaculate traits in Felidae species.

Our initial findings occurred by serendipity. During a survey of electroejaculate characteristics in a large population of cheetahs (*Acinonyx jubatus*) in southern Africa (14), we collected blood samples, almost as an afterthought. The latter were analyzed for allozyme polymorphisms by O'Brien and colleagues, who made the startling discovery that the cheetah was genetically depauperate, having no more genetic variation than inbred laboratory mice (15–17). The results were especially interesting considering that all male cheetahs were producing extraordinarily high proportions of structurally abnormal spermatozoa (~70% pleiomorphisms/ejaculate) (14, 18, 19). The findings also generated debate that the lost variability was somehow contributing to the cheetah's historic reluctance to reproduce in captivity and its tendency to succumb to infectious disease (16, 17, 19).

Two general questions became the focus of our research. First, is there a cause-effect relationship between reduced genetic variation and poor seminal quality in felids, especially an increased incidence of malformed spermatozoa? Second, what is the physiological significance and impact of pleiomorphic sperm in felid ejaculates? Specifically, do these structurally deformed cells participate in sperm-oocyte interaction and fertilization?

Addressing these questions has been difficult in the cheetah, in part because all cheetahs (captive and free-ranging) are not only genetically similar, but all produce high proportions of abnormally shaped sperm (14, 18). The lack of an outbred "control" population for study presents obvious limitations. In a recent detailed survey of captive cheetahs (n = 60), proven breeders produced comparable ejaculate quality (including proportions of pleiomorphic sperm) to age-matched, unproven males (19). Such findings only reinforce the uncertainty of the purpose or influence of these malformed cells. Is it possible to defend, from a physiological perspective, that pleiomorphic sperm in cheetahs are somehow normal? Based on evidence to follow, this appears not to be the case.

Incidence of Sperm Pleiomorphisms in Felids: A Relationship to Genetic Variability?

During the past 15 years, we have evaluated reproductive traits in more than 1000 individual felids representing 28 of the 37 known species in the family Felidae. Three consistent observations have emerged (7, 20, 21). First, there are species- or population-specific sperm concentration (number) and motility characteristics. Second, the taxon as a whole exhibits a higher incidence of teratospermia than most other mammals. Third, some felid species (or populations) ejaculate mostly structurally normal spermatozoa, whereas others largely produce malformed cells (Fig. 1.2).

These differences in sperm structure may be of genetic origin, but empirical, cause-effect studies have not yet been done. One can imagine the ethical problems of purposely inbreeding endangered species to verify such a hypothesis. Therefore, our investigations have been mostly opportunistic and retrospective, often taking advantage of unique populations that have become (for whatever reason) genetically compromised. Some evidence that felid ejaculate quality is sensitive to genetic influences is founded on brief case studies. Other findings are based on more systematic and longitudinal studies.

Evidence in a Leopard Cat Subspecies

One case report involves *Felis bengalensis euptilura*, a subspecies of leopard cat that has been readily propagated at the Tallinn Zoopark in Estonia (on the Baltic Sea coast). Similar to the common domestic cat in size, the *euptilura* subspecies is indigenous to eastern Russia, Manchuria, and Korea and generally unknown to North American captive breeding programs. Regimented electroejaculation of nine adult *euptilura* revealed total sperm numbers, motility ratings, and pleiomorphisms (15 structural types) (Table 1.1) similar to published values for normospermic domestic cats (14, 22). However, 2 age-matched males failed to conform to the

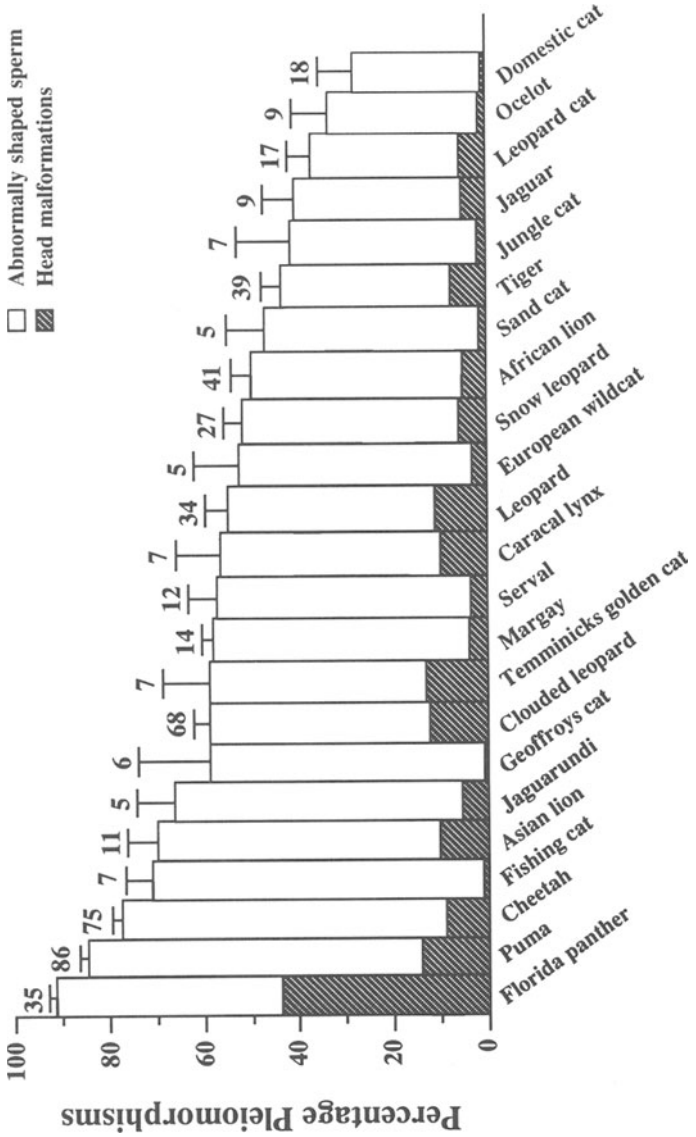

FIGURE 1.2. Proportion of total sperm pleiomorphisms and head defects (hatched area) in 23 species (or subspecies) in the Felidae family. Values are means ± SEM. The Florida panther is presented as a unique subspecies because of the high proportion of spermatozoa with head defects, primarily an acrosomal malformation. Domestic cat data are based on a normospermic population. Number above each bar indicates number of ejaculates.

TABLE 1.1. Electroejaculate traits of leopard cats (*Felis bengalensis euptilura*) known to be outbred ($n = 9$) versus 2 males produced by a sib-to-sib mating.

	Population mean (±SEM)	Male 10442	Male 10443
Total sperm number per ejaculate ($\times 10^6$)	9.9 ± 2.9	2.6	1.0
Sperm motility index*	63.6 ± 4.3	40.0	62.5
Structurally normal sperm (%)	74.4 ± 3.2	48.0	58.5
1. Abnormal acrosome	0.2 ± 0.1	0.0	1.0
2. Detached head	2.8 ± 1.2	3.5	2.5
3. Microcephaly	0.8 ± 0.4	0.5	0.0
4. Macrocephaly	0.3 ± 0.2	1.0	0.0
5. Bicephaly	0.1 ± 0.1	0.0	0.5
6. Bent neck region	0.4 ± 0.1	4.0	1.5
7. Bent midpiece with residual cytoplasmic droplet	6.0 ± 0.6	21.5	21.0
8. Bent midpiece without residual cytoplasmic droplet	1.4 ± 0.6	2.0	3.0
9. Deranged midpiece/sheath	0.5 ± 0.2	2.5	0.0
10. Tightly coiled flagellum	3.3 ± 1.5	11.0	3.5
11. Bent flagellum with residual cytoplasmic droplet	0.1 ± 0.1	0.0	0.0
12. Bent flagellum without residual cytoplasmic droplet	6.9 ± 2.0	4.0	3.0
13. Biflagellate	0.1 ± 0.1	0.0	0.5
14. Proximal cytoplasmic droplet	0.9 ± 0.4	1.0	5.0
15. Distal cytoplasmic droplet	1.8 ± 0.6	1.0	0.0

* Sperm motility index values were based on a calculation that considers overall sperm percent motility and type of forward progressive status, as described in reference 22.

population pattern (Table 1.1), producing only a fraction of the sperm number in the 9 counterparts. Although the sperm motility index was lower only for male 10422, both individuals produced more pleiomorphic sperm (26% and 16%, respectively) than the group mean, mostly an increased incidence of bent-neck and midpiece defects (Table 1.1).

The reason may have been related to the pedigree of the two outlier individuals. Both were siblings born to sibling parents, the sire being one of two brothers housed long-term with a full sister (Fig. 1.3). Because the parents of the sire and dam were unrelated (the male being wild-caught), the implication was that one generation of full-sibling matings may have adversely influenced both sperm numbers and sperm integrity in F1 offspring.

Evidence in Lion Subspecies

In collaboration with O'Brien and associates, we have examined genetic diversity and sperm characteristics in two populations of lions, one free-living in the Serengeti ecosystem in eastern African (*Panthera leo spp.*) and another (*Panther leo persica*, the Asian lion) in an isolated national park in western India. Compared to African lions, Asian lions have

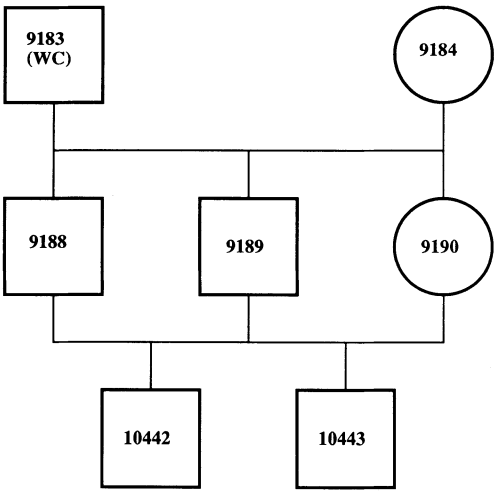

FIGURE 1.3. Lineage of 2 leopard cats (*Felis bengalensis euptilura*) known to produce poorer ejaculate quality than age-matched males managed under the same conditions (Table 1.1). Parents of both males were littermates not separated at sexual maturity. The sire may have been either male 9188 or 9189. The paternal ancestor was a wild-caught (WC) male from Korea.

markedly less heterozygosity based on allozyme polymorphisms and mitochondrial DNA fingerprint variation (23–25). These findings are reasonable because the ~300 extant Asian lions are descendents of ancestors that experienced a severe population contraction (to fewer than 20 individuals) in the first quarter of the twentieth century (26). These lions now strain the limited habitat of the Gir Forest Sanctuary and surrounding ecosystem (~1400 km^2), although a captive breeding program also exists at a nearby zoo.

In an earlier report (26), we documented that sperm number and structural integrity (but not sperm motility) were depressed in the genetically impoverished Asian lion compared to its African counterpart. More recently, we have reevaluated Asian males known to be fertile (e.g., proven breeders) versus those known to be infertile (based on serial couplings with multiple females and failure to produce young). Table 1.2 depicts sperm characteristics for (i) the original African "control" population, (ii) a group of 4 proven-breeder Asian males, and (iii) 2 Asian individuals (Ravi and Deep) known to be sterile. Again, Asian lions as a population tended to produce fewer total sperm of comparable motility, but with more structural deformities. Particularly striking, however, were the differences between the proven and sterile Asian males: Infertile individuals tended to produce many fewer total sperm, an overall poorer sperm motility index, and markedly higher proportions of defective-

TABLE 1.2. Comparison of electroejaculate traits for outbred African lions (*Panthera leo spp.*) free-living in Tanzania (n = 8), Asian lions (*P. leo persica*) that were proven breeders (n = 4), and 2 individual Asian lions known to be sterile.

	Population mean (±SEM)		Male	Male
	African lions	Asian lions	Ravi	Deep
Total sperm number per ejaculate ($\times 10^6$)	323.4 ± 29.9	224.3 ± 55.5	27.6	46.8
Sperm motility index*	80.5 ± 4.2	76.2 ± 4.8	55.0	50.0
Structurally normal sperm (%)	75.2 ± 7.2	51.8 ± 5.4	11.0	11.0
1. Abnormal acrosome	1.1 ± 0.3	4.2 ± 1.5	13.0	1.0
2. Detached head	0.0 ± 0.0	8.5 ± 2.3	16.0	25.0
3. Microcephaly	0.2 ± 0.0	0.3 ± 0.3	3.0	1.0
4. Macrocephaly	0.6 ± 0.2	0.2 ± 0.2	4.0	0.0
5. Bicephaly	0.2 ± 0.0	0.2 ± 0.2	0.0	0.0
6. Bent neck region	0.7 ± 0.1	1.2 ± 0.5	0.0	2.0
7. Bent midpiece with residual cytoplasmic droplet	2.3 ± 0.6	8.2 ± 3.0	18.0	33.0
8. Bent midpiece without residual cytoplasmic droplet	2.1 ± 0.6	2.8 ± 1.0	1.0	4.0
9. Deranged midpiece/sheath	1.9 ± 0.4	1.2 ± 0.9	11.0	2.0
10. Tightly coiled flagellum	2.3 ± 0.5	5.8 ± 1.6	8.0	11.0
11. Bent flagellum with residual cytoplasmic droplet	0.0 ± 0.0	0.0 ± 0.0	0.0	2.0
12. Bent flagellum without residual cytoplasmic droplet	0.9 ± 0.3	0.8 ± 0.2	1.0	4.0
13. Proximal cytoplasmic droplet	7.5 ± 1.1	11.0 ± 4.2	10.0	4.0
14. Distal cytoplasmic droplet	5.0 ± 1.8	1.0 ± 0.4	4.0	0.0

* Sperm motility index values were based on a calculation that considers overall sperm percent motility and type of forward progressive status, as described in reference 22.
Source: African lion data adapted from Wildt, Bush, Goodrowe, et al. (26).

appearing cells (predominantly detached heads, bent midpieces, and tightly coiled flagellum defects) (Table 1.2).

Are these observations relevant to the genetic history of the Asian lion? We contend that the most logical explanation for the significant differences in ejaculate traits is the outbred versus inbred genotypes of the African versus Asian population/subpopulations. The recent appearance of sterile Asian lion males suggests a continued cascade in gene loss, predisposing some males to physiological infertility. It is important to reemphasize that the entire Asian population (both wild and captive) is genetically invariant (23, 24). Both of Ravi's parents were wild-caught (Fig. 1.4), but the ejaculate traits of the sire Sundar (evaluated in 1986; total sperm/ejaculate: 74.6×10^6; sperm motility index: 51.8; structurally abnormal sperm: 71.8%, including 15.4% detached heads, 11.4% sperm with a bent midpiece, and 13.7% sperm with a tightly coiled flagellum) were poorer than proven breeder males listed in Table 1.2. Sundar also was a grandsire of Deep, who, in turn, was an offspring of a sib-to-sib

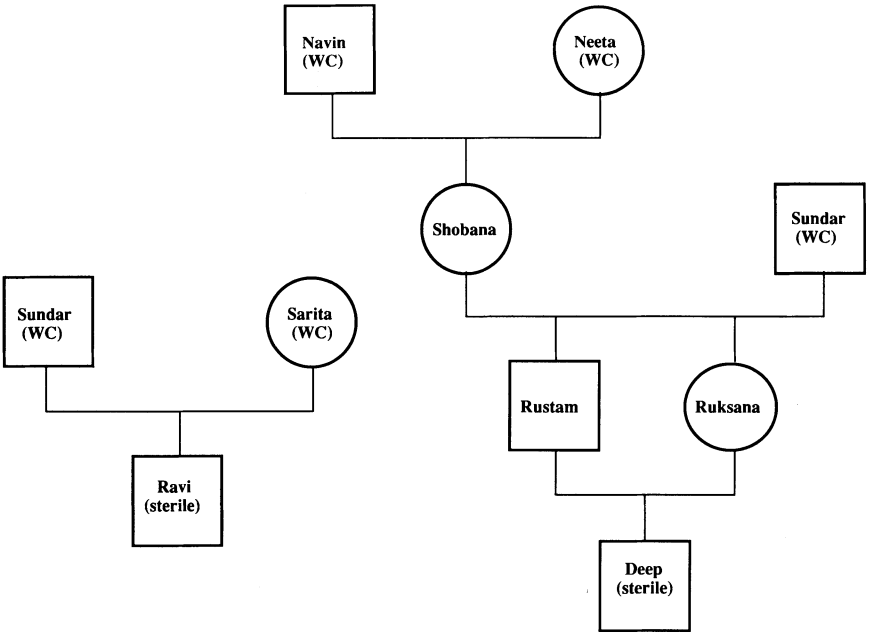

FIGURE 1.4. Lineage of 2 Asiatic lions (*Panthera leo persica*) known to be sterile and producing poor-quality ejaculates (Table 1.2). Sundar, the sire of Ravi and the ancestor of Deep, also was known to produce poor-quality semen, even though wild-caught (WC) (see text).

mating (Fig. 1.4). Therefore, considering that (i) there are quantitative differences in genetic variation and in ejaculate traits between African and Asian lions and (ii) sterile Asian lions tend to be offspring of deliberate inbreeding or sires with poor semen quality, then it seems likely that genotype is influencing reproductive fitness. In short, the genetic vigor of wild Asian lions is being compromised by a restricted habitat that inevitably promotes incestuous matings. The problem is accentuated by suboptimal captive management that is accelerating inbreeding and causing documented sterility.

Evidence in the Puma: Florida Panther Example

The Florida panther (*Felis concolor coryi*) is a subspecies of puma (cougar, mountain lion) on the brink of extinction. Historically, the panther ranged throughout the southeastern United States as far west as eastern Texas and as far north as Tennessee (27). However, human residential and agricultural development has squeezed the extant wild population, totaling 30 individuals, into southern Florida, principally within the Big Cypress Swamp and the adjacent Everglades National Park

ecosystems (28, 29). Although the critical status of the Florida panther has been known for 25 years and although legally protected, the population is not self-sustaining, and computer modeling has predicted subspecies extinction within 25 to 40 years (30).

The potential loss of this charismatic, large-sized predator has motivated federal and state authorities to mobilize a massive research effort into the natural history and biology of the subspecies. Original tracking, prey base evaluations, habitat utilization, and natural mortality studies (begun in 1976) were succeeded by more intensive investigations into population genetic structure, medical causes of mortality, habitat contamination, and in our case, reproductive function.

The Florida panther has several unique phenotypic and genotypic traits not found in other puma subspecies throughout North and South America. Panthers have a broad flat frontal region of the skull, a distinct whorl of hair (cowlick) on the mid-dorsal thorax, and a 90° kink in the vertebrate at the tail tip (31). On the basis of allozyme polymorphisms, the frequency of *polymorphic loci* (P) and the average heterozygosity estimate values are 4.9% and 1.8%, respectively (29). By contrast, pumas from other geographic regions have relatively abundant allozyme diversity, including 11 polymorphic loci (P = 27%) and average heterozygosity estimates from 1.8% to 6.7%. Overall genomic diversity also has been quantified using hypervariable minisatellite genetic loci (DNA fingerprinting). Using clones of two feline-specific minisatellite genomic families, O'Brien and associates have shown that the Florida panther expresses 85% less variation than other puma populations (29). The level of genetic variability in authentic Florida panthers is practically analogous to that measured in the homogeneous Asian lion (discussed earlier).

This genetic uniformity is exerting both a reproductive and general health cost. The Florida panther population is reproducing, but at a nonsustaining and poorly efficient level. For example, one adult male has been maintained long-term in captivity with female pumas of western US origin. Despite multiple pairings and observed copulations, no pregnancy has ever been established. We have systematically evaluated seminal and testicular characteristics in Florida panthers and free-living counterparts in four other geographic regions, as well as "generic" individuals in zoos (27). The results (Table 1.3) revealed several important findings. Regardless of site of origin, the puma as a species tended to ejaculate relatively high proportions of pleiomorphic spermatozoa. However, compared to all groups, Florida panthers produced poorer-quality electroejaculates, including ~94% of all sperm being malformed. The most prevalent defect was an acrosomal anomaly observed much less frequently in other felid species or populations. Compared to the other puma subspecies, more Florida panther sperm had this acrosomal malformation (>40%), as well as a defective midpiece that consisted primarily of an abnormal mitochondrial sheath (swollen, partial, or complete aplasia: >5%).

TABLE 1.3. Comparison of electroejaculate traits (mean ± SEM) for Florida panthers compared to free-ranging pumas from Texas, Colorado, and Latin America and captive generic males from North American zoos.

	Florida (n = 40)	Texas (n = 20)	Colorado (n = 8)	Latin America (n = 6)	Captive (n = 66)
Total sperm number per ejaculate ($\times 10^6$)	4.8 ± 2.1[a]	34.4 ± 13.7[a,b]	40.4 ± 11.2[a,b]	33.6 ± 20.5[a,b]	62.8 ± 11.6[b]
Sperm motility index*	45.6 ± 5.7[a]	56.9 ± 5.0[a,b]	73.8 ± 2.2[b]	71.9 ± 7.2[b]	58.5 ± 2.6[b]
Structurally normal sperm (%)	6.5 ± 0.7[a]	14.0 ± 3.5[b]	16.3 ± 2.1[b]	37.8 ± 3.3[c]	16.5 ± 1.9[b]
Abnormal acrosomes (%)	41.8 ± 2.0[a]	10.1 ± 2.8[b]	18.5 ± 2.5[c]	15.4 ± 4.3[b,c]	10.7 ± 1.8[b]
Abnormal midpiece†	5.4 ± 0.7[a]	1.3 ± 0.3[b]	3.5 ± 0.7[a,b]	1.8 ± 0.8[b]	2.2 ± 0.5[b]

Note: n = total number of ejaculates. Row values with different superscripts differ ($P < 0.05$).

* Sperm motility index values were based on a calculation that considers overall sperm percent motility and type of forward progressive status, as described in reference 22.

† Primarily consisting of a deformed mitochondrial sheath (swollen, partial, or complete aplasia).

Source: Adapted, in part, from Barone, Roelke, Howard, Anderson, and Wildt (27).

Transmission electron microscopy of the Florida panther spermatozoon revealed extraneous acrosomal material between the acrosome and the sperm plasma membrane, derangements in acrosomal continuity, and vesicular remnants of the Golgi complex in the head region (27). This deformity has many of the same characteristics as the miniacrosome defect in humans that has been attributed to degeneration during spermiogenesis or absorption of the acrosome by the Sertoli cells (32).

Florida panthers also exhibit an unusually high predisposition to *cryptorchidism* (one or both testicles retained in the body cavity). This developmental defect is heritable in other species and is suspected to result from a sex-limited autosomal gene (33, 34). The condition is uncommon in wild felid species and has been observed in only 2 of more than 50 captive males and never in free-living pumas in Texas, Colorado, British Columbia, or Latin America (29). Retrospective analysis has revealed an alarming increase of cryptorchidism in Florida panthers. From 1970 to 1974, only 1 of 6 males (16.7%) expressed the condition, but the incidence rose to 60% from 1980 to 1984, 72.8% from 1985 to 1989, and 83.8% (5 of 6 cubs) from 1990 to 1992 (27, 29). As a result, 90% of all living males are now afflicted, and recently, 2 young males from a consanguineous mating have been discovered to be bilaterally cryptorchid (and, thus, presumed sterile).

Genetic fixation is having other maladaptive consequences in the Florida panther. An atrial septal cardiac defect has been diagnosed recently as causing mortality in two young pumas, and heart murmurs have been discovered in approximately 80% of all new offspring (29). Heaped on these problems have been recent medical surveys indicating high seroprevalence of circulating antibodies to various infectious pathogens, including feline panleukopenia virus, feline calicivirus, puma lentivirus,

rabies virus, and feline corona virus. O'Brien and associates have argued previously that one consequence of genetic compromise is increased vulnerability to infectious agents, primarily due to homogenization of genes involved in immune defense (16). This scenario seems to be occurring in the Florida panther.

The overall findings provide a provocative example of the consequences of demographic reduction and genetic depletion on population survival. The results are quite clear: Once the genotype of a rare population becomes homogeneous, reproduction is only one of many physiological traits impaired. Furthermore, despite the best (late) efforts of government policymakers and science, heroic options for reversing the process are unavailable, and the population is at highest risk for extinction. Certainly, the primary threat to the Florida panther as a true subspecies continues to be direct demographic pressures imposed by humans. Continued habitat loss, prey depletion, and even the recent discovery of mercury toxicity in the environment likely are the clearest threats to survival. However, the by-product of genetic uniformity also contributes to what may be a calamitous finale for the Florida panther. Whatever the ultimate result, the subspecies offers a profound illustration of the absolute need for wildlife managers to be concerned about genetic depletion.

Influence of Teratospermia on Gamete Interaction in Felids

Do pleiomorphic spermatozoa ejaculated by some felid species or populations influence sperm-oocyte interaction or fertilization? In reality, it is likely that few of these sperm reach the oviduct, the site of fertilization. Nevertheless, almost a decade ago Mahadevan and Trounson (35) observed an indirect correlation between the incidence of structurally abnormal sperm in the inseminant and ovum penetration in human *in vitro fertilization* (IVF) trials. More recent studies have demonstrated that the binding ability of sperm to the *zona pellucida* (ZP) and the number of sperm within the ZP are diminished in samples from subfertile men that fail to achieve fertilization during IVF (36–38). The consequences of teratospermia in felids appear similar. In IVF trials we consistently observe a much higher incidence of fertilization and cleavage when inseminants contain high proportions of structurally normal spermatozoa (9). For example, embryo cleavage routinely occurs in 60%–80% of all oocytes cocultured with sperm from normospermic domestic cats (39, 40) or tigers (*Panthera tigris*) (41). In contrast, fewer than 20% of conspecific oocytes form cleaved embryos when co-incubated with teratospermic inseminants from the cheetah (42) or puma (43).

We have systematically examined the ability of sperm from different-quality ejaculates to bind, penetrate, and fertilize oocytes in vitro. These

studies have been facilitated by comparing two populations of domestic cats that consistently produce normospermic (<40% abnormally shaped sperm) or teratospermic (>60% pleiomorphisms) ejaculates (22, 44, 45). Subjecting these teratospermic ejaculates to swim-up processing increases the percentage of normal sperm forms recovered (22, 45). For example, teratospermic cat ejaculates routinely averaging only 33.3% normal sperm contained 66.5% normal cells after processing, a value no different from normospermic counterparts (70.7%) (45). When these sperm populations were co-incubated with conspecific, salt-stored oocytes, the number of sperm bound to the outer periphery of the ZP was increased by more than 3-fold using normospermic (mean: 26.2) versus teratospermic (8.3) inseminants. Compared to normospermic males, the sperm from teratospermic cats were less able to penetrate the inner, bilayered ZP or the *perivitelline space* (PVS) (Fig. 1.5a). Sperm from both groups also were capable of fertilizing in vivo-matured cat oocytes, but the incidence of embryo formation (cleavage) was 36% higher for the normospermic group (Fig. 1.5b). Although fertilization was compromised using morphologically normal sperm from teratospermic cats, all resulting embryos grew at a similar pace, and there was no effect of the origin of the sperm donor on the proportion of embryos eventually becoming morulae in vitro (Fig. 1.5b). Because sperm concentration and motility ratings were maintained constant between cat populations, we conclude that there is a fundamental functional deficit, even in normal-appearing sperm from teratospermic domestic cats (44, 45).

We have also examined the fate of pleiomorphic sperm in the teratospermic domestic cat and cheetah (Fig. 1.6) (9, 45, 46). Both study populations ejaculated more than 70% malformed sperm per inseminant that then were cocultured in vitro with salt-stored, domestic cat oocytes. Few of the pleiomorphic domestic cat sperm (<30%) bound to the ZP compared to >55% of the malformed cheetah sperm. In both species fewer than 20% of the abnormal sperm penetrated the outer layer of the ZP, and virtually none of the pleiomorphic cell types reached the inner ZP or the PVS. Obviously, the felid ZP plays a significant role in filtering structurally defective sperm. More recent data suggest that the ZP may act as more than a simple barrier/filter to pleiomorphic sperm. In an attempt to increase the incidence of oocyte penetration using teratospermic ejaculates, we mechanically pierced the ZP by micromanipulation, thus creating channels to potentially facilitate sperm entry (47). Teratospermic domestic cat ejaculates were swim-up processed and then cocultured with conspecific oocytes that were untreated or ZP pierced (6 channels/oocyte). Micromanipulation enhanced the proportion of oocytes with sperm in the inner ZP or the PVS without increasing the number of pleiomorphic cells at either site (Fig. 1.7). Thus, ZP piercing appears to compensate, in part, for the functional deficit preventing penetration by even structurally normal sperm in teratospermic ejaculates. Because the

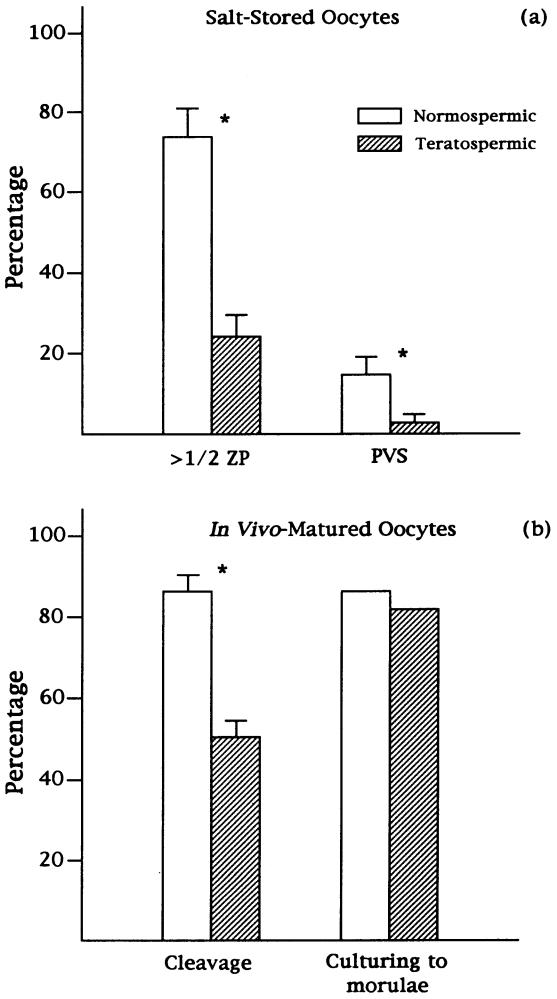

FIGURE 1.5. Penetration in vitro of salt-stored, domestic cat oocytes (*a*) (n = 202) or cleavage and embryo development after insemination of in vivo-matured oocytes (*b*) (n = 401) using sperm from normo- or teratospermic ejaculates. Categories of zona pellucida (ZP) penetration: >1/2 ZP = % oocytes with sperm in the inner half of the ZP; PVS = % oocytes with sperm in the perivitelline space. The asterisk indicates that means (± SEM) are different ($P < 0.05$). Adapted from Howard, Bush, and Wildt (44) and Howard, Donoghue, Johnston, and Wildt (45).

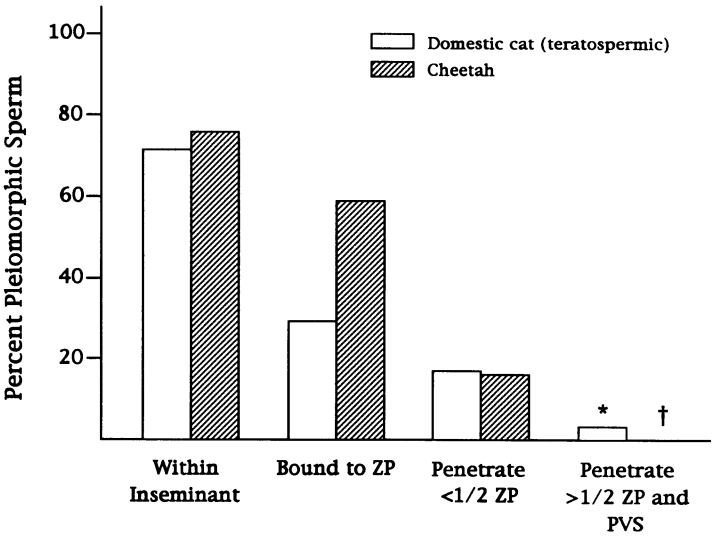

FIGURE 1.6. Illustration of the filtering role of the felid ZP. Teratospermic domestic cat (n = 6) and cheetah (n = 20) ejaculates were used to inseminate salt-stored domestic cat oocytes. Categories of ZP penetration: <1/2 ZP = % of oocytes with malformed sperm in the outer half of the ZP; >1/2 ZP and PVS = % of oocytes with malformed sperm in the inner half of the ZP or in the PVS. The asterisk indicates only sperm with a residual cytoplasmic droplet entering >1/2 ZP, not the PVS. The dagger indicates a zero value. Adapted from Howard, Donoghue, Johnston, and Wildt (45) and Howard, Barone, Bush, and Wildt (46).

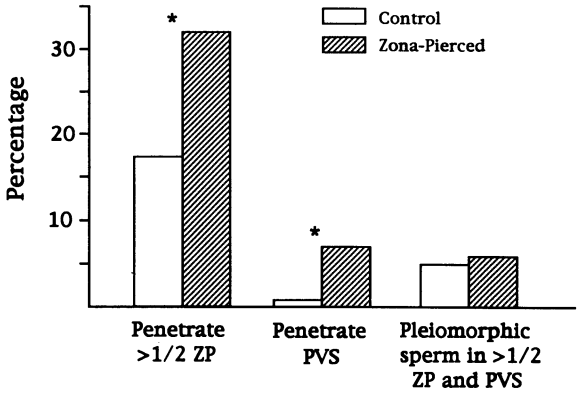

FIGURE 1.7. Impact of no treatment (control) or ZP piercing by micromanipulation on sperm penetration in vitro in the teratospermic domestic cat. Values represent the incidence of penetration of salt-stored oocytes (n = 122) and the proportion of structurally abnormal sperm in the inner ZP (>1/2 ZP) or PVS. The asterisk indicates that values are different ($P < 0.05$). Adapted from Roth, Howard, and Wildt (47).

technique does not allow indiscriminate penetration by malformed sperm, the ZP must selectively filter pleiomorphisms by multiple mechanisms (beyond simply acting as a barrier). Alternatively, perhaps these deformed cells are so structurally, biochemically, or metabolically compromised that fertilization will fail regardless of ZP integrity.

Based on these cumulative observations, we are now screening a host of specific factors that might help explain why normal and abnormal sperm from teratospermic males are inefficient at interacting with oocytes. These include sperm ultrastructure, motility/metabolism, ability to achieve the acrosome reaction, and type and concentration of sperm-binding receptors. We believe the findings will have obvious implications to conservation biology. First, there will be a continued affirmation and better understanding about how ejaculate quality relates to reproductive fitness. Second, we anticipate that these efforts will naturally give rise to developing assisted reproduction techniques for circumventing cases of suboptimal fertility. Finally, because men routinely produce high proportions of sperm pleiomorphisms,we also contend that the cat taxon serves as one of the most ethically attractive models available for studying the impact of teratospermia on fertilization as it relates to human reproductive health (48).

Meeting Management and Preservation Challenges Using Assisted Reproduction

The challenges to maintaining biological and genetic diversity can be met using an arsenal of scientific wisdom, logic, and technology. Because the essence of species survival is reproduction, the reproductive physiologist is destined to play an important role. For ex situ (zoo) breeding programs, assisted reproduction could be a powerful management tool for overcoming problems associated with sexual incompatibility, cases of organic infertility, and aged or underrepresented founders unable to contribute to species preservation. Especially important would be the ability to meet various SSP breeding mandates with fewer complications. (For example, transporting frozen semen, rather than living animals, would be less stressful and costly.)

Reproductive biology can also contribute to preserving species in native habitats. Perhaps the most profound impact could be achieved by developing systematic *genetic resource banks*, repositories containing germ plasm, blood products, tissues, and DNA (Table 1.4) (8, 10). Organized sampling and cryopreservation of, for example, spermatozoa from selected, free-living individuals would permit snapshot storage of existing diversity. The effect would be to provide insurance against future human-induced or natural catastrophes capable of reducing genetic diversity further or eliminating entire species. Because of habitat fragmentation

TABLE 1.4. Benefits derived from using assisted reproductive technologies (artificial insemination, IVF, and embryo transfer) in combination with the systematic banking (cryopreservation) of germ plasm/embryos from endangered species.

- Would help resolve the problem of too little space to preserve too many species, subspecies, populations, and individuals
- Would offer unique opportunities for circumventing management problems associated with a limited gene pool, sexual incompatibility, organic infertility, and aged, underrepresented founders
- Would provide a high level of protective insurance from catastrophes (natural disasters and disease epidemics)
- Would allow the interactive movement of biological material between living populations, thus maximizing genetic vigor

and the resulting isolation of populations, these germ plasm reserves could also be used for transferring genetic vigor. We recently described the logistical possibilities of interchanging genes by periodically capturing and artificially inseminating females from one isolated population using sperm from another (8). Each animal is producing surplus germ plasm, and given that this material is on reserve, there is no longer need to supplement zoo populations with animals from the wild. In essence, reproductive technologies could assist in keeping wild populations healthy and numerous. The most important by-product would be the sheer presence of a wild population that in turn justifies and promotes habitat protection.

The advantages of genetic resource banks go beyond supporting in situ populations. A natural benefit would be allowing the interactive movement of biological materials between living populations, especially transporting wild germ plasm to invigorate captive populations that have become genetically stagnant. Finally, sperm (and eventually embryo) repositories could help resolve the major crisis now facing zoos—lack of space. Of the many thousands of taxa deserving attention, biologists generally agree that zoos have sufficient space for conserving fewer than 1000 species of mammals, birds, and reptiles. Some of this problem could be alleviated by maintaining portions of needed genetic diversity in liquid nitrogen, thereby reducing the number of living animals required and freeing space for other species at high risk.

It is possible to use assisted reproductive technology and fresh or frozen-thawed germ plasm to generate offspring in rare wildlife species (reviewed in 9, 10). Table 1.5 lists 7 species in which young have been produced after artificial insemination or IVF/embryo transfer in our laboratory. Fresh sperm were used for artificial insemination in most cases, but it is noteworthy that offspring have been born to 3 endangered species (leopard cat, black-footed ferret, and Eld's deer) using thawed spermatozoa and to 2 species (cheetah and puma) known to be terato-

TABLE 1.5. Recent examples of producing offspring from rare or endangered species using artificial insemination (AI) or IVF followed by embryo transfer (ET).

Species	Technique	Fresh or frozen-thawed germ plasm	No. of pregnancies	No. of offspring
Leopard cat (*Felis bengalensis*)	AI	Fresh and frozen	2	3
Cheetah (*Acinonyx jubatus*)	AI	Fresh	3	7
Tiger (*Panthera tigris altaica*)	AI	Fresh	1	1
(*Panthera tigris tigris*)	IVF/ET	Fresh	1	3
Puma (*Felis concolor stanleyana*)	AI	Fresh	1	1
Clouded leopard (*Neofelis nebulosa*)	AI	Fresh	1	2
Black-footed ferret (*Mustela nigripes*)	AI	Fresh and frozen	3	7
Eld's deer (*Cervus eldi*)	AI	Frozen	9	10

Source: Adapted from Wildt, Monfort, Donoghue, Johnston, and Howard (9); Donoghue, Johnston, Seal, et al. (41); Howard and Doherty (50); Howard (51); Howard, Donoghue, Goodrowe, et al. (52); Donoghue, Johnston, Armstrong Simmons, and Wildt (53); and Monfort, Asher, Wildt, et al. (54).

spermic. We have emphasized in earlier reports (48, 49) that the rate of these successes is directly proportional to the amount of fundamental (basic) reproductive knowledge available for each species of interest. It is now well established that species-specific idiosyncrasies often prevent domestic livestock or laboratory animal technologies from being easily adapted to wild counterparts (9, 55). However, given a sound database, all evidence suggests that basic reproductive knowledge can be translated into living young. Our ability to produce multiple pregnancies in some species (Table 1.5) supports the assertion that these technical approaches hold exciting promise for conservation.

Summary and Concluding Perspective

Our overall challenge is not in conforming to the idea that more biodiversity is good (one would be hard-pressed to find disagreement on this point), but in meeting the staggering problem of identifying, prioritizing, and then preserving the earth's most valuable specimens. What is the role of the reproductive biologist? One high priority is generating the reproductive life history database for individual species in crisis, information critical for allowing successful natural or assisted reproduction to proceed.

Another seems to be in developing formal genetic resource banks useful for insuring existing diversity and managing more species than currently is possible in ex situ captive breeding programs. Certainly, because of continued fragmentation of in situ habitats, we also foresee infusions of genetic vigor into isolated wild populations via assisted reproductive technology as a viable option. Therefore, two additional contributions of reproductive biologists to conservation include (i) developing strategies to maximize genetic health while improving management efficiency and (ii) identifying when losses of genetic diversity appear to be influencing reproductive performance.

Although it has been difficult to define unequivocally a direct relationship between the level of genetic variation and fertility, we have provided evidence for compromised physiology and health in felid species, subspecies, and individuals known to be at least partially genetically impoverished. Such observations have led to a series of detailed studies on the impact of a specific unique characteristic (sperm pleiomorphisms) on sperm-oocyte interaction and fertilization in vitro. These studies produced evidence that both structurally abnormal and normal sperm from teratospermic ejaculates are compromised in fertilization ability.

Although of basic interest to gamete biologists, such results may be more important when considered in the context of conservation as a whole and the need for more research and improved management. For example, any evidence illustrating the potentially adverse effects of genetic loss certainly can be incentive for managers to manage better. However, more important is the utility of these types of findings for compelling policymakers to begin seriously supporting conservation biology research. In reality, the few reproductive biologists lured into this field are absolutely fascinated with the potential, but simultaneously panicked and impeded by the lack of any formal support mechanism. Amazingly, there is no National Institutes of Health for Endangered Ecosystems or Species, no government-based resource for considering competitive applications to fund conservation-oriented basic and applied research. No one can argue the critical importance of continued research funding to enhance the human condition; obviously, it should remain at the apex of the priority list. But considering that so much of the earth's biological and genetic diversity is approaching extirpation, should not this area of research at least be *on* a priority list?

In summary, there are three elements essential to allowing reproductive researchers to contribute to real conservation: (i) access to species in crisis, (ii) working models of how reproductive physiology can be used to generate genetically valuable young from rare species, and (iii) resources. Two of these elements now are available. First, increased interinstitutional collaboration among conservationists and the production of action plans that guide individual species preservation have become the norm. Second, examples are available demonstrating how high-quality basic research can

be translated (with assisted reproduction) to produce endangered young. Therefore, the only missing element is the resources needed to fund this new brand of research. Hopefully, this perspective will provide a greater understanding for the scientific community of the potential for preserving global biological and genetic diversity, as well as the dilemmas to be faced. For those scientists or their colleagues involved with policymaking, it is to be hoped that these words will provoke action.

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