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Spermatology for Understanding, Managing and Conserving Rare Species

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Abstract. Most conventional spermatology research involves common mammalian species including livestock, laboratory animals and humans. Yet, there are more than 4500 mammalian species inhabiting the planet for which little is known about basic reproductive biology, including sperm characteristics and function. This information is important, not just as adjunct knowledge, but because the majority of these species are threatened with extinction, largely due to human-induced pressures. The field of conservation is changing rapidly, and global cooperation is emerging among a variety of wildlife enthusiasts, ranging from management authorities of nature reserves to curators of rare zoological collections. Conservation progress depends on systematic, multidisciplinary research first to answer basic questions, with new data then applied to endangered species management plans. The reproductive physiologist is a crucial component of this scheme. Reproduction is the essence of species survival, and enormous effort needs to be directed at these 'untraditional' research species, subspecies and populations. Spermatology research combined with simultaneous efforts in endocrinology, embryology and cryopreservation (among others) can lead to the successful application of assisted reproduction. Examples from this laboratory include an array of wild felid species and a rare cervid and mustelid. Obstacles to success are formidable, including unique species-specificities, diminished genetic diversity and a general lack of resources. Nonetheless, the field offers tremendous opportunities for generating unique knowledge of comparative interest and with conservation utility.

Extra keywords: conservation, spermatozoa, endocrinology, assisted reproduction, artificial insemination, biodiversity, genetic variation.

Introduction

Traditionally, the reproductive biologist has been: *(1)* a basic scientist exploring the fundamentals of reproductive processes of readily available species; or *(2)* an applied problem-solver interested in coaxing more reproductive efficiency from valuable livestock or infertile humans or developing improved contraception.

There is, however, a real need for a different kind of reproductive biologist, one who primarily emphasizes basic and applied reproduction of rare animal and plant species. Ever increasing human growth and activity patterns exert constant pressures on our environment, especially the Earth's rare fauna and flora. We have described previously the enormous contributions to be made from assisted reproductive techniques and genome resource banks for managing endangered species both *ex situ* (in captive breeding programmes) and *in situ* (in nature) (Wildt 1989, 1990, 1992, 1994; Wildt *et al.* 1992*a*, 1993). The benefits range from the more efficient distribution of genes (to avoid the adverse effects of inbreeding) to enhanced preservation and 'insurance' of extant bio- and genetic diversity.

The differences between the traditional reproductive biologist and the conservation-oriented reproductive biologist are profound. The latter also must be a generalist with a knowledge and appreciation for the complexities of small population biology. After all, endangered species (by definition) are rare; usually few study subjects are available, and these are under strict regulations by local holding institutions and often regional and even international law. To-date, there also is no governmental funding source that promotes or widely entertains proposals focussing on single species conservation. Therefore, immediately there are logistical and political risks and a lack of essential financial support to systematically study rare species.

However, the most formidable problem has been a misalignment in research priorities. Too often, wildlife managers and researchers have considered their rare charges as simply closely-related counterparts of common laboratory and livestock animals (Wildt 1989; Wildt *et al.* 1992*b*). The general approach is to bypass understanding the fundamentals of reproduction for the 'quick fix'. However, 20 years of poor results in applying livestock

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technology to wildlife are having a convincing impression on the importance of species specificities (Wildt *et al.* 1992*b*). Although interfering with our ability to achieve rapid and repeated success, ironically it is precisely these unique physiological differences among species that make the field so fascinating.

In this paper, we illustrate how reproductive physiology (specifically spermatology) can contribute to both understanding and conserving wildlife species. Our strategy is to provide examples for the need for a multidisciplinary, holistic approach that emphasizes: *(1)* prerequisite basic research; and *(2)* the many novel variables influencing reproductive efficiency with examples focussing primarily on spermatology. Finally, we demonstrate that, when these data are in place, assisted reproduction techniques can be useful for producing offspring from endangered species.

The Importance of Endocrinology to the Wildlife Spermatologist

Conventional spermatology studies always are required before collected spermatozoa can be useful for active conservation; but, need goes far beyond the isolated discipline of sperm function.

It is not unusual for our laboratory to be asked on short notice to attend to a crisis — suddenly there are so few individuals of a species, subspecies or unique population that immediate remediation is required. Inevitably, the situation has two characteristics: *(1)* authorities waited too long before requesting assistance; and *(2)* almost nothing is known about the biology of the taxon in question. For us, the former is uncontrollable; for the latter, it is mandatory that systematic action be taken.

Often, the first issue is to establish the reproductive status of the extant population. From a spermatology perspective, established laboratory protocols can be adapted and used to collect, evaluate, process and understand sperm quality and function. But the usefulness of processed spermatozoa is only as good as our ability to simultaneously establish and monitor female reproductive status. Also important, male reproductive efficiency in many wildlife species is markedly influenced by endocrine-mediated seasonality.

Hormones, of course, drive reproductive success. With the advent of radioimmunoassays in the early 1970*s* came an abundance of publications elucidating the details of endocrine control of reproductive function. Such papers are uncommon in the literature today, even though this is exactly the kind of approach needed for a vast number of species that have never received endocrinology attention. Conventional approaches for tracking hormonal activity in domesticated animals (i.e. restraint and collection of serial blood samples) almost always are inappropriate for wild counterparts.

For this reason, recent advances in monitoring steroid hormone metabolites in voided faeces and urine provide an extraordinarily useful tool for tracking gonadal status in intractable wildlife species. The advantages are significant. Stress-sensitive and often timorous or aggressive individuals do not have to be restrained or sedated for blood collection. Furthermore, the quantitative measure on a given day represents a 'pool' of hormonal activity, thus eliminating some of the erratic dynamism associated with serial blood sampling. Finally, and most importantly, all data confirm the notion that, once the assays are validated, faecal or urinary hormone metabolite profiles reflect true gonadal function.

The Felids as an Example

Felids experience a wide range in reproductive efficiency in zoo breeding programmes. Some, like members of the *Panthera* genus (the great cats: lions, *Panthera leo*; tigers, *Panthera tigris*; jaguars, *Panthera onca*; leopards, *Panthera pardus*; snow leopards, *Panthera uncia*), reproduce with some reliability. Others, like the cheetah (*Acinonyx jubatus*), have a history of erratic-to-poor reproductive performance. Regardless, the taxon as a whole is notorious for being comprised of many individuals that fail to consistently demonstrate clear and overt signs of behavioural oestrus detectable by human managers. Even now, we have an amazingly poor understanding of the basic reproductive cycle, the impact of seasonality and the incidence of spontaneous *versus* induced (reflex) ovulation in the Felidae family.

For these reasons, and because felids (as predators) are facing enormous extinction pressures globally, our laboratory is devoting considerable effort to understanding their biology. The strategy is to develop a sufficient comprehensive database that will allow assisted techniques like artificial insemination (AI) to be useful.

The clouded leopard (*Neofelis nebulosa*), an endangered species weighing 16–23 kg (depending on gender), is indigenous to the forests of southeastern Asia. Historically, the clouded leopard has been difficult to propagate in captivity, because often the male and female are behaviourally incompatible. Consequences can be dire as it is common for the male to abruptly strangle-kill his proposed mate, even when she is in obvious oestrus. Therefore, species managers have recommended AI as an important tool for propagating the captive population. Our earliest basic studies centred on characterizing the seminal traits of the male clouded leopard throughout the year (Wildt *et al.* 1986*a*, 1986*b*). However, these data were of limited use to real conservation action without additional prerequisite knowledge on the general characteristics of the female's reproductive cycle. Certainly, the ability to track ovarian activity non-invasively had the potential of increasing AI efficiency. Therefore, there was a need to meld the disciplines of spermatology and endocrinology.

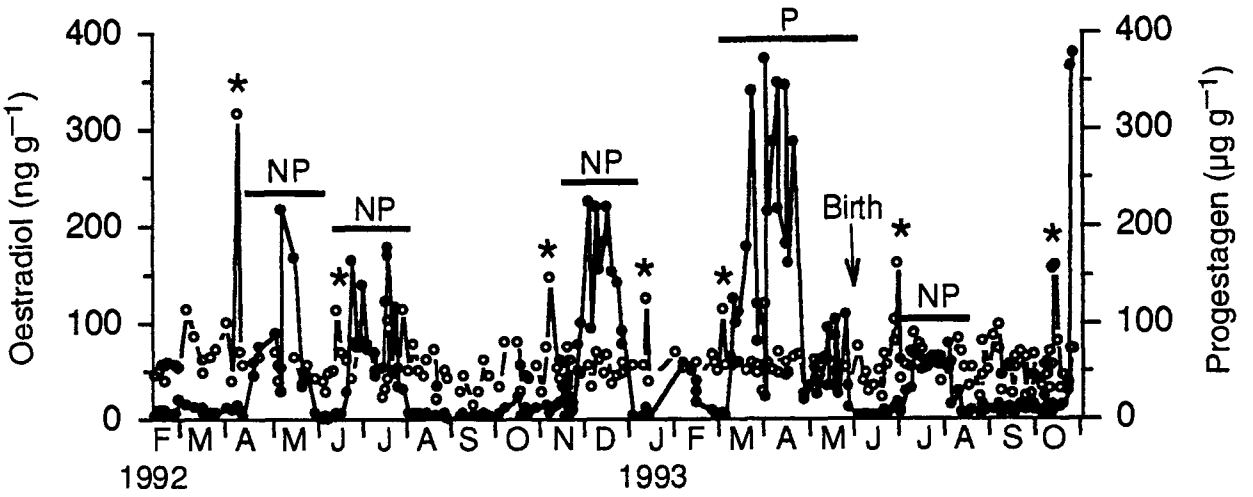

Fig. 1. Representative faecal oestradiol (○) and progestagen (●) metabolite profiles after several infertile matings (NP) and one pregnancy (P) in a clouded leopard housed continuously with a male. Faecal samples were collected and stored frozen until processed. Steroids were extracted from dried faecal material by boiling in 90% ethanol:water and were quantified by validated radioimmunoassays. Presumed preovulatory oestradiol surges are depicted by asterisks; behavioural oestrus was rarely observed in this female. Decreased ovarian activity was observed during the presumed non-breeding season (August–October). After an approximate 90-day gestation, a single cub was born in May 1993, but was rejected by the dam and subsequently removed for hand-rearing within 2 days of birth. Ovarian activity resumed within a month of cub removal. Data adapted from Brown *et al.* 1994.

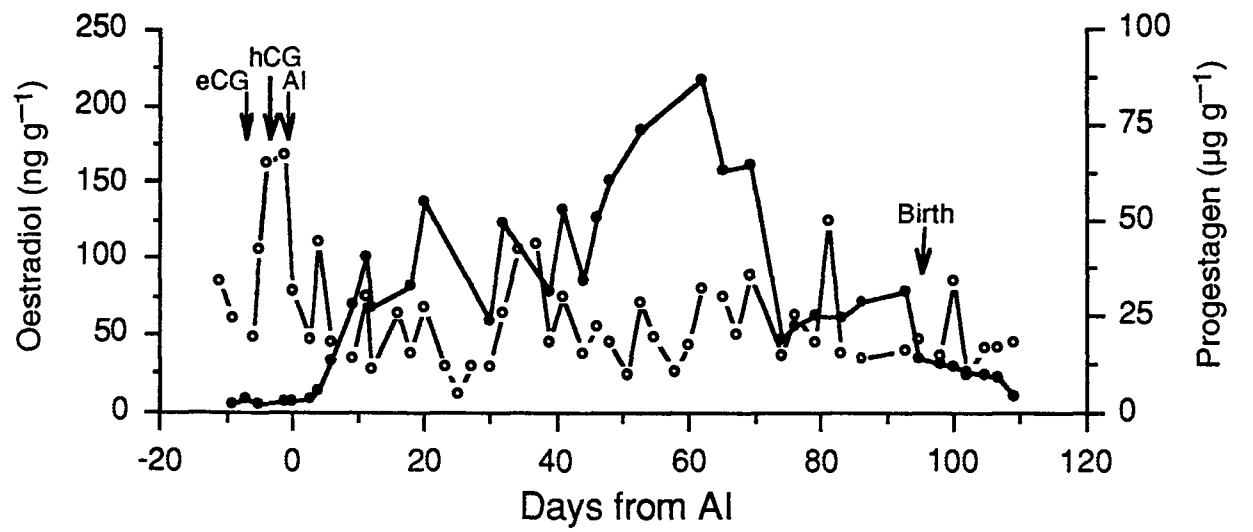

Fig. 2. Faecal oestradiol (○) and progestagen (●) metabolite profiles after ovulation induction and artificial insemination (AI) in a clouded leopard. Follicular development was stimulated by equine chorionic gonadotrophin (eCG), and ovulation was induced using human chorionic gonadotrophin (hCG) administered 80 h after eCG. Laparoscopic intrauterine AI was performed approximately 45 h after hCG. The female gave birth to two cubs after a 93-day gestation. All data are aligned to the time of insemination (Day 0).

Fig. 3. Clouded leopard cubs produced after intrauterine AI of a gonadotrophin-treated female. See Fig. 2 for a more detailed explanation and depiction of hormonal profiles of the dam. (Photograph courtesy of Rick Schwartz of the Nashville Zoo.)

After confirming (from radiolabelling studies) that most oestradiol and progesterone in felids was excreted in felid faeces, immunoreactive oestradiol and progesterone metabolite profiles were established by high performance liquid chromatography (Brown *et al.* 1994). Longitudinal analysis of faecal samples collected over time reflected natural and artificially-induced (after exogenous gonadotrophins) ovarian activity. Compared with baseline concentrations, approximately five-fold increases in oestradiol concentrations were observed during oestrus that occurred either naturally (Fig. 1) or after induction using exogenous gonadotrophins (Fig. 2). Several-fold increases in faecal progestagen concentrations also were observed after mating or AI (Figs 1 and 2). Although no differences were observed in progestagen concentrations between pregnant and nonpregnant females, the duration of elevated progestagen excretion during the nonpregnant luteal phase was only about half that observed during pregnancy (Figs 1 and 2). The data eventually provided the confidence that the 'artificial' cycle sufficiently simulated the natural cycle to begin AI studies. The result was the production of the first litter of clouded leopard cubs from AI with freshly ejaculated and processed spermatozoa (Fig. 3; Howard *et al.* 1993*a*). Endocrinology 'drove' the success of the applied sperm study.

The Cervids as an Example

As demonstrated above, spermatology research for endangered species frequently must involve a multidisciplinary, whole-animal approach that at least partially depends on understanding female endocrinology.

Seasonality plays a major role in regulating reproductive efficiency in wildlife species, usually in both the female and male. In many species, gonadal function is minimized for prolonged periods at certain times of the year. Without basic knowledge about seasonal reproduction, attempts at either natural or assisted breeding can be conducted inappropriately, wasting resources and sometimes risking animal health.

The Eld's deer (*Cervus eldi*) is one of 40 cervid species, and 32 of these contain subspecies formally listed as threatened or endangered. Although once ranging from eastern India through southern China, Eld's deer now exist primarily in small captive populations or in highly fragmented populations in the wild. We have argued that assisted reproduction could play a major role in the genetic management of these small, isolated populations that are at high risk for inbreeding (Monfort *et al.* 1994).

Our strategy has been much like that for felids, except that more extensive preemptive studies of both the female and male Eld's deer were required to better define the impact of reproductive seasonality. Initial studies demonstrated that most steroidal metabolites are excreted in urine rather than faeces, and that this tropical species is seasonally polyoestrous in North America with oestrous cycles commencing in late winter–spring and ending in September (Fig. 4) (Monfort *et al.* 1990).

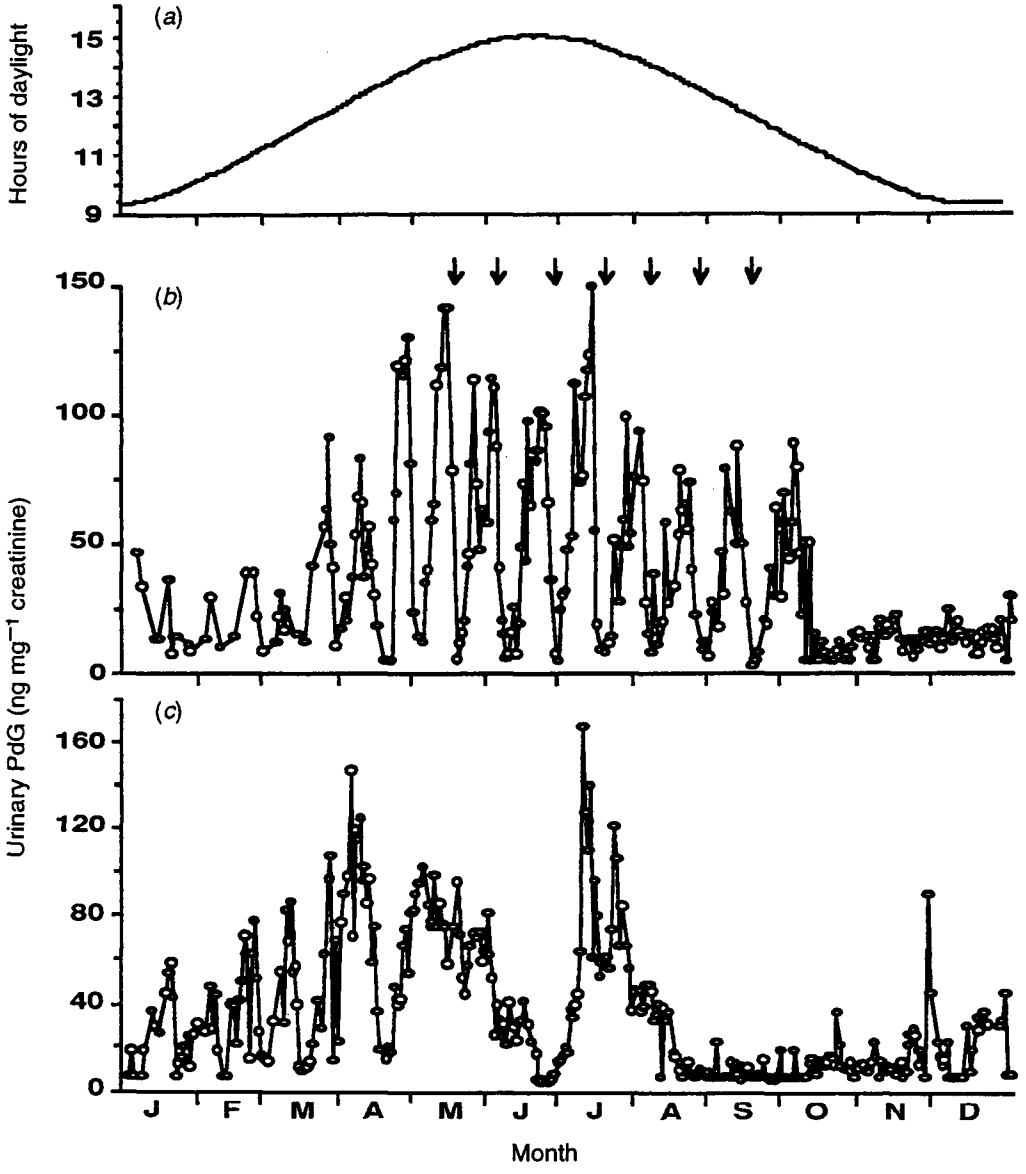

Fig. 4. Annual non-conceptive urinary pregnanediol-3-glucuronide (PdG) profiles for two Eld's deer hinds sampled daily: *(a)* annual changes in daylength; *(b)* a regularly cyclic female (arrows indicate days of behavioural oestrus); *(c)* female demonstrating aberrant cycles, predominately caused by prolonged luteal phases. Data adapted from Monfort *et al.* 1990.

More recently, extensive similar studies have been conducted in male Eld's deer demonstrating remarkable species uniformity in seasonal regulation of spermatogenesis that is related to daylength, the antler cycle, bodyweight, testes volume, circulating concentrations of testosterone, follicle-stimulating hormone and luteinizing hormone, and sperm quality (Fig. 5).

For the Eld's deer hind, our approach recognized those months of the year when the female was most amenable to oestrous synchronization and induced ovulation. For the male, our strategy identified optimal months to collect and cryopreserve viable spermatozoa. The result was precisely identifying the time of year that would maximize assisted reproduction success. Then exploiting methods previously developed for AI of farmed deer species (Asher *et al.* 1988), all information was integrated. Following oestrous synchronization and laparoscopic AI with genetically pre-selected, frozen–thawed spermatozoa, pregnancies were diagnosed and monitored by analysis of urinary hormone metabolites (Fig. 6). Multiple offspring were produced (Fig. 7), representing one of the first examples in which prospective sire and dam selection, germ plasm banking, AI and urinary hormone monitoring were used for a specific conservation goal.

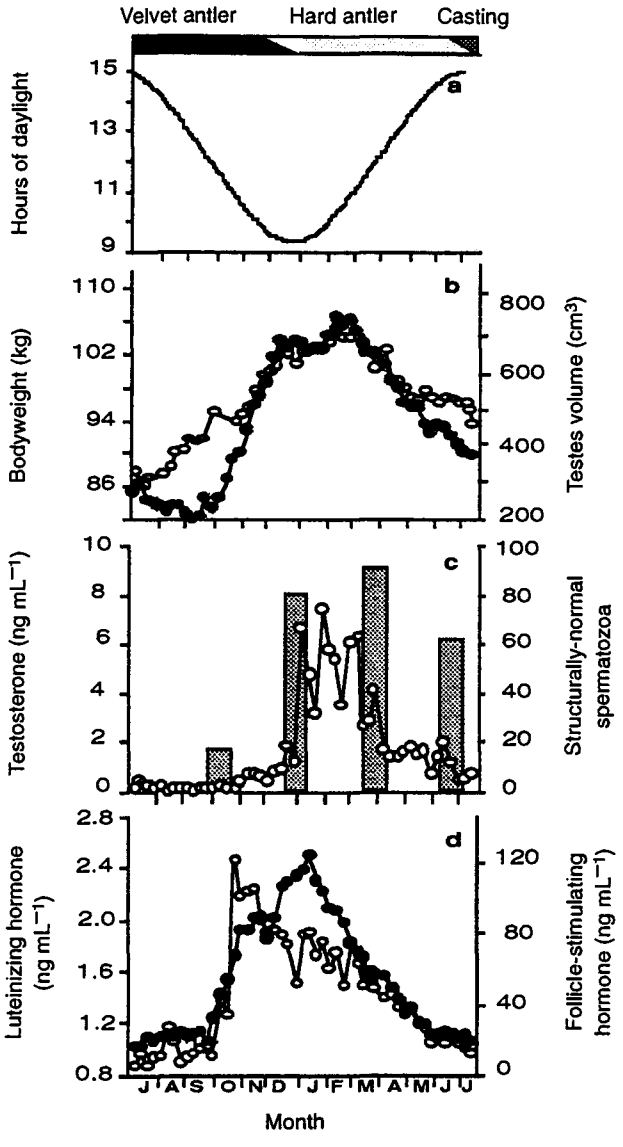

Fig. 5. The relationship of daylength, morphometric characteristics and reproductive and endocrine traits in 6 male Eld's deer maintained at 38°N latitude for 1 year. *(a)* Stages of antler development (velvet and hard antler), the approximate time that antlers were shed and hours of daylight; *(b)* mean weekly bodyweight (○) and testes volume (●); (*c* and *d*) direct relationship among circulating gonadotrophins (luteinizing hormone, ●; follicle-stimulating hormone, ○), testosterone (○) and number of structurally-normal spermatozoa (▦) per ejaculate. Data adapted from Monfort *et al.* 1993*a*.

Important Factors in using Spermatology Effectively for Conservation

Reproductive physiology and endocrinology techniques inevitably can be useful for conservation. The more important questions are — how soon and how efficiently? We have demonstrated above how a multidisciplinary, integrative approach is mandatory for establishing normative databases that eventually can facilitate producing living young. Even so, wild and rare species are influenced by a host of other variables not normally considered during studies with common livestock and laboratory animals. Our laboratory has focussed on two inter-related factors known to influence the utility of reproductive biotechnology for wildlife species management and conservation, species specificity and genetic variation.

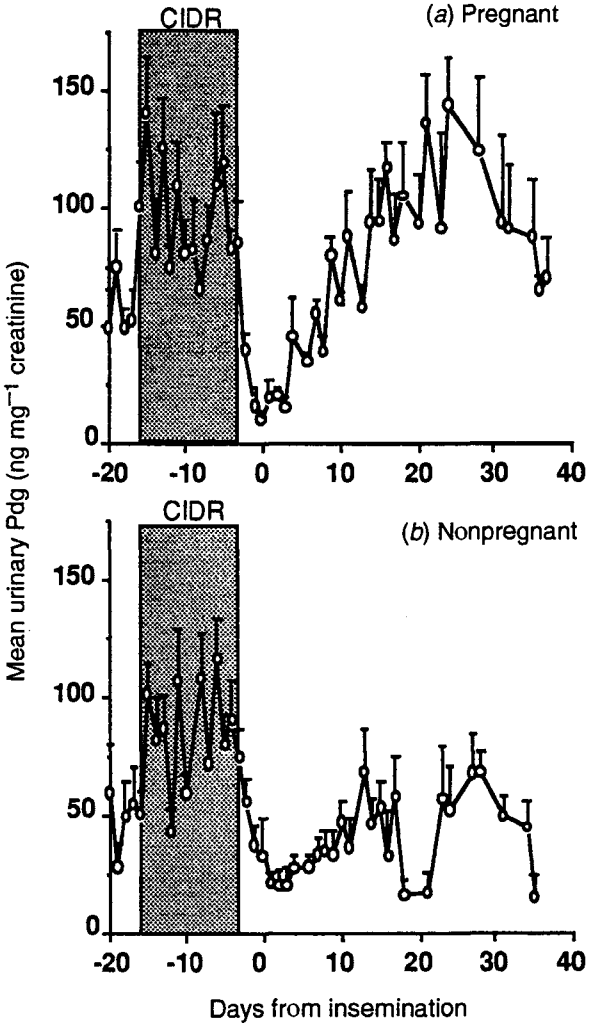

Fig. 6. Pregnanediol-3-glucuronide (PdG) concentrations (mean ± s.e.m.) for *(a)* pregnant ($n = 9$) and *(b)* nonpregnant ($n = 11$) Eld's deer hinds from 20 days before artificial insemination (AI) to 40 days after AI (values aligned to day of AI, Day 0). For oestrous synchronization, progesterone-releasing devices (CIDR-type G, 9% progesterone) were inserted intravaginally for a 14-day treatment interval (shaded region). In all hinds, semen ($7{\cdot}5–10 \times 10^6$ frozen–thawed motile spermatozoa per uterine horn) was deposited by laparoscopy performed 70 h after CIDR device removal. Data adapted from Monfort *et al.* 1993*b*.

Importance of Species Specificity to the Wildlife Spermatologist

Assisted reproduction does not offer simple solutions to species conservation, largely because techniques developed, for example, in cattle and swine, are ineffective in gorillas, giant pandas and Siberian tigers. Applying

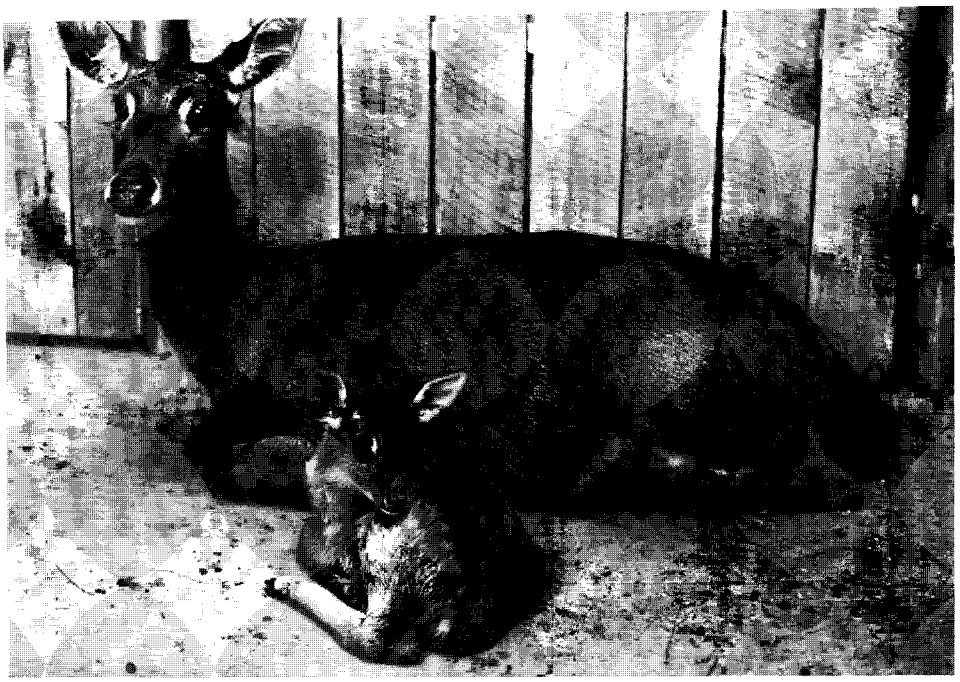

Fig. 7. Nine of 20 Eld's deer hinds delivered offspring after laparoscopic artificial insemination. Seven singletons (2 females, 5 males) were born alive and survived, and one singleton and one set of twins were stillborn.

existing knowledge about one species to another often fails, and even closely-related species within the same taxon express marked physiological differences in reproductive mechanisms. We have dealt with the impact of species and within-species genotype on gamete and embryo function in a previous paper (Wildt *et al.* 1992*b*). Within a given taxonomic group, it is not unusual to find vast species differences in: *(1)* seminal characteristics (Wildt *et al.* 1988; Wildt 1994); *(2)* sensitivity of females to exogenous gonadotrophins or oestrus-synchronizing protocols (Goodrowe *et al.* 1989; Schiewe *et al.* 1991; Howard *et al.* 1992, 1993*a*); and *(3) in vitro* oocyte maturation or *in vitro* fertilization (IVF) success using a standard culture system (Johnston *et al.* 1991; Wildt *et al.* 1992*a*).

Species differences can be expressed further at the level of the cell, both in terms of viability and function. One recent example comes from studying the snow leopard, one of 37 species in the Felidae family and a member of the *Panthera* genus which includes the tiger, lion, leopard and jaguar. One unique characteristic of snow leopard spermatozoa is an intolerance to tissue culture medium Ham's F10, the IVF medium of choice for the domestic cat and cheetah (*Acinonyx jubatus*) (Donoghue *et al.* 1992*a*), tiger (Donoghue *et al.* 1990), clouded leopard (Howard *et al.* 1994), puma (*Felis concolor*) and lion (Johnston *et al.* 1991). It is interesting that snow leopard spermatozoa from high quality electroejaculates abruptly lose motility in Ham's F10 (Fig. 8*a*) (Roth *et al.* 1994). In contrast, snow leopard spermatozoa exposed to simple phosphate-buffered saline (PBS) sustain a higher ($P < 0{\cdot}05$) sperm motility index profile *in vitro* for at least 6 h. This unique medium preference indicates a species-specific sensitivity not observed among other felids studied to-date.

Our approach for understanding this phenomenon further has involved systematically supplementing standard PBS with constituents of Ham's F10 to identify the factor(s) compromising snow leopard sperm survival. Preliminary results suggest that various energy sources (glucose, lactose, glutamine, pyruvate) have no effect, but supplementing PBS with sodium bicarbonate causes an abrupt loss in sperm motility (Fig. 8*b*). Therefore, snow leopard spermatozoa appear particularly sensitive to pH changes, the buffering system in the culture medium or to the sodium bicarbonate itself.

Spermatozoa from this species also appear to have specific requirements for becoming fully functional. For example, we routinely use an *in vitro* oocyte penetration assay (which relies on salt-stored domestic cat oocytes) to test the binding and fertilization potential of felid spermatozoa (Andrews *et al.* 1992). The assay is facilitated by the cat zona pellucida having two phenotypically distinct bilayers (Andrews *et al.* 1992), the inner layer (immediately adjacent to the perivitelline space) serving to prevent structurally-abnormal spermatozoa from fertilizing the oocyte (Howard *et al.* 1993*b*). Spermatozoa from the leopard cat (*Felis bengalensis*) (Andrews *et al.* 1992),

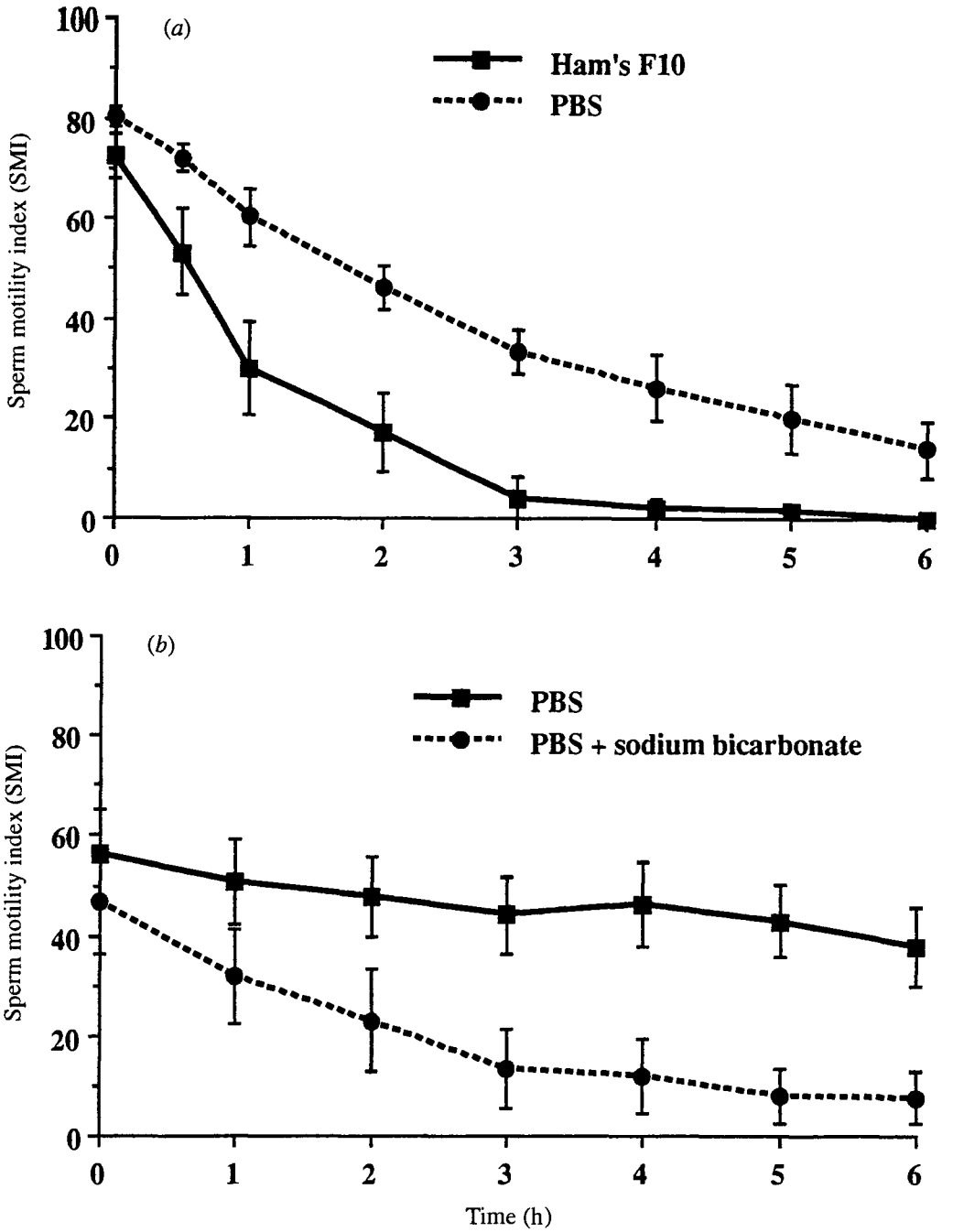

Fig. 8. Influence of *(a)* culture medium (PBS *versus* Ham's F10) at 37°C in 5% CO_2 and *(b)* buffer system (PBS *versus* PBS+sodium bicarbonate) at 25°C in air on motility longevity of snow leopard spermatozoa *in vitro*. Semen was collected by electroejaculation, washed and spermatozoa were resuspended in the appropriate medium. Sperm percent motility and forward progression were assessed, and a sperm motility index was calculated hourly for 6 h. Values are mean±s.e.m.; $n = 8$. Results demonstrate that *(a)* PBS enhances ($P < 0{\cdot}05$) sperm motility overall compared with Ham's F10 (data adapted from Roth *et al.* 1994) and *(b)* sodium bicarbonate supplementation to PBS has a profound detrimental effect on sperm motility.

cheetah (Donoghue *et al.* 1992*b*), tiger (Donoghue *et al.* 1992*c*) and clouded leopard (Howard *et al.* 1994) are capable of binding and penetrating both the outer and inner layers of the domestic cat zona *in vitro*. In contrast, although binding and achieving outer layer penetration, snow leopard spermatozoa virtually are incapable of entering the inner layer of the domestic cat zona (Fig. 9). This occurs regardless of culture in Ham's F10 or in simple PBS which supports sperm motility.

In this case, we suspect that this species-specific difference in sperm function may be related to capacitation mechanisms. Historically, sperm capacitation in the

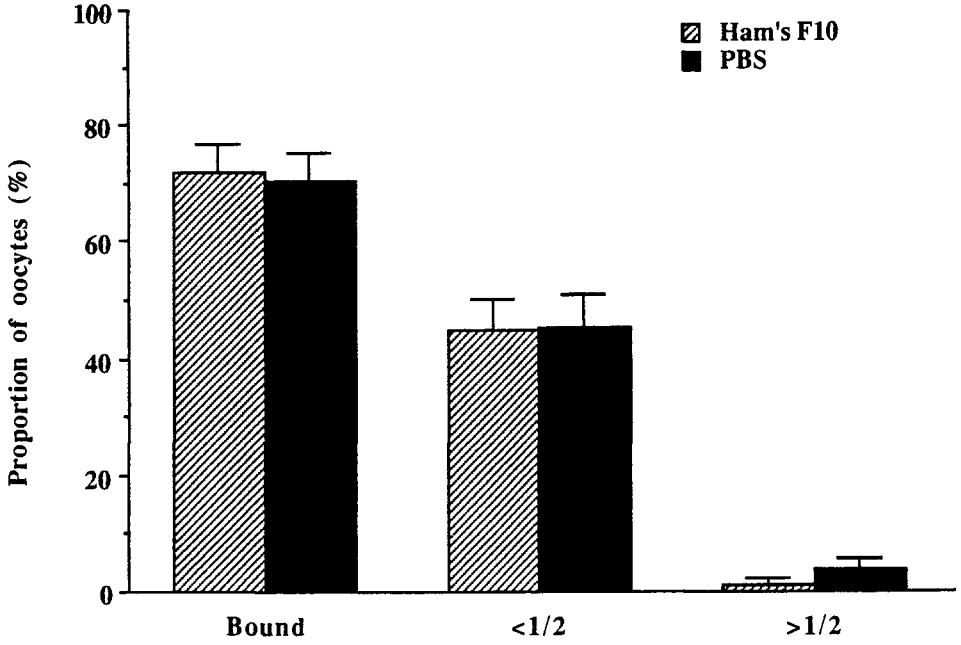

Fig. 9. Proportion of salt-stored domestic cat oocytes with snow leopard spermatozoa bound and penetrating the outer (<1/2) and inner (>1/2) zona pellucida. Oocytes ($n = 15$ per medium per ejaculate) were inseminated with 5×10^5 motile sperm mL^{-1} from ejaculates of 8 snow leopards. Gametes were co-incubated (37°C in 5% CO_2) for 6 h in Ham's F10 or phosphate-buffered saline (PBS), and then oocytes were fixed in 10% formalin and later evaluated for sperm bound to, or penetrating, the zona pellucida. Zona-binding and penetration did not differ ($P \geq 0{\cdot}05$) between media. Data adapted from Roth *et al.* 1994.

domestic cat and its wild relatives has been considered to occur readily in the presence of medium containing protein (Howard *et al.* 1991*a*; Andrews *et al.* 1992). Snow leopard sperm are different. Preliminary data have revealed that even after 6 h incubation in PBS containing either fetal calf serum (5%), bovine serum albumin (4 mg mL^{-1}) or heparin (20 μg mL^{-1}), followed by calcium ionophore treatment, approximately 70% of spermatozoa contain intact acrosomes. The same percentage of spermatozoa with intact acrosomes is observed in controls (no ionophore). Thus, true capacitation fails to occur following any of these treatments. Such findings reinforce the existence of even subtle species specificities. The consequence is that these intricacies must be discovered and then procedural remediations must be made to allow technology to have practical conservation value.

Importance of Genetic Variation to the Wildlife Spermatologist

Frequently, wildlife populations maintained *in situ* or *ex situ* are comprised of, or derived from, small populations. One of the most calamitous forces on natural habitat is fragmentation which eliminates natural corridors of genetic exchange. Populations become isolated, increasing the chances for diminished genetic variation as a result of incestuous matings (inbreeding). Zoos charged with managing 'insurance' populations face the same dilemma; there simply is too little space to breed the hundreds of species and subspecies deserving conservation attention. Further, when these captive animals are managed poorly with little attention to possible inbreeding, the negative impacts are profound (Ralls *et al.* 1979).

Although most popular scientific appeal has been directed at conserving global biodiversity (the sheer wealth of species on the planet) (Wilson 1992), we have argued for equal emphasis on preserving genetic variation within extant species and unique populations. Much of our research during the past decade has focussed on the precarious status of certain felid species or populations, the subject of a recent review (Wildt 1994). Certain populations not only demonstrate remarkably low levels of genetic variation, but ejaculate extraordinarily high numbers of structurally-malformed spermatozoa. These concurrent genetic and physiological findings appear to be more than coincidental. For example, African lions (*Panthera leo spp.*) free-living in the Serengeti ecosystem in Tanzania have relatively high levels of heterozygosity (based on allozyme polymorphisms and mitochondrial DNA fingerprint variation) compared with Asiatic lions (*Panthera leo persica*), another race living in India (O'Brien *et al.* 1987; Gilbert *et al.* 1991). This seems reasonable because the approximate 300 extant Asian lions are descendants of ancestors that experienced a severe population contraction (to fewer than 20 individuals) in the first quarter of the twentieth century (Wildt 1994).

These lions now strain the limited habitat of the Gir Forest Sanctuary and surrounding ecosystem. We have documented that both sperm number per ejaculate and the incidence of normal sperm forms are significantly higher in the genetically-diverse African lion compared with its Asian counterpart (Wildt *et al.* 1987; Wildt 1994). It seems likely that the difference in ejaculate traits between the lion subspecies is related to the highly outbred genotypes *versus* the inbred genotypes of the populations.

A more provocative example of the consequences of demographic reduction and genetic depletion is the Florida panther (*Felis concolor coryi*), recently reviewed by Roelke *et al.* (1993) and Wildt (1994). The Florida panther is a subspecies of the puma or mountain lion and the last of the 'big cats' living east of the Mississippi River in North America. The remaining 30–35 Florida panthers inhabit a single restricted region in southern Florida. Based on allozyme polymorphisms and hypervariable minisatellite genetic loci, the Florida panther expresses 85% less genetic variation than other puma populations in western North America (Roelke *et al.* 1993). The result has been documented male sterility which likely is related to a high incidence of sperm pleiomorphisms (>90%), low circulating testosterone concentrations and an unusually high disposition (~90% incidence) to cryptorchidism (Roelke *et al.* 1993; Barone *et al.* 1994; Wildt 1994). More than 40% of all ejaculated spermatozoa are afflicted with a severe acrosomal malformation (Barone *et al.* 1994). Genetic fixation is having other maladaptive consequences including the emergence of cardiac defects and a high seroprevalence of circulating antibodies to various infectious pathogens (Roelke *et al.* 1993). Taken together, the results are quite clear. When the genotype of a rare population becomes homogeneous, an array of physiological defects can surface. Almost all of these anomalies can adversely influence (either directly or indirectly) our abilities to successfully manage the population via natural or assisted reproduction.

By-Products of Holistic Wildlife Research: Opportunities for Studying Novel Mechanisms in New Species and Related Animal Models

There is a by-product to most discoveries in conservation-oriented research. In our experience, findings made during the course of endangered species studies have enormous potential for spawning novel questions and ideas that may not arise during livestock or laboratory animal studies. For example, our detailed investigations on the ejaculate characteristics of 23 species/subspecies in the Felidae family identified many taxa producing extremely high proportions of malformed spermatozoa. In addition to examining the potential relatedness of this phenomenon to genetic constitution, we have intensively explored the question — do pleiomorphic spermatozoa influence sperm–oocyte interaction and fertilization? Not only is this issue obviously relevant to the endangered species themselves, but findings have potential biomedical application and can be applied to humans where the male typically ejaculates many deformed spermatozoa (Overstreet *et al.* 1980).

In IVF trials, we consistently observe higher cleavage rates *in vitro* in the normospermic domestic cat and tiger than the teratospermic cheetah and puma (reviewed by Wildt *et al.* 1992*a*). These observations have motivated a series of detailed, systematic studies in the normospermic *versus* teratospermic domestic cat, a model useful for understanding the impact of teratospermia. Most domestic cats are normospermic, ejaculating fewer than 40% structurally-abnormal spermatozoa. However, occasionally male cats can be classified as teratospermic, consistently ejaculating more than 60% malformed spermatozoa (Howard *et al.* 1990, 1991*b*, 1993*b*). Swim-up processing of teratospermic ejaculates increases the number of normal spermatozoa recovered to levels comparable to those in normospermic ejaculates (Howard *et al.* 1990, 1993*b*); thus, it is possible to develop IVF inseminates from the two cat populations that have similar numbers of structurally-normal spermatozoa. When comparable numbers of motile spermatozoa from either population are co-incubated with salt-stored or *in vivo*-matured, conspecific oocytes, zona penetration success always is superior for the normospermic donors (Howard *et al.* 1991*b*, 1993*b*). Even though inseminates from the teratospermic donors contain similar numbers of structurally-normal spermatozoa, these cells are less able to penetrate the inner bilayer of the zona and less likely to induce embryo cleavage (reviewed by Wildt *et al.* 1992*a*; Wildt 1994). We conclude that there is a fundamental functional deficit, even in normal-appearing spermatozoa from teratospermic male cats.

For these reasons, we have been screening a host of factors that might explain why normal-appearing spermatozoa from teratospermic males do not efficiently interact with oocytes. One particularly exciting approach involves examining sperm proteins that may serve as receptors for binding zona pellucida (ZP) proteins and regulating fertilization. Receptor-coupled intracellular signalling mechanisms appear to exist that are mediated by a variety of second messengers triggering the acrosome reaction (Kopf 1990; Saling *et al.* 1990). Furthermore, protein phosphorylation and de-phosphorylation appear involved in regulating the acrosome reaction, at least in the mouse (Furuya *et al.* 1992) and human (DeJonge *et al.* 1991; Tesarik *et al.* 1993). Tyrosine-phosphorylated proteins also have been identified in mammalian spermatozoa, and at least one (a tyrosine kinase) is involved in ZP3 binding in the mouse (Leyton and Saling 1989; Leyton *et al.* 1992).

Our hypothesis has been that compromised sperm function in teratospermic male cats may be related to cellular mechanisms associated with diminished phosphorylation efficiency (Pukazhenthi *et al.* 1994*b*). Two tyrosine-phosphorylated sperm proteins (p95 and p160) have been identified in the domestic cat by immunoreactivity to a monoclonal anti-phosphotyrosine antibody (Pukazhenthi *et al.* 1994*a*; Fig. 10). Spermatozoa from normospermic domestic cats, when capacitated in the presence of 5% fetal calf serum, have higher ($P < 0{\cdot}05$) phosphorylation levels of both proteins (Fig. 11). However, the extent of phosphorylation is less ($P < 0{\cdot}05$) in spermatozoa from the teratospermic males (Fig. 11, Table 1), suggesting compromised cellular function in the teratospermic cat.

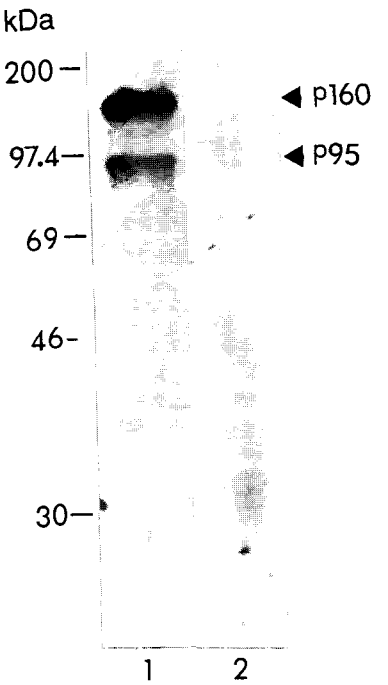

Fig. 10. Immunodetection of tyrosine-phosphorylated proteins p95 and p160 in domestic cat spermatozoa. Sperm plasma membranes were prepared by vortexing for 120 s, solubilizing in Laemmli buffer and subjecting to immunoblotting with an anti-phosphotyrosine monoclonal antibody (PY20) (Lane 1). Specificity of immunoreactivity was determined by pre-incubating PY20 for 1 h with 40 mM *O*-phosphotyrosine and then using the blocked antibodies for immunoblotting (Lane 2).

Swim-up-processed spermatozoa from the two populations also exhibit a similar difference in phosphorylation efficiency (Pukazhenthi *et al.* 1994*a*). This suggests that even morphologically-normal spermatozoa from teratospermic ejaculates are defective in phosphorylating sperm surface proteins, thereby possibly contributing to their diminished ability to fertilize oocytes. The question then arose — are these spermatozoa capable of activating cellular mechanisms that may regulate sperm function in the presence of zona proteins? When swim-up-processed, capacitated spermatozoa are exposed to solubilized cat zonae, phosphorylation of p95 is greater ($P < 0{\cdot}05$) in normospermic compared with teratospermic counterparts. However, p160 immunoreactivity is similar ($P > 0{\cdot}05$) between cat populations. To determine the interaction between the two proteins and ZP proteins, spermatozoa were solubilized, subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), transblotted to nitrocellulose and incubated with ^{125}I-labelled, whole cat ZP or individual ZP proteins. Both whole ZP and ZP3 bound p95, but none of the ZP proteins bound p160. Nevertheless, the involvement of p160 in the regulation of sperm function cannot be totally eliminated, because it's level of phosphorylation increased after both capacitation and ZP exposure.

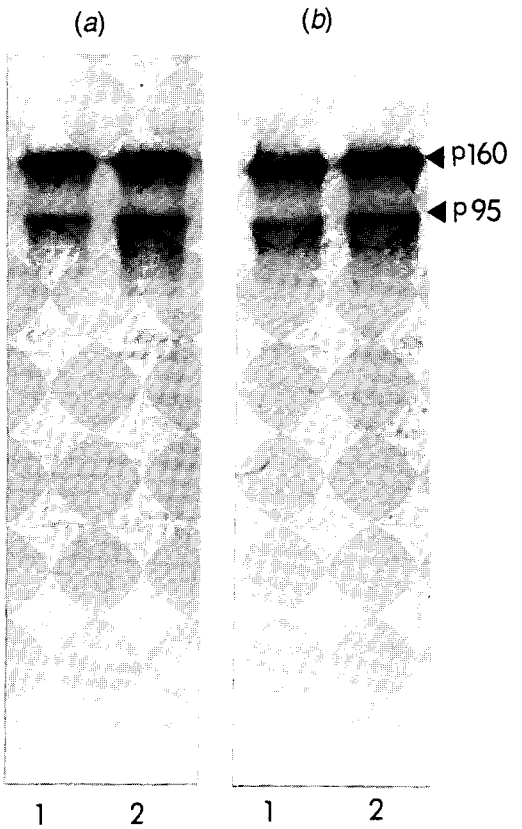

Fig. 11. Effects of capacitation on phosphorylation of tyrosine-phosphorylated proteins p95 and p160 from *(a)* normospermic and *(b)* teratospermic sperm membrane preparations. Domestic cat sperm plasma membranes from 1×10^6 cells were prepared by vortexing and subjecting to SDS-PAGE. After immunoblotting with the anti-phosphotyrosine monoclonal antibody (PY20) and detecting with enhanced chemiluminescence (ECL), regions of immunoreactivity were subjected to densitometric analysis to determine differences in phosphorylation after capacitation and between populations. Lane 1, uncapacitated spermatozoa; Lane 2, capacitated spermatozoa.

Based on these results, we speculate that the diminished phosphorylation in teratospermic males may compromise the ability of spermatozoa to undergo the acrosome

reaction. To further demonstrate the correlation between tyrosine phosphorylation and the acrosome reaction, we have begun to characterize the acrosome reaction both in the presence and absence of tyrosine kinase inhibitors in domestic cat spermatozoa. Simultaneously, we are beginning to screen spermatozoa from several normo- and teratospermic wild felids to determine if the sperm proteins and mechanisms are conserved within the family Felidae. Results may subsequently permit understanding the cellular basis and the significance of teratospermia on gamete interaction, fertilization and early embryogenesis in a host of endangered felid species.

Table 1. Effect of capacitation on phosphorylation of the tyrosine-phosphorylated sperm proteins, p95 and p160, from normospermic and teratospermic domestic cats

Autoradiographs were quantitated by scanning densitometry. Values are mean±s.e.m. Each cat population was represented by 12 ejaculates from 4 males. For each tabular cell, fold increases were calculated between the uncapacitated control and the capacitated aliquot. Within columns, means with different superscripts differ significantly ($P < 0{\cdot}05$)

Cat population	Fold increase	
	p95	p160
Normospermic	$3{\cdot}0\pm0{\cdot}24^{a}$	$2{\cdot}4\pm0{\cdot}15^{a}$
Teratospermic	$1{\cdot}75\pm0{\cdot}13^{b}$	$1{\cdot}84\pm0{\cdot}09^{b}$

Perspective and Conclusions

Most spermatologists focus on basic, mechanistic kinds of investigations or applied studies of importance for improving reproductive efficiency in humans or livestock. In this presentation, however, we offer evidence for another realm of reproductive research — establishing normative data, examining novel mechanisms using techniques ranging from whole animal to molecular and then applying new knowledge to conserving and managing endangered species. Because of recent reviews (Wildt, 1989, 1990, 1994), the use of assisted reproduction as a tool for actually managing endangered species has not been addressed extensively here. Suffice that zoo managers are beginning to propagate selected species strictly on the basis of genotype to avoid inbreeding and to ensure long-term vigour of captive collections. Zoo and wildlife administrators also are beginning to appreciate the need for multidisciplinary, basic research and the utility of reproductive techniques as methods for translocating and 'insuring' germ plasm (i.e. genes) and overcoming sexual incompatibility and infertility. Some minor resistance remains to the concept of 'manipulatory' research (i.e. anaesthesia and major surgery) for collecting data from endangered, stress-susceptible or highly charismatic species. However, these concerns are withering, in part, because of major advances in wildlife anaesthesia and the use of less invasive techniques like urinary and faecal hormone metabolite monitoring, laparoscopy and ultrasound.

The few researchers comprising the field also have made substantial progress, thereby providing real-life demonstrations of technology potential. For example, investigators from our laboratory have produced offspring using AI in the black-footed ferret (*Mustela nigripes*), Eld's deer, cheetah, tiger, puma, leopard cat, clouded leopard, ocelot (*Felis pardalis*) and snow leopard. IVF followed by embryo transfer has been successful in the tiger. The benefits of germ plasm cryopreservation also are beginning to emerge; we have produced living young using AI and thawed spermatozoa in the black-footed ferret, Eld's deer, leopard cat, ocelot and cheetah.

The key to expanded success is increased participation, if not total conversion, of the traditional reproductive physiologist to the field of conservation biology. Global networks among zoo and habitat managers are in place. There is a growing belief that this type of science really can contribute, largely because sufficient examples are available demonstrating that a strong biological database can be translated into consistent offspring production. Given the availability of sustained, large-scale funding for endangered species research, it is inevitable that spermatology (in combination with an array of other disciplines) will contribute substantially to the conservation of biological and genetic diversity.

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