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An overview of the assisted reproduction and genome banking activities of White Oak Conservation Center, Yulee, FL, in the service of species conservation

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Assisted reproduction includes simple strategies, such as oral progestin to maintain pregnancy and hormone monitoring to predict oestrus for breeding introductions, as well as complex procedures, such as oestrous synchronization and artificial insemination (AI). The primary focus of research at White Oak Conservation Center, Yulee, FL, has been to work towards techniques allowing movement of frozen semen to manage metapopulations rather than translocate animals. Using the Gerenuk *Litocranius walleri walleri* as a model for threatened antelope, oestrous synchronization and AI were refined to produce four live offspring (from six attempts) using hand restraint rather than anaesthesia for inseminations. Conversely, similar progress with Okapi *Okapia johnstoni* has been slower. Okapi sperm are highly susceptible to osmotic changes and the physical pressures of the freezing process, which has limited the ability to develop suitable cryopreservation protocols. Storage of frozen semen from highly threatened animals provides insurance against loss of the individual's genes to the population and, if used for future insemination, can potentially provide new 'founders'. Biomaterials from both species, including blood and blood products, are preserved in genome resource banks together with samples from other threatened species, including the Florida panther *Felis concolor coryi*. It is important to note that assisted reproduction in novel species requires significant commitment and continuity from zoological institutions for optimal results.

Key-words: artificial insemination; cryopreservation; genome resource banking; sperm.

INTRODUCTION

Over the last 12 years a research programme at White Oak Conservation Center (WOCC), Yulee, FL, USA, has been developed, initially to address a paucity of reproductive research on antelope species. Gradually the scope of the research has increased to include the disciplines of nutrition, behaviour, contraception and genetics, and although antelope remain one of the primary taxa studied, species as diverse as White rhinoceros *Ceratotherium simum simum* (Metrione *et al*., 2007; Christensen *et al*., 2009), Black rhinoceros *Diceros bicornis* (Christensen *et al*., 2009), Okapi *Okapia johnstoni* (Stanton *et al*., 2010), Flying fox *Pteropus hypomelanus* (Metrione, Verstegen *et al*., 2008) and lemurs (Chatfield & Penfold, 2007), and Cheetah *Acinonyx jubatus*, Jaguar *Panthera onca* and Kori bustards *Ardeotis kori* also have been investigated. Assisted reproduction can be a useful conservation management tool and includes an array of techniques, such as artificial insemination (AI), oestrous synchronization and embryo transfer. Although more often associated with the more complex strategies, assisted reproduction also encompasses simple strategies, such as the use of daily oral progestin to sustain pregnancies (Okapi: Schwarzenberger *et al*., 1999) or faecal hormone analysis to monitor oestrous cycles to time introduction to the ♂ for breeding (Okapi: L. M. Penfold, unpubl. data).

There is no doubt that captive management of non-domestic animals in a zoological setting can be enhanced by assisted reproduction, as it is critical for small-population management that genetic representation is obtained from as many individuals as possible (Lacy, 1994, 1997). Inbreeding in small, fragmented populations can be minimized in two ways. One is by intensive management and the frequent movement of animals among

populations for natural breeding. The other is to assist the natural breeding process by using, when appropriate and needed, assisted-breeding techniques. In particular, AI with cryopreserved sperm can augment genetic management. Genome resource banks (GRBs), containing frozen samples from genetically valuable ♂♂ would provide protection for existing captive populations (see also Clarke, 2009; Jewgenow *et al*., 2011; Saragusty *et al*., 2011). In the event of a disease, epidemic or natural catastrophe, sperm would be safeguarded in liquid nitrogen storage dewars. These samples then could be easily distributed from one holding institution to another to be artificially inseminated into genetically valuable ♀♀. This would eliminate the need to ship animals between institutions and, because cryopreserved sperm can be stored for decades, the genes of valuable ♂♂ could be used for years to come. AI combined with GRB can also be used with a goal of moving frozen semen between as well as within countries.

Imported ruminant species have the potential to carry any number of foreign animal diseases that are of concern to a country's agricultural industry and semen can also carry many of them (Kirkwood & Colenbrander, 2001). However, live animal imports have the added risk of arthropod transmitted diseases, such as trypanosomosis, theileriosis, African swine fever as well as foreign arthropod species, such as the Screwworm fly *Cochliomyia hominivorax*. Eradicated from the United States in the late 1950s (Wyss, 2000), the severe outbreak of Screwworm in 1972 demonstrated the vulnerability of US livestock to external parasites. Thus, there is inherent reduced disease risk importing frozen semen versus a live animal because semen cannot inadvertently contain arthropod species. One of the main programme areas at WOCC has been to develop a protocol for the importation of ruminant semen for AI to manage ruminant metapopulations. Efforts have focused on the Gerenuk *Litocranius walleri walleri*, a small antelope species with ♀♀ weighing *c*. 27–31 kg, as a model species for antelope, and the Okapi as a flagship species for both the Democratic Republic of Congo and WOCC.

Although GRBs are often associated with cryopreserved semen, it is important to remember that they may also store other biological samples, such as whole blood, blood products, tissue and urine. These biomaterials can be an important resource for future studies, including epidemiological studies of disease incidence, nutrition analyses and genetics studies. As such, a repository of biological samples can be an important resource for a threatened species. There are several excellent reviews on the full scope of genome resource banking (Wildt, 1992; Holt *et al*., 1996; Wildt *et al*., 1997), and this paper focuses briefly on two areas: biological sampling (Okapi) and long-term semen preservation (Florida panther *Felis concolor coryi*).

DEVELOPING A PROTOCOL FOR MANAGING METAPOPULATIONS BY MOVING FROZEN SEMEN

A prominent goal of WOCC has been to work towards ruminant semen importation to manage hoofstock populations. If frozen sperm together with AI can be used to move new genetic material between and within populations, theoretically this should not only have reduced risk but also should be less expensive than moving live animals. The Gerenuk was selected as an understudied species that could serve as a model species for other antelope. The effective use of assisted reproduction in antelope species is not simple. Techniques that work well in some species, such as cattle or humans, are not necessarily easily adapted to other species because of unique physiologies. Successful assisted reproduction is facilitated by first understanding the basic reproductive biology of an unstudied species; this was accomplished by longitudinal faecal hormone analysis of ♀♀ to identify that they were a polyoestrous, aseasonal species (Penfold *et al*., 2005). Knowing the basic reproductive biology of a species is important because seasonal species may not cycle all year. For example, the ♀ Jackson's hartebeest *Alcelaphus buselaphus jacksoni* are a

polyoestrous, seasonal species, undergoing a period of acyclicity in the spring (Metrione, Norton *et al*., 2008). It is important to note that although the Hartebeest *Alcelaphus buselaphus* and Gerenuk share the same geographic region and habitat, and are exposed to the same periods of light and rainfall, their reproductive strategies differ (see also Berger, 2011). Thus, although Gerenuk can potentially be inseminated year round, assisted reproduction in the Hartebeest must be avoided in the spring.

Further studies have revealed Gerenuk ejaculates contained high numbers of pleiomorphic sperm (Penfold *et al*., 2005) and genetic diversity of Gerenuk in captivity was reduced compared to free-ranging Kenyan Gerenuk; therefore supporting the theory that Gerenuk would benefit from the addition of new genetic material to the population. Studies to develop an AI protocol for Gerenuk, based on that of Domestic cattle *Bos taurus*, have used faecal hormone analysis to investigate oestrous synchronization protocols. Two prostaglandin injections administered 11 days apart were shown to be effective in inducing oestrus in the majority of ♀♀, and this was confirmed behaviourally using a vasectomized ♂ and noting mounting behaviour that occurred *c*. 48 hours after the second injection. Owing to their small stature, insemination was accomplished transcervically under anaesthesia, stabilizing the cervix with Allis forceps before inserting a catheter to deliver the semen (Morrow *et al*., 2009). Insemination of four ♀♀ after 72 hours with frozen–thawed sperm resulted in one pregnancy that went full-term but the calf died during the birth process after the ♀ went into dystocia. Subsequent inseminations ($n = 24$) over the next 9 years did not yield live offspring even when fresh rather than frozen semen was used; although early pregnancy, confirmed by faecal hormone analysis, was noted on at least three occasions. In 2009, the insemination protocol was changed to eliminate the anaesthesia component and, following synchronization, Gerenuk were hand restrained and inseminated in the field. Using this approach four out of six (67%) ♀♀ inseminated with fresh sperm gave birth to live offspring.

Parallel studies continued to work towards the importation of Gerenuk semen from Kenya. The political aspects of this project, namely working with governments to secure protocols and permits has proven extraordinarily challenging. Because no precedent existed for importation of ruminant semen from a country where foot-and-mouth disease (FMD) is endemic, a protocol defining how the animals should be handled in quarantine, together with disease testing requirements and other relevant procedures, was required to be generated by the US Department of Agriculture (USDA). Although a protocol was released for the project in 2004 (5 years after the process started), subsequent revisions to protocol by USDA still have not been completed to date, a total of 12 years since the initial request. Ironically, protocols for importation of live animals from the same region are readily available. So importing a live Gerenuk antelope is theoretically much easier than importing its semen alone. To provide balance to this issue it should be mentioned that a semen importation protocol would contain various requirements regarding the incidence of disease outbreaks in the country from which the semen was being exported. For example, one requirement is 'no case of FMD or Rinderpest has occurred in the last 5 years in the quarantine facility or within a radius of 5 km of the facility'. In turn, USDA is reliant on in-country expertise to provide assurances that there are no unacceptable disease risks with importing the semen, which may inherently introduce more 'risk' to the project. The reproductive scientist is thus also required to have an understanding of the disease landscape of the country of importation and disease-risk analysis, as well as some epidemiologic background in ruminant diseases. In the case of ruminant species, accomplishing a project involving the seemingly straightforward concept of semen importation, can be an enormous undertaking, requiring a broad understanding of not only the reproductive biology of the species but also multiple other

disciplines, including politics (of both the importing and exporting country), disease epidemiology and risk assessment and animal management during quarantine, all of which have high demands in terms of time, effort and funding.

Concerns about airport irradiation on the DNA of frozen semen prompted an investigation into the effects of X-radiation on Domestic bull sperm fertilizing ability and embryo development (Hendricks *et al*., 2010). No apparent effects were observed on Domestic bull sperm, which is encouraging for bovidae, although previous studies have indicated that X-radiation may be detrimental for felid sperm (Gloor *et al*., 2006). To date, Gerenuk sperm has not been imported for use in AI trials but this project highlighted difficulties with ruminant semen importation into the United States and WOCC has taken the lead with working with the USDA and other institutions interested in semen importation by developing a task force to liaise with the USDA on ruminant and ruminant biological sample importation.

CHALLENGES WITH ASSISTED REPRODUCTIVE TECHNIQUES

In contrast to the methodical progress with Gerenuk, culminating this year with offspring production, development of a similar programme with the Okapi has been far more challenging. In contrast to all other ruminants studied to date, Okapi semen has proven extremely difficult to collect, with average ejaculate volumes of only $1 \cdot 3$ ml, mean sperm motilities of 29% and normal morphologies of 48% (Penfold, 2008). Investigation of the effects of cryopreservation on Okapi spermatozoa revealed that they are highly susceptible to detrimental influences of the cryopreservation process, and possess an acute sensitivity to changes in osmotic conditions when treated with addition of anisosmotic phosphate-buffered saline (Penfold, 2008). This directly translated to poor post-thaw sperm survivability as large cell-volume excursions usually are expected upon addition of permeating cryoprotectants. Although these were minimized by adding cryoprotectants incrementally at 4 °C, further mechanical pressures induced during ice-crystal formation and the cryopreservation process damaged the majority of the Okapi sperm plasma membrane, resulting in an extremely poor yield of post-thaw motile and plasma-membrane intact cells. Current studies are investigating sperm-chilling studies rather than cryopreservation, with a goal to preserve sperm motility for 72 hours for international transport.

Similarly, oestrous synchronization protocols were not as easily adapted from Domestic cattle protocols, which are based on an average cycle of 21 days (Morrow *et al*., 2009). The 14 day cycle of Okapi eventually yielded a synchronization protocol of two prostaglandin injections 6 days apart (L. M. Penfold, unpubl. data). Although some Okapi cycle regularly, it has not been possible to predict oestrus accurately for natural insemination. AI trials conducted both at WOCC ($n = 5$) and at the Okapi Research Station, Epulu, Democratic Republic of Congo ($n = 3$), have not resulted in any pregnancies to date.

INSURANCE FOR THE FUTURE: GRB FOR OKAPI AND FLORIDA PANTHER

While assisted reproductive techniques were unsuccessful in the Okapi maintained in captivity in the Democratic Republic of Congo, these animals were utilized to collect valuable biological samples for GRB. An array of samples was collected from the Okapi, including serum, plasma, skin biopsies, urine and whole blood. Blood was also processed to bank serum for use in mineral analysis for nutrition studies. Faecal samples that were collected from the Okapi, stored in alcohol and sun-dried, and transported to the United Kingdom were useful in developing a microsatellite library for Okapi genetics studies (Stanton *et al*., 2010), demonstrating the advantages of multinational collaborations.

Unlike the relatively unknown Okapi, the plight of the Florida panther has been well documented (O'Brien *et al*., 1990; Culver *et al*., 2000; McBride *et al*., 2008). In brief,

low numbers of Florida panther in south-central Florida faced likely extinction before the introduction of eight Texas panthers *Felis concolor stanleyana*, five of which bred and produced 20 kittens whereon they were removed from the population. Various parameters (health, offspring production, population size) were used to study the effects of this genetic introgression, including reproductive fitness of the ♂, assessed by examining numbers of morphologically normal spermatozoa (Barone *et al*., 1994; Johnson *et al*., 2010). When a ♂ cat died, often through vehicular trauma or interspecific aggression, the testes were removed and shipped overnight on cold packs to the WOCC. Spermatozoa were recovered from the epididymides and a small fraction placed in 0 · 3% glutaraldehyde fixative for morphology analysis (Howard *et al*., 1986). If the samples were fresh enough, motile sperm could often to be recovered (a process known as gamete rescue), extended with an egg-yolk-based cryodiluent and frozen in liquid nitrogen using a protocol previously shown to be effective for felid species (Crosier *et al*., 2006). Analysis of sperm morphology revealed the spermatozoa of intergrade cats (Florida panthers containing Texas panther genes) was improved compared with that of pure Florida panthers. As sperm quality in felids is associated with genetic fitness (Wildt *et al*., 1983; Barone *et al*., 1994), this was one of several parameters that confirmed the success of the genetic introgression. Also, because Florida panther numbers are still low and there are no options for new genetic material to be introduced into the population, banked sperm samples may effectively serve as new founders through reintroduction into the future population via AI. Cryopreserved sperm from ♂♂ that subsequently died without producing offspring ensures that their genes are not lost to the population. If the samples were received within 24–36 hours, testicular tissue was also cryopreserved, further preserving genetic material for a future where more advanced techniques may potentially be able to reintroduce the genes back into the population.

CONCLUSION

Continuity and commitment are requisites for institutions if successful research in threatened species is to be accomplished. Species knowledge is gained in incremental steps over extended periods of time to build a database that, with persistence, can translate into successful assisted reproductive techniques. It is hoped that in the not too distant future the concept of genome resource banking can be put to practical use by transportation of frozen gametes to manage metapopulations through assisted reproductive techniques, such as AI. In the meantime, they serve as important repositories of valuable genetic material, conserving genes for the future.

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