CONSERVATION OF ENDANGERED ANIMALS BASED ON GENETIC POLYMORPHISM STUDIES AND ASSISTED REPRODUCTION

Final report

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1. TITLE : Conservation of endangered animals based on genetic polymorphism studies and assisted reproduction

2. BACKGROUND, INTRODUCTION AND DEFINITION OF THE PROBLEM

Human activities like habitat destruction and fragmentation, over exploitation, pollution and introduction of species into new locations, are directly or indirectly causing a rapid depletion of all biological diversity in the world. The rate of species loss is of such a great magnitude that it resembles a period of mass extinction, and species are lost at rates far greater than the origin of new ones. An unknown but definitely large number of species are already extinct, and several others are of such small population sizes that they are at a risk of extinction. These species are particularly vulnerable to accidental natural events like environmental fluctuations and catastrophes. Genetic stochasticity includes the harmful effects of inbreeding, loss of genetic diversity and accumulation of dangerous mutations. Inbreeding generally reduces birth rates and increases the death rates (inbreeding depression) in the inbred offspring, while loss of genetic diversity reduces the ability of the individuals to adapt to changing environments via natural selection. This vicious circle of reduced population size, loss of genetic diversity and inbreeding spins out of control in many cases resulting in an extinction vortex.

Such vulnerable species, in the near future may be incapable of surviving in their natural habitats, predominantly due to various human impacts, necessitating *ex-situ* conservation. Endangered species in captivity have to be systematically managed to maximize retention of genetic diversity over long periods, usually by minimizing kinship. These captive populations may provide individuals for reintroductions, whose success depends on off-setting inbreeding depression, loss of genetic diversity and genetic adaptation to captivity. With advances in conservation genetics it is now possible to clearly demarcate founder members and estimate numbers required to maintain a viable founding population in captivity. Later as the population approaches its target size, genetic issues in management like minimizing inbreeding and consequent inbreeding depression, and retaining genetic diversity can be addressed. Accumulation of deleterious mutations and genetic adaptation to captivity can be avoided so as to improve the success rate of reintroduction programs of the captive bred species into the wild.

In addition to genetic studies advances in reproductive biology have made several modern techniques available as aids in *ex-situ* conservation of endangered species. These techniques are especially suitable to overcome logistic problems or when the animals are not conducive to mating. Although numerous assisted reproduction techniques are available today, the ones most commonly used are artificial insemination with fresh or frozen/ thawed sperm, combined with extensive non-invasive monitoring of urinary or faecal hormones. Embryo technologies are less frequently used mostly due to the lack of basic knowledge about embryology in these rare species and also non-availability of surrogate mothers. Often these applied studies should be accompanied with systematic basic research studies like understanding sperm quality and its cryosensitivity in various species to eventually allow artificial insemination of thawed spermatozoa. Assisted reproduction techniques will play a significant role in both understanding the biology and saving endangered animal species. However, progress will take many years, as the successful use of assisted reproduction techniques in one animal species cannot easily be applied to another, because the way that animals reproduce is as different as their genetic diversity and physical appearances.

The ultimate aim of this project would be to evaluate the genetic and fertility status of the endangered animals and develop technologies to boost their numbers.

It may be noted that the specific objectives in the present proposal were developed based on the suggestions made by CZA in their letter F.NO. 9-2/2005-CZA (M) dated 5-8-2005 and 23-11-2006 wherein it was suggested that CCMB should develop proposals on the following themes:

1. **Monitoring of genetic variation by DNA fingerprinting.**
2. **Establishment of cell / gene resource bank.**
3. **Assisted reproduction involving semen analysis, ovulation monitoring, artificial insemination, in vitro fertilization and cloning.**

It was also agreed upon that species to work on would be identified in consultation with CZA and wherever possible common non-endangered species would be used as model systems for endangered species to develop and standardize techniques related to assisted reproduction.

3. OBJECTIVES

The main objectives are:

1. **Genetic polymorphism studies in endangered species for conservation breeding**
2. **Development of assisted reproductive technologies for the conservation of endangered species**
3. **DNA based diagnosis of wildlife diseases**
4. **Cryobanking of genes, tissues, gametes and embryos of endangered animals**

4. WORK DONE DURING 2012-2017

A. Genetic polymorphism studies in endangered species for conservation breeding

(i) Genetic polymorphism in Indian Rhinos

The population of Indian one horned rhinoceros (*Rhinoceros unicornis*) was severely threatened in the last century. Since then the species has been brought back from the brink of extinction by strict protection and a sustained conservation effort, and numbers have increased from under 200 in the 1950s to around 2,600 today. The species is inherently at risk because over 70% of its population occurs at a single site, Kaziranga National Park. Species with small and declining populations are faced with the loss of genetic variability due to the limited mating choices available and the smaller original gene pool. It therefore, becomes necessary to maintain a genetically healthy captive population of this threatened species.

Genetic studies have been initiated in Indian one horned rhinoceros (*Rhinoceros unicornis*). A total of 19 blood samples were received at CCMB-LaCONES. Among these, eight (8) samples were collected from individuals translocated from Pobitora Wildlife Sanctuary to Manas National Park, while eight (8) samples were collected from rhinos translocated from Kaziranga

National Park to Manas National Park. Two blood samples were collected from animals housed in Assam State Zoo and one came from Manas while the animal was being radio collared.

High molecular weight DNA was isolated from all the above blood samples using conventional Phenol-Chloroform extraction method. The DNA isolated from all the blood samples quantified and subjected to PCR amplification. Eleven microsatellite loci, moderately to highly polymorphic, were used for the genotyping of the blood samples. All these markers have been selected from those published in NCBI database. Preliminary results shows an average observed heterozygosity (H_O) of 0.049 amongst these individuals against an average expected heterozygosity (H_E) of 0.044. This may be due to the fact that major number of samples has come from two different populations namely, Pobitora Wildlife Sanctury and Kaziranga National Park. Further analysis for the heterozygosity and relatedness among these individuals is underway.

(ii) Genetic studies in captive clouded leopards

The clouded leopard (*Neofelis nebulosa*) is a medium-sized wild cat found in the forests of Asia. Their natural habitat stretches through southern China, the eastern Himalayas, northeast India, and Southeast Asia. In addition to habitat disturbance, the number of clouded leopards is also continuously dwindling as they are increasingly becoming targets of international trade for their skins, bones, and meat in lieu of tigers and leopards. This may lead to loss of genetic diversity in the existing population in captivity as well as in the wild. Therefore, it is very important to monitor the genetic health of the species and assess the extent of genetic polymorphism.

20 blood samples were received in different batches from Sephahijala Zoo, Tripura. High molecular weight DNA was isolated from all the above blood samples using conventional Phenol-Chloroform extraction method. The DNA isolated from all the blood samples quantified and subjected to PCR amplification. Thirteen highly polymorphic microsatellite loci were selected for the genotyping of the blood samples. All these markers have been isolated and

validated from a partial genomic library of leopard (*Panthera pardus*) at CCMB-LaCONES. Preliminary results shows an average observed heterozygosity (H_O) of 0.037 at five microsatellite loci amongst these individuals against an average expected heterozygosity (H_E) of 0.045. This indicates a closed, genetically less vibrant population of clouded leopards in captivity. Further analysis at remaining eight microsatellite loci is underway for the heterozygosity and relatedness among these individuals.

(iii) Genetic diversity study of captive and wild populations of Red Panda (Ailurus fulgens)

Red Panda (*Ailurus fulgens*) is a critically endangered animal and is listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). This animal is distributed throughout the Himalayas between 2,200 and 4,800 meters elevation in western China, Tibet, India, Nepal, Bhutan and Myanmar. The distribution range of this species should be considered disjunct, rather than continuous. It is the only species of the genus *Ailurus*. The subspecies *fulgens* is found in the Himalayas in Nepal, India, Bhutan, northern Myanmar and south-west China, and the subspecies *styani* occurs further to the east in south-central China. Red Panda is covered in Schedule I of the Indian Wild Life (Protection) Act 1972, the highest protection possible for a species in India.

Red panda in the near future may be incapable of surviving in their natural habitats, predominantly due to various human impacts, necessitating *ex-situ* conservation of this species. *Ex-situ* conservation of this animal has been taken up by the Central Zoo Authority, India with Padmaja Naidu Himalayan Zoological Park (PNHZP), Darjeeling as the co-ordinating zoo. This captive population may provide individuals for reintroductions, whose success depends on off-setting inbreeding depression, loss of genetic diversity and genetic adaptation to captivity. With advances in conservation genetics it is now possible to clearly demarcate founder members and estimate numbers required to maintain a viable founding population in captivity. Later as the population approaches its target size, genetic issues in management like minimizing

inbreeding and consequent inbreeding depression, and retaining genetic diversity can be addressed. Accumulation of deleterious mutations and genetic adaptation to captivity can be avoided so as to improve the success rate of reintroduction programs of the captive bred animals into the wild.

As a conservation breeding programme of Red Panda we received seven blood samples and four faecal samples housed at Padmaja Naidu Himalayan Zoological Park, Darjeeling (Table 1). We also analyzed two faecal samples labelled PNZP4a and PNZP4b from enclosure 4 which houses Samridhi and Kaijalee. Additionally we got total 151 faecal samples (41 in first sampling and remaining 110 in second sampling) from Neora valley national park and total 331 faecal samples (151 in first sampling and remaining 180 in second sampling) from Singalila national park.

Genomic DNA was isolated from blood samples by phenol-chloroform extraction method and from faecal samples with Qiagen stool kits. Total 109 (31+78) samples were found suitable for DNA isolation from Neora valley national park and total 265 samples (133+132) from Singalila national Park. Samples from Padmaja Naidu Himalayan Zoological Park were amplified at nine microsatellite loci and analyzed with Genemapper 3.1. The genetic status of each individual was accessed in terms of its being homozygous/ heterozygous at each locus and results are given below. Genotyping of the samples from Neora Valley national park and Singalila national park is in progress.

All samples from PNHZP listed in the table-1, except Siddharth, PNZP4a, PNZP4b, could be genotyped at seven or more loci out of nine loci. Various measures of genetic variation are presented in Table 2. Number of alleles observed across the microsatellite loci used for all individuals varied from 3 (L3 and L9) to 8 (L5) with an overall mean of 5.222±1.787. Observed number of alleles (5.222) across the loci was more than effective number of alleles (3.441). Shannon's information index showed that most of the loci were highly informative, with an overall mean polymorphism across the loci for Shannon's information index at 1.344±0.327. Expected heterozygosity (He) ranged from 0.455 to 0.826 with mean of 0.711±0.120; and observed heterozygosity ranged from 0.143 to 0.917 with mean of 0.666±0.250. Average expected gene diversity

(Nei's) within the population ranged from 0.439 to 0.791 with an overall mean of 0.680±0.114 (Table 2).

All captive animals from PNHZP exhibit good genetic variability (Table 2) and the overall observed heterozygosity (0.666). Through genetic analysis of blood and faecal samples, we observe that captive Red Panda at PNHZP, Darjeeling are genetically vibrant and can be used selectively for conservation breeding.

Table 1. Description of red panda samples analysed

S. No.	Zoo name	Sample
1	**Ram**	Blood
2	Durga	Blood
3	Sakya	Blood
4	Shainee	Blood
5	Janaki	Blood
6	Sheetal	Blood
7	Sahdev	Blood
8	Shaan	Faecal sample
9	Rigsel	Faecal sample
10	Rahul	Faecal sample
11	Siddharth	Faecal sample

Table 2. Measures of genetic variation at studied microsatellite loci in captive Red Panda

Locus	Sample size	Observed number of alleles	Effective number of alleles*	Shannon's information index[!]	Observed heterozygosity	Expected[a] heterozygosity	Nei's heterozygosity	Heterozygote deficiency[b]
L1	9	5	3.5217	1.3785	0.4444	0.7582	0.716	-0.4139
L2	12	7	4.8	1.7235	0.9167	0.8261	0.7917	0.7582
L3	12	3	2.6667	1.0397	0.75	0.6522	0.625	0.8261
L4	12	5	2.4828	1.166	0.75	0.6232	0.5972	0.6522
L5	16	8	4.3761	1.7104	0.8125	0.7964	0.7715	0.6232
L6	11	7	4.4815	1.6682	0.9091	0.8139	0.7769	0.7964
L7	11	5	2.9512	1.2743	0.7273	0.6926	0.6612	0.8139
L8	11	4	3.9032	1.373	0.5455	0.7792	0.7438	0.6926
L9	14	3	1.7818	0.7589	0.1429	0.455	0.4388	0.7792
Mean	12	5.2222	3.4406	1.3436	0.6665	0.7107	0.6802	0.455
St. Dev		±1.7873	±1.0329	±0.3273	±0.2496	±0.1199	±0.114	±0.7107

*Effective number of alleles (Kimura and Crow, 1964)

[!]Shannon's Information index (Lewontin, 1972)

[#]PIC (Polymorphic Information Content)

[a]Expected heterozygosities were computed using Levene (1949) and Neiõs (1973) expected heterozygosity

[b]Heterozygote deficiencies were expressed as D= (Ho ï He)/He

(iv) Genetic Studies in Pheasants

Pheasants and humans have been closely associated. Aside from the material benefits to be derived from pheasants, they have been absorbed into human cultural traditions over the centuries. Several species feature prominently in the art, religion, social customs, and folklore of different ethnic groups in Asia. The red jungle fowl has been associated with humans for centuries, and has (possibly) been in domestication as the progenitor of the domestic fowl for nearly 5,000 years. They have adapted well to human-made habitats and are often found in disturbed agricultural habitats around human settlements. This widespread proximity to people renders this jungle fowl vulnerable to hybridization with the domestic fowl, to which it gave rise, potentially threatening the genetic purity of wild jungle fowl populations in some areas.

A total of thirteen blood samples were received from Nehru Zoological Park, Hyderabad, to assess the genetic health of these animals for future involvement in the Conservation Breeding program of CZA. High molecular weight DNA was isolated from all the above blood samples using conventional Phenol-Chloroform extraction method. The DNA isolated from all the blood samples were quantified and subjected to PCR amplification using eight microsatellite loci, moderately to highly polymorphic chosen from public database. Results showed an average observed heterozygosity (H_{obs}) of 0.468 at eight microsatellite loci amongst these individuals against an average expected heterozygosity (H_{exp}) of 0.534. Based on the genetic studies conducted, it was concluded that the two males (M1 and M2) and one female (F3) were genetically more vibrant than the other individuals and were recommended to be used for selective conservation breeding.

Further, a total of nine blood samples were received from Blyth's Tragopan Conservation and Breeding Centre, Kohima, Nagaland to assess the genetic health of these animals for future involvement in the Conservation Breeding program of CZA. High molecular weight DNA was isolated from all the above blood samples using conventional Phenol-Chloroform extraction method. The DNA isolated from all the feather samples were quantified and subjected to PCR amplification using mitochondrial D-loop marker. Results showed average nucleotide diversity per site (pi) is 0.00266 and Haplotype diversity (Hd) is 0.800. Based on the DNA sequence

comparisons, it was concluded that samples 181, 182, 183, 188 and 189 were genetically similar to each other, whereas, samples 184, 185, 186 and 187 were genetically more vibrant and were recommended to be used for the selective conservation breeding program.

(vi) Genetic Studies in captive Snow Leopards

The snow leopard (*Panthera uncia*) is among most elusive felid, classified as an endangered species by the IUCN Red list. The snow leopard is a single species at the top of the food chain, which is considered an indicator of healthy mountain ecosystems. The endangered snow leopard occurs in rugged, high-altitude regions of central Asia. This cat species has an important role in the Himalayan ecosystem and it is the only species studied very least in the felid. The population has been declining as a result of natural habitat fragmentation, degradation of prey populations, poaching and other anthropogenic threats.

A total of seven blood samples were received from Padmaja Naidu Zoological Park, Darjeeling, to assess the genetic health of these animals for future involvement in the Conservation Breeding program of CZA. DNA samples were subjected to PCR amplification using microsatellite markers and genotyping was done. Results showed an average observed heterozygosity of 0.643 at seven microsatellite loci amongst these individuals against an average expected heterozygosity of 0.758. On the study of genotype and relatedness of the seven snow leopard samples, it was concluded that snow leopards Porabhat (Male) and Kim (Female) were genetically more vibrant when compared to other samples and were recommended for conservation breeding.

(vii) Genetic Studies in captive Tibetan Wolves

Wolves are the second largest mammals next to humans. They are widely distributed in Northern Russia, Europe, Middle East, India and Nepal. In India there are 2 sub species of *Canis lupus a*nd are represented by two isolated populations *i.e. Canis lupus* and *Canis lupus himalayensis*. Tibetan wolf is one of the least known mammals and studies have confirmed that it is a genetically unique population drifted from the general wolf-dog group for quite some time ago. In recent years,

Tibetan Wolves are under nomadic threats and the population is continuously declining.

A total of twelve blood samples were received from Padmaja Naidu Zoological Park, Darjeeling, to assess the genetic health of these animals for future involvement in the Conservation Breeding program of CZA. DNA samples were subjected to PCR amplification using nine microsatellite markers. The mean observed heterozygosity was 0.453 at nine microsatellite loci amongst these individuals against an average expected heterozygosity was 0.463. Based on these genetic studies conducted, it was concluded that male Denzong was genetically more vibrant and could be used for selective breeding with the females DIKF and Donka. Similarly, male Deepam could be used for control breeding with females Yankee and Pushpa.

(viii) Genetic polymorphism studies in Indian Mouse Deer

Fifty four blood samples of Indian Mouse Deer were received from Nehru Zoological Park, Hyderabad, to assess the genetic health of these animals for future involvement in the Conservation Breeding program of CZA. High Molecular weight genomic DNA was extracted using phenol-chloroform extraction procedure. DNA samples were subjected to PCR amplification using cross species amplification in mouse deer and were found to be polymorphic. The average number of alleles for the eleven microsatellite loci, per locus was found to be 5. The observed heterozygosity was found to be more than 60 %. Based on the study of genotype and relatedness of 54 Indian Mouse Deer samples, it was concluded that Balaji, Rakesh, Shravan and Venkat were more heterozygous and unrelated to most heterozygous females in the population and can be used for further selective breeding purpose.

(ix) Genetic polymorphism studies in Asiatic lions

A total of 18 blood samples were received from Nehru Zoological Park, Hyderabad, to assess the genetic health of these animals for future involvement in the Conservation Breeding program of CZA. High molecular weight Genomic DNA was isolated from the 18 blood samples of Asiatic lions by Phenol chloroform method and checked on 0.8% agarose gel. The DNA isolated from all the samples were quantified and subjected to PCR amplification using microsatellite markers which are moderately to high polymorphic were chosen and genotyping was done as

described in Gaur *et al. 2006*. The average number of alleles for the eight microsatellite loci, per locus ranged from 2-3. The observed heterozygosity was found to be more than 40%. Based on the study of genotype and relatedness of the 18 Asiatic lion samples, it is concluded that five individuals, Crazy, Akash, Anthony, Soniya and Jyothi are found to be more heterozygous and Crazy is unrelated to all females. These individuals can be used for selective breeding program. The relatedness is depicted in Fig. 2. The matrix includes un-related male and female Asiatic lions which are heterozygous at more than 50% of the eight loci used for genotyping. The lines show that the male is un- related to the female in the matrix. The connecting lines show that the male is un-related to the female in the matrix.

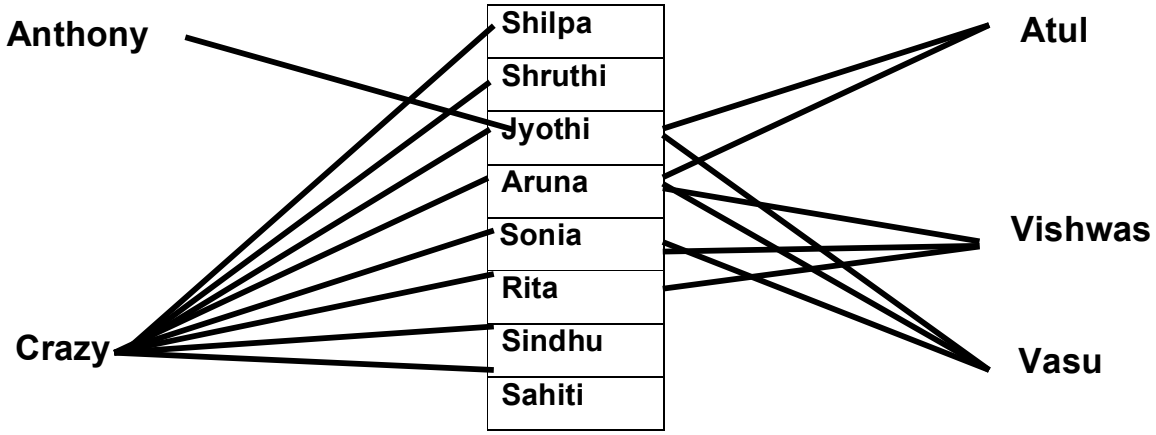

Fig. 2. Relatedness matrix for the male and female Asiatic lions

x) *Genetic diversity of captive lion-tailed macaques with reference to conservation breeding programme in Indian zoos*

Lion-tailed macaque (LTM) is one of the most endangered primates in the world. It is endemic to the wet evergreen forests of southern Western Ghats (Sharavati river valley in Karnataka to Kalakkad in Tamil Nadu), but a vast majority of its distribution occurs in fragmented landscapes. Habitat fragmentation and its consequences like loss of genetic diversity are the major threats to its survival in wild. Moreover, owing to its low growth rate, any potential demographic crash would result in a relatively slow recovery and further depletion of genetic diversity. Therefore, ex-situ conservation of the species is of paramount importance, and it entails the

maintenance of a healthy captive population as a source of reproductively fit and genetically-diverse individuals.

Lion-tailed macaque is one of the priority species for the Central Zoo Authority's Conservation Breeding Programme (CBP). For captive-breeding of LTM, CZA identified Arignar Anna Zoological Park (AAZP), Chennai as the CBP's coordinating zoo, meanwhile Sri Chamarajendra Zoological Garden (SCZ), Mysore and Thiruvananthapuram Zoo (TZ) were identified as the two participating zoos. Laboratory for the Conservation of Endangered Species (LaCONES), Hyderabad was identified as a coordinating institute and has been given the mandate to provide scientific guidance to the participating zoos. Our objective was to evaluate individual LTMs in all the three aforementioned zoos for use in CBP. Blood samples were collected from 23 LTMs from the three zoos: 7 each from AAZP and SCZ, and 9 from TZ for genotyping and sequencing. Where blood samples could not be obtained, fresh faecal samples were collected. Only sequencing analysis was carried out with the faecal samples. Genotyping was done using a panel of 11 polymorphic microsatellite markers to estimate the genetic heterozygosities of 23 the individuals. The average expected and observed heterozygosities were also calculated for the three zoos. Sequencing was done to estimate the haplotype diversity and to determine the genetic affiliation of captive individuals to the wild population. 900 base-pairs of mitochondrial DNA was sequenced: 400 bp of mitochondrial cytochrome b (MCB) and 500 bp of hypervariable region-I (HVR).

Genetic heterozygosity

Mean no. of alleles ranged from 3.364 to 6.727 and mean observed heterozygosity ranged from 0.623 to 0.766. AAZP had the lowest allelic diversity and one out of the 11 markers was monomorphic in the AAZP population. However, counter-intuitively, AAZP population showed the highest observed heterozygosity among the three zoos. This could be explained by the fact that the founders for the AAZP population were derived from two different wild populations. SCZ population had the highest mean no. of alleles but lowest mean observed heterozygosity. The genetic distance between AAZP and SCZ was the highest, whereas that between SCZ and TZ was

lowest. Individual heterozygosities ranged from 0.45 to 0.91.

Genetic affiliation

A study conducted by LaCONES has identified an ancient divergence in wild LTMs with the Palghat gap acting as a barrier to gene flow. Populations north and south of Palghat gap form clearly defined monophyletic clades. Sequencing analysis revealed that the captive individuals also follow this pattern by falling in to one of the two clades according to their place of origin (Fig. 3). Both SCZ and TZ have LTMs belonging to both clades. AAZP, on the other hand, has individuals only from the south clade.

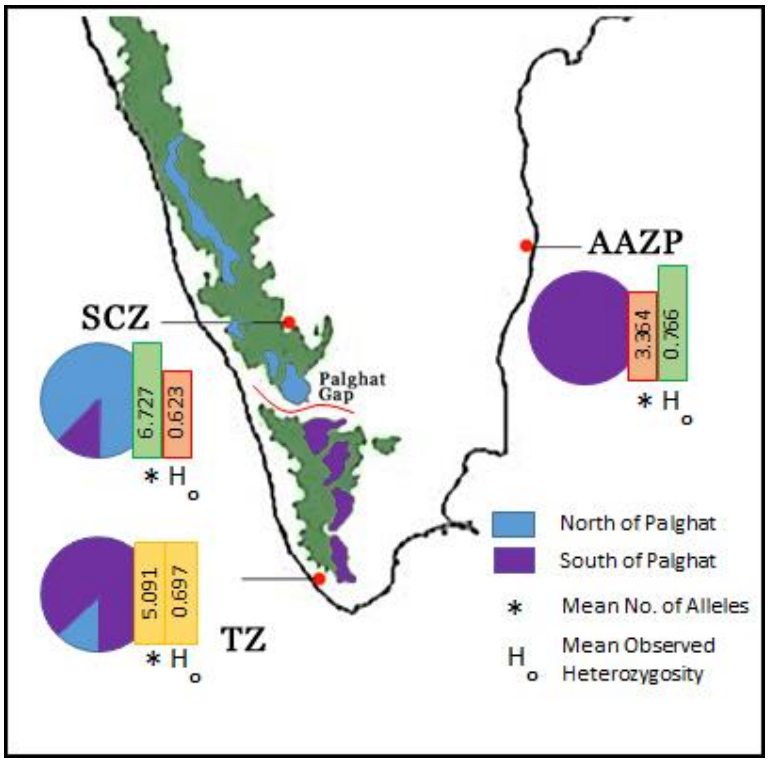

Fig. 3. Showing two different genetic lineage of captive lion-tailed macaques in three south Indian zoos

The European Endangered Species Programme (EEP) and the U.S. Species Survival Plan (SSP) have seen mixed success in captive breeding of LTM. The SSP population plummeted after selective breeding of only the highly heterozygous individuals (thereby reducing the effective population size), whereas EEP concentrated on better troop management and increased coordination between

participating zoos including frequent exchanges when required, and was met with success.

In India, captive LTMs occur in at least eight zoos, only three of which have a potentially viable population. The remaining zoos either have LTMs solely for display purpose or lack sufficient space or expertise to maintain a viable population. The CZAâs CBP has envisioned the maintenance of a captive metapopulation that can serve as a viable reserve of LTM gene pool. This requires greater coordination between the three zoos chosen for CBP. AAZP has had the greatest success in breeding LTMs and has consequently been chosen as the coordinating zoo. Our results indicate that AAZP has the lowest allelic diversity among the three zoos. This presents a peculiar problem which can only be solved by greater coordination between these zoos.

LTM is a genetically diverse species and the populations north and south of Palghat gap have diverged from each other around 1 million years ago. These populations most likely have unique evolutionary potentials and represent two evolutionary significant units (ESUs). Unlike in EEP and SSP, the wild origin of almost all Indian captive individuals is known and those of confiscated individuals can be determined using molecular tools. Since the ultimate purpose of any conservation breeding programme is the release of captive individuals in to the wild if and when required, the genetic affiliation of all captive individuals must be determined, breeding between those belonging to different ESUs must be avoided and reintroductions must be made in the most appropriate place. Although individual genetic heterozygosities are important, they should not be used a reason to eliminate individuals with low heterozygosities from the breeding programme.

Recommendations: LTMs belonging to North and South of Palghat gap should be managed as separate breeding units to avoid outbreeding and to facilitate easy reintroduction in to the wild if and when required. Accordingly, individuals could be exchanged between the participating zoos before using them in the CBP. Since genetic diversity is distributed unequally among the zoos, these must be a coordinated effort with an aim to maintain the best possible captive gene pool of

LTMs. Assisted reproductive technologies may be used for individuals with high genetic value that fail to breed.

B. Development of assisted reproductive technologies for the conservation of endangered species\

(v) Detection of pregnancy and fertility status in big cats using an enzyme immunoassay based on 5a-pregnan-3a-ol-20-one

Reproductive potential of animals is normally monitored by radioimmunoassay (RIA) or enzyme-linked immunoassays (ELISAs) based on measurement of steroids circulating in the blood, because circulating hormone concentrations are generally accepted to be the most accurate indicators of the physiological status of animals. However, repeated blood sampling is not advised or encouraged in wild animals due to stress caused by anesthesia. Therefore, non-invasive fecal reproductive hormone analysis could be used as an alternative method for hormonal analysis in wild animals. This method has several advantages since it is non-invasive, represents pooled value of hormonal activity and facilitates long-term assessment of ovarian status and reproductive activity. In the present study, as part of a conservation breeding program for endangered animals in India, attempts were made to develop a common fecal ELISA for monitoring reproductive function in big cats. We initially established that 5Ŭ-pregnan-3Ŭï ol-20-one is a common progesterone metabolite in the feces of lion, tiger and leopard. In this study, we developed and standardized an ELISA against 5Ŭ-pregnan-3Ŭï ol-20-one, one of the progesterone metabolites, and compared the efficiency of the developed ELISA with RIA kits that were available commercially. In addition, the developed ELISA was used to detect pregnancy in big cats.

The present ELISA showed a high sensitivity, low intra- and inter- assay, higher recovery rate and low cross reactivity with other steroid metabolites and a significant correlation with commercially available RIA based on progesterone antibodies. The sensitivity of the present ELISA (6 pg/ well) is comparable to that

reported in previous studies for other big cats (15 pg/ well, 30 pg/ml. Cross reactivity of the present antibody to estrogens was below 1%.

Apart from monitoring estrus cyclicity in big cats, the 5Ŭ-pregnan-3Ŭï ol-20-one ELISA could also be used pregnancy detection and monitoring in lion, tiger and jaguar because concentrations were significantly higher in feces of pregnant animals compared to non-pregnant animals across the pregnancy period. All the results consistently showed that the levels of 5Ŭ-pregnan-3Ŭï ol-20-one in feces of pregnant tigers (P<0.001), lions (P<0.001) and Jaguar (P<0.001) were significantly higher compared to the levels in non-pregnant animals all across the pregnancy period (Fig. 1). Mean values in pregnant aqnimals ranged from 7,000 to 32,000 ng/g for Asiatic lions, 14,000 to 34,000 ng/g for tigers, and 8,000 to 26,000 ng/g for jaguars.

In the present study, we demonstrate that an ELISA for 5Ŭ-pregnan-3Ŭï ol-20-one, a major progesterone metabolite in the feces of big cats, can be used to detect and monitor ovarian function and pregnancy in several large cats species (Fig. 1). However, our ELISA may prove to be a universal and versatile assay for assessment of fecal progesterone metabolite profiles in a wide range of animals, and including other species of big cats.

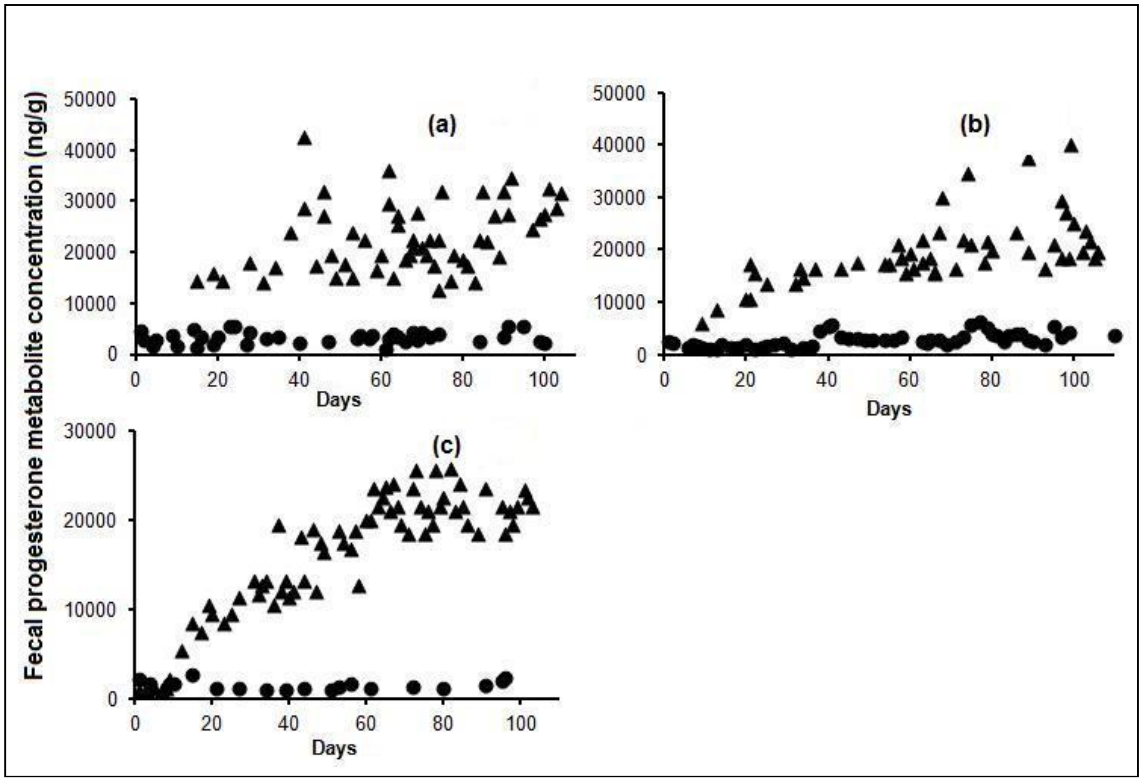

Fig. 1. Fecal progesterone metabolite (5a-pregnan-3a-ol-20-one) concentrations (ng/g) in pregnant (triangles) and non-pregnant (circles) Asiatic lions (a, pregnant = 7; nonpregnant = 4), Indian tigers (b, pregnant = 8 and non-pregnant = 4) and jaguar (c, pregnant = 2, non-pregnant = 1).

(ii) Development and standardisation of enzyme immune assays for non-invasive hormone monitoring in musk deer

Musk deer (*Moschus* sp) are very shy, solitary animals living in the forested and alpine scrub habitats in the mountains of southern Asia, notably the Himalayas. Musk deer is presently endangered owing to over-harvesting for its valuable musk, listed into Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).There is an urgent need to study the reproductive physiology of Musk Deer to enhance the success with which it breeds in captivity. This is the first attempt to standardize procedure for predicting the reproductive status using non-invasive techniques in Musk Deer by fecal steroid hormone analysis. For the purpose, we used three immune assays based on progesterone and its metabolites either developed by LaCONES or commercially available.

A total of 309 fecal samples was collected from five captive female musk deer housed at the Regional Research Institute of Himalayan Fauna (RRIHF), Ranikhet, Uttarakhand through a collaboration program with NCBS, Bangalore as part of a long term program on hormone monitoring of Himalayan ungulates for a period of one year. The fresh fecal samples were dried and pulverized and stored in the field before sending them to Laboratory for Conservation of Endangered Species (LaCONES) for fecal steroid hormone analysis. Fecal extraction was carried out by weighing approximately 0.2g of dried, pulverized fecal samples, boiled in 5ml of 90% of ethanol for 20mins. After centrifugation at 500g for 10mins the supernatant was recovered and the pellet was resuspended in 5ml of 90% ethanol. The samples were then vortexed for 1min and re-centrifuged to recover the supernatant. Both the

supernatants were pooled, dried in an oven at 40°C, resuspended in 1ml of absolute methanol and used for EIA. The fecal extracts were quantified by Enzyme Immunoassay (EIA).

The mean inter-assay coefficients of variation were 8.4% for PdG, 7.8% for progesterone, 10.2% for 5-Ŭ-pregnan-3Ŭ-ol-20-on. The intra-assay coefficients of variation were 5.6% for PdG, 3.4% for progesterone, 6.2% for 5-Ŭ-pregnan-3Ŭ-ol-20-one. Parallelism was demonstrated between serial dilutions of fecal samples and standards for all the hormones. The assay sensitivity at 90% binding was 0.39 pg/well for Progesterone, 6pg/well for 5-Ŭ-pregnan-3Ŭ-ol-20-one and 39pg/well for pregnanediol-3-glucuronide (PdG). EIA was carried out for three hormones pregnanediol-3-glucuronide (PdG), progesterone (P4), 5-Ŭ-pregnan-3Ŭ-ol-20-one.

Of the three EIAs, the pregnanediol-3-glucuronide (PdG) EIA was more efficient, sensitive and accurate for detecting progesterone metabolites in the fecal sample than other two EIAs. The PdG EIA kit had detected pregnancy in three out of five musk deer that were monitored. All the pregnancies were preceded by non-conceptive cycles. The hormone concentrations of all the pregnant animals were elevated throughout gestation and were significantly higher during pregnancy than non-pregnant animals. The average gestation period was 198 ± 8.3days with a range of 184 to 218 days. This PdG EIA offers a reliable, sensitive and accurate method for detecting pregnancy and monitoring reproductive cycle in the endangered species musk deer.

(iii) Observations on reproductive performance of Indian mouse deer (Moschiola indica) in captivity

The mouse deer or the Indian chevrotain (*Moschiola indica*) is a primitive deer, belongs to a distinct family Tragulidae. It shares pig like characters such as the presence of four toes, large hooves, absence of facial scent glands and mating behaviour. Mouse deer play a major role in forest ecosystems as seed disperse and form prey for many carnivores. It has been included as an endangered species in the Indian Wildlife Protection Act (1972) due to frequent hunting. Information on reproduction and behaviour of this endangered species is limited. Nehru zoological park, Hyderabad initiated a conservation breeding program on mouse deer with six deer, supported by the Central Zoo Authority, Government of India.

A total of 31 births (17 females; 14 males) was observed between March 2010 and February 2013 (Fig. 5) though births occurred throughout the year. Most of the births occurred during the post monsoon season (September to February) than other months (P=0.041) and a fewer births was observed during the summer months (May and June). Female mouse deer came to oestrus at an age of 145 days (mean = 162 ±18.52 n = 5) and gestation length ranged from 150 to 163 days (mean = 154 ± 3.7days; n=13). Age at first fawning was 304 days (mean 318.6 ± 15.3 days, n = 5). The inter-birth interval ranged from 150 to 170 days. The litter size was one, except in one instance where a female gave birth to twins. The mean birth weight of the fawns was 468.8 ± 63.3 g (n = 9) and the average height was 10 ± 3.3 cm (range 9 to 12 cm; n=9).

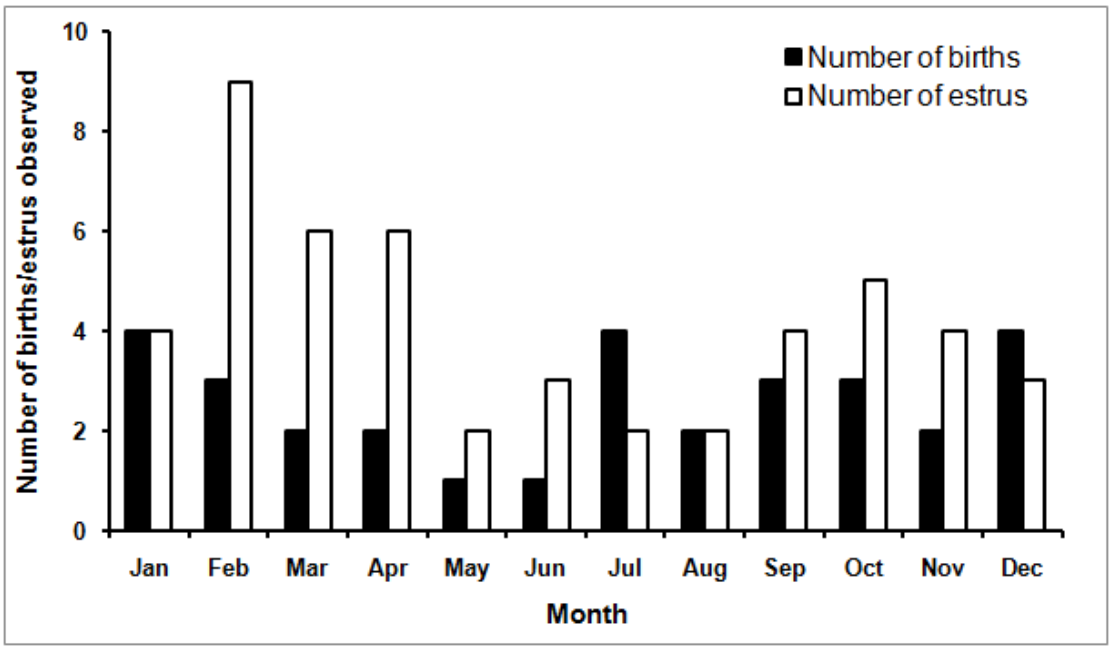

Fig. 5. The number of oestrus and births observed in mouse deer during the study period (April 2010ï February 2013) at captive breeding facility, Nehru Zoological Park, Hyderabad.

A female mouse deer at conservation breeding centre, Nehru Zoological Park, Hyderabad

Overall 50 oestrus episodes were observed from 11 females, resulting in 24 births. Oestrus was observed during February to April and September to November in 68% of cases (Fig. 3) and the duration ranged between 2 to 3 days for the deer which came to oestrus for the first time. Prior to a successful copulation, multiple mountings by the male were observed. The successful copulation (coitus) lasted 15-20 minutes (9 observations). During coitus, the female lied recumbent and was quiet, as when resting, with the male straddling her. The male then grasps the female with his forelegs, while most of his weight rests on his back legs flexed. As copulation proceeded, the female lay quietly, her ears back. From time to time, she turned her head and neck with normal alertness. This observation was similar to that of mating behaviour of camels. After successful copulation, no interaction was noted and the pair parted away.

All the females showed signs of post-partum oestrus within 4-6 hrs of fawning until successful copulation occurred. There was an instance where a male was involved in mating successfully with two females that came to post partum oestrus in a single day. The female mouse deer did not show any significant change in physical appearance even after 3-4 months of conception until 10 days prior the fawning when the abdomen started to descend along with enlargement of udder and vulva.

The present study showed some interesting phenomena like post-partum oestrus and mating behaviour in mouse deer. Although births were observed

throughout the year, higher percentages of births were recorded following monsoons. Similarly, females came into oestrus mostly during pre-monsoon periods. Kusuda *et al.* reported that the lesser mouse deer in Japan gave births in two seasons viz. May and November- December though other workers have reported that no such specific breeding season for lesser mouse deer in the wild or captivity. The mouse deer bred throughout year with less number of births during peak summer (May-June).

The mean gestation length was 154.1 days and it ranged from 150 to 163 days and the present observation is higher than that of lesser mouse deer, which had a gestation length of 140ï 145 days and 132-136 days. The difference in the gestation period might be attributed to differences at species level. The earliest age of first birth was at 304 days in the present study; while in the lesser mouse deer it was 258 days. As reported in other mouse deer species, the litter size was one in the present study except on one occasion. A similar observation was made in lesser mouse deer where, one in 58 births were twins.

Copulation timings and mating behavior were different from other deer species. Most of the ungulates mate multiple times during oestrus unlike single successful mounting in this deer. A similar and long mating behaviour (10- 15 minutes) was observed in camels, however it mates multiple times during oestrus period. The present observations on breeding characteristics of mouse deer can assist in future breeding programs in other zoos in India and elsewhere.

(iv) Reproductive monitoring of captive Snow leopards in Darjeeling zoo

Snow leopard (*uncia uncia*) is listed as endangered in the IUCN red list of threatened animals 2015. The snow leopard is also now listed in appendix I in the convention on the international trade in endangered species of wild (CITES). They are confined to the northern part of central Asia, Himalayas in India and mostly found in China. They generally inhabit at elevations between 5,700-18,000 feet, but may be seen at lower elevations while following prey in the winter. Due to wild prey depletions, the snow leopards resorts to hunting domestic livestock which puts it at risk of being killed by farmers. Information about the reproductive physiology and behavior of snow leopard is limilted. Previous studies have strongly suggested that snow leopards are

seasonal breeders and the animal shows estrous only during the breeding season of the year (Dec-Mar). The present study was aimed to develop and standardize the enzyme immuno assays (EIA)s to study the reproductive physiology and pregnancy detection in captive snow leopard using non-invasive method.

About 1584 faecal samples were collected from five females over a period of one year (Jan 2013 to Jan 2014) from Padmaja Naidu Himalayan Zoological Park and Conservation Breeding Centre (CBC), Darjling, West Bengal (Table 3). Partially processed or dried fecal samples were sent to LaCONES, CSIR-Centre for Cellular and Molecular Biology for further analysis. All the samples were extracted as described in Brown et al (1994). Dried fecal samples were pulverized and made it in powder form. About 0.2g of dried fecal powder boiled in 5ml of 90% ethanol for 20 min and centrifuged at 1000 rpm for 10 min. Clear supernatant transferred in another tube and pellet re-suspended in 5ml of 90% ethanol, vortex for 1 minute, re-centrifuge it. Both the supernatants combined, dried and re- suspended in 1ml of methanol and kept in -20 untill further analysis. Progesterone and estradiol metabolites were quantified by in housed developed direct and indirect competitive Enzyme Linked Immuno-Sorbent Assay (ELISA).

EIAs were validated by demonstrating parallelism between the serial dilution of pooled faecal extracts (endogenous antigen) and respective standards (exogenous antigen) curves. Assay sensitivity was calculated at 90% binding. The assay sensitivities were found to be 6 pg/well and 1.95 pg/well for progesterone metabolites and estradiol EIAs, respectively.

Of the five snow leopards moniroted using fecal progestogen and estrogen, three of them (Yasmin, Kim, Ritu) were found reproductively active and cycling, the other two females either irruglarly cycling or non-cycling (Rare and Teesta). Yasmin (Aged - 9.10 yrs) was showing estrous cycle and mated with male snow leopard (Karan), suspected to be pregnant as evidenced by elevated levels of progesterone metabolites after mating but progesterone metabolites showed sudden decrease till half period of gestation and animal did not deliver due to unknown reasons. Ritu (Aged-10 yrs) and Kim (Aged-4 yrs) were cycling as evidenced by sudden peaks of estrogens during the breeding season but no signs of mating recorded during the

study period. Rare (Aged-1.2yrs) was not showing any cycle or reproductively active sign as this animal was sexually immature. Teesta (Aged-12) showed irregular cycling and sudden increase and decrease of progesterone metabolites, no signs of mating recorded during the study period. From this study it is concluded that three females are fertile and could be used for conservaton breeding program.

Table 3. Details of area, age cycling status and number of samples collected and analyzed in female snow leopards and red pandas from January 2013 to January 2014

S.No	Name of the Animal	Area (Display/ CBC*)	Age as on March 2014 (yrs)	Samples collected	Samples analyzed	Fertility status
1	Yashmin	Display	9.10	220	189	Cycling
2	Teesta	CBC	12	249	229	irregular
3	Kim	CBC	4	299	282	Cycling
4	Ritu	CBC	10	249	232	Cycling
5	Rare	CBC	1.10	251	222	Immature

*****CBC ï Conservation Breeding Centre

(v) Reprodutive assessment of captive Red panda in Darjeeling zoo

Red Panda *(Ailurus fulgens*) is listed vulnerable by IUCN red list and also under Schedule I species of the Indian Wild Life (Protection) Act 1972. Their population is distributed along Himalayas and their range includes north eastern parts of India (Meghalaya, North West Bengal, Sikkim). The major threats for decline of their population are habitat loss and poaching. As part of conservation measures the CZA and in collaboration Darjeeling zoo, a conservation breeding program was initiated. This present study was aimed to assess the reproductive status captive of Red panda which are part of CZA conservation breeding program.

A total of 1417 fecal samples were collected from four captive female Red Panda housed at Padmaja Naidu Himalayan Zoological Park, Darjeeling (Table 4). The samples were collected over a period one year from January 2013 to January 2014. The fecal samples were dried, pulverized and sent to Laboratory for Conservation of Endangered Species (LaCONES) for fecal steroid hormone analysis. Fecal

extraction was carried out by weighing approximately 0.2g of dried, pulverized fecal samples, boiled in 5ml of 90% of ethanol for 20mins. After centrifugation at 500g for 10mins the supernatant was recovered and the pellet was resuspended in 5ml of 90% ethanol. The samples were then vortexed for 1min and re-centrifuged to recover the supernatant. Both the supernatants were pooled, dried in an oven at 40°C, resuspended in 1ml of absolute methanol and used for EIA. The fecal extracts were quantified by Enzyme Immunoassay (EIA).

EIAs were carried out for 5-Ŭ-pregnan-3Ŭ-ol-20-one and estradiol (E2). The mean inter-assay coefficients of variation were 9.32% for 5-Ŭ-pregnan-3Ŭ-ol-20-on and 4.09% for estradiol. The intra-assay coefficients of variation were 2.4% for 5-Ŭ-pregnan-3Ŭ-ol-20-one and 3.2% for estradiol. Parallelism was demonstrated between serial dilutions of fecal samples and standards for all the hormones. Assay sensitivity was calculated at 90% binding. The assay sensitivities were found to be 6 pg/well and 1.95 pg/well for 5-Ŭ-pregnan-3Ŭ-ol-20-on and estradiol EIAs, respectively.

Enzyme Immunoassay revealed one (sheetal) of the four red panda was pregnant which successfully gave birth to a cub during the study period. This was confirmed by elevated concentrations of progestogen metabolites in fecal samples during the gestation period till parturition after which it returned to baseline values.The estradiol values showed that this animal was cycling.

Among the other animals, mating was observed in an animal (Janaki) but it could not sustain its pregnancy for the entire gestation length which was evidenced by an abrupt decrease in the progesterone concentration after mating. The other two animals (Smile and sambridhi), one was a juvenile so no regular estrous cycles were observed during the period of study. During the last phase of our study, estrous peaks were observed during the breeding season in the other animal which resulted in successful mating. Our results indicated that the pregnancy and estrous cycle can be detected by non-invasive fecal steroid hormone analysis. The presnt study reveals that two females (Sheetal and Janaki) are reproductively and these could be used for breeding program

Table 4. Details of animal ID, area, age, cycling status and number of samples collected and analyzed in red pandas from January 2013 to January 2014

S.No	Name of the Animal	Area (Display/ CBC*)	Age as on March 2014 (yrs)	Samples collected	Samples analyzed	Cycling status
1	Sheetal	Display	10.8	362	167	Cycling
2	Sambridhi	CBC	5.8	285	126	Irregular cycle
3	Janaki	Display	3.9	354	177	Cycling
4	Smile	CBC	1.9	293	142	Immature

(vi) Artificial insemination leopard

Attempts have been made for intra vaginal and intra-uterine inseminations in leopards. Female leopards were induced into estrus by administering equine gonadotropic hormone (eCG) followed by human chorionic gonadotropic hormone (hCG). Insemination was carried 36-40 h after administration of hCG. Semen was collected by electro ejaculator and evaluated prior to insemination for motility. Semen was deposited either in the posterior portion of the cervix through vaginal catheter or in the uterine horn through laparoscopy. So far three female leopards were inseminated with two different protocols for induction and ovulation (Fig.6a and b) with no success.

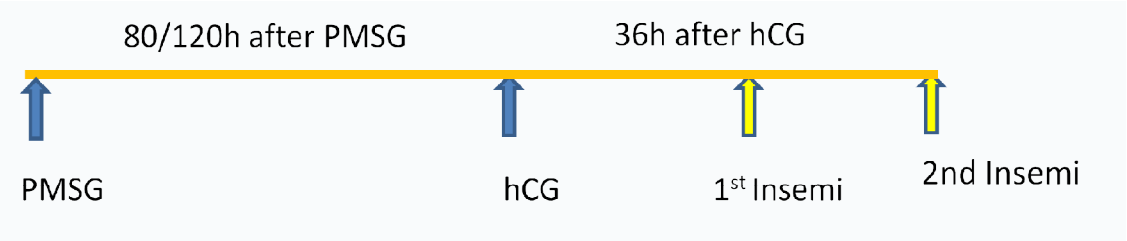

Fig. 6a. Protocol followed for induction of estrus and ovulation in leopard

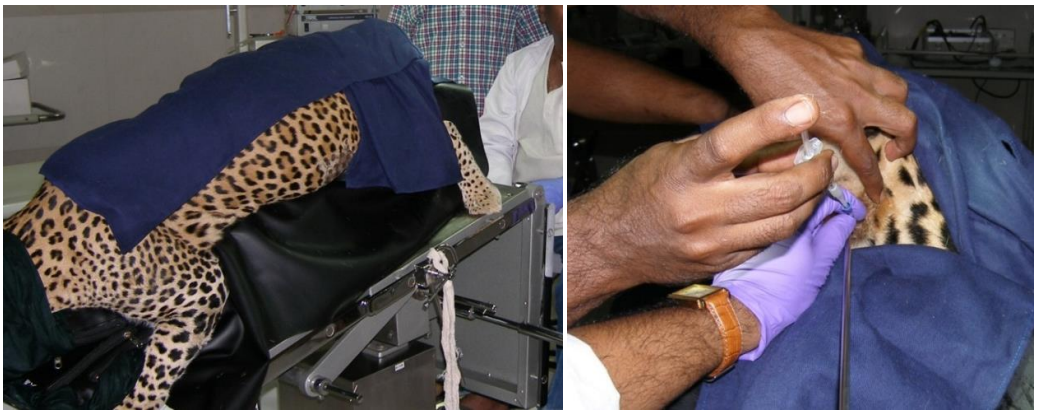

Fig. 6b. Insemination of leopard through intra-vaginal approach.

vii) Non-invasive pregnancy diagnosis in big cats using the PGF2α metabolite assay.

In felids it is quite important to reliably diagnose pregnancy and, in particular, to distinguish it from a pseudopregnancy. Pseudopregnancies or non-pregnant luteal cycles occur in cat species after an infertile mating or early abortion. Induced ovulation leads to the formation of corpora lutea (CLs) which produce progesterone. At mid-pregnancy, feline CLs require extraovarian luteotropic signals which most likely come from the placenta. After parturition or a failed pregnancy (pseudopregnancy), the CLs undergo regression and cease progesterone production. Thus, as an alternative to progesterone, placental hormones are suitable to be used for pregnancy detection. For several felid species, relaxin has been shown to be a reliable pregnancy marker during mid-pregnancy and can be detected in urine after a substantial ultrafiltration. The main disadvantage of relaxin is its pregnancy-related time course, being highest at mid-pregnancy and falling to baseline during the last trimester, and hence, prediction of parturition appears unachievable with this approach.

Another type of placental hormones - the prostaglandins - are also indicators of pregnancy, and metabolites of this hormone class are also detectable in urine and feces. Prostaglandins are involved in regulating ovarian, uterine and placental functions. Placental prostaglandin F2Ŭ (PGF2Ŭ) acts as a luteolytic agent in domestic cats and in ruminants. In carnivores, PGFM has been applied as a useful analytical marker of PGF2Ŭ and in dogs PGFM is elevated just 2-4 days prior to

parturition. In many felid species, however, prepartal PGFM peak in urine and feces occurs several weeks before parturition, which thus indicates PGFM as a clear signal differentiating between pregnant and pseudopregnant luteal cycles in domestic and nondomestic felid species. However, big cats from the panthera lineage seem to behave differently from any other lineage of the Felidae family. In particular, measurements of fecal PGFM in an Indochinese tiger (Panthera tigris corbetti) and a Persian leopard (Panthera pardus ciscaucasica) revealed quite low levels of this pregnancy marker during the last trimester of pregnancy and the absence of a characteristic peak around parturition. At that time, we conclude that more samples collected during a time course from individual cats of the panthera linage are needed to conclusively establish the suitability of the PGFM- pregnancy test in big cats. Therefore, the objectives of our study were (1). to assess the reliability of PGFM as a marker for pregnancy detection in big cats based on additional samples from pregnant females and to compare patterns of immunoreactive PGFM metabolites among three species of the panthera lineage.

Between 1 ï 4 fecal samples from known pregnant Asiatic lions (n=2), Bengal tigers (n=4) and jaguars (n=2) (a total of 217 samples) were collected weekly (except over a few weeks in Asiatic lions and tigers) over a period of 18 (lion and tiger) and 17 weeks (jaguar), also including several samples following parturitions. Samples were collected in the morning between 8 and 9 am, without urine contamination, at the Nehru Zoological Park (NZP), Hyderabad. Approximately 10 g of fecal samples were collected and stored in a freezer (-20ºC) until further processing. Information on mating and parturition were collected regularly from Nehru Zoological Park during the study period.

Analyses of PGFM were carried out in samples from two pregnant lions, four pregnant tigers and two pregnant jaguars. Fig. 7a depicts the weekly (1-4 samples /week, mean + SEM) PGFM concentrations generated from the two Asiatic lions which varied significantly among the weeks (Friedman test χ^2= 18.00, P=0.006). The mean basal PGFM levels during the first 7 weeks following conception ranged between 830 and 1100 ng/g. Further, PGFM concentrations started to increase from week 8 onwards (1512 ± 236 ng/g) until parturition (4987 ± 615 ng/g). Compared to basal concentrations (830 ï 1100 ng/g), this increase in PGFM concentration was

significant from week 9 onwards ($P<0.05$). Altogether, PGFM concentrations varied significantly among early, mid and last term of pregnancy (Friedman test, $G2=13.56$, df = 2, $P=0.001$; Fig. 8).and increased significantly from early to last term of pregnancy (M-W test, $P<0.001$).

A similar profile was obtained from the 4 pregnant tigers (Fig. 7b). Baseline levels of PGFM ranged from 1133 to 1665 ng/g during the first 5 weeks of pregnancy, then rose significantly after the 9th week of pregnancy, remained elevated during the course of pregnancy and peaked the week prior to parturition (6650 ± 623.8 ng/g). Thereafter, PGFM concentrations dropped to baseline levels (1500 ng/g) the week following parturition. PGFM levels showed significant differences between the terms (Friedman test, $G2=30.12$; df=2, $P=0.001$) and significant increases from the first to last term (M-W test $P<0.001$; Fig. 8).

The course of PGFM from the two jaguars revealed a different pattern (Fig. 7c). Mean basal levels of PGFM ranged between 546 to 875 ng/g during the first 6 weeks of pregnancy, showing steadily increasing concentrations beginning at week 7 until week 12 followed by a distinct increase towards peak levels the week prior to parturition (5875 ± 216.5 ng/g). Thereafter, PGFM dropped to basal levels. PGFM concentrations varied significantly between the first term (675.5 ± 42.1 ng/g) and the last term of pregnancy (3378 ± 346.2 ng/g, Friedman test, $G2=30.47$, df=2, $P=0.001$, Fig. 8).

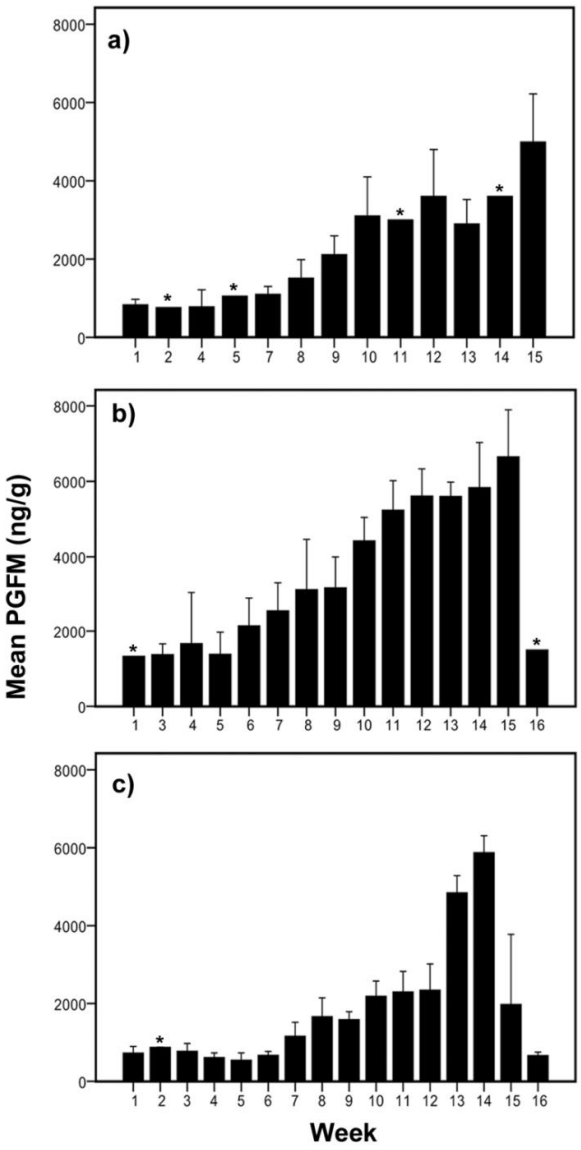

Fig. 7. Fecal PGFM concentrations measured by EIA in samples of two pregnant lions (a), four pregnant tigers (b) and two pregnant jaguars (c), given as weekly means ± SEM. Asterisks indicate single sample per week.

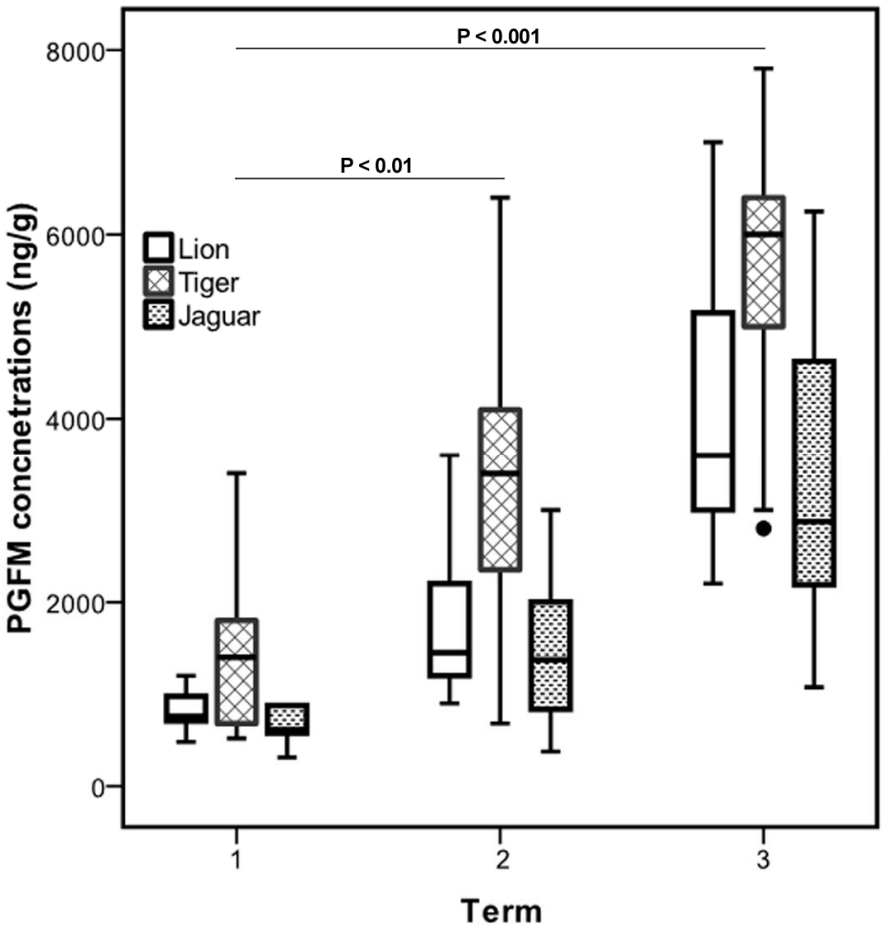

Fig. 8. Term-wise PGFM concentration measured by EIA in Asiatic lion, Bengal tiger and jaguar. To examine differences in PGFM concentrations between different gestation periods, we divided pregnancy into three equal periods as early (1-5 weeks), mid (6-10 weeks) and late term (11-15 weeks) for Asiatic lion, Bengal tiger and jaguar.

Non-invasive methods using urine and feces have been used to detect pregnancy in many species by determining the levels of pregnancy-specific hormones. Elevated levels of progesterone (P4) and its metabolites (5□-pregnan-3□-ol-20-one) can be used for pregnancy detection in several species of felids. However, these hormones remain elevated even during pseudo-pregnancy, thus hampering pregnancy diagnosis. Among all pregnancy-related hormones, PGFM has been found to be a reliable indicator of pregnancy in several felid species, whereas discrepancies were

found among the big cats of the panthera lineage. In this study, the fecal PGFM concentrations remained distinctly elevated during the last trimester (last 30 days) of pregnancy, peaking towards parturition in all animals studied, the pregnant lion, tiger and jaguar. Compared to previous results in felids of the panthera lineage, e.g., the Sumatran tiger, the black panther and Chinese leopard showed elevated PGFM during the last weeks of pregnancy. Overall fecal PGFM concentrations in our study species were higher than in previously described felids, but at the same time we did not find any significant differences in concentrations between extraction methods used in this study (dry method) and a previous study (wet method). Irrespective of the chemical character of the unknown major PGF2 metabolite in feces, our results confirm that the PGFM test can be used in big cats for pregnancy diagnosis. In all three *panthera* species analyzed here, an elevation of fecal PGFM metabolites over a threshold level of 1.5 µg/g dry feces can serve as pregnancy sign, allowing diagnosis with very few samples if not just a single one. Another management tool in high demand is the prediction of parturition. Our data suggest that elevations in PGFM over 5 µg/g feces might be indicative for delivery within a week.

viii) Non-invasive assessment of reproductive status in captive Indian rhinoceros (Rhinoceros unicornis)

Indian rhinoceros or greater one-horned Asian rhinoceros (*Rhinoceros unicornis*) is schedule I species of Endangered in India and listed vulnerable B1 in the IUCN 2015 red list of threatened species. The Indian rhinoceros habitats are riverine grasslands and confined to the northern part of the Indian subcontinent. The population of Indian rhinoceros is declining due to loss of habitat and poaching for horn (the most important reason for declining Indian rhinoceros in India). Only few individuals are left in the wild, northeast in India and Nepal. Information about the reproductive physiology and behavior of Indian rhinoceros is limited. Sexual maturity is reached at 5 years of age in captivity but in wild they usually start breeding only at or after 6 years of age. The average gestation period is about 470 days. The present study was aimed to develop and standardize the enzyme immuno assays (EIA) s to study the reproductive physiology and pregnancy detection in captive Indian rhinoceros using non-invasive method.

About 1137 dung samples were collected from four females over a period of one year (March 2014 to March 2015) from Sanjay Gandhi Jaivik Udyan, Patna (3 females) and Nehru Zoological Park, Hyderabad (1 female Table 5). Partially processed or dried fecal samples were sent to LaCONES, CSIR-Centre for cellular and molecular biology for further analysis. The dung samples were extracted according to the procedure described earlier in Galama et al (2004) with some modifications. The dried samples were pulverized, sieved and 0.2 g of the resulting faecal powder sample was transferred to 15 ml falcon tubes to which 5 ml of 80% methanol was added, and the sample vortexed for 20 min. After this, samples were centrifuged at 3300 *g* followed by collection of supernatant in 5 ml plastic cryovials, which were stored at -30° C until further assay.

Progesterone and estradiol metabolites were quantified by in housed developed direct and indirect competitive Enzyme Linked Immuno-Sorbent Assay (ELISA). EIAs were validated by demonstrating parallelism between the serial dilution of pooled faecal extracts (endogenous antigen) and respective standards (exogenous antigen) curves. Assay sensitivity was calculated at 90% binding. The assay sensitivities were found to be 6 pg/well and 1.95 pg/well for progesterone metabolites and estradiol EIAs, respectively.

In all the four females, Two of the females (Saraswathi and Hartali) were reproductively active and cycling as evidenced by estrogen peaks during the estrous cycle. Saraswathi was mated in July 2014 and suspected to be pregnant as high levels of progesterone metabolites were measured compared to baseline concentration after 4 months of mating. Unfortunately, one of female (gairi) was died in November 2014 due to unknown reasons during the study period.

Table 5. Details of area, age cycling status and number of samples collected and analyzed in Captive female Indian rhinoceros from March 2014 to March 2015

S.No	Name of the Animal	Zoo	Age as on March 2014 (yrs)	Samples collected	Samples analyzed	Reproductive cycling status
1	Saraswathi	Hyderabad	7	193	193	Cycling and became pregnant
2	Hartali	Patna	26	364	364	Cycling
3	Gauri	Patna	13	327	163	Cycling
4	Gairi	Patna	14	253	150-	Cycling

Viii) Non-invasive assessment of faecal progestagens and pregnancy detection in Himalayan Musk Deer (*Moschus chrysogaster*)

Introduction

The Himalayan musk deer (*Moschus chrysogaster*) is an endangered, solitary and primitive ungulate, inhabiting high elevation areas (3000 m ï 4500 m) in its distribution range. The species is one of the seven species in the family *Moschidae* of which all except one (*M. moschiferus*) have been categorized as endangered. Musk deer occur in at least 13 countries in South Asia, East Asia, Southeast Asia and eastern part of the Russian Federation. Musk deer populations have been experiencing a drastic decline all across their distribution range due to large scale poaching for musk and extensive habitat destruction and fragmentation in the wild. The Himalayan musk deer, distributed from the highlands of central China to south and west Himalaya [2] has been particularly affected.

Musk deer are commercially exploited for their musk, a secretion of the male preputial gland that is used in traditional medicines and perfumery. In East Asian countries, especially China, natural musk is used extensively as a sedative and stimulant in Chinese Traditional Medicine (CTM) to cure many ailments [6]. In China alone, the demand of musk for pharmaceutical industry is between 500 kg and 1000 kg per year, which is equivalent to 100,000 deer killed annually from the estimated

populations of 600,000 in wild. Since 1979, all populations of musk deer species have been listed under appendices of the Convention on International Trade in Endangered Species of Wild Fauna.

As a part of the conservation breeding program of Indian endangered animals, the present study aimed at standardizing and validating an enzyme immunoassay for pregnancy detection and monitoring in the Himalayan musk deer using faecal steroid hormone analysis.

2. Materials and Methods

Animals and sample collection

We collected fresh faecal samples weekly, once or twice, from five adult females apparently aged between 4 and 6 years housed at Musk Deer Research Centre, Mahroori, Bageshwar, Uttarakhand, India, between November 2011 and December 2012. The collected faecal samples were immediately dried, pulverised and stored in -20º C until hormone assays. Both males and female musk deer were housed in a large enclosure (22m X 10m) during day time with sufficient natural cover (grass) and water in a semi-natural environment, while in the night they were housed separately in small enclosures (2m X 4m). The animals were fed locally available forage of grass and forbs, at dawn and dusk and water was provided *ad libitum.* All animals were closely monitored by the animal keeper for any signs of behavioural estrous and mating during the study period.

Extraction of faecal steroid metabolites

Faecal hormone metabolite extraction was carried out following the procedures described previously [13,14]. Approximately 0.2 g of pulverized faecal samples were weighed and boiled for 20 mins in 5ml of 90% ethanol. The samples were centrifuged at 500 X g for 10 min. The supernatant was recovered and the pellet was resuspended in 5ml of 90% ethanol. The samples were then re-centrifuged after vortexing for 1 min. The supernatants were pooled, dried completely in an oven at 40°C, re-suspended in 1ml of absolute methanol and used for Enzyme Immunoassay (EIA). Extraction efficiency was determined by adding a known amount of radiolabelled steroid, ^{3}H progesterone (P4) tracer and calculated as total percentage of labelled hormone observed relative to the amount expected as

previously described. Extraction efficiencies ranged from 81.0% to 86.0% and were obtained after addition of ^{3}H progesterone tracer before extraction.

Hormone assays

Pregnanediol-3-glucuronide concentration was measured using polyclonal anti-pregnanediol-3-glucuronide antiserum (R13904; provided by Dr.Coralie Munro, University of California, Davis) diluted to 1:10,000, horseradish peroxidase (HRP)-conjugated PdG label diluted to 1:4,00,000 and PdG standards (39-5000 pg/well). This antibody had a cross reactivity of 100% with PdG, 45% with 20Ŭ hydroxy-4-pregnen-3-one, and less than 10% with other progestagens [26]. Progesterone concentration was measured using a monoclonal anti-progesterone antiserum (Quidel No. CL425; provided by Dr.Coralie Munro, University of California, Davis) which was diluted to 1:6000, HRP-conjugated P4 label diluted to 1:1,00,000 and P4 standards (0.039-2000pg/well). This antibody cross reacts with P4 (100%) and other pregnane metabolites.. Faecal 5-alpha pregnane was measured using previously standardised EIA [14]. The enzyme labelled goat anti-rabbit IgG antibody (Genei, Bangalore) was diluted to 1:10,000. EIAs for PdG and P4 were performed as described.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). The interval between day of mating and parturition was considered as the gestation period. For pregnant and non-pregnant females, peak and baseline values were determined using an iterative process previously described by Brown et al. [31]. The mean (+SEM) of faecal progestagens for early (63 days after mating), middle (64-126 days) and late pregnancy values (126 till parturition) was calculated by taking the dates of parturition of three pregnant deer into consideration. Due to the small number of weekly samples, faecal PdG concentrations in pregnant animals were compared monthly to identify the month of significant increase . Differences in faecal progestagen concentrations between the months and the EIAs were examined using Studentôs *t*-test. We used general linear model (GLM) procedures with repeated measures to examine changes in hormone concentrations across the three EIAs,

three terms of pregnancy and among the individuals. The Pearson's correlation coefficient was used for analysis of relationships among the EIAs. All the analyses were carried out using SPSS ver 17.1.

3. Results

We collected 302 faecal samples from five adult females over a period of 12 months (October 2011 to October 2012). Three of these females were recorded mating and gave birth, while two were seen mating but did not give birth during the period of observation.

3.1. Assay validation

The presence of PdG, P4 and 5-alpha-pregnane was confirmed by HPLC and the collected fractions showed immunoreactivity to respective assays. However, the faecal pregnanediol-3-glucuronide (PdG) peak did not co-elute with standard PdG, which may be due to faecal pregnanediol-3-glucuronide (PdG) being more non-polar compared to standard PdG.

All the three assays (PdG, P4 and 5-alpha pregnane EIAs) were validated using the parallel displacement curves between pooled faecal extracts and their respective standards, thus demonstrating immunological similarities between the standards and the faecal hormone metabolites (Fig. 1) The recovery of known amounts of unlabelled steroids was 90±3.82%, 84.4±1.83% and 88 ± 2.14% for PdG, P4 and 5-alpha-pregnane, respectively. The correlation (r^2) and slope (m) values for the recovery of exogenous PdG, P4 and 5-alpha-pregnane were r^2=0.99 and m=0.94; r^2=0.99 and m=0.84; and r^2=0.99 and m=0.85, respectively. The assay sensitivities at 90% binding were 39 pg/well for PdG, 0.39 pg/well for P4 and 6 pg/well for 5Ŭ-pregnane. The mean inter-assay coefficients of variation (CV) were 8.4% for PdG 8.5% for P4 and 10.2% for 5-alpha-pregnane. The intra-assay CV for PdG, P4 and 5-alpha-pregnane were 6.8%, 3.4% and 6.2%, respectively.

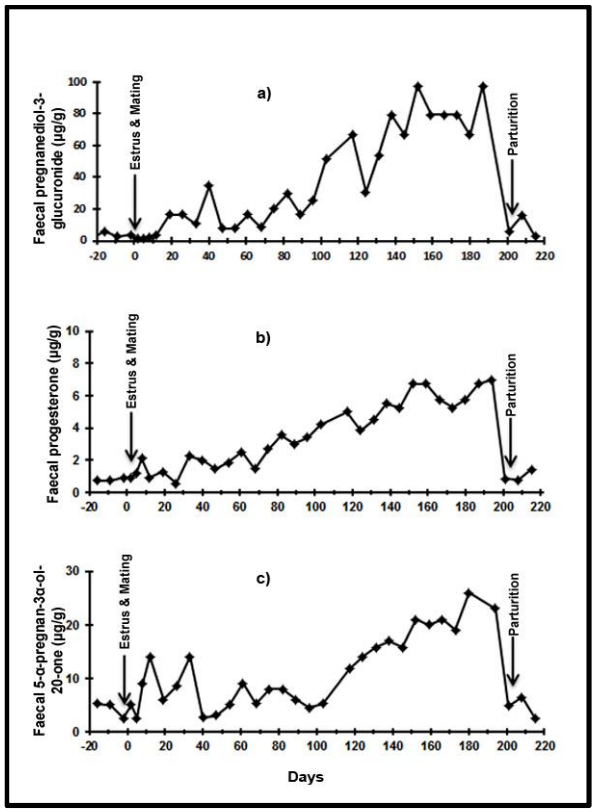

Fig. 1 . Comparison of faecal progestagen profile of a pregnant female musk deer measured by three different immunoassays: (a) PdG, (b) P4 and (c) 5-alpha pregnane (c).

3.2. Comparison of immuno assays

Representative faecal hormone profiles of PdG, P4 and 5Ŭ-pregnane in an individual pregnant female are shown in Figure 3. All the three progestagens followed a similar pattern where the faecal progestagen concentrations increased significantly following mating until parturition. Faecal progestagen levels, measured using three EIAs in five females, were significantly correlated to each other (PdG vs P4 r=0.75, P=0.0001; PdG vs 5Ŭ-pregnane r=0.49, P=0.001; P4 vs 5Ŭ-pregnane r=0.51, P=0.001, n=247). Quantitatively, the PdG concentrations in faecal samples were higher than those of P4 and 5Ŭ-pregnane by an average factor of 9 and 3, respectively (PdG vs P4, P=0.001; PdG vs 5Ŭ-pregnane, P=0.001). Progestagen concentrations measured using all the three EIAs showed significant differences between pregnant and non-pregnant individuals (PdG-P=0.001,P4-P=0.01, 5Ŭ-

pregnane- P=0.01 Table 1). Further, faecal progestagen concentrations varied significantly among the EIA assay types ($F_{2,206}$ = 141.68, P=0.001), terms of pregnancy ($F_{2,206}$ = 61.14, P=0.001) but not among the individuals ($F_{1,206}$ = 0.86, P=0.35). The faecal PdG increased significantly with increase of terms ($F_{2,69}$ =121.38 P=0.001) but no significant difference was observed among the individuals ($F_{2,69}$= 2.34, P=0.90; Table 1). Moreover, the average PdG concentration was found to be higher in early pregnancy compared to other progestagens (Table 1).

Table 1

Faecal progestagen concentrations (εg/g, mean±SE) in non-pregnant (n=2) and pregnant (n=3) females using three different EIAs.

Assay	Non-pregnant (n=220)	Pregnant (n=69)	Early pregnancy (µg/g feces)	Mid pregnancy (µg/g feces)	Late pregnancy (µg/g feces)
PdG	5.02 ± 0.25	45.4 ± 3.91[#]	13.68 ± 1.82[a]	50.66 ± 4.79[b]	82.45 ± 3.15[c]
P4	1.33 ± 0.06	4.18 ± 0.23[#]	2.05 ± 0.13[a]	4.97 ± 0.25[b]	6.09 ± 0.18[c]
5-alpha-pregnane	8.82 ± 0.37	13.43 ± 0.71[#]	8.90 ± 0.64[a]	13.14 ± 0.89[b]	20.59 ± 0.67[c]

#- Significantly different from the non-pregnant values at P=0.001;

Columns with different letters are statistically significant at P = 0.01

3.3. Hormone profiles using PdG

As PdG showed a clear pattern in all the sampled individuals, with higher concentrations in pregnant animals, we used the PdG EIA for monitoring and detecting pregnancy in all the five musk deer females. Based on observation of mating and parturition, the mean gestation length was estimated to be 189 ± 5.03 days (n=3) and ranged from 183 to 199 days. All the pregnant females showed

similar progestagen profiles during pregnancy (Fig. 2). The mean faecal PdG concentration for non-pregnant females ranged from 2.56±0.33 εg/g to 7.64±0.92 εg/g that of individual pregnant musk deer ranged from 41.3±6.71 εg/g to 51.5±7.57 εg/g, showing a 6-7 fold increase (Table 2).

To detect early pregnancy, the faecal PdG concentrations were grouped into monthly means and ranged from 6.17 ± 1.3 εg/g (1st month) to 83.77 ± 3.9 εg/g (6th month). The monthly mean concentration elevated gradually from the second month until parturition and a significant increase from the baseline value was observed third month onwards (1st month vs 3rd month P=0.001, 1st month vs 4th month P=0.001, 1st month vs 5th month P=0.001). The PdG concentrations of the first two months did not show significant difference from the basal level (basal level vs first month P=0.10; basal level vs second month P=0.09). Overall, the PdG concentrations in the faeces of pregnant individuals were consistently higher during the entire period of pregnancy than that of the non-pregnant animals (P=0.001).

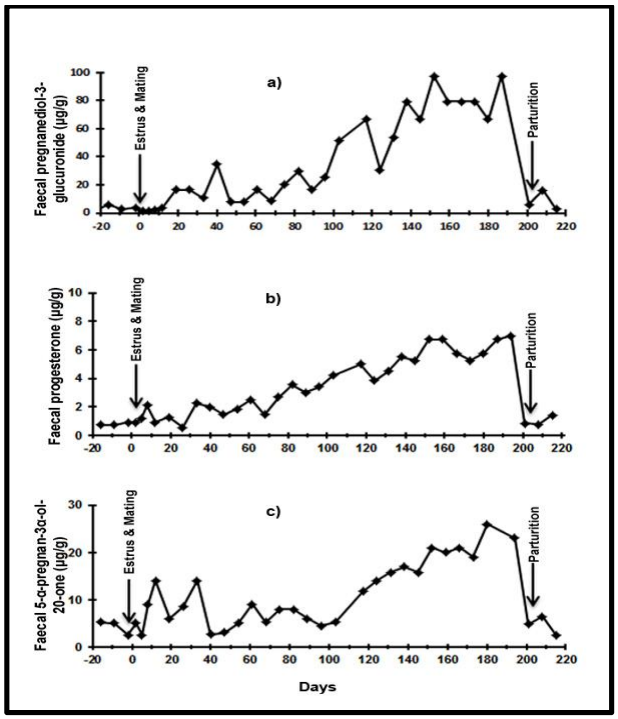

Fig 2. Faecal PdG profiles of the five sampled musk deer individuals (a, b, c ï pregnant individuals, d, e ï non-pregnant individuals) using PdG EIA (arrows indicate oestrus and mating or otherwise mentioned).

4. Discussion

The present study reports the first measurement of faecal hormone metabolites for the assessment of reproductive status in Himalayan musk deer. We observed the presence of all three progestagens in the faecal extracts which was confirmed by HPLC as single immunoreactive peaks. Of the three EIAs performed, the PdG EIA was found to be the most accurate for faecal hormone monitoring in the species. This assay could detect pregnancy at three months after mating and with a much higher reliability from 4th month onwards.

The mean gestation period of 189 days as indicated by faecal progestagen analysis lies well within the range of 185 to 195 days as reported earlier by behavioural observations. However, the gestation period has been reported to increase with increasing body size among the subspecies of genus *Moschus* and it ranged widely from 178 to 198 days.

Among the three assays, PdG EIA could be used to assess the pregnancy status of musk deer from 3rd month after mating. Moreover, the faecal progestagen concentrations were found elevated significantly from the early pregnancy till middle to late pregnancy in all the sampled individuals. In a recent study on the two-toed sloth (*Choloepus didactylus*), the PdG EIA could detect pregnancy as early in the second month of gestation, which was two weeks earlier than using P4 EIA. Thus, pregnanediol-3-glucuronide could be used for pregnancy diagnosis in Himalayan musk deer at an early stage of 3 months after mating and monitoring the reproductive status of these species.

This study provides the first account of the progestagen profiles of Himalayan musk deer through non-invasive faecal hormone analysis. Faecal progestagen was found in all the females with varied concentrations during different terms of the reproductive cycle along with significant differences between pregnant and non-pregnant individuals. Pregnanediol-3-glucuronide EIA was found to be the most appropriate assay to monitor pregnancy status in the species. The progestagen profiles generated through the study would prove an essential tool in monitoring reproduction in wild populations of the species as well as for captive breeding programmes.

C. DNA based diagnosis of wildlife diseases

Disease has not traditionally been regarded as a significant driver of species extinction. In addition to environmental change, habitat loss, climate change and overexploitation, diseases can also cause directly or indirectly to the extinction or significant decline in wild populations. Eighty eight per cent of mammals listed by IUCN as threatened by infectious diseases belong to two orders of mammals: carnivores and artiodactyls, in particular, those species more closely related to domestic animals. Historically, wildlife diseases have been considered important only when agriculture or human health have been threatened. Out of 1415 human pathogens, 61% are Zoonotic, 70% of zoonotic emerging infectious diseases (EIDs) events were caused by pathogens with a wildlife origin. These figures indicate the importance of wildlife and their diseases causing infections in humans. The major limitations to diagnose wild life diseases are lack of information for particular disease, approach, availability of biological material from affected animals, collection and preservation of the biological samples from dead animals in wild. Early detection of diseases in wild animals is very important for management/prevention of disease outbreaks in wild animals as well as EIDs in human. PCR based detection of pathogens in fecal samples is the easiest and rapid diagnostic method in wild animals.

We have collected fecal samples of felids and canids from NZP, Hyd to initiate the detection of canine parvo virus (CPV), feline parvo virus (FPV) and infectious canine hepatitis (ICH) using PCR method. DNA was isolated from fecal samples using Qiamp DNA mini stool kit. The DNA isolated from vaccines is used as postive control. The DNA was amplified by known sequences of viral protein 2 gene and protein E3 gene for CPV (427bp) and FPV(165bp) and ICH (1030bp) respectively using polymerase chain reaction (PCR). The PCR products were run in 1.5% agarose gel for respective product sizes. A total of 150 samples were collected from felids and canids and screened during three seasons (winter, summer and rainy seasons) from NZP, Hyd. None of the sample was found positive for the above diseases.

We have detected canine parvo virus in one of the post mortem samples of rusty spotted cat in NZP, Hyderabad using PCR based method (Fig. 9).

We have screened 14 samples from skin lesions of morbid fresh water turtles from NZP, Hyd. Based on the Bacterial 16S rRNA gene sequences, the isolates were closeley related (99.73) to *Aeromonas veronii* and *A. ichthiosmia.*

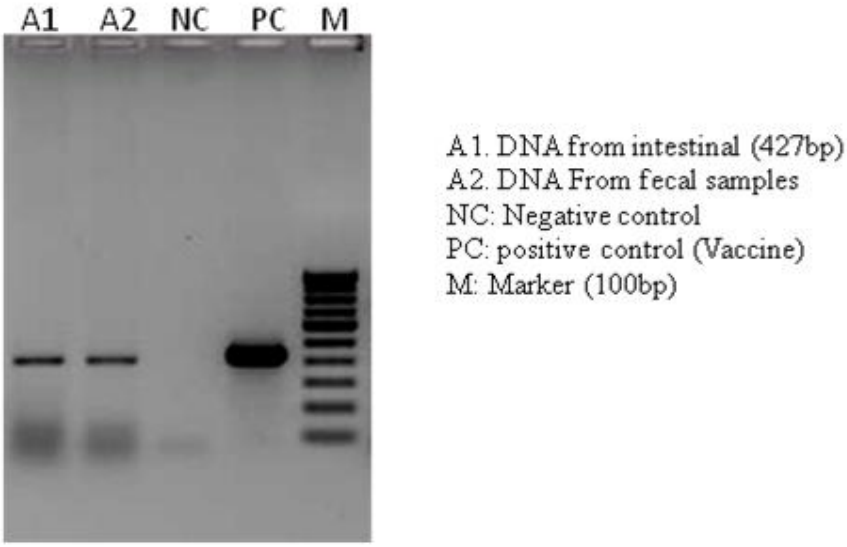

Fig. 9. Agarose Gel (2%) picture showing positive for CPV from intestine and fecal samples of rusty spotted cat collected from post-mortem

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using polymerase chain reaction (PCR). The PCR products were run in 1.5% agarose gel for respective product sizes.

A total of 120 individual fecal samples of various felids and canids were collected for screening of CPV, FPV and CAV from the zoos of Andhrapradesh in the months of summer and winter (Table 6). None of the sample was found positive for the above diseases except one jaguar fecal sample from NZP, Hyddrabad was positive for CPV.

Table 6. Details of the fecal samples collected from various Zoos during 2014-15

S.No	Name of the Zoological Park	Collection period	No of individual fecal samples collected
1	Indira Gandhi Zoological Park, Visakhapatnam.	April 2014	45
2	Sri Venkateshwara Zoological Park,Tirupati.	April 2014	31
3.	Nehru Zoological Park, Hyderabad.	Dec 2014 - Jan 2015	45
	Total		121

Canine parvo virus was detected from one of the post mortem samples of rusty spotted cat in NZP, Hyderabad using PCR based method. Further, screening for Endotheliotropic Elephant Herpes Virus (EEHV) was carried out using postmortem samples (Heart, Liver, Tongue and Blood) of elephant calf that received from Maharastra Forest department. Based on the EEHV specific PCR and sequence, Heart, Liver, Tongue samples were positive for EEHV1A strain.

D. Cryobanking of genes, tissues, gametes and embryos of endangered animals.

i) Rescue of gametes from dead wild/endangered animals

The ability to recover oocytes from postmortem ovaries of endangered or wildlife species holds tremendous potential for conservation using assisted

reproductive technologies. In addition, *in vitro* maturation and fertilization of immature oocytes collected from wild/endangered animals which have died due to accidents or medical reasons would allow one to establish species specific protocols for assisted reproductive technologies.

Immature oocytes were collected from the ovaries of dead barasingha, thamin deer, swamp deer and spotted deer and matured *in vitro. T*he developmental ability of these *in vitro* matured oocytes following parthenogentic activation was demonstrated (Fig. 4). The proportion of oocytes that matured *in vitro* as judged by the presence of extruded 1st polar body and had reached telophase-I and metaphase-II in the four deer species were not significantly different. Only swamp deer oocytes developed to blastocysts (5.7%) following activation. The effect of time delay between the death of animals and excision of ovaries on recovery and developmental ability of the recovered oocytes was also studied. The effect of the time interval that lapsed between the death of animal and recovery of ovaries on recovery of culture grade oocytes, *in vitro* maturation and development to blastocyst stage was also not significant.

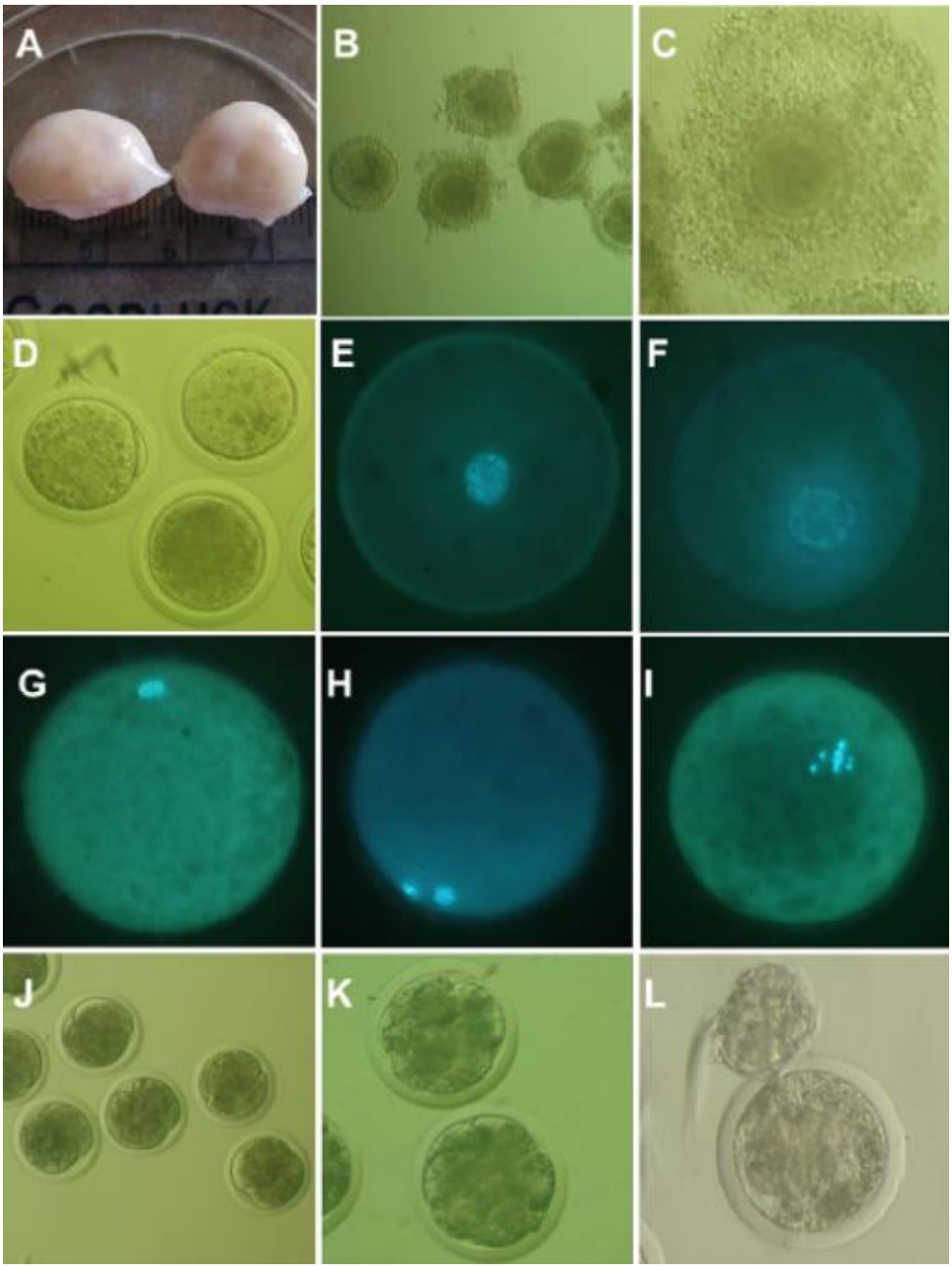

Fig. 4. Representative photographs of *in vitro* maturation and activation of oocytes of swamp deer collected from the ovaries of dead animals. (A) ovaries; (B) Immature cumulus oocyte complexes; (C) cumulus cell expansion; (D) extrusion of 1st polar body 27 h after *in vitro* maturation. Different meiotic stages of swamp deer oocytes 27 h after *in vitro* maturation. (E) germinal vesicle stage oocyte; (F) germinal vesicle break down; (G) MI stage oocyte; (H) MII stage oocyte; (I) degenerated oocyte. *In vitro* development of parthenogenetic activated swamp deer oocytes (J-L). (J) 2 ï cell stage; (K) morulae; (L) blastocyst.

(i) Establishment of cell bank for wild/endangered animals

A total of 10 ear samples of various animals were collected from NZP, Hyderabad at postmortem. Fibroblast cell lines were developed and cryopreserved from 6 animals (chousingha, sambar deer, leopard, chimpanzee and marmoset) during April 2013 to March 2014. (Fig. 10).

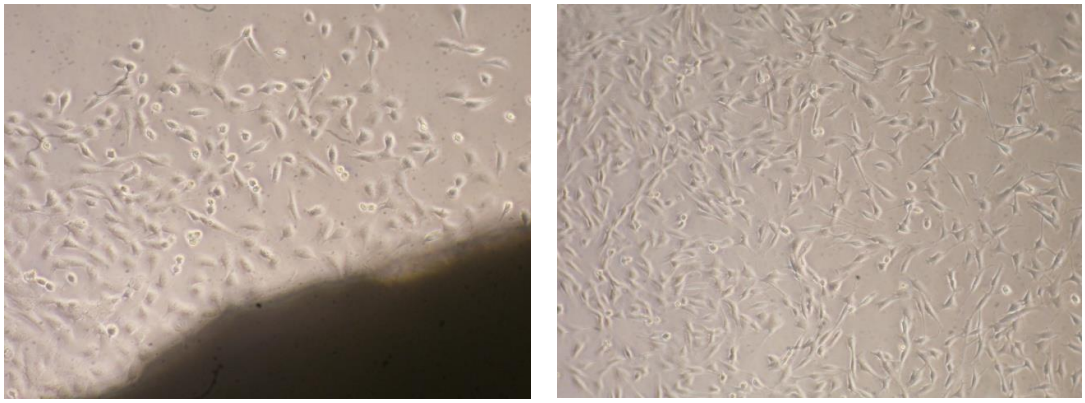

Fig. 10. Outgrowth of leopard ear skin fibroblast (a) and fibrobalsts at 60% confluence.

A total of 10 postmortem skin samples of ten individual species were collected from NZP, Hyderabad (Table 7). Fifty percent (5/10) of the samples developed to fibroblast cell lines and were cryo preserved.

Table 7. Details of cell lines developed during 2014-2015

Sl. No	Name of the species	Number of individual samples received	Number of individuals samples developed cell lines
1	Jaguar	1	1
2	White Tiger	1	1
3	Leopard (Maharastra)	1	-
4	Indian wolf	2	1
5	Wild Dog	1	-
6	Sambar deer	3	2
7	Gharial (Trachea)	1	-
	Total	10	5

Somatic cell nuclear transfer technology is the one of the existing assisted reproductive technologies to clone or resurrect a species if need arises. This technology depends on the availability of somatic cells of the species to be cloned. Thus establishment of cell cultures of endangered animals could serve as a bio-resource for resurrection of wild animals. Such cell cultures once developed could be stored in liquid nitrogen for posterity.

A total of 39 individual skin samples of 16 species of various animals were collected post-mortem at NZP, Hyderabad and cryopreserved (Table.8). Fibroblast cell lines were developed and cryopreserved from 33 individual animals (33/39).

Table 8. Details of animals and cell lines developed

	Name of the Species	No. of Individuals	Growth Yes/NO
Herbivores			
1	Mouse Deer	7	5/7
2	Chousingha	3	3/3
3	Thamin Deer	2	1/2
4	Sambar Deer	4	3/4
5	Hog Deer	3	2/3
6	Swamp Deer	2	2/2

7	Barking Deer	8	8/8
Primates			
1	LTM	1	1/1
2	Chimpangee	1	1/1
3	Mandrill Monkey	1	1/1
4	Baboon	1	1/1
5	Capped Langur	1	1/1
Carnivores			
Felids			
1	Jaguar	1	1/1
2	Tiger	1	1/1
3	Leopard	1	1/1
Canids			
1	Indian Wolf	2	1/2
		39	33/39

(ii) Recovery and cryopreservation of gametes from dead wild/endangered animals

The development of species specific protocols for assisted reproductive technologies (ARTs) holds tremendous potential for conservation of wild/endangered species. Lack of basic knowledge about reproductive functions of wild animals is the one of the major concern to develop ARTs for species concerned. The ability to recover and preservation of oocytes from postmortem ovaries of endangered or wildlife species is the one of the promising approach to build basic biology of gametes for further development of ARTs. In addition, in vitro maturation and fertilization (IVM-IVF) of immature oocytes collected from wild/endangered animals which have died due to accidents or medical reasons would allow one to develop embryos of wild /endangered species.

We have attempted to generate embryos using oocytes and spermatozoa collected from post mortem ovaries and testicles of sambar deer by IVM-IVF. The ovaries and testicles were collected following the unexpected death of male and

female sambar deer at NZP, Hyd. The ovaries were processed immediately for oocyte recovery, the testicle was preserved in PBS with antibiotics at 4^0C for 24h in order to synchronize oocyte maturation for *in vitro* fertilization. A total of 22 immature sambar oocytes were collected, 11(50%) out of 22 oocytes were culture grade and subjecteed to maturation for 24 h. The sperm was collected by retrograde flushing of the cauda epidydimis and prepared for IVF. The matured oocytes and sperm were co-incubated for 12h. Subsequently the oocytes were cultured for 6 days to develop into morula/blastocyst. None of the oocyte was cleaved. This may be due to the effect of postmortem changes on the fertilizable capacity of the spermatozoa.

Immature cumulus oocyte complexes were collected from the postmortem ovaries of rusty spotted cat (*Prionailurus rubiginosus)* collected postmortem. A total of 55 oocytes were collected, 32 out of 55 oocytes were culture grade oocytes. These culture grade oocytes were matured in tissue culture medium 199 (modified with sodium bicarbonate) supplemented with 0.3% BSA (fatty acid-free) (w/v), 10 εg/ml FSH, 6 IU/ml LH, 1 εg/ml 17ϐ-estradiol, 0.36 mM pyruvate, 2.2 mM calcium lactate, 2.0 mM L-glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin in an incubator with 5% CO_2 under humidified air at 38.5°C for 36 h (Rao et al 2015). A total of 18 oocytes were matured (56%) and these matured oocytes were cryopreserved using cryotop method (Rao et al 2012).

A total of 134 immature oocytes were recovered from the postmortem ovaries of seven species (Table 3). Out of 114 immature oocytes, 53 oocytes were matured in vitro (46.0%). A total of 43 oocytes were cryoprserved after in vitro maturation and 15 immature oocytes of sacrel baboon were cryopreserved (Table.9).

Table.9. Rescue and Cryopreservation of oocytes from dead wild animals

S.No	Name of the species	Total no of oocytes recovered	Total no of oocytes matured (%)	No of matured oocytes Cryopreserved
1	Mouse Deer	19	10 (52.0)	--
2	Rusty Spotted Cat	55	18 (32.0)	18
4	Indian Wolf	20	11 (55.00)	11

5	Wild Dog	06	4 (66.00)	4
6	Leopard	14	10 (47%)	10 matured
7	Sacrel Baboon	20	--	15 immature
		134	53/114 (46%)	58

(iii) Vitrification of Mouse deer oocytes

Immature cumulus oocyte complexes were collected from the ovaries of mouse deer collected postmortem. A total of 40 oocytes were collected by slicing method, 50% (20/40) of the oocytes were grades as good quality oocytes. A total of twenty oocytes were cryopreserved using cryotop method.

(iv) Cryopreservation of testes from wild animals

Testis cryopreservation can aid in preservation of germplasm of male animals that die before sexual maturity. This is especially of significance in conservation of rare and endangered animals whose dwindling population is also affected by high neonatal/juvenile mortality. Immature testis cryopreservation in conjunction with testis tissue xenografting provides a powerful approach for the conservation of endangered species. Cryopreservation of testis tissue is challenging owing to its structure and presence of different cell types that differ in size and membrane permeability.

Cryopreservation is a method of preserving biological samples at subzero temperatures for a longer period of time where cryoprotectants play a crucial role in maintaining cellular integrity. Among penetrating cryoprotectants, dimethyl sulfoxide (DMSO) was proved to maintain architecture of immature testicular tissue, and provides superior cryopreservation with fewer morphological alterations. Fetal bovine serum (FBS), the most widely used supplement in cell culture also acts as non-penetrating cryoprotectant in combination with DMSO.

Testes from several wild animals were collected following their death from Nehru Zoological Park (NZP), Hyderabad and cryopreserved. Table 10 summarize the testes collected and cryopreserved. Two concentrations of DMSO (0.7 and 1.5M) and FBS (20 and 80%) are currently being used so that there is superior morphological preservation, germ cell survival and robust cell proliferations status of

the cryopreserved testicular tissues. We have also initiated xenografting to demonstrate if cryopreserved immature testis tissues can mature and produce differentiated germ cells *in vivo* that can be used for assisted reproduction.

Cryosensitivity of testis tissues vary with species and it would be imprudent to define just one formulation of cryo-medium that would be able to cryopreserve testis from various species. Therefore, we are currently in process to define different combinations of DMSO, FBS and other cryoprotectants that would cryopreserve testis with superior morphological preservation, germ cell survival and robust cell proliferations status from a given family.

Table 10: Details of testes collected and cryopreserved from Nehru Zoological Park, Hyderabad

S. No	Common name / scientific name	Family	Age*	Weight of testis (gm)	Date of preservation	Initial viability (%)
1	Black buck *(Antilope cervicapra)*	Bovidae	Adult	-	23-04-13	-
2	Chousingha *(Tetracerus quadricornis)*	Bovidae	Adult	18.5	18-07-13	36.2
3	Indian mouse deer *(Moschiola indica)*	Tragulidae	Young (1 month)	0.42	07-08-13	86.2
4	Colobus monkey *(Colobus guereza)*	Cercopithecidae	Adult	7.58	16-08-13	68
5	Chimpanzee *(Pan troglodytes)*	Hominidae	Adult	76.53	20-08-13	54
6	Indian mouse deer *(Moschiola indica)*	Tragulidae	Young (15 days)	0.24	20-08-13	98
7	Leopard *(Panthera pardus)*	Felidae	Young (6 month)	2.7	23-08-13	54
8	Chousingha *(Tetracerus quadricornis)*	Bovidae	Adult	8.73	03-09-13	60
9	Nilgai *(Boselaphus tragocamelus)*	Bovidae	Young (cryptorchid)	2.72	02-09-13	4
10	Nilgai *(Boselaphus tragocamelus)*	Bovidae	Adult	49	04-09-13	39
11	Sambar deer *(Rusa unicolor)*	Cervidae	Adult	15.6	06-12-13	56.4
12	Rusty spotted cat *(Prionailurus rubiginosus)*	Felidae	Young	0.44	17-12-13	79
13	Indian mouse deer *(Moschiola indica)*	Tragulidae	Young (15 days)	0.44	15-01-14	72
14	Marmoset *(Callithrix)*	Callitrichidae	Adult	3.1	19-02-14	41
15	Mandrill *(Mandrillus sphinx)*	Cercopithecidae	Adult	62.01	25-03-14	43

Please note that it was impossible to know exact age of animals in most cases due to lack of records. However in few cases, age was told and is mentioned.

(v) *Germ Cell Differentiation in Cryopreserved Immature Indian Spotted Mouse Deer (Moschiola indica) Testes Xenografted onto Mice*

Death of immature animals is one of the reasons for the loss of genetic diversity of rare and endangered species. Since sperm cannot be collected from immature males, cryo-banking of testicular tissue combined with testis xenografting is a potential option for conservation. The objective of this study was to evaluate the establishment of spermatogenesis in cryopreserved immature testicular tissues from Indian spotted mouse deer (*Moschiola indica*) after ectopic xenografting onto immunodeficient nude mice. Results showed that testis tissues that were frozen in cryo-media containing either 10% DMSO with 80% FBS (D10S80) or 20% DMSO with 20% FBS (D20S20) had significantly more ($P < 0.01$) terminal deoxynucleotidyl transferase-mediated dUTP nick end labelled (TUNEL)-positive interstitial cells when compared with fresh testis tissues (46.3 ± 3.4 and 51.9 ± 4.0 vs 22.8 ± 2.0). Xenografted testicular tissues showed degenerated seminiferous tubules 24 weeks post-grafting in testes that had been cryopreserved in D20S20 (Fig. 11C); alternatively, pachytene spermatocytes were the most advanced germ cells in testes that were cryopreserved in D10S80 (Fig. 11B, D). Proliferating cell nuclear antigen (PCNA)-staining confirmed the proliferative status of spermatocytes, and the increases in tubular and lumen diameters indicated testicular maturation in xenografts (Fig. 11E, F). However, persistent anti-Mullerian hormone (AMH)-staining in Sertoli cells of xenografts revealed incomplete testicular maturation. This study demonstrates that cryopreserved testis tissue that had been xenografted from endangered animals onto mice resulted in the establishment of spermatogenesis with initiation of meiosis. These findings are encouraging for cryo-banking of testicular tissues from immature endangered animals to conserve their germplasm.

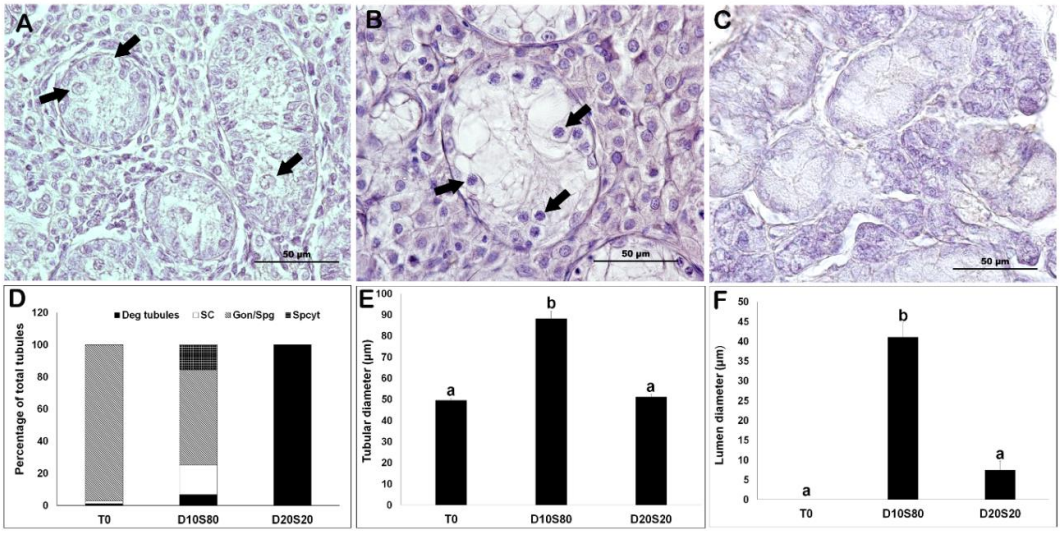

Fig. 11. Histological and morphometric evaluation of donor tissues and grafts with most advanced germ cell types. Typical morphology of the most advanced germ cell types in each tubule is indicated by arrows. (A) Donor tissue from 15-day-old Indian mouse deer showing gonocytes/spermatogonia. Grafts from recipient mice that were grafted with testicular tissues cryopreserved in (B) D10S80, which contain pachytene spermatocytes as the most advanced germ cells, and (C) D20S20, which contain all degenerated tubules after 24 weeks. (D) Percentage of seminiferous tubules with the most advanced germ cell type in xenografts. Deg tubules, degenerated tubules; SC, Sertoli cell only; Gon/spg, gonocytes or spermatogonia; and Spcyt, pachytene spermatocytes. (E) Average tubular diameter and, (F) tubule lumen diameter of grafts. T0, fresh testicular tissue as a representation of starting material. Data represent mean ± SEM. Bars with different letters are significantly different at $P < 0.05$. Scale bar = 50 µm.

(vi) Cryopreservation of Sperm in Testis of Adult Wild Mammals

Preservation of sperm could be an option for the conservation of the genetic potential of endangered animals whose population is rapidly declining because of illegal hunting, loss of habitats, and inbreeding-related problems. It is possible to collect and cryopreserve spermatozoa before or after death in a sexually mature males, and these sperm cells can be used later for assisted reproduction. Although electrostimulation is the usual method for the collection of spermatozoa from wild animals, which has been used for assisted reproductive technologies in domestic and wild cats, the use of this method is not applicable for animals that would unexpectedly die. Therefore, for such animals only spermatozoa recovered from cryopreserved epididymis and testicular tissue, can be used for embryo production. In cases of unsuccessful epididymal sperm retrieval, the testis is the only source of sperm. Compared to epididymal sperm, testicular sperm have higher potential to retain viability after freezing and thawing. Several pregnancies have been reported

after intra-cytoplasmic sperm injection (ICSI) with testicular sperm. Over the period of 2 years, we have cryopreserved testis from several adult wild mammals in different combinations of DMSO and FBS (Table 11). We are currently evaluating the combination in which the sperm cells are optimally preserved by assessing their viability, morphological defects. Early apoptosis and expression of proteins (Fig. 12).

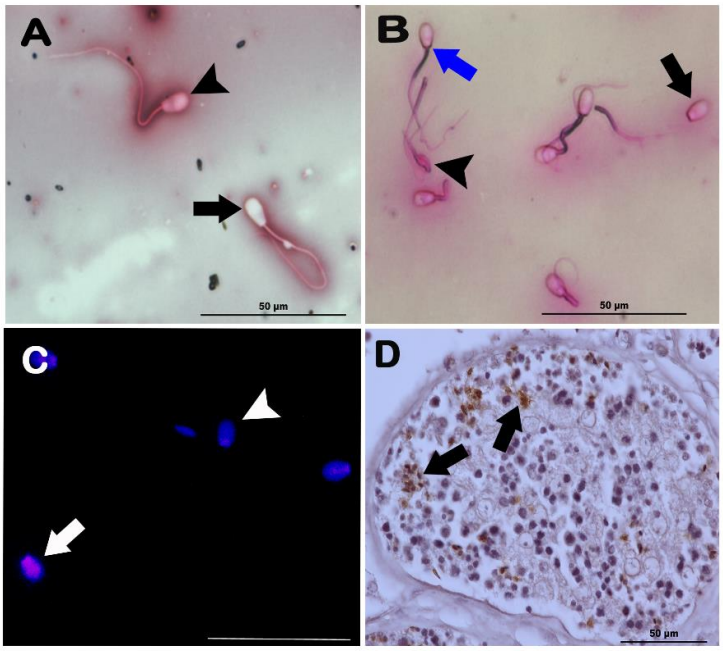

Fig. 12. Evaluation of cryopreserved adult non-human primate testis for sperm viability and quality, early apoptosis and expression of sperm-specific proteins. (A) Viability assessment of sperm by eosin-nigrosin staining. Arrow indicate a live spermatozoa with white head and an arrowhead indicates a dead sperm with pink head. (B) Morphological abnormalities as determined by rose bengal staining. Blue arrow shows a normal, black arrow a tail damaged and black arrowhead a head damaged spermatozoa respectively. (C) Annexin V staining (red) of sperm. An Annexine V-positive spermatozoa is indicated by an arrow and a negative spermatozoa by an arrowhead. The sperm are counterstained with DAPI. (D) Immunohistochemical analysis of testis for the expression of sperm specific protein protamine 2. Scale = 50 µm

Cryopreservation of Adult Testis as an Aid to Preservation of Germplasm

For wild animals that may unexpectedly die, spermatozoa recovered from the epididymis can be used for assisted reproductive technologies (ART). However, in cases of unsuccessful epididymal sperm retrieval, adult testes are the only source of sperm. In the past year we demonstrated cryopreservation of testicular tissue of three adult primates (mandrill, chimpanzee, and marmoset) and three adult cervids (sambar deer, barking deer and hog deer) using uncontrolled slow freezing protocol. For successful cryopreservation, optimization of concentrations of commonly used cryoprotectant such as dimethyl sulfoxide (DMSO) and combinations of foetal bovine

serum (FBS) and DMSO is essential. Testicular tissues were cryopreserved in 10% DMSO (D10) with 80% FBS (D10S80) or 20% DMSO (D20) with 20% FBS (D20S20). The cryopreserved testes were evaluated for efficacy of cryopreservation by assessing the viability and acrosome status of retrieved sperm, expression of

spermatozoa- and spermatid-specific proteins (PRM2 and TNP1) by immunohistochemistry, and expression of pro- and anti-apoptosis- (BAX/BCL2 ratio), early apoptosis (AnnexinV), and cell proliferation-specific (PCNA) proteins by western blot analysis. The results suggest that mandrill testis can be effectively cryopreserved in D10S80, marmoset testis in D10S80 and D20S20 whereas, chimpanzee testis in D20. D20S20 is optimal for cryopreservation of testis of three cervid species. The present study provides the basis to develop field-friendly cryopreservation protocol for the testis cryopreservation for primates and cervids, and for closely related species.

Table 11. Details of animals from which testis were collected and cryopreserved *post-mortem* in 2014-15.

S. No.	Common name / scientific name	Family	Age	Weight of testis (gm)	Date of preservation	Initial cell viability (%)
1	Indian Jackal *(Canis aureus indicus)*	Canidae	Adult (7years)	3.8	07-04-2014	42
2	Giant squirrel *(Ratufa indica)*	Sciuridae	Adult	0.35	15-04-2014	64
3	Thamin deer (*Panolia eldi)*	Cervidae	Adult	-	14-05-2014	18
4	Sambar deer *(Rusa unicolor)*	Cervidae	Adult	35.7	07-07-2014	26
5	Sambar deer *(Rusa unicolor)*	Cervidae	Adult	43.6	23-07-2014	26
6	Mandrill *(Mandrillus sphinx)*	Cercopithecidae	Adult (8years)	-	24-07-2014	-
7	White headed marmoset (*Callithrix geoffroyi)*	Callitrichidae	Adult	0.42	08-10-2014	52
8	Jaguar *(Panthera onca)*	Felidae	Adult (12 years)	28.57	01-11-2014	18
9	Indian wolf *(Canis lupus pallipes)*	Canidae	Adult	7.83	19-01-2015	61

Please note that it was impossible to know exact age of animals in most cases due to lack of records. However in few cases, age was told and is mentioned.

E. Services

1). Pregnancy Diagnosis of Captive Wild Animals

About 100 fecal samples from eight species (tiger, lion, leopard, jaguar, elephant, jungle cat, Himalayan black bear and olive baboon) were analyzed for pregnancy detection from various zoos (Nehru Zoological Park, Hyderbad, Bhopal zoo, Mudhumalai elephant camp, Mysore zoo and Patna zoo) and reported to zoo management.

2) Diesease screening

We have provided services for screening of CDV in post mortem samples of felids from Maharastra forest Department. Six tigers and three leopards were screened for CDV disease. Five blood samples of lions from NZP, Hyderabad were screened for CDV.

F. Symposium / Workshop Organized

A three-day workshop / conference on ñReproduction and Welfare of Endangered Animals in Conservation Breedingò for Scientists, Biologists and Zoo veterinarians at LaCONES, CCMB during January 28 ï 30, 2015.

G. Training of Personnel

Fourteen project JRF, SRF and RA were trained in the area of biotechnological use in wildlife conservation. Similarly, 30 B.Tech. / M.Sc. dissertation students were also trained for three to six months.

H. Publications

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