SPERM CRYOPRESERVATION, *IN VITRO* FERTILIZATION AND FAECAL OESTROGEN ENZYME IMMUNOASSAY VALIDATION IN THE AFRICAN LION (*PANTHERA LEO*)

By

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Dedicated to my parents, Tiens and Henriette,
and my loving companion, Cas.

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DECLARATION

I hereby declare that this dissertation submitted by me to the University of the Free State for the degree, **Magister Scientiae Agriculturae**, is my own independent work and has not previously been submitted for a degree at any other university. I furthermore cede copyright of the thesis in favour of the University of the Free State.

Henriette Stander

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May 2004

LIST OF KEYWORDS

Spermatozoa

Lioness

In vitro maturation

Endocrine

Glycerol

Steroid hormone

DMSO

Electro-ejaculation

Assisted Reproductive techniques (ART)

Biladyl®

Zoletil®

Oestrus

Felid

Oocytes

TL-Hepes

LIST OF ABBREVIATIONS

DMSO – Dimethyl sulfoxide

IVF – *In vitro* fertilization

IVM- *In vitro* maturation

COC's – Cumulus oocyte complexes

ART – Assisted reproductive techniques

ET – Embryo transfer

AI – Artificial insemination

PBS – Phosphate buffer solution

HRP- Horse radish peroxidase

E_2 – Oestradiol 17β

ZP – Zona pellucidae

IUCN – International Union for Conservation of Nature and Natural Resources

BLH – Bovine luteinizing hormone

P. t. sondaica – Panthera tigris sondaica

P. t. balica – Panthera tigris balica

P. t. amoyensis – Panthera tigris amoyensis

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CHAPTER 1

1. GENERAL INTRODUCTION

The African lion (*Panthera leo*) belongs to the family *Felidae* (cats) that is listed in appendix II of the Convention on International Trade in Endangered Species (CITES, 1973). This animal is one of the large predators found in Southern Africa. Lions once used to roam across Europe, the Middle East, India and Africa. Today, however, wild lions are only found in one region in India and in scattered areas south of the Saharan desert in Africa. Due to loss of habitat and conflict with man, the number of lions remaining in the wild has decreased dramatically in recent years. Although, lions are currently not yet endangered they remain extremely vulnerable in terms of their existence (Bauer *et al*., 2003).

There are currently only an estimated 15 000 to 27 000 lions left in Africa under natural conditions. However, the free movement of these wild animals is restricted by man-made fences and most lions in Africa may be considered as captive (Bauer *et al*., 2003). These forms of captivity limit the natural development and maintenance of genetic diversity that is vital to the long-term survival of any specie. In South Africa, lions are mainly confined to the national parks, game reserves and privately owned game farms. The genetic pool assosiated with a small lion population is very limited and could lead to inbreeding, due to limited genetic variation within that population. Inbreeding may lead to fertility problems, resulting in further deterioration in the African lion numbers.

In general, genetic diversity can be maintained by the introduction of new genes, which means introducing unrelated male or female lions, into a small population. This genetic diversity is critical to the survival of a population (WBRC, media release, 2001). However, the introduction of new animals (in this case lion) into a population is not always very practical, due to the risks assosiated with the immobilization and transportation of wild animals. Introducing a new lion or lioness into a pride has the risk of the animal and/or its cubs being rejected or killed by the animals currently in the group. The risk of diseases being transmitted across country borders has placed a restriction on the movement of animals from one country to another. These restrictions place further limitations on efforts to promote genetic diversity in captive lion populations.

The application of assisted reproductive techniques can provide a solution to obtaining genetic diversity in a wild population of captive lions (Wildt *et al*., 1997). The application of techniques such as artificial insemination (AI), embryo production and transfer (ET) can bypass the risks involved in transferring animals between populations but at the same time introduce new genetic material into a lion population. Before these techniques (currently being implemented in human and livestock species), can successfully be implemented in lions, a knowledge of the lion's basic reproductive physiology should be generated. Furthermore, the endocrine events regulating the female's oestrus cycle and gestation period need to be fully understood, as well as the techniques involved in successful semen and embryo cryopreservation. Cryopreservation of feline spermatozoa has been found to be specie specific and a protocol for the cryopreservation of semen from one feline specie will not necessarily apply to the other species (Nelson *et al*., 1999). Therefore, it would seem that specie

specific research with regards to the *Panthera leo* is needed in order to be able to apply assisted reproductive techniques in the *Panthera leo*.

Virtually all conservation biologists agree that habitat preservation is the best way to conserve biodiversity (Wildt *et al*., 1997). The cat specie that has been adversely affected by the loss of its natural habitat is the tiger (*Panthera tigris*). Of the 8 tiger sub-species that were recognized as endangered in 1969, three (*Panthera tigris vigata, P.t. sondaica and P.t. balica*) are now extinct and one (*P.t.amoyensis*) is currently critically endangered. As natural habitats become smaller and more isolated, the species occupying these habitats become more vulnerable to increased inbreeding, disease epidemics, natural disasters and social change. Biotechnology, however, does have a tremendous potential as a tool for assisting in the conservation of endangered species and therefore could be applied even to the wild felids (Wildt *et al*., 1997).

The aim of this study was thus to contribute to a better understanding of the reproductive characteristics of the African lion and exploit the possibility of using techniques such as semen cryopreservation and *in vitro* fertilization that would assist in developing a genetic bank. This could then eventually contribute to the development and use of these assisted reproductive techniques in the African lion and contribute to the long-term survival of this species.

This study was performed in two phases. Firstly a novel protocol for the cryopreservation of African lion (*Panthera leo*) spermatozoa was evaluated and a heterologous *in vitro* fertilization assay using bovine oocytes was performed to test the fertilizing capacity of the spermatozoa following cryopreservation. This was followed by

a second phase in which a faecal oestrogen enzyme immunoassay for monitoring ovarian activity during the oestrus cycle in the African lion (*Panthera leo*) was validated. Once this technique has been validated, it can be used as a potential aid in monitoring the stage of the oestrus cycle of the lioness.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

There are 37 feline species in the world and most of them, with the exception of the domestic cat, are threatened to some extent by extinction, as reported by the Convention of International Trade of Endangered Species (Luvoni *et al*, 2003). The African lion (*Panthera leo)* is one of the largest cat species in the world and is classified as vulnerable, but not endangered, on the Red List of Threatened Species of the World Conservation Union, (Bauer *et al*., 2003). The lion (*Panthera leo)* once roamed large parts of Africa, Europe, the Middle East and Asia The species disappeared from Europe during the first century AD and from North Africa, the Middle East and Asia between 1800 and 1950, except for the one isolated population in India, containing approximately 250 lions of the sub-species *Panthera leo persica*. The remaining lion populations in the world are mainly confined to the African countries south of the Sahara Desert with the majority of the natural African lion population limited to East and Southern Africa (Bauer *et al*., 2003). Currently it is estimated that there are approximately only 15 000 to 20 000 lions left in Africa. This may give reasons for concern regarding the long-term survival of the species (Bothma, 1998). In Southern Africa, the African lion is mainly limited to national parks, game reserves, privately owned game farms and zoos, with the remaining free roaming lion populations classified as enclosed populations due to fences separating populations and limiting movement. These enclosed lion populations are thus susceptible to limited genetic variation. In felids, low genetic variation within species populations has been associated with poor reproduction (Goodrowe *et al.,* 2000). Inbreeding was said to

occur in the Serengeti lion population due to restricted migration, and this led to low reproduction levels of this specific population (Wildt *et al.,* 1987). It is thus of great importance to secure genetic variation inside such an isolated lion population or for that matter any captive lion population to reduce the risk of inbreeding that could lead to fertility problems. Reproductive failure in the African lion will be detrimental to the remaining lion population as this could further increase the rate of inbreeding.

There is without a doubt a need to protect the number of lions in the remaining population and the development of intensive breeding programs in lion populations, as well as the application of assisted reproductive techniques. These techniques may contribute to securing and stabilising the number of lions remainig in Southern Africa.

Understanding the basic reproductive processes of the African lion is essential and will assist in these breeding programs and the application of assisted reproductive techniques in the specie. An animal can only reproduce if it is fertile. Female animals demonstrating cyclic activity and having the potential to conceive are considered fertile. Male animals that are considered to be fertile on the other hand have the capability to produce sperm of good quality with the capacity to fertilize the oocytes (Jainudeen and Hafez, 1987). In the process of evaluating the reproductive endocrinology of the lioness, or any other feline specie, a knowledge could be gained regarding the cyclic activity (ovarian activity), including the length of the oestrus cycle, the time of ovulation and the type of ovulation (spontaneous or induced) (Goodrowe *et al.*, 2000). However, the monitoring of the ovarian activity in wild animals is not always very practical. Endocrinological studies require obtaining blood samples which is in turn a practice which could be stressful to the animal due to the required immobilization. To counter

this stress, non-invasive techniques for assessing steroid hormonal levels in wild animal have been developed (Brown *et al.*, 1996). One of these non-invasive techniques entail collecting faecal and/or urinary samples of an animal over a period of time and assessing the steroid hormone (or their metabolites) levels by radio- or enzyme immunoassay techniques (Czekala *et al*, 1994). This non-invasive faecal or urine analysis for hormone determinations has been found to be effective for the monitoring of reproductive events in wildlife species, and eliminate potential stressors associated with blood sampling (Goodrowe *et al.*, 2000). Faecal steroid analyses can demonstrate whether a female is cyclic (episodic fluctuations in oestrogen) and assist determining the appropriate time for introduction of the male to the female in breeding programmes. Assessing the hormonal status of the female through faecal immunoassays could thus assist in breeding programs. The analysis of faecal progestins could confirm ovulation and subsequently diagnosis of pregnancy or pseudopregnancy in a lioness (Goodrowe, 1992). Confirmation of ovulation and subsequent pregnancy diagnosis can save time and money. Goodrowe *et al.* (2000) used this approach to diagnose ovulation and pregnancy in a lioness.

Fertility examinations of male domestic animals include the evaluation of semen quality as well as testicular characteristics (Jainudeen and Hafez, 1987). It would seem logical that assessing the fertility status of wild animals should also include a semen and testicular evaluation. Research has been done on seminal characteristics of a number of wild feline species as well as the domestic cat (Howard *et al.*, 1990; Donoghue *et al.*, 1992; Goodrowe, 1992; Gilmore*, et al.* 1998; Da Paz, *et al.*, 2002; Damiani, *et al.* 2004). These results obtained by these researches on basic seminal characteristics of various feline species can be used as reference values for this and other further

studies. Different techniques are used to obtain sperm from male animals, e.g. electro-ejaculation, dissection of the epididymis from live or dead animals and the use of an artificial vagina (Sojka, 1986). Once semen has been obtained using these various techniques, and before its use in assisted reproduction techniques, the evaluation of sperm quality is essential in order to know whether the sample of semen is suitable for preservation treatment (Luvoni *et al*., 2003).

Semen evaluation generally includes the determination of the overall sperm motility, rate of forward progression, sperm abnormalities, total sperm concentration, acrosome integrity, semen pH, color and volume of the ejaculate. Felids are generally also affected by the occurrence of teratospermia, a condition in which more than 60% of the spermatozoa show aberrant forms (Pukazhenthi *et al*., 1999). This high proportion of abnormalities present in felid ejaculates can negatively affect the motility of the sperm. The etiology of teratospermia in the domestic cat is unknown, but structurally defective spermatozoa observed in wild felids such as the cheetah and geographically isolated lion populations have been related to decreased genetic variation of enclosed populations (Luvoni *et al*., 2003). According to Pukazhenthi *et al*., (1999), a high incidence of damaged acrosomes generally occur in felid sperm. Once the sperm cells are harvested and evaluated, it can be used fresh, cooled or as frozen-thawed semen for artificial insemination or in *vitro* embryo production. Cooled and cryopreserved sperm undergo several types of damage, which could alter the motility and morphology of the sperm and are responsible for the low pregnancy rates recorded following AI (Luvoni *et al*., 2003). Both cooled and cryopreserved felid sperm used in *in vitro* fertilization (IVF) have been proven capable of producing embryos, even though the results are still poor (Pope *et al*., 1991; Donoghue *et al.,* 1992; Lengwinat and Blottner,

1994; Nelson *et al.*, 1999; Bartels *et al.,* 2000; Damiani, *et al.* 2004). In felids, fertility following artificial insemination with frozen semen is poorer than with fresh semen (Luvoni *et al.*, 2003). Artificial insemination has also been used to produce cubs in captive big wild cats, including the cheetah, ocelot and the tiger (Brown *et al*, 1996).

The cryopreservation of feline semen has been studied and has been successful in certain cases (Sojka, 1986; Bartels *et al.*, 2000; Howard *et al.,* 1990). However, in a study conducted by Pukazhenthi *et al.* (2001), they reported that feline semen does not freeze very well. Feline sperm seem to be very susceptible to acrosomal and membrane damage during the freezing process. The cryoprotectant agent usually used in freezing diluents (glycerol/DMSO) provides protection to the cells in terms of intra cellular ice crystal formation, by replacing water in the sperm cell. The osmotic effect of this cryoprotectant (glycerol) may result in membrane damage and the toxicity of glycerol for cat spermatozoa has been previously reported which could further reduce the number of viable sperm (Nelson *et.al.*,1999). Pukazhenthi *et al.* (1999), reported that sperm from teratospermic males (<40% normal sperm per ejaculate) are more susceptible to cold and osmotic stress than those from normospermic (>60% normal sperm per ejaculate) males. The cooling process during cryopreservation could also lead to decreased progressive motility of sperm (Pukazhenthi *et al.*, 1999). It is thus essential to develop a semen cryopreservation protocol with optimum cryoprotectant inclusion levels as well as optimal cooling rates.

The reason for the preservation of semen is to preserve genetic material in order to develop assisted reproductive techniques (ART) that could increase the chances of survival of endangered species. The success of a cryopreservation protocol is usually

expressed in terms of the pregnancy rate. There are 4 different techniques generally used by researchers to determine the fertilizing capacity of frozen-thawed sperm and therefore indicate the success of the semen cryopreservation protocol (Goodrowe *et al.*, 2000). The first is the homologous zona pellucida (ZP) adhesion technique. According to Goodrowe *et al.* (2000), this technique can only be used in capacitated sperm. A loss in acrosomal integrity of the sperm through cryopreservation will reduce adhesion by sperm to the zona pellucida. The second technique is a homologous or heterologous ZP penetration assay, where the sperm cells not only attach to the ZP surface, but also penetrate. During this technique domestic cat oocytes may be used for testing the fertilizing capacity of wild felid sperm (Howard *et al.*, 1986). The third technique is the zona free egg penetration test. The ZP is removed from the oocyte with the aid of trypsin (enzyme). Homologous oocytes from endangered species may not always be available and therefore this assay may allow saving these valuable oocytes (Nelson *et al.*, 1999). In the fourth, a homologous oocyte fertilization, the oocytes and sperm used are from the same specie and in vitro fertilization of the post-thawed sperm is determined through a homologous fertilization assay (Bartels *et al.*, 2000).

Assisted reproduction has become an important aspect in feline reproduction and with advances in biotechnology it can show tremendous potential as a tool for assisting the conservation of endangered cat species (Goodrowe *et al.*, 2000).

2.2 AFRICAN LION BEHAVIOUR WITH EMPHASIS ON REPRODUCTION

Behavioural studies have been reported on the African lion and the information obtained in these studies assist in placing laboratory results in perspective with the

natural behaviour of the species in the wild. It should however be kept in mind that research done on zoo animals is not necessarily representative of those still remaining in the wild (Guggisberg, 1961).

Lions are usually found in open, sparcely wooded savannahs, where their solid tawny pelts blends in with their surroundings. The lion's colouring and habitat selection thus allow it to hunt and survive successfully. However, sometimes a pride can be located in mountainous or sub-desert areas (Packer and Pusey, 1982). Lions generally do not inhabit forest and jungle areas and do not often roam deserts due to a scarcity of prey. The African lion is typically an animal roaming the dense, dry regions and can survive for some time without water, as they obtain their moisture from their prey and wild melons (Smuts, 1982). Lions can therefore survive in very arid environments.

2.3 SOCIAL BEHAVIOUR OF THE AFRICAN LION

The lion differs from other members of the cat family, in that it is the only wild cat that shows social behaviour (Bothma, 1988). The African lions form groups or prides in which they exist. These prides consist of a group of related females that are controlled by coalitions of usually unrelated (to the females) male lions that mate with the females, eat from the female's kills and defend their territories. The number of females in a pride is normally stable (Bothma, 1988). Bothma, (1988) recorded as many as 39 lions in a pride found in the Kruger National Park. The size of the lion pride is dependent on the availability of food. The more arid areas in South Africa support fewer lions and smaller prides due to lower numbers of prey found in these areas. According to Bothma (1988), there is also a lower percentage of cub survival rates in areas with low food availability. Caraco and Wolf (1975) reported that larger lion groups have been known

to break up into smaller groups during the onset of the dry season. These smaller groups are energetically more efficient in obtaining food and hence their chances for survival are increased.

It is generally found (or observed) in the social herachy structure of a pride, that the pride is dominated by one or two large male. The dominant male drive the subordinate (younger) males born in the pride, out of the pride at the age of two and a half to three years (Bothma, 1988). These males driven out of a pride may form a group together with other young male lions although female lions have the tendency to remain with their natal pride. As much as one third of the female cubs grow to adulthood and leave the group to become nomads and start their own prides (Packer & Pusey, 1982).

The reign of the male in dominating the pride is relatively short and may last for 2 to 3 years. To secure the territory, the dominant male marks his territory by spraying urine on tree trunks and bushes as a warning to other intruding males (Bothma, 1988). The spraying of the urine (containing pheromones) also acts as an attraction to the females. Once the reign of the dominant male has expired (this happens when the dominant male losses a fight with younger male), the pride is taken over by the new dominant male. The take-over of a pride by a new dominant male may result in most of the cubs present in the pride at that time being killed by the new leader. The lioness comes in heat 3 to 5 days after the cubs were killed. The female is then mated by the dominant male. This ensures that the consequent offspring born in the pride are genetically related to the new reigning dominant male (Bothma, 1988).

2.4. REPRODUCTION IN THE AFRICAN LION:

The complex social organization of a lion pride and the typical cat-like reproductive physiology of the lion has an important effect on the reproductive behaviour and the sexual activity of the African lion (Bothma, 1988). Current available information on the reproduction of the African lion is based on behavioural observations of wild African lions, as well as reproductive studies on captive African lions (Guggisberg, 1975). The information obtained by using captive animals in research studies cannot always be extrapolated to the wild population and situation due to factors such as a difference in diet, the complex group dynamics (i.e. hunting), environment and stress factors. All of the above mentioned factors may influence the reproduction physiology of animals in captivity.

According to Bothma (1988) none of the cat species, including the African lion, maintain permanent bonds throughout their life. By nature the lion is polygamous, which means that a male will mate with more than one female (Guggisberg, 1961). . Male African lions start to sexually relate to females at the age of 5 years, while females become sexually mature at the age of 4 years (Smuts *et al.*, 1978). When a lioness exhibits oestrus, the dominant male forms a courtship bond with her and prevents other males from getting too close to the female (Packer & Pusey, 1982). Two males might reach a female in heat at the same time and this might lead to a serious fight resulting in injuries to one or both males. This factor stresses the difficulty of introducing new males into a lion population due to dominance in lion prides and the chance of a male being killed after being introduced into a new population (Guggisberg, 1975).

The lioness exhibits oestrus once she has reached puberty at the age of 4 years (Bothma, 1988). This lioness in oestrus will develop a strong odour and spray tree trunks and bushes with her urine (Guggisberg, 1961). The urine and the cheek gland secretions of the lioness generally contains pheromones. These pheromones are the chemical substances that inform the males about the hormonal phase or receptivity of the lioness (Bothma, 1988).

Once the lioness starts to exhibit oestrus, the dominant male will follow her wherever she goes.and copulate frequently at short intervals (Bothma, 1988). According to Eloff (1973), the lion and lioness stay together for days, copulating 2 to 3 times every hour. Goodrowe *et al.* (2000), also reported a lioness only to ovulate after repeated copulations. It would seem as if copulation itself and the frequency of copulation affect ovulation in the African lion. Currently it is not clear if the lioness is regarded as a spontaneous or induced ovulator as copulation seem to stimulates ovulation in the lioness. More research redarding this subject is needed. No spontaneous ovulations have been reported in e.g. the tiger (Seal *et al.*, 1985) or snow leopard (Schmidt *et al.*, 1993), whereas occasional spontaneous ovulations have been recorded in clouded leopards (Brown et al., 1995). The incidences of spontaneous ovulation may vary among species, but for the cheetah it appears to be extremely low (Brown *et al.*, 1996). The puma (*Felis concolor*) and the jaguar (*Panthera onca*) have been classified as induced ovulators (Shille and Wing, 1986). The domestic cat is an induced ovulator and does not spontaneously ovulate during oestrus (Banks, 1986). The study by Shille and Wing (1986), on patterns of gonadal hormone secretions of the lioness, suggest that the lioness is a spontaneous ovulator. Spontaneous ovulations have been observed in lions by Schramm *et al.*, (1994), but more information in this regard is needed. It is

important to know the time and type of ovulation that occur in the felids, as this will assist in the success of applying artificial insemination and other assisted reproductive techniques in wild felids.

There is uncertainty of the exact duration and frequency of the oestrous cycle in the lioness. Shille and Wing (1986) reported on the oestrus behaviour and patterns of gonadal hormonal secretions in the puma (*Felis concolor*), the jaguar (*Panthera onca*) and the lioness (*Panthera leo*). According to these studies, the oestrus cycle interval for the lioness was recorded to be between 3 and 8 weeks. This study emphasized the idea of combining behavioural studies with laboratory results to better understand the basic reproduction of the African lion. After close observations of wild lions, Guggisberg (1961) reported the lioness to exhibit oestrus every two and a half months. In the Okavango lion conservation project (Namibia), the oestrus period of the lioness, based on behavioural observations, was found to last between 1 and 5 days. A lioness could also exhibit oestrus within a short time period following the loss of her dependent cubs. During a take-over of the pride by a coalition of males, all the cubs are killed and therefore all the females exhibit oestrus within a few days after the take-over. It is uncertain whether the lioness exhibits oestrus all year round or is a seasonal breeder (Packer & Pusey, 1982).

Studies (Briggs *et al*., 1990) have been conducted regarding the seasonality of the lion, in other words if the lion will be sexually active all year or only during a specific season. Captive lions have been used in studies with regards to determining the reproductive seasonality. In a study by Briggs *et al.* (1990), with the aim of determining whether the frequency of oestrus in the lioness varies between seasons, it was found not to differ

between seasons. Again, the artificial environment of the captive lion could have an influence in the results obtained in the study.

According to Bothma (1988), there is no clear mating season in the lions of the Kgalakgadi Transfrontier Park in South Africa. Guggisberg (1961), reported a higher frequency of copulation to occur between male and female lions in the Kruger National Park, mainly from the end of March (beginning of autumn) to the end of July (mid winter), but copulation as such could occur all year round. These results suggest an all year round cyclic sexual activity in the lioness, with a period of higher seasonal activity during autumn.

After copulation and successful fertilisation, the gestation period begins. It is reported that the gestation period of the lioness can last 90 to 110 days, as determined by visual studies (Guggisberg, 1961). The large variation (20 days) reported in gestation length demonstrates how little is known regarding the African lion's basic reproductive physiology (Schaller, 1972).

Once the cubs are born, the lioness nurtures the cub until the age of 10 weeks, when the cubs start to eat meat (Bothma, 1988). At this age, the lion cub is still very vulnerable to other predators. Schaller (1972) reported an overall cub mortality of 67% for lions in the Serengeti, while the lion cub mortality is estimated to be 29% in the Kruger National Park. Once a lion cub has reached 2 years of age, its chances of survival increases drastically (Bothma, 1988). At this age, the male lions usually leave the pride to avoid conflict with the dominating male lion. These younger males then

form coalitions of 2 or 3 animals and may later form their own prides (Packer & Pusey, 1982).

2.5 GENERAL

The African lion in South Africa is mainly restricted to national parks and game reserves, which leads to a restriction in movement and number of animals in a population and therefore a limited gene pool. Breeding African lions in captivity has become a popular activity in South Africa but the introduction of lions into an established population has been identified as a high-risk operation. The chances of the new animal being rejected by the pride, or even worse, being killed is high because of the social dominance factor in the pride. Immobilisation and the transfer of an animal to a new population are very stressful to the animals and pose certain additional risks. Often the control of movement or the prohibition of importing live animals from certain countries or areas makes it simply impossible to introduce new genetic material into isolated populations. A solution to the risks involved in transferring animals and genes (sperm, oocytes or embryo) has to be found. This can be achieved by implementing assisted reproductive techniques such as artificial insemination, embryo production and embryo transfer in a species. Biotechnology alone will never accomplish saving endangered species, as conserving and management of the natural resources is also essential for the long-term survival of the wildlife in Southern Africa.

Assisted reproductive techniques are not a new concept in animal science, although only a few studies have been conducted on the application of assisted reproductive techniques in the African lion (Briggs *et al.*, 1990; Schramm *et al.*, 1994; Bartels *et al.,* 2000). Assisted reproductive techniques were originally developed to overcome human infertility and to improve livestock production. The results of these studies in

livestock provide the basis from which specific studies for each wildlife specie can be designed (Wildt *et al.*, 1997). Currently, there are also reports of reproductive studies on other wild cats, such as the ocelot, tiger, cheetah, jaguar and snow leopard (Goodrowe, 1992; Nelson *et al*, 1999; Brown and Wildt, 1997; Wildt *et al.*, 1997). The reproductive physiology of each wildlife specie needs to be fully understood before assisted reproductive techniques can be successfully implemented in the specie (Wildt *et al.*,1997). Laparoscopic intra-uterine inseminations have resulted in the birth of offspring in the cheetah, tiger and puma (Goodrowe, 1992). AI in non- domestic felids however is rarely successful due to compromised sperm transport and the uncertainty of the ovulatory status of the females (Dresser *et al.*, 1983). Through further research, the success rate of AI in felids can increase dramatically. The application of ART in the African lion seems to be achievable and could ensure the long-term survival of the species. If this is to be achieved the reproductive patterns and assisted reproductive techniques need to be perfected.

Chapter 3

Testicular and semen characteristics in African lions (*Panthera leo*)

3.1 Introduction

The wild African Lion population has declined rapidly over the last decade (Nowell and Jackson, 1996). A recent unpublished inventory by the African Lion Working Group, the African Lion Database, shows the number of lions left in Africa is between 18 000 and 27 000.

The expansion of human activities such as agriculture and poisoning are the main threats to the African lion population (Bauer *et al.*, 2003). The lions left in Southern Africa are confined to privately owned farms, national parks and game reserves. Fences isolate these remaining lion populations. The exchange of genetic material between these populations is limited or non-existing. All kinds of problems could arise in such closed lion populations, such as inbreeding, which could lead to fertility problems and low reproduction levels of these animals (Wildt *et al.*, 1997). Inbreeding has been shown to have deleterious affects on reproduction in a wide range of species, including laboratory mice, domestic livestock and zoo exotics (Hafez,1987). The introduction of new genetic material into a lion population with limited genetic variation could reduce the level of inbreeding. This will ensure greater survival rates of the lion population and prevent deterioration of lion population numbers left in Southern Africa. Genetic material can be exchanged between populations by introducing new sires into the population (Wildt *et al.*, 1997). It is not easy to introduce a male lion into a new pride. The current dominant and reigning male of the pride will challenge the new incoming male. The challenge could be fatal to the incoming male (Guggisberg, 1961). The stress involved with restraining and immobilising a wild animal such as a lion to

transfer between populations makes it very difficult to reintroduce lions into new prides. An alternative to moving animals between populations is to use the gametes (oocytes, sperm) to introduce new genetic material into a population (Wildt *et al*, 1997). Assisted reproductive techniques such as artificial insemination, *in vitro* and *in vivo* embryo production assist in the transfer of genetic material between populations. Information on the natural reproduction mechanisms of an animal as well as the fertility status of the male and phase of reproductive cycle of the female is needed before any attempts can be made to apply the reproductive techniques (Wildt *et al*., 1997). At present very little is known about the reproductive biology and physiology of the African lion. More research is needed on assisted reproduction techniques such as artificial insemination, embryo production and embryo transfer used in the African lion. Assisted reproduction techniques could assist in the transfer of genetic material between populations of African lions, securing the long-term survival of the species.

This study is aimed at assessing the fertility status of the African lion male, mainly by focussing on testicular as well as semen characteristics. A fertile male should have normal functioning testicles that produce viable sperm with a high fertilising capacity (Jainudeen *et al*., 1987). There are no references available describing the normal testicle and epididimys of the African lion. A few studies have been conducted on semen characteristics of the African lion (Bowen *et al.,* 1982, Howard *et al*., 1984, Byers *et al*., 1989, Donoghue *et al*., 1992, Bartels *et al*, 2000, Patil *et al*., 2002). These studies will thus provide reference values for seminal characteristics of the African lion.

3.2 Materials and methods:

Eight captive male African Lions (*Panthera leo*) with ages ranging from 1 to 4 years and a mean weight of 171kg were used during this trial. Six of the lions (free-ranging) belonged to a privately owned game farm near Bothaville in the Free State, while the other two lions (caged) were from the Johannesburg Zoo, South Africa.

All animals were chemically immobilised with Zoletil 100 (Virbac®, RSA) with the aid of a carbon dioxide-powered darting rifle. Each powder vial of Zoletil contained a 250mg tiletamine and 250mg zolazepam base. On reconstitution of the powder with 5 ml solvent, the solution contained 50 mg/ml tiletamine and 50 mg/ml zolazepam base. A ratio of 1:1(tiletamine and zolazepam) allows efficient general anaesthesia in large carnivores with limited side effects. A Zoletil dosage of 4-5mg/kg body weight was used to chemically immobilise the animals. The animals were darted one at a time and moved out of their camps into a shaded area (to prevent hyperthermia) for handling (plate 1- appendix E).

3.2.1. External sexual organ evaluation:

The external characteristics of both testes and epididimys of all 8 lions were examined. The 2 testicles from each animal were palpated to determine the texture and the development of the cauda epididimys. The length and width of both testicles (right and left) were recorded (cm) and its volume was estimated using these measurements.

3.2.2 Semen collection:

Semen was collected from the each lion while chemically immobilised (plate 1-appendix E) by rectal electro-stimulation (electro-ejaculation), using the technique described for

tigers (*Panthera tigris*) by Loskutoff *et al.*, 2001. A Beltz electroejaculation unit and a rectal probe (24 cm long and 2.5cm wide with three 9-cm long electrodes) were used. The rectal probe was well lubricated with a non-spermacidal lubricant (HR lubricating jelly, Carter – Wallace, New York) and carefully inserted into the rectum of the animal with the electroejaculation at a low voltage (0.5V). The rectal probe was placed so that the middle electrode ran over the accessory sex glands in the ventral midline, at approximately 7 cm from the anal sphincter. To achieve proper placement of the probe, care was taken that both hind legs were equally extended on stimulation to get the most efficient response to the electric stimulations. To avoid leg soreness following electroejaculation (lactic acid build-up), care was taken not to hyperextend the leg muscles by using excessively high voltage or prolonged electrostimulations. A low voltage (0.5V) was used for the initial stimulation to induce penile erection. Once erection was achieved a sterile 20 ml conical tube (30 x 115 mm; Falcon 352095, Becton Dickenson) was placed over the glans penis. The next step was to start the cycles of electric stimulations that would result in ejaculation and sperm collection. The stimulations were administered by increasing voltage of 0.5 volt increments until 2 V was reached. The voltage was then reduced to 0. Another cycle of 0.5V incrementation was started and a higher peak voltage was used (3V) and then returned to 0V. This cycles was implemented over a period of 3 seconds. The cycles with increasing voltage peaks was repeated until ejaculation occurred.

Multiple collection tubes were used (5 to 10 per lion) and these were quickly changed whenever some collected semen was visible in the tubes. The used tubes with collected semen were kept at body temperature (37°C) in a water bath until evaluated. Another cycle of electrical stimulations was repeated after a few seconds of rest, (each

time at an increased voltage) until an ejaculate containing a dense semen concentration was collected. No more than 15 electrostimulation cycles were used on each animal.

3.2.3 Semen evaluation:

Immediately after collection the samples in the tubes were evaluated for the presence of sperm cells using a microscope and the samples containing sperm cells with an overall motility higher than 50% were pooled, while the other tubes were discarded (plate 4 – appendix E).

The pooled sample from each lion was evaluated according to the procedure described by Loskutoff and Crichton (2001), for *volume, colour, pH, linear forward progression and overall motility* within 5-10 minutes after collection.

Volume: The total volume of the pooled samples collected (containing >50% motile sperm cells) was recorded as the total volume (ml) of ejaculate per animal.

Colour: A colour was encoded to the pooled semen sample of each animal that would describe the appearance of each pooled samples the best, namely, watery, cloudy, milky or yellowish.

pH: The pH of the pooled semen sample was measured by placing approximately 5 µl of the pooled sample on a piece of pH measuring paper. The colour of the paper area covered with the semen sample indicated the pH of the pooled semen sample.

Overall motility: The overall motility was determined from the pooled samples approximately 10 minutes after collection. A 1:100 dilution of semen: medium was made by using semen from the pooled sample and using TL Hepes medium, BioWhittaker, (buffered Tyrode's medium containing albumin, lactate and pyruvate). Approximately 10 µl of the 1:100 dilution was placed on a prewarmed microscope glass slide and covered with a coverslip. A total of 200 sperm cells were counted as moving or non-moving in several fields of the microscope slide. The slides were made in duplicate for each animal and the mean percentage sperm cell motility of the two slides recorded using a compound microscope with 400x magnification (Loskutoff and Crichton, 2001).

Linear forward progression: A scale of 0-5 was used to grade the linearity of the forward movement of sperm cells (Loskutoff and Crichton, 2001).

0 = no movement

1 = head movement (no forward progression)

2 = slow forward progression (laboured head movement)

3 = fast forward progression

4= faster forward progression

5 = fastest, linear progression

Approximately 10µl of pooled semen sample was placed on a prewarmed (37°C) microscope glass slide and covered with a coverglass. The slide was placed under a microscope and the linear forward progression established using 100x magnification. A total of 100 cells were individually evaluated and the average score used to describe the linear forward progression of the semen sample. Two slides were made for each lion and the mean motility of the two slides recorded.

Concentration: A 1:100 sperm: water dilution was made of the pooled semen samples from each animal. Ten microliters of sperm:water dilution was placed on a Newbauer hemacytometer grid. The hemacytometer was placed under a compound microscope at 400x magnification. The numbers of sperm cells contained in the five diagonal squares (each compromising 25 smaller squares) in the middle large square of the grid were counted and multiplied by 5 in order to correct for the dilution factor. This equalled the total number of sperm (multiplied by one million) per millilitre. The total number of sperm cells per ejaculate was obtained by multiplying the concentration per millilitre by the total volume (ml) of the pooled sample (Loskutoff and Crichton, 2001).

Structural morphology, viability and acrosome integrity: The eosin B-fast green vital staining technique, Loskutoff and Crichton, 2001, was used to prepare a microscope slide from the pooled semen sample from each animal.
Approximately 10µl of the pooled samples from an animal were combined with approximately 5 µl of eosin B-fast green dye on a microscope slide. A thin smear was made and dried immediately. Two slides were made per animal. A total of 100 sperm cells were evaluated per slide (using a compound microscope) and assigned to four different categories, namely: Live/Acrosome intact, Dead/Acrosome intact, Live/Acrosome reacted, Dead/Acrosome reacted (Loskutoff and Crichton, 2001). The totals in each class were expressed as a percentage of the total number of sperm counted. The mean of the two slides was recorded.

The same slides were used to assess structural morphology of the spermatozoa. A total of 100 sperm cells were evaluated per slide. The total abnormalities detected

were expressed as a percentage. The structural abnormalities of the sperm cells were also assigned to 3 different categories: primary abnormalities, secondary abnormalities and tertiary abnormalities (Loskutoff and Crichton, 2001). The mean of the two slides was recorded.

The data obtained during the examination of the external sex organs and the semen evaluation was used to determine if there is a correlation between the external organ characteristics and semen quality. A one-way Proc ANOVA of SAS (1995) was used to compare the most important characteristics of semen collected with testicular characteristics.

3.3 Results and Discussion

3.3.1 External sexual organ evaluation

The results of the external sexual organ examinations on the 8 lions examined are set out in Table 3.1. The ejaculates of the last 2 animals (#7 and #8) contained no sperm and were thus excluded from the evaluation of sperm characteristics. Both these animals were from the game farm near Bothaville. The semen from the remaining six animals was analysed.

Table 3.1 External sexual organ characteristics of male lions

#	*Age (years)*	*Length Left Testicle(cm)*	*Length Right Testicle(cm)*	*Width Left Testicle(cm)*	*Width Right Testicle(cm)*	*Testicle texture*	*Development of cauda epididymis*
1	3	5	5.5	3.5	4.4	Firm	Well developed
2	3	6.6	6.8	3	3	Both testicles flaccid	No structure palpable
3	4	5.5	5.5	3.6	3.1	Right testicle flaccid	Well developed
4	3	5.2	5.2	4	4.2	Firm	Well developed
5	4	6.5	4.8	4.8	3.6	Firm	Well developed
6	5	5.9	3.2	3.2	3.8	Left testicle flaccid	Well developed
7	2	3	3	1	0.8	Firm	No structure palpable
8	4	6.6	6.8	3	3	Right testicle flaccid	Well developed
MEAN	3.5	5.5	5.1	3.3	3.2		
SD	0.9	1.2	1.4	1.1	1.1		

The mean length of the left and right testicles was 5.5±1.2cm and 5.1±1.4cm respectively and the width of the left and right testicles was 3.3±1.1cm and 3.2±1.1cm respectively. There were no significant differences between the left and the right testicles in respect of length and width.

The texture of both testicles from animals #1, 4, 5 and 7 was firm. One or both testicles from animals # 2,3,6 and 8 were flaccid. A flaccid testicular texture is an indication of testicular degeneration (Hafez, 1987). A well-developed cauda epididimys was detected in both testicles from animals # 1,3,4,5.6 and 8. No cauda epididymis was detected by palpation in animals # 2 and 7.

3.3.2 Semen collection and evaluation:

The overall results of semen collection and evaluation of the African lion are summarised in Table 3.2.

Table 3.2 –The maximum voltage used for electro-stimulation and seminal characteristics of the African lion (Panthera leo)

	Lion #1	*Lion # 2*	*Lion #3*	*Lion # 4*	*Lion #5*	*Lion # 6*	*Mean*	*Standard deviation (+/-)*
Maximum voltage used(V)	7	10	6	7	6	9	7.50	1.64
Erection	Yes	Yes	Yes	Yes	Yes	Yes	-	-
Volume (ml)	5	3	5	6.5	6	6.2	5.28	1.28
Colour	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	-	-
pH	7	7	7	7.2	7.2	7	7.07	-
Urine	None	None	None	None	None	None	-	-
Overall motility (%)	95	85	95	90	88	90	90.50	3.94
Linear forward progr (0-5)	4	4+	4+	4+	4	4	4.00	-
Total concentration (x million/ml)	110	89.99	109	99	97	101	101.00	7.57
Total normal sperm (%)	40	64	76	68	74	72	65.67	13.29

The maximum voltage for effective electric stimulation varied between the lions. The mean peak effective voltage during the trail was 7.5 ±1.6V. Howard *et al*., (1984) reported electrical stimuli of similar voltage range (2-8V) for effective semen collection in 28 non-domestic felids species.

The mean volume of the ejaculate (pooled fractions containing >50% motile sperm) collected from the African lions was 5.2±1.28ml. Lehloenya *et al*. (2002) reported a volume of 0.2-2.1ml for semen collected from cheetahs. Similarly Howard *et al*.. (1991) reported a mean ejaculate volume of 1.6ml in male cheetahs. The mean volume of domestic cat semen collected using an artificial vagina has been observed to be 0.04ml (ranging from 0.03ml to 0.12ml), while electro-ejaculation produced a mean volume of 0.23ml of semen (Sojka, 1986). A mean ejaculate volume of 1.6±1.3ml was recorded in the Indian leopard (Jayaprakash *et al*., 2001). The data from this study suggests that the lion produce a higher volume ejaculate that some other cat species.

The colour of the lion ejaculates *was* recorded to ensure that there was no urine contamination. All the collected pooled semen samples had a cloudy, whitish colour and a pH between 7.0 and 7.2. This indicated no urine contamination that could possibly be lethal to the spermatozoa. The colour of feline semen is usually milky white in appearance unless contaminated with urine or blood (Howard *et al*., 1986). The mean pH for the domestic cat semen (ranging from 7.0 to 8.2) was reported to be 7.4 (Sojka, 1986).

The mean overall motility of the lion pooled samples recorded immediately after collection was 90.5±3.94%. Patil *et al.* (2002) reported an overall motility for lion semen (*Panthera leo*) of > 80%, while Donoghue *et al*. (1992) reported an overall motility for tiger (*Panthera tigris*) semen of 90%. According to Sojka (1986), the overall motility for domestic cat semen (*Felis catus*) is 78% for semen collected with an artificial vagina and only 60.5% for semen collected by electro-ejaculation. The overall sperm motility of the Indian leopard (*Panthera pardus)* was reported to be 57.1±17.0% (Jayaprakash *et al*., 2001).

The linear forward progression in all of the semen samples recorded ranked between 4 and 5. This indicates virile and fast forward movement of the spermatozoa. Virile semen is essential in both natural and artificial insemination to achieve acceptable fertilisation rates.

A mean sperm concentration of 101 x 10^6 sperm per ml was recorded from 6 lions from which semen could be collected. This count is much higher than the average sperm concentration for the domestic cat of 56 x 10^6 sperm/ ml (Herron *et al*., 1986). A

concentration of 80.3 x 10^6 sperm/ml has also been recorded in domestic cats by Goodrowe and Hay (1993), while Lehloenya *et al.*, (2002), reported a mean sperm concentration of 13.5 x10^6 sperm/ml in cheetah males. Roth *et al*. (1995) reported the total number of cheetah sperm to range from 1.9 to 71.0 x 10^6 sperm/ml. The total sperm concentration of Indian leopards is reported to be at 55.78±38.67 x10^6sperm/ml (Jayaprakash *et al.*, 2001). The results obtained in this study indicate that lions have one of the highest sperm concentrations among the feline family.

The morphology of the sperm cells in the ejaculates collected from the 6 lions was evaluated. An overall mean of 34.33% abnormal spermatozoa was recorded in the lion ejaculates for this study. These abnormalities were divided into primary, secondary and tertiary abnormalities. Primary abnormalities originate in the seminiferous tubules of the testes during spermatogenesis and may be heritable; secondary abnormalities usually occur during sperm transport in the epididimys and are usually not heritable; while tertiary abnormalities usually result due to semen manipulation (Loskutoff and Crichton, 2001). The main primary abnormalities detected in the lion sperm were bent midpieces (18.2±6.5%), coiled tails (20.8±26.3%) and detached heads (5.3±3.9%). The most common secondary abnormalities detected were retained protoplasmic droplets (4.5±3.15%) and bent tails (28.5±10.1%). The summary of the results of the structural morphology of the lion sperm cells observed in the pooled ejaculates is set out in Table 3.3.

Table 3.3 – Sperm cell morphology in lion ejaculates collected by electro-ejaculation

Lion #	Total abnormal cells (%) (*)	Primary defects (% of *)			Secondary defects		Tertiary defects
		Bent midpiece	Coiled tail	Detached head	Protoplasmic droplet	Bent tail	
1	60	23	5	0	6	19	47
2	36	9	70	2	0	19	0
3	24	16	7	9	5	37	26
4	32	20	7	6	2	42	23
5	26	27	4	5	5	33	26
6	28	14	32	10	9	21	14
Mean	34.33	18.17	20.83	5.33	4.50	28.50	22.67
SD	13.29	6.49	26.30	3.88	3.15	10.11	15.51

Felines are known to have a high proportion of abnormal sperm cells present in their ejaculates. Neither the etiology nor the significance of the high incidence of abnormal spermatozoa in *Felidae* is known (Howard *et al*, 1984). In the study conducted by Howard *et al.* (1984) on morphological abnormalities of spermatozoa taken from 28 non-domestic felid species, the majority of the species recorded relatively high proportions of morphologically aberrant spermatozoa. The mean total of abnormalities per ejaculate recorded for the 28 different feline species ranged from 16.5% to 84.3%. These authors reported means of 47.9% normal sperm in lion ejaculates and 69.6% normal sperm in tiger ejaculates. The most common primary sperm abnormalities reported in the feline species were pleiomorphic spermatozoal heads, midpieces or acrosomes, a tightly coiled tail and a bicephalic/biflagellate cell. The most common secondary abnormalities observed included bent midpieces, necks or tails or retained protoplasmic droplets. In a study aimed at evaluating the fertility of the Indian leopard Jayaprakash *et al.* (2001) reported 71.9±15.32% normal spermatozoa in 11 males. The results of the current study are in line with the available literature with regard to sperm morphology.

The high percentage of abnormal spermatozoa can sometimes be related to a severe inbreeding problem, as has been observed in cheetah populations found in South Africa (O'Brien *et al.,* 1983). It should be kept in mind that a high proportion of the animals used in fertility trials are kept and bred in captivity. The probability of inbreeding occurring in such a population is thus higher than in the wild. This is particularly true for breeding sites with poor record keeping systems commonly recorded in most private lion breeding farms in South Africa.

Howard *et al* (1984) drew the conclusion that the high incidence of spermatozoal defects in Felidae are a physiological norm that may be attributable to genetic variation within the species or to factors associated with the captive environment. No significant correlations could be established in this study between the testicular characteristics (volume) and the seminal characteristics (sperm concentration, motility and abnormalities). The results of the semen evaluation (volume, concentration and morphology) of lions with normal testes and developed epididimys were compared by ANOVA with those of lions with abnormal testes or undetected cauda epididimys. No significant differences could be determined. This means that the animals that showed abnormalities on the external organs, such as a flaccid testicle or undetected cauda epididimys, did not necessarily produce semen of poorer quality (lower sperm concentration, lower motility or higher abnormal spermatozoa). No spermatozoa could be detected in the ejaculate collected from one of the lions with firm testicles and a well developed cauda epididimys. Normal testicle texture does not guarantee good semen production. This emphasises the importance of examining the ejaculate of a male lion that is intended to be used for breeding.

3.4 Conclusions:

There was considerable variation in the testicular and seminal characteristics observed between individual African Lion (*Panthera leo*) males studied. The African lion produces a grater volume of ejaculate than the other members of the cat family, with one of the highest semen concentrations and motility. The percentage of sperm abnormalities is grater in the African lion compared to other Felidae species. Therefore a pre-breeding and pre-sale reproductive examination should be routinely conducted. This reproductive examination should also include semen collection and a comprehensive examination of the ejaculate, as the results from this study indicate that a simple examination of the external sexual organs does not correlate with semen quality. The results of this study could form a guideline for breeding soundness examinations. It is recommended that further research be conducted before consistent reference values can be established, as the results from this study were from a limited number of animals.

Chapter 4
Lion Sperm Cryopreservation

4.1 Introduction:

Genetic variability is the key to long-term survival of any species (Wildt, 2000). As most lion populations in Africa are kept within fenced borders, these animals face the risk of inbreeding due to a closed population and limited genetic variation. The introduction of new genes into these lion populations is not only desirable, but also vital for the long-term survival of the species. Natural breeding of non-domestic species is desirable, but not always possible and difficult to manage due to the aggressive behaviour between males, the complex social order found within groups, limited gene pools and problems associated with geographically separated lion populations (Howard *et al.,* 1981). Assisted reproductive techniques could offer a solution as a mechanism to introduce new genetic material into a population of lions with a limited gene pool. These assisted reproductive techniques include artificial insemination, in vitro embryo production and embryo transfer. These techniques are currently being used extensively in domesticated animals (e.g. cattle, sheep, goats, etc.), but wild carnivores present a new challenge due to inherent factors such as immobilisation and availability of wild animals (Goodrowe *et al*., 2000).

The importance of assisted reproduction techniques as part of a multifaceted captive breeding program for selected wild cat species is thus gradually gaining acceptance and momentum (Pope, 2000). It is important that effective semen collection and preservation techniques be developed to facilitate the use of these assisted reproductive techniques in both captive and wild lion populations (*Swanson et al.,*1999).

The cryopreservation of sperm has become an important concept especially when it was established that certain biological systems could endure storage in ultra cold temperatures of -196°C (Hammerstedt *et al.*, 1990). Cryopreservation of semen extends the storage period of spermatozoa, allowing the use of semen collected in one location from an animal to be used later in a distant location. This also allows the rapid improvement of genetic variation in a population and could facilitate the use of breeding and selection techniques, currently used in livestock, to be applied in wildlife species (Nelson *et al*., 1999). Limited information is available on the domestic cat and other wild felids regarding semen cryopreservation (Swanson *et al*., 1996; Sojka, 1986; Goodrowe, 1992; Nelson *et al*., 1999; Da Paz *et al*, 2002; Bartels *et al*, 2000; Patil *et al*., 2002; Morato *et al*., 2003) which could possibly be used as guidelines to develop a semen cryopreservation protocol for the African lion (*Panthera leo*).

In general, the cryopreservation and thawing of cat sperm with the aid of cryoprotectants results in extensive membrane damage. This limits the survival rate in the post-thawing of sperm and compromises the use of AI (Wildt *et al*., 1997). This demonstrates the need to develop a specific and effective lion semen cryopreservation protocol that would limit sperm cell damage during the freezing process (Pukazhenthi *et al*., 1999).

The freeze and thawing of sperm cells is a very delicate process that requires a fine balance, correct timing and involves complex biological events. If the survival rate (in terms of overall percentage motility) of the sperm cells post-thawing is acceptable, the semen can be used for AI and for *in vitro* embryo production. Semen of a number of cat species have previously been successfully frozen and thawed and these include the

domestic cat (Sojka, 1986), siberian tiger (Nelson *et al*, 1999), the cheetah, the leopard and ocelot (Howard *et al*., 1986) indicating that cryopreservation in the family *Felidae* is possible and viable. Two cryoprotectants, namely, glycerol and dimethyl sulfoxide (DMSO) are often added to semen extenders in wild cat sperm cryopreservation protocols (Nelson, *et al*, 1999). The aim of this study was to develop and test a protocol for the successful cryopreservation and thawing of lion semen using glycerol and DMSO as cryoprotectants at different concentrations. This could contribute to the application of assisted reproductive techniques such as artificial insemination and embryo production in the breeding of the African lion (*Panthera leo).*

4.2 Materials and Methods:

Semen from 6 chemically immobilised adult captive lions (age 1 to 4 years) was collected by electro-ejaculation (as described in Chapter 3) and cryopreserved using 5 different cryopreservation protocols. Dimethyl sulfoxide (DMSO) and glycerol were the two cryoprotective agents evaluated at different concentrations (4% and 8% final concentrations) against the control treatment where no cryoprotectant was used.

4.2.1 Preparation of the media:

All the media used for the different cryopreservation protocols were prepared prior to semen collection.

4.2.1.1 Cocktail AB® (Minitub GmbH, Germany)

The antibiotic cocktail used in this protocol (Biladyl Cocktail AB*®*) is commercially available from Minitub (GmbH, Germany). Twelve ml double distilled, sterile water was

added to the freeze-dried antibiotics for reconstitution. The final composition of the reconstituted antibiotic cocktail (expressed as active units of antibiotics per 0.02ml) was as follows: 100µg Tylosin, 500µg Gentamicin, 300µg Lincomycin and 600µg Spectinomycin.

4.2.1.2 Biladyl A solution

Biladyl®, a commercially available Tris-citrate buffered cryodiluent (Minitub GmbH, Germany), was used in the study. Biladyl (49g) was diluted with double distilled water to a volume of 390ml. Ten ml of the reconstituted antibiotic Cocktail AB® (Minitub GmbH, Germany) and a volume of 100ml clean egg yolk (20% of final volume) from fresh chicken eggs was added to the 390 ml Biladyl ®. This formed the Biladyl solution A (Biladyl A) thar was stored at 4°C until used. The total volume of Biladyl A®, made up using the above-mentioned process, was 500 ml.

4.2.1.3. Biladyl B solutions (cryoprotectant solutions)

The solutions containing the cryoprotectant to be tested (glycerol and DMSO) Biladyl B solutions, were prepared at an 8% and 16% Glycerol and DMSO concentration respectively, by adding 4ml and 8ml glycerol and DMSO to 46ml and 42 ml of Biladyl A solution respectively (as described in 4.2.1.2) to make up a total volume of 50 ml.

4.2.2. Semen cryopreservation protocol

Each of the semen samples collected from each of the 6 lions was diluted to a total volume of 8 ml with TL Hepes solution (Bio Whittaker), containing 10% fetal calf serum (at room temperature).

A 1 ml of the diluted semen sample from each animal was placed into each of 5 (a control and 4 treatments) 15 ml conical tubes (Fisher Scientific Co.). A volume of 1ml of Biladyl A solution (37˚C) was added to each of these 5 conical tubes containing the diluted semen bringing the total volume to 2ml in each conical tube. The tubes were placed into a beaker containing water at room temperature (22°C) and placed into a refrigerator (4°C) for one hour. This procedure allows a slow decline in temperature preventing a thermal shock. The 4 different Biladyl B solutions (50ml each) containing the glycerol and DMSO cryoprotectants at a 8% and 16% concentrations, respectively, were also placed in the refrigerator (4°C) at this time.

The control cryopreservation diluent consisted of 2ml of diluted semen combined with Biladyl A and another 2ml of Biladyl A (containing no cryoprotectant). Then 2ml of each of the 4 Biladyl B solutions containing 8% and 16% glycerol or DMSO were added slowly (drop by drop) to each one of the other 4 treatment tubes containing semen from each animal to give a final cryoprotectant concentration of 4% and 8% Glycerol and DMSO, respectively. These treatments are set out in Table 4.1.

Table 4.1 –The cryopreservation treatments used for lion semen

Tube	**Treatment**	**Contents**
1	Control	Lion semen, Biladyl A
2	1	Biladyl, 20% egg yolk, 4% Glycerol(Biladyl B fraction)
3	2	Biladyl, 20% egg yolk, 8% Glycerol (Biladyl B fraction)
4	3	Biladyl, 20% egg yolk, 4% DMSO (Biladyl B fraction)
5	4	Biladyl, 20% egg yolk, 8% DMSO (Biladyl B fraction)

Immediately after the Biladyl B solution being added, 12 straws (0.25ml) per treatment per animal (previously identified with a permanent marker pen) were loaded with semen and sealed with PVP powder. These 12 straws per treatment (1 to 5) for each animal were prepared and left in a walk-in cold room (4°C) for another 2-hour equilibration period.

Following the equilibration period, the straws were placed on a styrofoam boat at a height of 2.5 cm above the liquid nitrogen level in a large styrofoam box. After 10 minutes in the liquid nitrogen vapour, the straws were plunged directly into the liquid nitrogen and transferred into a liquid nitrogen flask until thawed and analysed for viability.

4.2.3 Semen thawing protocol

After a period of 5 months of cryopreservation in liquid nitrogen a total of 6 straws per treatment from of each lion were thawed according to 3 different protocols and evaluated microscopically for viability. Two straws per treatment from each animal were thawed by one of the 3 following methods:

- Room temperature for 2 minutes
- Placed in a water bath for 2 minutes at 36°C
- Placed in a water bath for 8 seconds at 50°C

The thawed samples were then immediately examined microscopically for overall motility and forward progression (*see par. 3.2.3.4*). The data were analysed by setting up a 5x3 factorial experimental design and using a one-way ANOVA procedure of SAS

(1995). Tukey's studentized range test (SAS,1995) was used to determine which treatment(s), if any, differed significantly from the others.

4.3 Results and discussion:

The post-thaw motility and forward progression results of the semen samples cryopreserved using the five different methods and thawed by one of three methods are set out in table 4.2.

Table 4.2 Post-thawed motility (%) of lion semen frozen-thawed using different protocols.

Thawing protocol	**Cryodiluents**				
	Control	**T_1**	**T_2**	**T_3**	**T_4**
	Biladyl A	**4% Glyc**	**8% Glyc**	**4% DMSO**	**8% DMSO**
Ambient temp	0^{a}	13.65 ± 5.22^{b}	6.99 ± 1.5^{b}	11.8 ± 3.97^{b}	10.34 ± 4.12^{b}
32'C	0^{a}	15.05 ± 4.9^{b}	13 ± 3.2^{b}	21 ± 6.3^{b}	12.8 ± 3.7^{b}
50'C	0^{a}	18.5 ± 6.3^{b}	12 ± 2.8^{b}	13.3 ± 2.1^{b}	14.6 ± 5.17^{b}

** a,b, values within columns and rows for the same parameter with different superscripts differ significantly($P<0.01$)

Table 4.3 Post-thawed forward progression (scale1-5) of lion semen frozen and thawed by different protocols

Thawing protocol	**Cryodiluents**				
	Control	**T_1**	**T_2**	**T_3**	**T_4**
	Biladyl A	**4% Glyc**	**8% Glyc**	**4% DMSO**	**8% DMSO**
Ambient temp	0^{a}	1^{b}	1^{b}	1^{b}	1^{b}
32'C	0^{a}	1.5^{b}	1.5^{b}	1.5^{b}	1.5^{b}
50'C	0^{a}	2^{b}	1.5^{b}	2^{b}	1.5^{b}

** a,b, values within columns and rows for the same parameter with different superscripts differ significantly($P<0.01$)

The results of the ANOVA used to compare significant differences in post-thaw sperm motility of lion semen cryopreserved with different cryoprotectants is set out in Table 4.4.

Table 4.4 – ANOVA on differences in motility of the sperm cells post-thawed between cryoprotectant treatments and thawing temperatures.

Source	**DF**	**Mean Square**	**F Value**	**P value**
Cryoprotectant medium	4	432.139	4.45	0.003
Temperature	2	84.307	0.87	0.424

There was a significant difference (P<0.01) in motility and forward progression of the semen treated with different cryodiluents. None of the sperm cells cryopreserved with Byladyl A (containing no cryoprotectant-control), survived the freeze-thawing process as indicated by no motility after thawing. The motility recorded for the control group was significantly (P<0.01) lower than that of treatments 1 to 4 (with cryoprotectants). This result was expected as literature showed that sperm would not survive cryopreservation without using a cryoprotectant. However, no significant differences were recorded between the treatments containing the cryoprotectants, glycerol and DMSO at 4 or 8%(T_1 to T_4).

The results show a sharp decline in overall motility of the lion sperm following the freeze-thawing protocols (all treatments). The freeze-thaw protocols reduced the average motility of the lion spermatozoa from 90.5%(fresh semen) to between 0% and 21% (post-thawing) for the 3 thawing protocols used (ambient temperature, 30°C and 50°C). This dramatic reduction in sperm motility is actually very common for cat semen (Sojka, 1986). Donoghue *et al.* (1992) showed that the freeze thawing protocol

reduces initial tiger sperm motility at collection (80%) by about 5 to 45% post thawing. Byers *et al*. (1989) also found that in Siberian tigers the initial sperm motility at collection decreased dramatically from 61.12% (fresh) to 40.4% immediately after thawing. Malo *et al. (*2004), cryopreserved epididymal lion sperm (motility of fresh semen - 15%) in glycerol and recorded a post-thawing motility of 5% for motile sperm. This indicates that freezing felid semen result in a considerable reduction of motility. The loss in motility can compromise forward movement of the sperm cells inside the female reproductive tract. Sperm cells with a low motility can, however, be used in IVF programs, although the chances are low that fertilisation may occur during artificial insemination due to compromised motility (Wildt *et al.,* 1997). The results of this study indicate that the freeze-thawed protocols using glycerol or DMSO at 4% or 8% seem to be satisfactory to cryopreserve lion semen to be used during IVF or intrauterine insemination. The motility below 20% of the freeze-thawed sperm cells (from 90% on fresh semen) clearly indicates the need for improvement in cryopreservation. A large number of factors could have influenced the lion sperm survival during this specific cryopreservation protocol. One of the aspects being the equilibration period. The equilibration period alters the physical properties of the sperm cell membrane (by temperature change) for infiltration of cryoprotectant into the sperm cells (Pukazhenthi *et al*.1999). The concentration level of the cryoprotectants used during a semen freezing protocol is also essential for sperm survival. Inclusion levels of the cryoprotectants in a cryopreservation protocol that are to high can be toxic to sperm, while too low inclusion does not protect the sperm. Further studies varying all these factors are warranted in order to find more efficient cryopreservation protocols for lion semen. The survival rate as indicated by the percentage of motile sperm does not

ensure fertilisation capacity of the semen. The fertilising capacity of the frozen and thawed lion semen is another aspect that has to be considered in future studies.

4.4 Conclusions:

The results of this trial proved that it is possible to cryopreserve lion semen using Biladyl ®, containing Glycerol or DMSO at 4 or 8%. There were no significant differences in sperm motility and forward progression between the 2 different cryoprotectants (Glycerol and DMSO) used at 2 different levels (4 and 8%) to cryopreserve the lion semen. Semen not cryoprotected did not survive the freezing protocol.

There were also no significant differences in post-thaw sperm cell motility between the 3 thawing protocols compared in this trial. However, a very dramatic reduction in lion sperm motility and forward progression can be expected in freeze-thawed lion semen using either DMSO or Glycerol at 4 or 8% and thawed at either room temperature, 32ºC for 2 minutes or at 50ºC for 8 seconds. It remains to be evaluated what effects this reduction in motility may have on the fertilising capacity of the lion semen. More studies are warranted to improve the survival rate of freeze-thawed lion semen and to ensure satisfactory post-thawed fertilisation capacity.

CHAPTER 5

In vitro heterologous fertilizing capacity of frozen/thawed African lion (*Panthera leo*) semen

5.1 Introduction

Most non-domestic felids are either threatened or endangered with extinction in their natural environment (Swanson *et al.*, 1999). Reproductive biotechnology in endangered felid species has the potential of making a significant impact on the conservation of the world's most endangered cat species (Da Paz *et al.*, 2002). Refined techniques for cryopreservation of felid sperm can assist in developing efficient systems for artificial insemination and embryo production in endangered cat species (Nelson *et al.*, 1999). Cat sperm, however, is very sensitive to cryopreservation and the freezing of cat sperm in cryoprotectants results in extensive sperm membrane damage (Pukazhenthi *et al.,* 1999). The changes induced in sperm cells of tigers and lions during the freezing processes affects not only the viability and motility parameters, but also the fertilising capacity (Patil *et al.*, 2002). The physiological mechanisms involved in the capacitation and fertilisation of sperm are compromised during these cryopreservation and thaw processes (Wildt, 2000). The viability and fertilising capacity of sperm post-thawing, is then an indication of the efficiency of a cryopreservation technique (Loskutoff and Crichton, 2001).

The post-thawing fertilising capacity of sperm can artificially be determined by setting up an in vitro fertilising assay (Loskutoff & Crichton, 2001). Homologous (from the same specie) *in vitro* fertilisation tests require large numbers of oocytes and sperm for potential endangered species. Although desirable, these assays are not always

feasible and it is not always possible to obtain large numbers of oocytes from especially rare species, in this case the African lion. For practical reasons, heterologous *in vitro* fertilisation assays are used to evaluate the fertilising capacity of frozen-thawed sperm using zona-free oocytes from a mother specie (bovine or hamster oocytes are commonly used for this purpose) to test sperm fertilisation from a different specie (e.g. lion semen) (Loskutoff and Crichton, 2001).

The aim of this study was to determine the efficiency of different sperm cryopreservation protocols for the African lion, by setting up a heterologous *in vitro* fertilising assay. The successful fertilisation rate during the *in vitro* heterologous assay could give an indication of the fertilisation ability of the post-thawed lion semen.

5.2 Materials and methods:

5.2.1 Setting up a heterologous *in vitro* fertilisation (IVF) assay:

A standard bovine oocyte maturation and fertilisation protocol was used as described by Loskutoff and Crichton (2001).

5.2.1.1 *Collection of the bovine ovaries*

Bovine ovaries were collected from a local abattoir and transferred to the laboratory in a sterile saline solution (0.9%) at 36°C. Follicles with a diameter of between 1 and 10mm were aspirated with the aid of a 18G 2.5cm needle and a 20 ml syringe within 6 hours following collection. The follicular fluid collected was filtered, with the aid of a 70µm filter unit (Gelman, Acrodiscs). Tissue adhering to the filter was sprayed from the filter with a pre-warmed TL Hepes (BioWhittaker) medium at 36° using a 20 ml syringe fitted with a 20G needle. The precipitate was collected in petri dishes (20 x 100 mm),

containing pre-warmed TL Hepes medium at 36°C. A stereomicroscope was used to search and identify the cumulus-oocyte complexes (COC's) and these were then microscopically graded as good, fair or poor quality COC's according to the number of cumulus cells attached to the oocytes (Loskutoff and Crichton, 2001). Large numbers of cumulus cells attached to the oocytes indicated an oocyte of good quality, whereas, an oocyte with relatively low numbers of cumulus cells attached would be graded as fair. Only oocytes of good and fair quality were selected to be used in the IVF program.

5.2.1.2 *In vitro Maturation (IVM) of oocytes*:

A 500 hundred μl bovine *in vitro* maturation medium (IVM) with 4.4ml 199 TCM (Earle's salt), 25μl bFSH stock solution, 25μl bLH stock solution, 50μl Penicillin-streptomycin, 0.5ml heat-inactivated fetal calf serum and 25 μl maturation pyruvate, was dispensed into each well of a 4-well Nunclon plate. The maturation medium was gassed with a humidified triple gas containing 5%CO_2, 90%N_2 and 5%O_2 at a physiological temperature of between 38.0 to 38.5°C . The good and fair quality oocytes were divided into 4 groups consisting of 20 COC's per group and placed into the gassed IVM medium. The COC's were then incubated in the triple gas at a temperature of 38.5°C for approximately 22 hours.

5.2.1.3 *Preparation of zona-free oocytes*

For the heterologous fertilizing assay, the zona pellucidae of the in vitro matured bovine oocytes were removed with a 0.5% pronase enzyme under a stereomicroscope using a process described by Loskutoff and Crichton (2001). Briefly, the process entailed placing approximately 10 μl of a 0.5 % pronase on a prewarmed microscope slide at 37°C. The oocytes were then placed inside the pool of pronase with the aid of a

capillary tube connected to a 1ml syringe. The oocytes were left in the pronase drop on the glass slide for 3 to 5 seconds until the zona pellucidae had been digested. The zona-free oocytes were then removed from the pronase solution and placed into a Nunclon 4-well dish containing *in vitro* fertilising (IVF) medium already gassed with triple gas. The feline IVF medium (500µl) was made up of 2.22ml Tyrode's salt solution (Sigma T2397), 218 µl BSA (7.5%) (Sigma A8412), 33.5 µl sodium bicarbonate (Sigma S8761) and 25 µl of a fertilisation supplement. The fertilisation supplement was prepared using 0.438g glutamine, 0.12g pyruvate and 0.15g gentamycin made up to a volume of 30 ml with nanopure water and filtered through a 0.22µm filter (Acrodiscs, Gelman).

5.2.1.4 In vitro fertilization of the zona-free bovine oocytes

Two straws from each of the 6 lions containing cryopreserved semen containing a 4% and 8% glycerol and DMSO cryopreservative respectively were thawed at 36°C for 2 minutes and then pooled. The motile sperm were separated from the dead (non - motile) sperm by Percoll density centrifugation (Nelson *et al.*, 1999). In this technique, two Percoll fractions with different densities are used. The 90% Percoll fraction was prepared by combining the following: A volume of 45 ml Percoll (Sigma®), 5 ml Tyrode's salt solution, 0.0985 ml of 1M CaCl, 0.197 ml 0.1M MgCl, 0.184 ml lactic acid (60% syrup) and 104.5 mg $NaHCO_3$ were combined using an aseptic technique. To prepare the 45% Percoll solution, 1 part of the 90% Percoll solution was mixed with 1 part of a Hepes TL solution (Bio Whittaker). A volume of 2 ml of the 90% Percoll at 22°C was pipetted into a 15 ml conical sterile tube (Fisher Scientific Co.) and 2 ml of the 45% Percoll gently layered on top of the 90% Percoll layer. The pooled sample of thawed semen was then carefully layered on top of the 45% Percoll layer. The tube

was capped and gently centrifuged for 30 minutes at 700G. After centrifugation 4 layers could be distinguished. The first layer from the top being the 45% Percoll solution, then a second layer containing the dead sperm cells, the third layer being the 90% Percoll solution and at the bottom a pellet of concentrated live sperm cells was visible. The top 3 layers (45% Percoll, dead sperm and 90% Percoll) were aspirated and discarded from the tube. Approximately 10 µl of the live sperm pellet (without dilution) was placed in a hemacytometer chamber and counted to determine the sperm concentration. The bovine zona free oocytes (n=20/well) were placed into 4 different wells and each well was inseminated with 20µl of the live post-thawed lion sperm cells (cryopreserved in 4 different treatments) pooled from 2 straws/animal. After being incubated for 14 hours at 38.5°C the oocytes were microscopically evaluated for the formation of pronuclei. The presence of pronuclei served as an indication of successful fertilization (Loskutoff and Crichton, 2001).

The whole mount procedure as described by Loskutoff and Crichton (2001) was used to evaluate oocytes for the formation of pronuclei. The oocytes or embryos (inside a small amount of IVF medium) were placed on microscope slides prewarmed at 37°C using capillary tubes connected to a 1cc syringe with a light sucking force. Petroleum jelly was carefully dabbed to the 4 sides of a separate coverslip and this gently placed over the microscope slide with medium containing the oocytes to be evaluated. Two sides of the coverslip (transversal to the length of the slide) were sealed using rubber cement. The slides were placed in a combined solution of methanol (90%) and glacial acetic acid with a 3:1 solution in a vertical position with the closed sides of the coverslip preventing the oocytes from being lost in the liquid. The slides were kept submerged in this solution for approximately 20 hours. After being removed, a drop of filtered 1%

aceto-orcein solution (100mg orcein stain, Sigma, in 4.5 ml glacial acetic acid and 5.5ml distilled water) was placed on each open side of the coverslip. The aceto-orcein solution stained any pronuclei present in the embryo's. All the oocytes/embryo's were then evaluated for visible pronuclei using a compound microscope at 1000 x magnification.

5.3 Results and discussion:

The *in vitro* fertilization rates of the zona-free bovine oocytes inseminated with thawed lion semen using glycerol and DMSO at concentrations of 4% and 8% respectively, are set out in Table 5.3.

Table 5.3-Bovine heterologous *in vitro* fertilization rates of cryopreserved lion semen

Cryopreservation Treatment	Fertilization rate	Fertilization (%)
4% Glycerol	4/16	25.0
8% Glycerol	0/12	0
4% DMSO	4/12	33.3
8% DMSO	2/15	13.3

The presence of pronuclei in the embryos after 14 hours of incubation at 38.5ºC indicated successful fertilization. The bovine zona-free oocytes inseminated with lion sperm cryopreserved in 4% DMSO had a 33.3% fertilization rate while the 4% glycerol had a 25% fertilization rate. The fertilization rates with lion sperm cryopreserved in DMSO and Glycerol at 8% were (13.3%) and (0%) respectively. These results are lower than those obtained in other domestic animals (±55%) (Loskutoff and Crichton, 2001). The inclusion of DMSO and glycerol at 8% seems to reduce the fertilization rate of post-thawed lion sperm cells. In a study conducted by Nelson *et al.* (1999), pronuclei formed in zona-free bovine oocytes inseminated with tiger semen cryopreserved in 4% DMSO and 4% glycerol at rates of 11/20 (55%) and 10/20 (50%) respectively. In the same

study, the jaguar showed *in vitro* fertilization rates of 3/17 (17.6%) and 3/8 (37.5%). Bartels *et al*. (2000) reported a 12.7% in vitro fertilization rate in the African lion using homologous oocytes. Goodrowe (1992), compared in vitro fertilization rates in several feline species and found IVF rates of 43.5% for the puma, 25% in the cheetah, 17.5% in the leopard and 63.8% in the tiger. These results indicate some variation between feline species. The IVF (heterologous) rates recorded during this study on lion are comparable with previous IVF rates recorded in other feline species.

5.4 Conclusions:

In vitro fertilization rates in feline species are relative low. Acceptable fertilization rates can be expected from African lion semen cryopreserved using Biladyl® extender, containing 4% DMSO or a 4% Glycerol. An increase in cryoprotectant concentration seemed to be detrimental to post-thaw fertilization ability of the sperm cells. Although the fertilization rate obtained during this study are relatively low, the cryopreservation protocols using Glycerol or DMSO at a 4% concentration hold promise for future use in homologous *in vitro* fertilization programs for the African lion (*Panthera leo*).

Chapter 6

The validation of an oestradiol-17β enzyme immunoassay for the African lioness (*Panthera leo*)

6.1 Introduction:

Studies on reproductive physiology in felids began in the 1970's (Goodrowe, 1992). In general, pioneer studies focussed mainly on gaining knowledge regarding the female reproductive cycles. Monitoring the reproductive activity of females (fluctuating endocrine patterns) helped to determine the length of the female oestrous cycle, the time and type of ovulation (induced versus spontaneous), duration of gestation and parturition (Goodrowe, 1992). Once sufficient knowledge regarding the basic reproductive physiology was known, assisted reproductive techniques (ART) in wild felids or any other species became possible. ART includes artificial insemination, *in vitro* fertilisation (IVF) and embryo transfer (ET).

The oestrus cycle of a female animal can be assessed by monitoring the fluctuations of the steroid hormones present in blood, urine or faeces. There are five major classes of steroid hormones. These are the progestagens, glucocorticoids, mineralcorticoids, androgens and oestrogens (Brown and Wildt, 1997). Oestrogens, such as oestradiol and oestrone, are derived primarily from testosterone or to a lesser extent from androstenedione. Oestradiol -17ß(E_2) is the major oestrogen produced by the ovarian follicle in many species. Oestrogen is responsible for certain behavioural changes during oestrus. The concentration of E_2 increases during the follicular phase (development of follicles) of the oestrus cycle. The fluctuation of oestrogen (indicating

oestrus and possible ovulation) can be expressed as an endocrine profile after monitoring steroid hormone levels (Nachreiner, 1986).

Radio and enzyme immunoassays (RIA and EIA) are techniques used to monitor steroid endocrine levels in blood, urine and faeces (Goodrowe *et. al.,* 1989). The basic principle of RIA depends on the competition of a constant amount of radiolabelled antigen (Ag) with a variable amount of unlabelled Ag for a limited number of antibody (Ab) binding sites. The data may be graphed and this is called the standard curve. Hormones of unknown quantity in samples are determined by extrapolating the values from the standard curve. In the case of the EIA, enzymes have been applied as labels of the antigen and antibodies in the place of radioisotopes (Brown *et al*., 1996).

Physiological endocrine blood levels and profiles during the oestrous cycle have not yet been established for all the wild felids. This is mainly due to the difficulty associated with the collection of blood on a regular basis from a wild animal (Brown and Wildt, 1997). The ideal would be if endocrine profiles (determined using RIA or EIA) could correspond with appropriate oestrus behaviour observed from females. Very few reproductive and endocrine studies have been conducted specifically on the African lion. Schmidt *et al*., (1979), assessed blood plasma hormone levels (oestrogen and progesterone) of the African lion to determine seasonal variation in sexual activity in the lioness, while Briggs *et al*., (1990), conducted a study to determine the endocrine profile of the African lion during oestrus, in pregnant and psuedopregnant animals using blood samples and the RIA technique.

Goodrowe (1992), reported on an alternative method for monitoring steroid hormone levels in wild felids. By measuring the hormonal metabolite concentrations excreted in the urine and or faeces of wild felids could be used in assessing a endocrine profile of the animal. The primary advantage of monitoring faecal hormonal metabolites is that it requires entirely non-invasive techniques (Brown and Wildt, 1997). The use of non-invasive techniques in wild felids has the advantage of minimising stress because it does not require anaesthetics to be administered to the animal and is less dangerous to the researcher. Most of the reproductive studies on wild cats have been focussed on zoo animals, therefore obtaining faecal samples from a zoo animal to assess endocrine levels can be a routine task for the zoo keeper with very little if any stress to the animal (Wildt *et al*., 1983). In summary, faecal steroid metabolite monitoring enables the assessment of pubertal status, generation of data regarding seasonality, duration of pregnancy, the incidence of spontaneous versus induced ovulation, prediction of parturition and the identification of pregnancy in felids (Brown and Wildt, 1997). Schille *et al*. (1991) reported the validation of faecal radioimmunoassays to characterise the oestrous cycle dynamics in the tiger, cheetah, clouded leopard, snow leopard, lion, leopard and the ocelot.

Although RIA has been used to determine hormonal profiles of the lioness, this study will aim to validate a faecal enzyme immunoassay for determining oestradiol 17β levels in the lioness. The technique used in this chapter is not entirely new, but is unique in the sense that it is used on the lioness for the first time. Ballitti *et al*., 1999, Graham *et al.,* 1996 and Brown *et al*., 1994, based the materials and method used in this study.

6.2 Materials and method:

6.2.1 Collection of faecal samples

Faecal samples were collected and processed as described by Czelaka *et al*. (1994). Faecal samples from one adult African lioness, held at the Bloemfontein Zoo, were collected on a weekly basis for a period of 3 months. A 2.5 cm long section of the faeces was collected as a faecal sample as soon as possible after defecation and frozen. A sub-sample of 0.5g of faeces from each sample were placed into 80% methanol in a glass vial to extract all the lipids from the faecal sample. The methanol acted as a solvent for all steroids present in the faeces. The vials were capped tightly to prevent the evaporation of the methanol. Approximately 18 hours later the solution containing the extracted steroids from the faeces dissolved in methanol was aspirated from the sample and the remaining solids in the glass vial discarded. The aspirated solution containing unknown amount of steroid hormone (oestradiol 17-β) in methanol were diluted 1:5 with a Phosphate buffered solution (PBS).

6.2.2 Preparation of buffers and solutions

The preparation of all the buffers and solutions used in the assay is set out in appendix **A**.

6.2.3 Working volumes of antibody and labelled hormone

The optimum working dilution of the oestradiol-17β antibody and the oestradiol-17β - HRP (labelled hormone) had to be determined before setting up the standard curve. One aliquot of oestradiol-17β antibody stock (1:50) was removed from the freezer and thawed. Three different working concentrations (1:10 000; 1:15 000; 1:20 000) were prepared by diluting the stock with a coating buffer. A minimum of 1 ml final volume of

each concentration was prepared. Each antibody dilution was used to coat 12 wells of a 96-well plate (50µl per well) according to the template in appendix **B.** The remaining wells were filled with PBS (50µl per well), after having ensured the bottom of each well was thoroughly coated. The plate was then sealed and incubated overnight at room temperature.

After 18 hours a 1ng/ml solution of oestradiol (unlabelled) was prepared by diluting the 25ng/ml stock (oestradiol) with PBS (1:25). The 1 ng/ml oestradiol stock was expected to have a binding level of 50% to the antibodies specific to the hormone (oestradiol-17β) as this specific concentration is in the middle of the range of standard concentrations to be used later in the assay. The standard (1 ng/ml oestradiol) was added to the plate (50 µl) according to appendix **C** and the plate incubated at room temperature for 30 minutes. After incubation the E_2-HRP (labelled hormone) was prepared at 3 different working dilutions by diluting the E_2-HRP stock (1:100) with PBS to give final concentrations of 1:10 000;1:15 000;1:20 000 after which time the E_2-HRP was added to the plate (50 µl) according to appendix **D**. The plate sealed and incubated for a further 2 hours (room temperature) and then washed and emptied from all remaining fluids in order to remove all unbound labelled hormone. Volumes of 100µl substrate was added to each coated well and after one hour the optical density of each well were recorded using a spectrophotometer (Tecan, SA). As a result the optimal dilution ratio of the antibody to labelled hormone complex were determined as 1:20 000 (Ab) and 1: 15 000 for the labelled hormone. However, for convenience, all further assays were run using an antibody dilution ratio of 1:20 000 and label diluted ratio 1:16 000 as this ratio was easily achieved from the stock concentrations previously prepared.

6.2.4 Setting up the standard curve

All wells of a 96-well plate except those for non-specific binding (NSB); (A1 and B1-see Table 6.2) were coated with 50 µl antibody (1: 20 000) and after ensuring that the bottom of the wells were completely covered with the Ab, incubated overnight. Thereafter the wells was washed with a solution of Tween 20 (Sigma) to remove all antibody not stuck to the bottom of the well. An additional 50 µl of PBS was added to all the wells after the washing procedure was completed. Ten standards were prepared by serial dilution of 25ng/ml stock oestradiol (for final concentrations see Table 6.1). Then 50 µl of each of the standards was added to the wells according to Table 6.2. After adding of the standards a 50 µl PBS was added to all remaining wells. It should be noted that the total volume of fluid in each well after adding the PBS was 100µl. The plate was then sealed and incubated for 30 minutes at room temperature. After the incubation period of 30 minutes, 50 µl of E_2-HRP (1:16 000) were added to each well in the plate and incubated for another 2 hours after which time the plates were completely emptied and washed with washing solution. A volume of 100 µl substrate was added to each well, the plate resealed and placed on a shaker for up to an hour. The plate was then placed inside the spectrophotometer (Tecan,SA) and the optical density of the wells read at a wavelength of 405nm.

The standard curve was established by fitting the best possible line through the optical density values obtained for each standard concentration. The y- axis of the standard curve was set as the optical density and the x-axis as the log of the standard concentrations. The quality control values was derived from the standard curve and prepared at 3ng/ml (oestradiol stock diluted with PBS) for the high value quality control

(QCH) and the low value at 0.5ng/ml (QCL). The same QCH and QCL concentrations were used during all further assays.

6.2.5 Parallelism test

A parallelism test was perform to investigate if the hormone (oestradiol-17β) in the standard concentration and the hormone present in the lion faecal samples (oestradiol-17β) binded to the antibodies specific to that hormone at the same rate. A serial dilution was prepared from the pooled lion faecal samples (extraction), because the standard concentrations were also prepared by serial dilution. The same procedure to assay the serial diluted samples together with the standards was used as described in paragraph 2.4. The slopes of the two curves were compared using a statistical analysis of covariance. Lines of equal slopes demonstrate no error in binding to the antibodies between the hormone present in the standards and the hormone in the faecal samples.

Table 6.1 - The 10 standard concentrations containing known amounts of oestradiol -17ß used for setting up a standard curve.

Standard	Oestradiol-17ß concentration
1	0.05ng/ml
2	0.09ng/ml
3	0.19ng/ml
4	0.39ng/ml
5	0.78ng/ml
6	1.56ng/ml
7	3.125ng/ml
8	6.25ng/ml
9	12.5ng/ml
10	25ng/ml

Table 6.2- Plate layout for assessing the standard curve for oestradiol17β

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS (NSB)	PBS (Bo)	Std 1	Std 2	Std3	Std4	Std5	Std 6	Std 7	Std 8	Std 9	Std 10
B	PBS (NSB)	PBS (Bo)	Std 1	Std 2	Std3	Std4	Std5	Std 6	Std 7	Std 8	Std 9	Std 10
C	QCH	QCL	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10
D	QCH	QCL	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10
E	S 11	S 12	S 13									
F	S 11	S 12	S 13									
G												
H												

Abbreviations for Table 6.4:

PBS – Phosphate buffer solution (50µl)
NSB – Non-specific binding well (50µl) (contains no Ab or hormone)
Bo – Zero binding of the unknown hormone with the Ab;only binding between labelled hormone and Ab
Std – Standard concentration (50µl)
S - Lion faecal sample (50µl)
QCH - Quality control high (50µl)
QCL –Quality control low (50µl)

6.2.6 Sample assay

The protocol used to determining the oestradiol concentrations in the faecal samples followed the same procedure used for setting up the standard curve. The plate was coated with oestradiol antibody dilution (1:20 000) except the NSB (non-specific binding) wells (A1 and B1) which represented the background colouring, because of the enzyme substrate reaction. The plate was incubated overnight and then the excess antibodies were washed out with Tween 20 (Sigma) washing solution (Appendix A). The plate was loaded with the 10 standard concentrations, the samples and quality controls (50µl per well) according to table 6.3. The rest of the wells were filled with PBS (50µl per well) after which the plate was sealed and incubated for 30 minutes at room temperature. The E_2-HRP (labelled hormone) was then added to all the wells (50µl per

well) followed by a further two-hour incubation at room temperature. Thorough washing of the plate followed the incubation period to remove all unbound labelled hormone and then 100 µl of the substrate was added to the wells. The plate was sealed and placed on the shaker for an hour before the optical density of each well was determined by the spectrophotometer (Tecan,SA) at a wavelength of 405nm.

Table 6.3 - Plate layout for 13 lion faecal samples assayed

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS (NSB)	PBS (Bo)	Std 1	Std 2	Std3	Std4	Std5	Std 6	Std 7	Std 8	Std 9	Std 10
B	PBS (NSB)	PBS (Bo)	Std 1	Std 2	Std3	Std4	Std5	Std 6	Std 7	Std 8	Std 9	Std 10
C	QCH	QCL	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10
D	QCH	QCL	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10
E	S 11	S 12	S 13	—	—	—	—	—	—	—	—	—
F	S 11	S 12	S 13									
G												
H												

Abbreviations for Table 6.3:
PBS – Phosphate buffer solution (50µl)
NSB – Non-specific binding well (50µl) (contains no Ab or hormone)
Bo - Zero binding of the unknown hormone with the Ab;only binding between labelled hormone and Ab
Std – Standard concentration (50µl)
S - Lion faecal sample (50µl)
QCH - Quality control high (50µl)
QCL –Quality control low (50µl)

6.3 Results and discussion:

During the parallelism test, the binding of the standard oestradiol 17β concentrations to the Ab was compared with the binding of the hormone present in the faecal samples (oestradiol 17β) to form a E_2 17 β - antibody complex. The gradients of the two curves were compared. The log curves of the standard and the faecal antibody results are shown in figure 1.

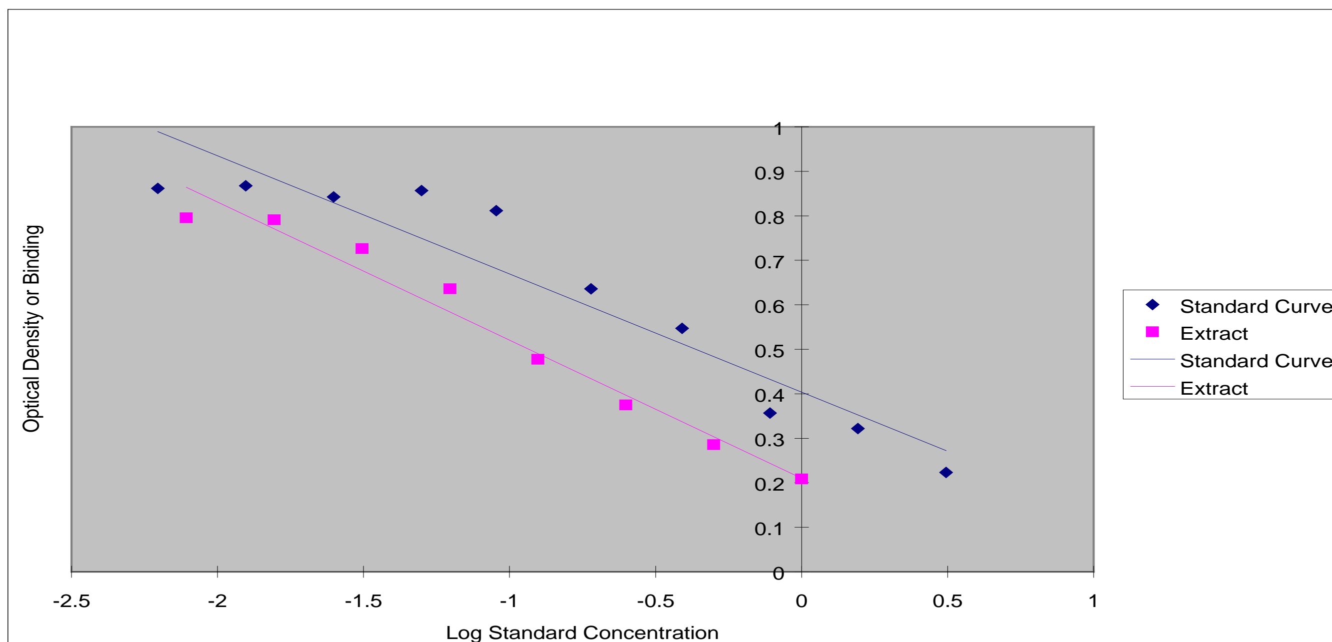

Figure 1: The binding of standard oestradiol - 17β concentrations and hormone present in pooled lion faecal samples with oestradiol - 17 β antibodies

There were no significant difference in the gradients and therefore the two lines could be considered are parallel indicating that no error in binding to the Ab by standard concentrations and the hormone present in the faecal samples occurred. This faecal oestradiol -17β enzyme immunoassay developed could be considered validated.

The lion faecal - 17β(E_2 - 17ß) levels determined through this assay are set out in Figure 2. The graph shows a rise in E_2 - 17β lion faecal levels on day 20 [2.94ng/ml] and day 60 [3.87 ng/ml]. Shille & Wing (1986), reported that the oestrous interval of a lion to be 3 to 8 weeks after oestrogen levels was determined in blood samples of the lioness. Guggisberg (1961), observed an interval of 2 months between the 2 consecutive behavioural oestrous periods in lionesses in the Kruger National Park . Schramm *et al.* (1994), reported a large variation in the interoestrus intervals of 6 lions ranging from 18 days to 66 days (blood oestrogen levels). According to Figure 1 an interval of exactly 43 days can be seen between oestradiol peaks. This could mean either that the oestrous interval of the lioness in this case was 43 days or this interval

could represent two cycles of about 21,5 days each. In the second case, another E_2 - 17β peak could have been missed due to the long interval between faecal collections. During the present study, not enough faecal samples were collected to set up a reliable hormonal profile for oestradiol 17 β. More frequent faecal samples need to be taken from more females over a longer period for the assay to form a representative hormone profile of the lioness. It would be of interest to compare the blood oestradiol levels of the lioness with those obtained from the faecal samples. Nevertheless, these results are in line with those reported in the literature.

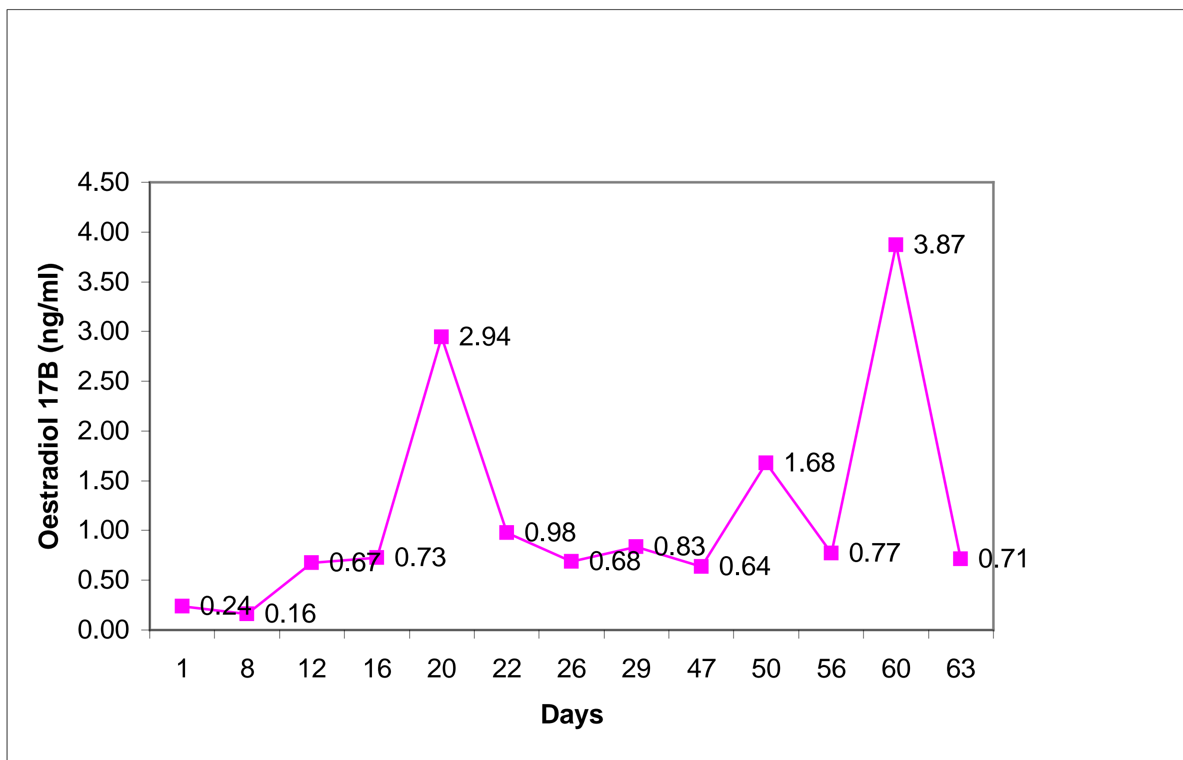

Figure 2: Faecal oestradiol -17β concentration in one African lioness over 63 days

6.4 CONCLUSIONS:

The faecal oestradiol -17 β enzyme immunoassay proposed in this study was validated. This assay is adequate to assay the oestradiol-17β level in the faeces of a lioness.

This non-invasive technique represents a practical way of assessing E_2 -17 β hormone levels in captive lions. More frequent faecal collections are however needed to characterise the hormonal profiles. The same principle may be used to develop enzyme immuno assays for other steroid hormones. Once the hormonal profiles have been determined for the lioness, they may be of value in applying assisted reproductive techniques, such as AI and embryo production in the African lion. Important events such as the time of ovulation or when implantation take place could be monitored and assist reproductive techniques to be used with greater efficiency. Once reproductive techniques are successfully applied in the African lion (*Panthera leo*), they may contribute to the long-term survival of the species.

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Appendix A

1. Coating buffer 0.05M carbonate buffer(pH 9.6)

1.59g Sodium Carbonate (Na_2CO_3, Sigma S7795) and 2.93g Sodium Hydrogen Carbonate ($NaHCO_3$, Saarchem 582 28 20 EM) were made up to 980 ml with deionised water (dH_2O). The pH was adjusted to 9.6 using NaOH and a final volume of 1000 ml was made up with dH_2O. The buffer was stored overnight at 4°C.

2. Phosphate buffer solution (PBS) 0.1M, pH 7.0, with 0.1% Bovine serum albumin (BSA)

5.42g Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$, Saarchem 582 26 70 EM), 8.66g diSodium Hydrogen orthophosphate (Na_2HPO_4 Anhydrous, BDH BB102494C), 8.7g Sodium Chloride- (NaCl, Saarchem 582 23 20 EM) and 1.0g Bovine serum albumin (Fraction V, Sigma A4503) were used to make up the PBS solution. The salts were combined and dissolved in approximately 800ml dH_2O and the BSA added and allowed to dissolve slowly, without mixing (to prevent foaming). The pH was adjusted to 7.0 by slowly adding small amounts of 3M NaOH. The solution was made-up to a final volume of 1000ml with dH_2O and stored overnight at 4°C, until used in the assay.

3. Citrate buffer (for substrate solution) 0.05M, pH 4.0

9.61g Citric Acid (Anhydrous, Sigma C0759) were dissolved in approximately 950ml distilled H_2O (dH_2O) and the pH adjusted to 4.0 with 3M NaOH. The solution was then made up to 1000ml with dH_2O and stored at 4°C.

4. ABTS(Azino –bis-diammonium salt) solution (for substrate solution) 40mM, pH 6.0

0.329g of 2,2'-Azino-bis(3-Ethylybenzthyoline-6-sulphonic acid) diammonium salt) (ABTS, Sigma A1888)was mixed with 12.5 ml dH2O. The pH of the solution was carefully set to 6.0, using a very weak solution of NaOH. The solution was made up to 15 ml with distilled water and stored in a dark area (light excluded) until used for the assay.

5. Hydrogen peroxide solution (for substrate solution) 0.5M, 2.0% v/v

500µl of Hydrogen peroxide (H_2O_2, 8M 30%, Sigma H1009) were diluted in 7.5ml of dH_2O.

6. Washing solution 0.05% v/v

0.5 ml Tween 20 (Polyoxyethylenesorbitan monolaurate, Sigma P1379) was carefully pipetted and slowly added to 1l dH_2O. The solution was thoroughly mixed as the Tween 20 is much denser than water and will settle at the bottom of the flask unless properly mixed. This solution was stored overnight at 4 °C until used.

7. 3M NaOH

12g of Sodium hydroxide (NaOH pellets, Saarchem 582 32 00 EM) pellets were dissolved in 100ml of distilled water and kept at room temperature for use during the assay.

8. The Substrate

The substrate mixture was prepared by combining the Citrate buffer, ABTS and H_2O_2. Enough substrate for two plates was prepared for each assay from 25ml Citrate Buffer, 250ul ABTS and 80ul H_2O_2. The substrate is very sensitive to light and therefore it was used immediately once the catalyst (H_2O_2) had been added.

9. Antibody working volumes

The neat oestradiol 17-β antibody stock was diluted 1:50 with coating buffer (15μl antibody diluted in 750 μl coating buffer) and aliquoted into volumes of 30 μl. The 30 μl volumes of oestradiol17-β were frozen at -20°C until needed.

10. Labelled hormone working volumes

The pure E_2 – HRP (Oestradiol with Horse Radish Peroxidase attached as label) was diluted 1:100 with PBS (10μl pure E_2 – HRP diluted in 1 ml PBS in an Eppendorf tube). This was stored at 4°C until needed.

Appendix B

Layout for coating the wells with Antibody (E_2)

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	B	B	C	C	PBS	PBS	PBS	PBS	PBS	PBS
B	A	A	B	B	C	C	PBS	PBS	PBS	PBS	PBS	PBS
C	A	A	B	B	C	C	PBS	PBS	PBS	PBS	PBS	PBS
D	A	A	B	B	C	C	PBS	PBS	PBS	PBS	PBS	PBS
E	A	A	B	B	C	C	PBS	PBS	PBS	PBS	PBS	PBS
F	A	A	B	B	C	C	PBS	PBS	PBS	PBS	PBS	PBS
G	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

A – 1:10 000 dilution of E_2 - 17β antibody (50 µl)
B – 1: 15 000 dilution of E_2 - 17β antibody (50 µl)
C – 1:20 000 dilution of E_2 - 17β antibody (50 µl)
PBS – Phosphate buffer solution (50 µl)

Appendix C

Layout for coating the wells with 1ng/ml E_2 standard concentration

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	E_2 std	E_2 std	E_2 std	E_2 std	E_2 std	E_2 std	PBS	PBS	PBS	PBS	PBS	PBS
C	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
D	E_2 std	E_2 std	E_2 std	E_2 std	E_2 std	E_2 std	PBS	PBS	PBS	PBS	PBS	PBS
E	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
F	E_2 std	E_2 std	E_2 std	E_2 std	E_2 std	E_2 std	PBS	PBS	PBS	PBS	PBS	PBS
G	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

E_2 std – oestradiol standard concentration 1 ng/ml (50µl)
PBS – Phosphate buffer solution (50µl)

Appendix D

Layout for coating wells with labeled hormone (E_2 – HRP)

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	X	X	X	X	X	PBS	PBS	PBS	PBS	PBS	PBS
B	X	X	X	X	X	X	PBS	PBS	PBS	PBS	PBS	PBS
C	Y	Y	Y	Y	Y	Y	PBS	PBS	PBS	PBS	PBS	PBS
D	Y	Y	Y	Y	Y	Y	PBS	PBS	PBS	PBS	PBS	PBS
E	Z	Z	Z	Z	Z	Z	PBS	PBS	PBS	PBS	PBS	PBS
F	Z	Z	Z	Z	Z	Z	PBS	PBS	PBS	PBS	PBS	PBS
G	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

X – 1 : 10 000 dilution of E_2 – HRP (50 µl)
Y – 1 : 15 000 dilution of E_2 – HRP (50 µl)
Z – 1: 20 000 dilution of E_2 – HRP (50 µl)
PBS – Phosphate buffer solution (50 µl)

Appendix E

Plate 1 - A chemically immobilized African lion (*Panthera leo*)

Plate 2 - Semen collection from an African lion by electroejaculation

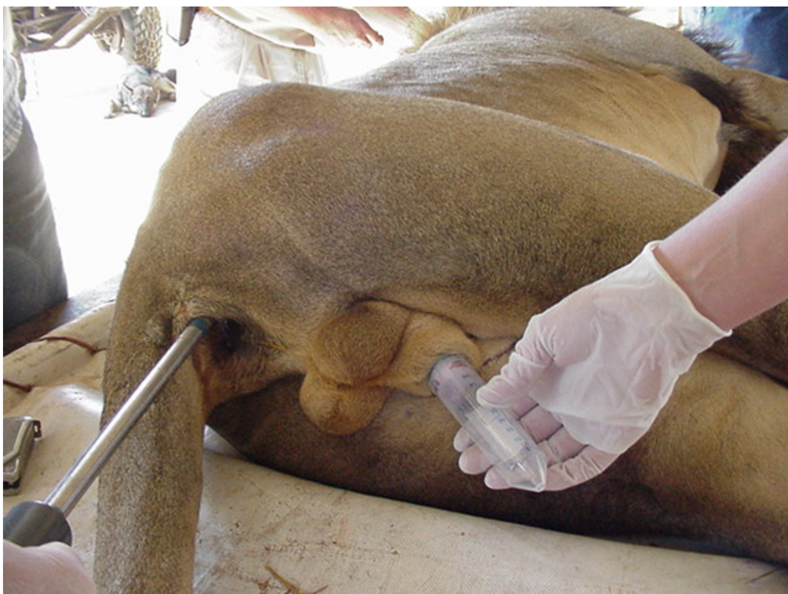

Plate 3 - Semen collection from an African lion (*Panthera leo*)

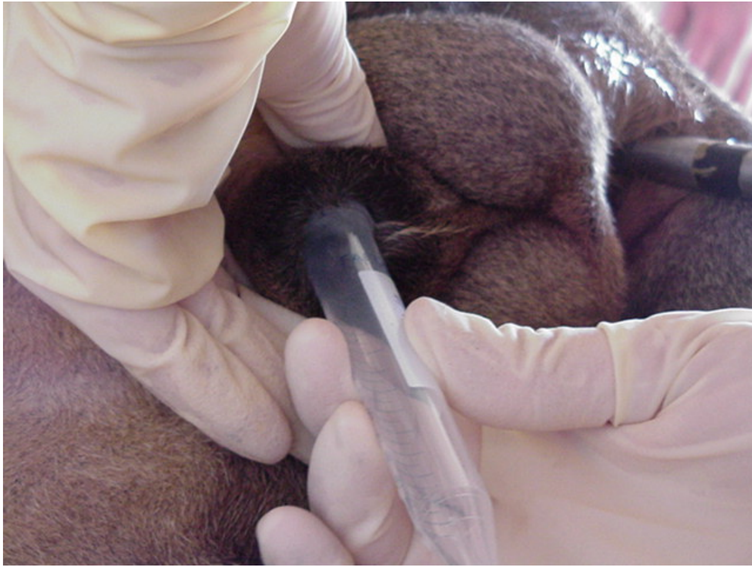

Plate 4 - Lion semen evaluation

ABSTRACT

SPERM CRYOPRESERVATION, *IN VITRO* FERTILIZATION AND FAECAL OESTROGEN ENZYME IMMUNOASSAY VALIDATION IN THE AFRICAN LION (*PANTHERA LEO*)

By

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Eight captive male African lions aged 1 to 4 years (*Panthera leo*) from Bothaville district (n=6) and Johannesburg Zoo (n=2), as well as 1 lioness from the Bloemfontein Zoo were used during this study. The 8 male lions were chemically immobilized using Zoletil® (Virbac,SA). External sexual organ characteristics were determined by palpation. The length and the width of the testis were recorded. Results showed that 4 lions had testis with a firm texture, while the other 4 animals had either 1 or both testis flaccid. Well developed cauda epididymis were detected in 6 of the lions. The mean length of the testis was 5.3 ± 0.5 cm and the width 3.1± 0.39 cm.

Semen was succesfully collected from 6 chemically immobilized male lions by means of electroejaculation. The semen was evaluated after collection for volume, pH, overall motility, linear forward progression, concentration and structural morphology using standard procedures.
A mean of 5.28 ± 0.6 ml of semen with a whitish color was collected from the 6 animals. The mean pH of the samples was 7.1± 0.05. A mean of 90 ±1.6% motile sperm with a linear progression of 4 was recorded for the semen samples collected. The semen

samples had a mean concentration of 101 x10^6 ± 3.1 sperm/ml and 65.7 ± 2.14% structurally normal sperm.

Five different treatments were used to cryopreserve the collected lion semen. Biladyl®, a Tris-citrate buffered extender, was used in combination with either DMSO or Glycerol at inclusion levels of 4% and 8%. After adding the cryoprotectant, the extended semen was loaded into 0.25ml straws (n=12) and equilibrated for 2 hours at 4°C. After equilibration, the straws were frozen in liquid nitrogen vapour (±-70°C) and stored in a liquid nitrogen flask at -196°. After 5 months of cryopreservation, 2 straws per animal, per cryopreservation treatment were thawed by one of 3 methods: i) room temperature (22°C) for 2 minutes, ii) 36°C for 2 minutes (waterbath) and iii) 50° for 8 seconds (waterbath). The semen was evaluated post-thawing for overall motility and forward progression. No post-thaw motility or forward progression was recorded in the semen samples thawed without a cryoprotectant (control). Treatments containig Glycerol or DMSO at 4% and 8% recorded a significant higher motility and forward progression. A mean post-thaw motility of 18±2.4 % was recorded for semen (cryopreserved in DMSO and Glycerol) thawed at three different temperatures. There were no significant differences between the 4 treatments containing cryoprotectants or between the 3 thawing methods used.

A heterologous in vitro fertilization assay was set up to evaluate the fertilizing capacity of the lion sperm cells post-thaw. Semen from the 4 cryoprotectant treatments (4 and 8% DMSO and Glycerol) were thawed at 36°C for 2 minutes and used to fertilize *in vitro* matured zona pellucida free bovine oocytes. The results showed a fertilization rate of 25% and 33% for lion semen cryopreserved in 4% Glycerol and DMSO respectively. The sperm cryopreserved in 8% Glycerol showed fertilizing rates of 0% and sperm cryopreserved in 8% DMSO a rate of 13% fertlization. Although the fertilzation rate obtained during this study are relatively low, the cryopreservation protocols using 4% Glycerol and DMSO hold promise for *in vitro* fertilizatrion programs for the African lion (*Panthera leo*) using in homologous oocytes.

A faecal oestradiol- 17β enzyme immunoassay for the African lioness was developed and tested. Faecal samples were collected from one adult lioness on a weekly basis

over a period of 3 months. The assay was validated for the African lion and the oestradiol -17β level present in the faeces was determined. The oestradiol - 17β levels ranged between 0.01ng/ml to 3.8ng/ml. An interval of 43 days between oestradiol -17β peaks was determined. A representative oestrogen profile could not be set up due to limited faecal samples

From the results presented in this study it could be concluded that lion semen can be successfully cryopreserved in Biladyl® cryodiluent containing either 4% or 8% Glycerol or DMSO. The results of the post-thaw heterologous IVF assay suggest that the 4% Glycerol or DMSO inclusion in the semen extender for cryopreservation, results in better fertilization rates than at concentrations of 8% glycerol or DMSO.

OPSOMMING

SPERM BEVRIESING, *IN VITRO* BEVRUGTING EN DIE VALIDASIE VAN 'N FEKALE OESTROGEEN ENSIEM IMMUNOLOGIESE- TOETS IN DIE AFRIKA LEEU (*PANTHERA LEO*)

deur

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Tydens hierdie studie is 8 manlike Afrika leeus en 1 vroulike Afrika leeu gebruik. Die leeus was in aanhouding in die Johannesburg en Bloemfontein dieretuine asook op 'n plaas in die Bothaville distrik. Die 8 manlike leeus is chemies verdoof deur middel van Zoletil®. Na verdowing is die tekstuur van die testis en die cauda epididymis ondersoek. Die lengte en breedte van die testis is bepaal. Ferm testis het by 4 manlike diere en goed ontwikkelde cauda epididimis by 6 van die 8 manlike diere voorgekom. Die gemiddelde lengte en breedte van die testis was 5.3± 0.5 cm en 3.1±0.39 cm onderskeidelik.

Semen is suksesvol van 6 manlike leeus (onder verdowing) gekollekteer deur middel van elektro-ejakulasie. 'n Standaard prosedure is gebruik om semen te evalueer vir volume, pH, konsentrasie, strukturele morfologie, beweging en tempo van lineêre beweging. 'n Gemiddeld van 5.3 ±0.6 ml semen met 'n pH van 7.0 is per dier gekollekteer. 'n Gemiddeld van 90 ±1.6 % beweeglike sperme met 'n lineêre

progressie van 4 was teenwoordig in die semen monsters. Die gemiddelde konsentrasie van leeu semen gekollekteer was 101 x 10^6 ±3.1 sperme/ml met 65.7 ± 2.1 % morfologies normale sperme

Vyf verskillende behandelings is getoets tydens die bevriesing van leeu semen. Biladyl® is gebruik as semenverdunningmiddel. Die oorblywende 4 behandelings het bestaan uit 'n kombinasie van Biladyl® met 1 van die volgende as kriobeskermings middel: 4% en 8% gliserol en DMSO byvoegings onderskeidelik. Die verdunde semen is in 0.25ml strooitjies opgetrek. Na ‘n ekwilibrasie tydperk van 2 uur by 4°C, is die semen in vloeibare stikstof damp gevries en in vloeibare stikstof geberg.

Na ‘n periode van 5 maande van bevriesing, is 2 strooitjies/dier/behandeling ontdooi by een van 3 temperature, i) 36°C (waterbad) vir 2 minute ii) kamertemperatuur en iii) 50°(waterbad) vir 8 sekondes. Die semen is ge-evalueer vir beweeglikheid na ontdooiing. Geen beweging van sperme is waargeneem by semen gevries in slegs Biladyl® sonder gliserol/DMSO. ‘n Gemiddelde beweging van 18.0 ±2.4% na ontdooiing het voorgekom by leeu semen gevries in 4% en 8% gliserol en DMSO. Geen betekenisvolle verskille het voorgekom tussen behandeling vir leeu semen bevriesing of tussen verskillende metodes vir die ontdooi van leeusemen.

Die bevrugtingspotensiaal van die bevrore semen in 4% en 8% gliserol of DMSO is bepaal deur ‘n heterologiese in vitro bevrugtings toets. Leeu semen gevries in 4% of 8% gliserol en DMSO is ontdooi by 36°C vir 2 minute en gebruik om bees zona pellucida- vrye eierselle te bevrug. Resultate het bevrugtingspeile van 25% en 33% getoon vir semen gevries in 4% gliserol en DMSO onderskeidelik. Semen gevries in 8% gliserol en DMSO het gelei tot 0% en 13% bevrugting.

‘n Fekale oestradiol-17β ensiem immuun- toets is gevalideer vir die vroulike Afrika leeu. Mismonsters is op ‘n weeklikse basis oor ‘n tydperk van 3 maande van ‘n vroulike leeu gekollekteer. Die toets is gevalideer en die vlakke van oestradiol-17β teenwoordig in die mismonsters is bepaal. Oestradiol-17β vlakke het gewissel tussen 0.01ng/ml en 3.8ng/ml. ‘n Interval van 43 dae tussen oestradiol pieke het voorgekom. ‘n

Verteenwoordigende hormoon profiel vir oestradiol uitgeskei in die mis van die vroulike leeu kon nie bepaal word nie vanweë ‘n tekort aan monsters.

‘ n Algemene gevolgtrekking kan gemaak word dat leeu semen suksesvol gevries kan word in Biladyl® semenpreserveermiddel wat gliserol en DMSO teen 4 en 8% onderskeidelik bevat. Die resultate van die heterologiese *in vitro* bevrugtings-toets het daarop gedui dat beter bevrugting plaasgevind het met leeu semen gevries in laer konsentrasies van gliserol en DMSO (4%) as met hoër insluitingspeile (8%).