SEMEN ANALYSIS AND INDUCTION OF ACROSOME REACTION IN SPERMATOZOA OF TANA MANGABEY (*Cercocebus galeritus*) AND DE BRAZZA'S (*Cercopithecus neglectus*) MONKEYS'

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ABSTRACT

Semen from Tana Mangabeys (*Cercocebus galeritus* n=4) and De Brazza's monkeys (*Cercopithecus neglects,* n=3) was evaluated for routine parameters to establish fertility potential following electro-ejaculation. The correlations of semen parameters were analysed. Acrosomal status of sperm obtained from Mangabeys was monitored following challenge with dbcAMP and calcium ionophore A23187 *in vitro* by Fluorescein labeled *Pissum sativum* agglutinin (FITC-PSA) assay. The proportion of spermatozoa undergoing spontaneous and artificially induced acrosome reaction (AR) were compared.

Twenty-six out of 33 (78.7%) attempts in Mangabeys and 17/26 (65.3%) in De Brazza's monkeys produced ejaculates. Semen pH ranged from 8.0 to 9.0. Among Tana Mangabeys, the mean ± s.e.m percentage rapid progressive sperm motility was 65 ± 3, slow progressive motility was 13.5% ± 8.6%, non-progressive motility was 10.2% ± 1.2% while nonmotile sperm was 10.2% ± 0.6%. Respective grades of sperm motility were 50% ± 2%, 11.5% ± 1.7%, 14.2% ± 1.3% and 23.2% ±2.6% in De Brazza's monkeys. The mean ± s.e.m ejaculate volume was 57.4 ± 6.8 µl in Mangabeys showing a correlation {r= 0.94 (P< 0.05)} with semen weight. The mean ± s.e.m ejaculate volume in De Brazza semen was 39.2 ± 3.6 µl. There was a correlation (r= 0.96) between weight of ejaculate and volume of its liquefied fraction among De Brazza's monkeys. The weight of liquefied semen fraction showed a correlation (r= 0.87) to its volume and a correlation (r=0.92) to the weight of fresh ejaculate in De Brazza's monkeys. The mean (Mean±SE) sperm concentration (1989 ± 200) $\times 10^6$ per ml in Mangabeys was significantly different (P <0.01) from (64.1± 12) $\times 10^6$ per ml recorded in De Brazza. There was no correlation {r= 0.067, (P=0.7)} between sperm concentration and volume in Mangabeys. Mean

sperm abnormality was 29% in De Brazza's monkeys compared to 16.2% in Mangabeys while abnormal sperm heads occured at rates of 10.1% compared to 4.5% respectively.

Three fluorescence patterns were observed namely; Pattern IA (intact Acrosome), with fluorescence uniformly distributed over the anterior 2/3 of the sperm head, Pattern PA (patchy acrosome), with fluorescence as above, but in a patchy manner and Pattern AR (acrosome reacted), with a flourescence band across the sperm head along the equitorial segment after staining with FITC-PSA. The mean percentage of sperm with pattern IA at time, t = 0 minutes to time t = 150 minutes, decreased from 99% to 67.1% in control compared with 100% to 34% in experimental tubes while pattern PA increased from 1% to 19.4% in control compared with 0% to 29% in experimental. Mean percentage of pattern AR sperm increased from 0 % to 13.4% in control compared with 0% to 37% in experimental. Significant differences ($P<0.05$) between control and experimental tubes for the three sperm patterns where noticed throughout except at time, t = 120 minutes. where ($P=0.0504$). The highest inducible AR in sperm was 37%.

This study showed that;

i) Rectal probe electro-ejaculation can retrieve semen from the two species of monkeys although the volumes recovered were too low. Semen recovered from De Brazza's monkeys showed poor parameters which may explain their low fertility. Very high sperm concentration from Mangabeys reveals high spermatogenetic potential.

ii) The fluorescent isothiocynate labelled lectin, *Pissum sativum* agglutinin (FITC-PSA) which is specific for sperm head glyco-conjugates can be used to study time course acrosomal loss in Mangabey sperm.

iii) Dibutyrly cAMP/CaIA23187 reagent mixture induces a higher proportion of acrosome reaction in sperm compared with the spontaneous reaction.

DECLARATION

This thesis is submitted as my original work to best of my knowledge for the award of M.Sc. degree of the University of Nairobi

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This thesis is submitted for examination with University approval of the following personalities as supervisors.

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DEDICATION

This work is dedicated to all people involved in the field of conservation research and to the animal kingdom whose continued survival shall remain a special obligation to man and woman. Special dedication is directed to all primate species that shall continue to die as result of scientific exploration for the benefit of man.

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ABBREVIATIONS AND SYMBOLS

AC	Adenyl Cyclase
ATPase	Adenosine Triphosphatase
AR	Acrosome Reaction
BSA	Bovine Serum Albumin
BWW	Bigger's Whitten Whittingham
cAMP	Cyclic Adenosine Monophosphate
CASA	Computer Assisted Motion Analysis
CTC	Chlortetracycline
DAG	Diacylglycerol
dbcAMP	Dibutyryl Cyclic Adenosine Monophosphate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethelene Diamine Tetra Acetic Acid
ET	Embryo transfer
FITC-PSA	Fluorescein Isothiocynate-Pissum Sativum Agglutinin
GalTase	β-1,4-Galactalsyltransferase
GSK-3	Glycogen Synthase Kinase-3
H258	Hoesch 33258 (Bis benzimide)
HEPES	N-(2-Hydroxyethyl) Piperazine- N'-(Ethanosulfonic Acid)
I (I1, I2)	Phosphatase Inhibitors
IBMX	3-Isobutyl-1-Methyl Xanthine
IP4	Inositol tetraphosphate
IVF	In Vitro Fertilizatioin
LH	Leutinizing Hormone
LPAT	Lysophosphotide acyl transferase
ODF	Outer Dense Fibres
PIP2	Phosphatidylinositol Diphosphate
PKA	Protein Kinase A
PLA2	Phospholipase A2
PLC	Phospholipase C
PPs (PP1, PP2)	Protein Phosphatases
PTP	Protein Tyrosine Phosphorylation
RPE	Rectal Probe Electro-Ejaculation
TALP	Tyrodes' Salt supplemented with Albumin, Lactate Pyruvate
ZP (ZP2, ZP3)	Zona Pellucida glycoproteins
µl	Microlitre
µM	Micromolar
ml	Millilitre
g	gram
mM	Millimolar

CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Most conventional reproductive research involves the use of common species including livestock, laboratory animals and non-human primates. Reproductive biology of more than 4000 mammalian species including non-human primates inhabiting the planet remains unknown (Wildt, 1995). This information is important, not just as adjunct knowledge, but because the majority of primate species such as, Tana Mangabeys and De Brazza's monkeys' are threatened with extinction largely due to habitat destruction, hunting and other human activities such as, agriculture and timber logging (Batynski, 1996; Brennan 1985; Wildt 1995; Karere *et al.*,1997; Morrell *et al.*, 1997).

Conservation and management of primate species both wild and captive, are major concerns of primatologists. The latter are being addressed in several ways, including, study of habitat ecology and housing design, assisted reproductive techniques and mating strategies, fertility and reproductive success (Gould, 1995; Morrell *et al.*, 1997; Ramon 1997; Wildt 1995). Reproduction is the essence of species survival and reproductive success is best determined by observing parents over the entire life span and or the number of their offsprings living to the age of reproduction (Ramon 1997). However, this is often practically impossible. Captive breeding programs are needed urgently to help rescue these species, as an adjunct to habitat conservation. This is especially the case where the challenge is greatly exacerbated by sheer numbers of species in crisis. Unfortunately,

primates may not breed well in captivity as has been observed with De Brazza's monkeys and the Tana Mangabeys at the Institute of Primate Research Karen (Adoyo, P. personal communication). Therefore, assisted breeding techniques are needed to increase the number of individuals and to facilitate their genetic management.

There is evidence to suggest that primate conservation can be expanded and enhanced using assisted reproductive technologies such as, artificial insemination (Morrell *et al.*, 1997). Techniques such as semen collection and sperm cryopreservation, artificial insemination (AI), *in vitro* fertilisation (IVF), embryo cryopreservation and embryo transfer (ET) that have already gained widespread application in livestock have potential usefulness in endangered species. The technique by Wilmut *et al.*, (1997) in which viable lamb offsprings have been produced from cells either derived from adult mammary gland, fetal tissue or embryo-derived cells as nuclear donors to an enucleated oocyte also provides another potential in many species to generate neonates.

Management of endangered species require that particular attention be given to the fertility of individual animals and controlled breeding of the population. Application of such techniques to endangered species has been restricted by paucity of breeding stock, concomitant restriction of selection of animals, and inadequate understanding of their reproductive physiology. There is little data concerning reproductive traits in non-human primates including; ejaculate or semen characteristics, optimal site and timing of insemination, spermatozoa *in vitro* longevity and metabolism. This is largely due to species diversity (Wildt, 1986). Yet a potential for recovery of spermatozoa for study or for later

use exists. For endangered species such as, the De Brazza's monkeys and the Tana Mangabeys, enhancement of reproductive potential and maintenance of genetic diversity requires a thorough understanding and characterisation of semen parameters. Fresh semen has been used for insemination as an alternative to natural breeding in monkeys and great apes and the results indicate that conception and births are possible (Czaja *et al.*, cited by Morrell *et al.*, 1997; Kreamer and Cruz 1969; Gould *et al.*, 1985; Wildt 1986; Morrell *et al.*, 1997). However, the success rate in the range of 4 - 40 % is still very low. Sperm cryopreservation methods similar to those employed in human and livestock have been applied to the semen of several primate species but with a low recovery rate and a shorter storage period (Russel and Austin, 1967b) as cited by Wildt, (1986). Attempt to transfer AI technology to primates has met with limited success, in part, due to little knowledge concerning manipulation of a variety of primate gametes. Success in assisted reproductive technologies requires full knowledge of spermatozoal physiology in an individual animal or species of animals. It is this lack of information that provides strong impetus for further research.

1.2 LITERATURE REVIEW

1.2.1 Spermatogenesis

Spermatogenesis is an elaborate complex process of cell differentiation starting with a non-differentiated spermatogonial germinal stem cell and terminating with a fully differentiated highly specialised motile cell called the spermatozoon. The process starts with embryonic colonisation of the germinal crests by primordial germ cells and is followed by differentiation and multiplication of stem spermatogonia (Byskov 1982). The process, as elaborated by Bellve' and O'Brien (1983) consists of two phases; spermatocytogenesis and spermiogenesis. Spermatocytogenesis is a process that involves several complex events occurring in a strict programmed order in which primordial germ cells are transformed sequentially through gonocytes, spermatogonia, primary and secondary spermatocytes, to spermatids (Bloom and Fawcett 1975; Setchell 1982). During spermatocytogenesis, spermatogonia undergo several mitotic cell divisions characteristic of each species after which some of the formed daughter cells stop proliferating and differentiate into primary spermatocytes. Primary spermotocytes (2n) then enter a prolonged first meiotic prophase, in which paired homologous chromosomes duplicate and participate in crossing over, before proceeding with completion of division I of meiosis to produce two secondary spermatocytes, each containing 22 duplicated autosomal chromosomes and either a duplicated X or Y chromosome (2n). At this stage, each chromosome still consists of two sister chromatids. Subsequently, each of the two secondary spermatocytes undergo division II of meiosis with resultant total production of four spermatids, each consisting of a haploid number (n) of chromosomes.

Spermiogenesis, is characterised by; a) inactivation of the genome, b) condensation of chromatin, c) development of locomotory apparatus and d) differentiation of haploid spermatid cells into spermatozoa (De Reviers *et al.,* 1990). During differentiation, spermatids undergo complex morphological, biochemical, and physiological changes that result into formation of flagellated mature spermatozoa. These are then released from Sertoli cells into the lumen of the seminiferous tubules through a process known as spermiation (Halmiton 1987; Hermo and Clermont 1995). Spermatozoa are then transported to the epididymis, where they are stored and undergo further maturation changes to increase their fertilisation potential.

The quantitative and qualitative efficiency of sperm production are under the control of gonadotrophins, androgens, testicular paracrine and autocrine factors (Hamamah and Mieusset 1996; Pescovitz *et al.,* 1994). Although little is known of how all the factors that regulate spermatogenesis act, evidence points at both local and circulating factors. The various testicular cell types communication systems such as, Sertoli cell, germ cell, peritubular cell, seminiferous epithelium, and Sertoli-leydig cell are now known (Hamamah and Mieusset 1996; Pescovitz *et al.,* 1994). Albeit an array of hormones (hypothalamic-pituitary and gonadal hormones), nutrients growth factors, proto-oncogens, and transcriptional factors have also been implicated. To date, only follicle stimulating hormone (FSH), leutinizing hormone (LH), testosterone, vitamin C, 'c-fos', and stem cell factors have been shown to play a definite and essential role in modulating spermatogenesis (Pescovitz *et al.,* 1994). The complexity of spermatogenesis explains its marked sensitivity (among other factors) to toxic substances, hormonal

imbalances and genetic disorders that may result in malformed and or malfunctional spermatozoa that have been associated with poor fertilisation potential.

1.2.2 Epididymal Sperm Maturation

During epididymal maturation, spermatozoa undergo additional changes in morphology, metabolic patterns, enzyme activities, chemical and physical properties of the plasma membrane (Voglmayr 1975; Jones 1989; Yanagimachi 1994). However, the biochemical mechanism underlying sperm maturation remain largely undefined. Among the known spermatozoa maturational changes in the epididymis include;

a) Development of sperm motility; initiation of sperm motility occurs following spermiation (Bedford *et al.*, 1973). Initiation of sperm motility involve changes in intra-sperm levels of cyclic adenosine monophosphate, calcium ions and pH plus maturation of the sperm plasma membrane (Cooper 1986). Inability of testicular sperm to move has been associated in part, with immaturity of the plasma membrane. Plasma membrane changes involve intramembraneous components such as, enzyme systems, plasma membrane stabilisation by cholesterol and a general decline in plasma membrane phospholipids, cholesterol, and protein content (Hall *et a.,l* 1991; Suzuki, 1981; Seki *et al.*, 1992; Huszar *et al.*, 1997). These changes stabilise the sperm plasma membrane. Failure in any of the above pathways may result in production of sperm with a functional defect. As spermatozoa transcend the epididymis, percentage progressive sperm motility, ability to fuse with the ovum and their concentration all increase (Haidl 1994; Weissenberg *et al.*, 1995; Nirmal and Rai 1997). These changes have been attributed to the changes in biochemistry of epididymal fluid.

b) Transfer of substrates such as, glycerol-3-phosphorylcholine, a forward motility protein, alteration in the cAMP-modulated fluid kinase system and the development of a mechanism that keeps intracellular Ca^{2+} low (Infante and Huszagh 1985; Vernon *et al.*, 1987; Wooten *et al.*, 1987).

c) Morphological changes such as, modification of the acrosome, and loss of cytoplasmic droplet (Bedford 1979; Hoffer and Shalev 1981).

d) Disulfide cross linking of proteins of the outer dense fibres and fibrous sheath, modification of dynein ATPase, disulfide bridging of nucleoproteins, and condensation of chromatin (Morcardi *et al.*, 1995; Weissenberg *et al.*, 1995; White and Voglmayr 1986; Yoseffi *et al.*, 1994)

1.2.3 Morphology of Mammalian Spermatozoa

Spermatozoa consists of two functionally and morphologically distinct region enclosed by a single plasmalemma; the head and the tail (Bloom and Fawcett 1975). The junction of the head and the tail is formed at the site of an implantation fossa at the base of the nucleus. The head houses a highly condensed haploid nucleus and the acrosome. The nucleus comprises of condensed chromatin which is primarily composed of deoxyribonucleic acid complexed with cystein-rich protamine and has a species-specific shape (Ashikawa *et al.*,1987; Beatty 1975). The acrosome is a specialised membrane-limited vesicle closely appossed to the anterior two thirds of the nuclear envelope which posteriorly narrows to form the equatorial segment. The inner acrosomal membrane overlies the nuclear envelope and the outer acrosomal membrane overlies the plasma membrane (Russell *et al.*, 1979, cited by De Jong *et al.*, 1989). The acrosome size and form can vary according to species (Bedford and Hoskin 1990). The equatorial region is

characterised by an absolutely parallel arrangement of the outer and inner membranes of the acrosome. The compartment of the acrosome is apparently maintained at a pH below five, possibly through action of a Mg^{2+}-dependent proton-translocating membrane ATPase (Meizel and Deamer 1978; Working and Meizel, 1982). Its matrix is a complex of several hydrolytic enzymes that enable the sperm to penetrate the egg vestments during fertilisation.

The sperm tail consists of an axoneme composed of a 9+2 pattern of microtubules surrounded by a portion of the cell membrane. The axoneme is arranged as two central single microtubles surrounded by nine microfibrils that occur as doublet microtubules (Gibbons 1968, cited by Gibbons 1979). The two microtubules in a doublet are designated as subtypes A and B. Subtype A microtubules bare two short proteinous arms, one being free (referred to as dyenin arms) and the other, nexin, forming a bridge with the adjacent B microtubule. Dyenin is a another divalent cation-dependant ATPase isoenzyme that occur associated with axonemal microtubules (Gibbons and Rowe 1965; Gibbons *et al.,* 1976). The axoneme is the kinetic apparatus of the sperm tail which generates propulsive force essential for sperm motility.

The tail, usually divided into the mid-piece, the principle piece, and the end piece terminates in a tapering manner. The mid-piece houses spirally arranged mitochondria. The principle piece is characterised by a fibrous sheath around the fibrilar structures. The sperm tail has nine outer dense fibres (ODF) relatively larger than the microtubules of the axoneme (Bloom and Fawcett 1975). The outer dense fibres represent up to 30% of the

protein portion in human sperm and are involved in sperm progressive motility. Poorly developed ODF, are associated with poor sperm motility (Henkel *et al.,* 1994). The ODF have been hypothesised to have passive elastic functions rather than an active role in generation of sperm motility (Baccetti *et al.,* 1973; Henkel *et al.,* 1994). According to Baltz *et al.,* (1990), there is a connection between the flagella length and the thickness of the ODF. Species with long spermatozoa tail (hamsters, rats, and guinea pigs) have significantly thicker ODF. When sperm are being transported in the epididymis, especially during ejaculation, shear forces by which flagella damage may occur arise. It has therefore been suggested by Baltz *et al.,* (1990) that ODF serve to increase the tensile strength of sperm so as to protect them against such forces.

1.2.4 Sperm Motility:

The ability of mammalian spermatozoa to fertilise an egg requires a state of active sperm motility. Sperm motility depends on the availability of adenosine triphosphate (ATP) within the axoneme (Bedford and Hoskins 1990). The basic mechanochemical mechanisms which defines flagella motility is the ATP induced sliding of microtubules (Gibbons 1968 cited by Gibbons 1979). Energy for microtubule sliding is derived from the ATPase activity associated with the dynein arms on the outer doublet microtubules (Gibbons, *et al.,* 1978). Forward motility in sperm results from the propagation of co-ordinated bending waves from the neck region along the length of the flagellum. The pattern of motion results from active forces within the tail working against both the internal resistance of the flagellum and the viscosity of the surrounding medium. However the variety of wave forms that produce diverse types of sperm motility such as progressive and hyperactivated motility suggest in addition, a regulatory mechanism that modulates the basic dynein

microtubule sliding mechanism. Tail bending movements that generate forward velocity is an outcome of two operation systems having different intracellular messengers. One system, the sliding filament system generates the force for bending wave, the vigour of which relies on cAMP-dependent kinase phosphorylation (Katz and Yanagimachi 1980). The other system, (poorly understood) appears to be modulated by a calcium-dependent mechanism, co-ordinates sliding and regulates the flagella beat.

Mature sperms stored in the initial regions of the epididymis are sluggish and irregular in motility but show erratic movements with vigorous flagella beat as they move towards the distal region of the epididymis (Yeung *et al.,* 1996). Active sperm motility develops only at ejaculation often instantly, as a result of neutralization of suppressive factors that are not yet even fully identified. Additionally, the quality of sperm motility (pattern and frequency of tail beat) undergoes further change as a concomitant factor of capacitation in the female reproductive tract. This is because penetration of the cervical mucus and the egg vestments require specific sperm movement characteristics referred to as hyperactivated motility (Morales *et al.,*1988a). Sperm motility pattern can differ according to species. This probably reflects the difference in length and thickness of the tail, as well as the relative proportion of the intracellular organelles, in particular, the outer dense fibres and their disposition in relation to the axoneme (Fawcett 1970; Philips 1972).

1.2.4.1 Sperm Kinetic Energy

Flagella movement is powered by sliding of adjacent microtubule doublets generated by the interaction of ATP hydrolysis and dynein-ATPase arms of one doublet

microtubules with the adjacent doublet microtubules (Gibbons and Gibbons 1976; Gibbons *et al.*,1976). This active sliding of microtubules which is still obscure, is generated by the inner arms of dynein in a calcium-dependent bi-directioanal power stroke manner that in part may cause sperm motility (Ishijima *et al.*, 1996). Dynein is known to provide ATPase activity in the sperm axoneme and can be activated by divalent cations such as calcium. However, Na^+ and K^+ ATPase activity that occurs in the plasma membrane of the flagellum and in the nine outer fibre is also a source of ATPase (Ishijima *et al.*, 1996),.

The exact mechanism of how the microtubule-sliding systems converts chemical energy derived from ATP hydrolysis into mechanical work is still obscure. However, kinesin, a motor protein that modulates a similar energy transition to transport cellular components along microtubules, has been documented by Shirakawa and colleagues (1995). It is possible that a similar operation may exist in sperm axoneme. An interaction between the central pair microtubules and the radial spokes that provides a regulatory function in the conversion of doublet microtubule sliding to bending waves was demonstrated by Whitman *et al.*, (1978). It is this interaction that regulates the dynein-mediated sliding between the outer doublets tubules. The role of nexin in flagella movement is not absolutely clear, but the interaction of dynein with tubulin (the main structural protein making up axonemal microtubules) has been established in the propagation of the bending wave.

1.2.4.2 Biochemical Basis for Sperm Motility

Intracellular calcium, cyclic adenosine monophosphate (cAMP) and pH changes have been identified as biochemical mediators of sperm motility and fertility (Cheng and Boettcher 1981; Chinoy *et al.,* 1983; Yanagimachi 1994). Other modulators such as, calmodulin, and protein phosphatases are involved in the initiation and regulation of sperm motility, but a unifying hypothesis of how all these interact to produce co-ordinated flagella activity is still lacking (Ahmed *et al.,* 1995; Cheng and Boettcher 1981; Hong *et al.,* 1985; Yanagimachi 1994). Cyclic adenosine monophosphate and cAMP-dependent protein kinases (PKAs) are key elements in regulation of sperm functions such as, sperm maturation, motility, capacitation and the acrosome reaction (De Jonge *et al.,* 1991). Cyclic adenosine monophosphate is produced from ATP by catalytic action of sperm adenyl cyclase (AC) which itself is stimulated by calcium (Hyne and Garber 1979). Calcium stimulates sperm AC in a dose dependent manner in which both cAMP content production and sperm motility are increased (Morton *et al.,* 1974). The identification of calmodulin in both sperm head and tail, has led to the suggestion that it is involved in this process since many calcium-regulated processes are mediated by calmodulin (Jones *et al.,* 1980; Means *et al.*, 1982). However, it is not known whether calmodulin mediates all the effect of Ca^{2+} on sperm motility. One role played by calmodulin is the regulation of dynein ATPase, an enzyme that interacts with ATP to promote sliding of outer doublets of the axoneme (Gibbon 1981; White and Voglmayr 1986). In addition, calmodulin has been shown to act on a Ca^{2+}-stimulated magnesium-ATPase in the sperm flagella membrane (Forrester and Bradley 1980). Forrester and Bradley (1980) suggest that in caput epididymal sperm the pump is relatively inactive but during sperm transit and ejaculation, the enzyme becomes

activated presumably by adsorbing the calmodulin-like ATPase activator. The physiological significance of such an enzyme in sperm appears to be the maintenance of calcium levels within the flagellum below that which inhibit motility. How Ca^{2+} moves across the plasma membrane is unclear however, the sperm plasma membrane has been shown to contain an active calcium pump that becomes activated during sperm epididymal transit. According to Forrester and Bradley (1980), this Ca^{2+}-Mg-ATPase pump, binds Ca^{2+} with high affinity and that the pump is activated by calmodulin-like protein. Therefore, activation of the calcium pump combined with a drop in the external concentration of Ca^{2+} may be an important regulatory aspect of the initiation of sperm motility.

The motility of mature and immature sperm can be improved and induced respectively *in vitro* by elevation of intracellular cAMP levels, using phosphodiesterase inhibitors such as, caffeine or cAMP analogues (Boatman and Bavister 1984; Jaiswal and Majumder 1996; Tesarik *et al.*,1992). The role of cAMP in sperm is probably to mediate cAMP-stimulated phosphorylation of proteins essential for initiation or maintenance of sperm motility (Garber and Kopf 1980). Elevated cAMP stimulates protein kinase A which subsequently activates trypsin kinase. Activated trypsin kinase phosphorylates tyrosine residues of an axonemal 15-Kda protein and the resultant protein phosphorylation allows the axoneme to slide and bend (Morisawa *et al.*, 1990).

Protein phosphorylation which is increased by agents that elevate sperm cAMP has been found to be directly related to sperm motility (Gary *et al.*, 1996). Protein phosphatases (Pps) isolated from other tissues sources have been shown to inhibit

motility of demembranated sea urchin sperm when included in the reactivation media (Takahishi *et al.*,, 1985). Protein phosphatase activity has been shown in sperm extracts of bovine, human and non-human (Ahmed *et al.*, 1995; Gary *et al.*, 1996; Tang and Hoskin 1975). In somatic cells, serine /threonine specific protein phosphatases are involved in a wide range of cellular functions such as dephosphorylation of phosphoproteins by phosphate group hydrolysis and thus oppose the actions of protein kinases (Shenolikar and Nairn 1991). Classification of protein phosphatases based on substrate specificity and response to a defined set of inhibitors and activators is given by Cohen (1991). Accordingly four types of protein phosphatases(PPs), PP1, PP2A, PP2B and PP2C are recognised. Protein phosphatase 1 (PP1) is sensitive to the heat and acid-stable Inihibitors 1 and 2 (I1 and I2), where as PP2 are insensitive to I1 and I2. Inactivation of PP1 by I1 is dependent on I1-phosphorylation of a single threonine residue by cAMP-dependent protein kinase A (PKA) where as I2 does not require phosphorylation for PP1 inhibition (Yeung 1994). The inactive PP1-I2 complex formed is activated by dissociation of PP1 and I2, a reaction controlled by glycogen synthase kinase-3 (GSK-3) phosphorylation of I2 (Resink *et al.*, 1983). It is Srinivasan *et al.*, (1996) who has shown that the development of sperm motility in the epididymis is associated with decreased GSK-3 and protein phospatase1 (PP1) activity.

The immotility of caput epididymal sperm as opposed to caudal epididymal sperm which move with forward progressive pattern as previously described by Burgos and Tovar (1974) can be explained in part by the fact that caput epididymal sperm contain levels of protein phosphatase activity two fold higher than do mature motile caudal sperm

(Srinivasan *et al.*, 1996). This supports earlier postulation that certain components of the contractile system such as phosphorylation of 9+2 axonemes may be modified during sperm maturation leading to the change in motility (Tongkao and Chulavatnatol 1978). Although both caput and cauda epididymal sperm posses protein kinases and can form phosphoproteins (Hoskin *et al.*, 1974). It seems that tubulin of the sperm from the caput epididymis is not phosphorylated unlike caudal spermatozoa suggesting that tubulin becomes accessible and serves as substrate for protein kinases during sperm maturation.

*1.2.4.3 **Hyperactivated Sperm Motility***

Mammalian spermatozoa exhibit characteristic motility patterns associated with capacitation either *in vivo* or *in vitro* collectively referred to as hyperactived motility (Morales *et al.*, 1988a). The most common expression of hyperactivation involves a change from progressive motility to a highly vigorous non-progressive random motion with large lateral displacement of the sperm head and wide amplitude flagella beats (Yanagimachi 1994). Initiation and maintenance of sperm hyperactivation requires energy substrates, HCO_3^-, K^+, and extracellular Ca^{2+} ions (Boatman and Robbins 1991; Cooper 1984; Fraser 1982, 1983; Fraser and Quinn 1981; Neill and Olds-Clark 1987). Several biological functions have been proposed for hyperactivated sperm motility and these include;

a) Increasing flexibility for moving sperm out of cervical mucous fold pockets to enhance the chances of sperm to encounter an egg in the oviduct (Suarez *et al.*, 1983).

b) Disengaging adherence of sperm from oviduct epithelium (Dermott and Suarez 1992).

c) Facilitating sperm passage through viscous or viscoelastic mucus of the oviduct and the cumulus matrix (Suarez *et al.*, 1991).

d) Facilitating sperm penetration through the zona pellucida (Carmen *et al.*, 1995).

To date only (c) and (d) have been tested experimentally and verified. Hyperactivated motility serves to generate increased force against surfaces and that this additional thrusting power facilitate sperm penetration of the zona pellucida (Carmen *et al.*, 1995). Premature sperm hyperactivation in the female tract may result in sperm exhaustion and failure of these sperm to fertilise (Lineman *et al.*, 1990).

Yanagimachi (1994) has proposed that sperm hyperactivation takes place upon completion of capacitation; suggesting that the loss or alteration of the surface coat of the sperm tail during or at the end of capacitation exposes or activates putative receptors. The receptors, when activated either spontaneously or by ligands (using some medium component), stimulates a G-protein, which in turn activates Ca^{2+} channels allowing a transient Ca^{2+} influx. It is the Ca^{2+} influx that stimulates AC to initiate cAMP-protein kinase cascade. Activated G-protein may also activate Na^{+}/H^{+} channels, allowing a rise in intracellular pH. If sperm fail to generate adequate levels of cAMP, hyperactivated motility may not be initiated and in such cases addition of membrane-permeable dibutyrly cAMP or a phosphodiesterase inhibitor such as caffeine to the medium allows the sperm to exhibit hyperactivated motility (Boatman and Bavister 1984)

1.2.4.4 Assessment of Sperm Motility

Sperm motility and the concentration of motile sperm are important indicators of fertility potential (Francavilla *et al.,* 1990). Assessment of sperm motility in particular is difficult with considerable subjective and between technician variability. There are several difficulties in categorising and analysing motility in ejaculated sperm with any clarity. Philips (1972) observed three sperm movement categories in a sample released from the mouse epididymis and from the uterus as; **i)** A circling type characterised by an asymmetrical beat. **ii)** One that proceeds on a rectilinear path. **iii)** A third which crawls along surfaces in a snake like manner. The WHO (1992) grades sperm motility as follows; rapid progressive motility, grade a: slow progressive motility, grade b: Nonprogressive motility, grade c: and Immotile spermatozoa grade d:.

Following the computerisation of sperm kinematics, several studies have shown the importance of both the qualitative and quantitative evaluation of the motile sperm population in a semen sample using computer-assisted semen analysis (CASA). Although computer assisted motion analysis of sperm motion provides comprehensive objective and reproducible analysis, the technique is rarely available and is therefore not a routine procedure for semen analysis (Katz and Overstreet 1981; Comhaire and Vermenlen 1995). In such circumstances, the WHO (1992) manual for grading sperm motility is usually adopted.

Many environmental factors may affect sperm motility and careful account must be taken of the conditions under which motility is observed. For clear observation, its

necessary to confine sperm to a narrow plane between two glass surfaces which themselves may modify the outcome by exertion of functional drag on the sperm. It is important that the volume of semen and the dimension of the coverslip used are standardised so that the analysis are always carried out in a preparation with a fixed depth of between 25 - 30 micrometres. Such depth allow full expression of the rotating movement of normal spermatozoa. A fixed semen volume of 10 to 12 microlitres on a slide covered with a coverslip of 20x20 or 24x24 mm is recommended by Comhaire and Vermenlen (1995). Birks *et al.*, (1994) have shown that the temperature at which motility is carried out greatly influences sperm motility profiles. Since sperm are exposed to a temperature of 37 ^{o}C during their passage through the reproductive tract, it has been proposed that 37 ^{o}C rather than room temperature be the standard temperature of examination in order to be more physiologically relevant.

1.2.5 Sperm Capacitation and Acrosome Reaction

Mammalian sperm released from the male reproductive tract are capable of moving actively but lack immediate capacity for fertilisation. The physiological changes underlying the acquisition of fertilisation ability collectively, have been referred to as capacitatio (Austin 1960). In vivo these changes take place in the female reproductive tract (vagina, cervix, uterus and oviduct) over a period of some hours depending on species (Bedford and Hoskin1990). However capacitation does not seem to depend in any specific way on secretions of the female tract (Yanagimachi 1994). Once capacitated, sperm can undergo acrosomal exocytosis in response to oocyte-associated molecules, penetrate the zona pellucida and fuse with the oocyte plasma membrane (Wassarman

1987;1994). Three acquired abilities of capacitated sperm are recognised; the transition from linear to a non-linear vigorous motility referred to as hyperactivated motility, the ability to bind to the zona pellucida and the ability to undergo an acrosome reaction (Katz and Yanagimachi 1980; Yanagimachi 1981; Yanagimachi 1994).

Capacitation results from poorly understood series of morphological and molecular events involving transmembrane and intracellular signalling (Luconi *et al.*,1998). One of the principle events involved in capacitation is believed to be a biochemical alteration of the sperm plasma membrane (Yanagimachi 1989; Cohen-Deyang and Eisenbach 1995). This involves removal and alteration of sperm plasma membrane components in the anterior portion of the sperm head so that the acrosome reaction can occur. Gradual removal or alteration of the peripheral glycoproteins, rearrangement of integral glycoproteins, reduction in membrane cholesterol, and changes in the distribution and composition of certain membrane phospholipids all seem to contribute to this process (Cohen-Deyang and Eisenbach 1995; Eddy 1988; Luconi *et al.*,1998; Yanagimachi 1981). Known biochemical changes occurring during capacitation include; increased levels of adenylate cyclase and cAMP, increase in intracellular pH, calcium influx, loss of surface components, modification of sperm plasma membrane and changes in the lectin-binding pattern of spermatozoa (Ahuja 1985; Fraser 1984; Lee and Ahuja 1987; Mrsy *et al.*, 1984; O'Rand 1979; Parrish *et al.*, 1994; Singh *et al.*, 1978; Vredeburgh and Parrish 1995). Capacitation is correlated with changes in sperm membrane fluidity, intracellular ion concentration, metabolism and sperm motility (Yanagimachi 1994; Myles and Primakoff 1997).

According to Cohen-Deyang and Eisenbach (1995), there are no obvious characteristics that can be evaluated to allow measurement of the transition towards the fully capacitated state. No gross morphological changes, which might be monitored easily do occur, and at present no biochemical parameters have been defined that could give such information. There are however, two terminal pre-fertilisation events which do give some indication of fertilisation potential; the acrosome reaction and hyperactivated sperm motility.

The acrosome reaction (AR) is an exocytotic event that constitutes progressive fusion and vesiculation of the outer acrosomal membrane with the overlying sperm plasma membrane with concomitant release of acrosomal matrix (Chang 1951). Since the fused membranes are lost, the AR is taken as a unique form of exocytosis which releases acrosomal contents, exposing and or modifying the inner acrosomal membrane, and modifying the sperm plasma membrane (Emilliozzi and Fenichel 1997; Galantino, *et al.*, 1997; Luconi *et al.*, 1998; Ward and Storey 1984). The functional significance of the AR is two fold; as an absolute requirement for sperm penetration through the zona pellucida, and for sperm fusion with the oolemma. (Yanagimachi, 1989)

During the AR, hydrolytic proteases associated with acrosomal matrix such as, acrosin, are released (Johnson *et al.*, 1983). As these enzymes have both zona-binding and proteolytic activities their function is to disperse cells of corona radiata and to digest the zona in order to ease sperm penetration (Topfer and Henschen 1987; Yanagimachi 1989). As the sperm head passes through the zona, it displays both slicing (side to side)

and rocking (back and forth) motion, providing the sperm head enough mechanical force which together with the enzymatic means are used for zona penetration (Carmen *et al.*, 1995; Katz *et al.*, 1988; Yanagimachi 1989). Only acrosome-reacted sperm fuse with the egg plasma membrane. During the acrosome reaction, the plasma membrane over the equatorial segment of the acrosome gains the ability to fuse with the egg plasma membrane through some as yet unclear mechanism. Abnormal sperm like round headed spermatozoa without acrosomes are unable to fuse with the egg plasma membrane (Lalonde *et al.*, 1988). The occurrence of the physiological AR is considered as the end point of capacitation, hence, ability of the sperm in a population to undergo an AR has been equated with fertilisation potential.

1.2.5.1 Sperm-Egg Interaction at Fertilization

Under *in vitro* conditions, binding of the sperm head plasma membrane to the zona pellucida involves an acrosome-intact cell (Wolf, 1989). Such binding is mediated by a specific glycoprotein receptor, ZP3 of the zona pellucida which then triggers the acrosome reaction in the zona-bound spermatozoa (Wassarman, 1987). The acrosome-reacted spermatozoon remains on the zona through a relatively weak binding of its inner acrosomal membrane to another zona glycoprotein, ZP2 (Bleil *et al.*, 1988). Sperm from a variety of mammalian species express β-1,4-Galactalsyltransferase (GalTase) on their surface (Larson and Miller 1997). Initial gamete recognition is mediated by the binding of GalTase to glycoprotein ZP3 of zona pellucida. When sperm binds to ZP3 of the zona pellucida, it triggers the AR by aggregating a receptor in a G-protein-dependent fashion. Once bound by ZP3, GalTase activates G-proteins leading to exocytosis of the acrosome

(Gong *et al.*, 1995). Released acrosome contents digest a penetration slit in the zona pellucida through which the sperm penetrates to fertilize the egg. Thus, the sequence of events, occurring in sperm prior to or during fertilisation are; transition to hyperactivated sperm motility, acrosome-intact sperm binding to the zona pellucida, occurrence of acrosome reaction and finally sperm penetration through the zona pellucida. (Wasserman 1987, Wolf 1989, Yanagimachi 1994)

1.2.5.2 Factors affecting sperm capacitation:

Capacitation *in vivo and in vitro*

Capacitation does not appear to be species-specific since the female reproductive tract can support sperm capacitation of quite different species (DeMayo *et al.*, 1980; Hirst *et al.*, 1981; Saling and Bedford 1981). The environment of the uterus and oviduct may synergize to hasten the onset of capacitation. The site where sperm begin and end capacitation may vary according to species. In species where sperm are deposited in the uterus during coitus, sperm seem to complete most parts of capacitation in the lower segment of the isthmus. In species where sperm are deposited in the vagina at coitus, capacitation may begin as sperm pass through the cervical mucus.

Capacitation can be achieved in balanced salt solutions containing appropriate concentrations of electrolytes, metabolic energy sources and serum albumin, in a composition that approximates that of the oviduct fluid (Yanagimachi 1994). To date commonly used media for IVF and *in vitro* sperm capacitation include, modified Tyrode's, Kreb's-Ringer solution and other commercial tissue culture media such as BWW (Kholkute *et al.*, 1990; Cummins *et al.*, 1991). Supplementing media with

appropriate energy sources such as, glucose, lactate, or pyruvate and correct concentrations of ions such as, calcium with optimum osmolarity, pH, and the right antibiotic are necessary prerequisites (Miyamoto and Ishibashi 1975; Miyamoto and Chang 1973; Miyamoto *et al.,* 1974; Roger and Yanagimachi 1975). On the other hand media devoid of some components such as, energy substrates or HCO_3^- and Ca^{2+} ions also supports capacitation (Fraser 1982:1985:1987; Lee and Storey 1986; Santoss and Gordon 1982). In the absence of serum albumin sperm capacitation proceeds very slowly (Uto and Yamaha 1996). This suggests that the absence of some components may affect the proportion and speed of spermatozoa that undergo capacitation. Although, no single medium has been found to support *in vitro* sperm capacitation and fertilization in all species, sperm from some species such as, the golden hamster, require a special ingredient (a sperm motility factor), other wise their sperm simply die (Yanagimachi 1970).

Temperature

Temperature difference has been found to significantly alter the physical state of sperm plasma membrane lipids and to affect sperm capacitation (Holt and North 1986). Incubation temperature of 37-38 °C is adequate to support *in vitro* sperm capacitation but in the sheep and pig, capacitation proceeds far more efficiently at the body temperature of these animals (38.7-39.7 °C) than at 37 to 38 °C (Cheng *et al.,* 1986).

Inter- and Intra-species variation

The minimum time required for sperm capacitation *in vitro* or *in vivo*, is not rigidly fixed. Some species (mice and human) are fast capacitators (within an hour) while others like rabbits take several hours (Bedford and Hoskin 1990; Lambert *et al.,* 1985).

The time varies depending on the physiological state of the animal, and the physical and chemical composition of the medium. In general, conditions that keep the sperm membranes stable prolong capacitation, whereas those conditions that destabilise membranes shorten the capacitation time. In dogs, ferrets, and horse, where insemination precedes ovulation by days rather than a few hours, their sperm are maintained in a partially capacitated state in the cervix and the lower segment of the fallopian tube. Capacitation continues to completion as sperm move towards the upper ampullary segment in the periovulatory period (Bedford and Hoskin 1990). In the rat and hamster, sperms must at least pass on into the oviduct in order to complete capacitation. While the uterus initiates the first step(s), it alone can not complete it. In the rabbit the uterus or the oviduct each can independently accomplish sperm capacitation in 10 to 15 hours (Bedford 1969). However, capacitation occurs much faster (4 to 5 hours) when sperm traverse both the milieu of the uterus and the oviduct in this species.

Epididymal sperm versus ejaculated sperm

The plasma membrane of ejaculated sperm is more stable than that of epididymal sperm rendering capacitation easier in the later (Yanagimachi 1994). Epididymal sperm were found to fertilise eggs *in vitro* more easily than ejaculated sperm (Shalgi *et al.*, 1981). Spermatozoa obtained from regions distal to the corpus have greater fertilising capacity than sperm obtained from the caput epididymis (Yanagimachi 1994). Epididymal glycoproteins and seminal plasma components bind firmly to the sperm plasma membrane, stabilising it further upon ejaculation (Hunter and Nornes 1969, Volglmayr *et al.*, 1983). Mixing of caudal epididymal sperms with accessory seminal

fluids prior to ejaculation modifies the sperm plasma membrane so that premature destabilisation of the membrane does not occur during the extended residence time in the female reproductive tract. Seminal plasmin, an inhibitor of calcium uptake, and the poorly characterised decapacitation factor(s) are some of the proteins that have been suggested to delay sperm capacitation (Rufo *et al.,* 1984 cited by Pablo *et al.,* 1995; Yanagimachi; 1994).

1.2.5.3 Cellular Aspects of Sperm Capacitation

Although mechanisms regulating mammalian sperm capacitation and the acrosome reaction are not fully understood, there has been substantial evidence to suggest that, receptor-coupled intracellular signal transduction system(s) that may proceed through a series of sequential changes in enzymes and second messengers are involved (Garbers and Kopf 1980; Yanagimachi 1994: Plabo *et al.,* 1995). To date, the definitive understanding subcellular changes on sperm capacitation are not well understood. (Cohen-Deyang and Eisenbach 1995). However, it is known that the sperm surface has an active role in the event of fertilisation. The definition of sperm surface in both its composition and domain organisation begins during spermatogenesis and continues until the moment of sperm-egg fusion. Alteration of the sperm surface proceeds as a result of internal programming and environmental cues from both the male and female reproductive tracts including interactions with the egg (Myles and Primakoff 1997). Capacitation has been shown to be accompanied by changes in sperm membrane composition and structure, as assessed by changes in the distribution of intramembranous particles such as, membrane phospholipids (cholesterol) and glycoproteins (Davis 1980;

Davis *et al.*, 1979). These workers demonstrated that the cholesterol/phospholipid ratio of the sperm plasma membrane decreases upon incubation of sperm *in vitro*, suggesting that such a change facilitates the transformation towards capacitation. This change may influence membrane fluidity which increases the rate at which intrinsic programmed membrane events such as, changes in sperm ionic movements, metabolism and motility that follow capacitation occur.

Although the molecular basis of mammalian sperm capacitation is still poorly understood, the involvement of the diacylglycerol and protein kinase C pathway and the adenylate cylcase/cyclic adenosine-3'5'-monophosphate (cAMP) pathway has been demonstrated (De Jonge *et al.*, 1991). It has been shown that under a variety of different incubation conditions, capacitation is correlated with tyrosine phosphorylation of a subset of proteins of molecular weight 40-120KDa (Galantino, *et al.*, 1997; Plabo *et al.*, 1995,). According to Plabo and colleagues (1995), components of culture media such as, Ca^{2+}, $NaHCO_3$ and serum albumin that are required to support capacitation also play an important obligatory role in bringing about changes in protein tyrosine phosphorylation (PTP) in a time and concentration dependent manner. Calcium, serum albumin, and $NaHCO_3$ act to induce PTP during capacitation and in turn protein tyrosine phosphorylation may ultimately be important in controlling events leading either to the capacitated state or to events that occur as a consequence of this maturational process. The pattern of PTP appears to be regulated by a cyclic adenosine monophosphate (cAMP) pathway which is correlated with the time course of capacitation. Protein tyrosine phosphorylation can be induced by heparin and or other biologically active

cAMP analogues such as, dibutyryl cAMP,(db cAMP), 8-bromo cAMP, and the phosphodiesterase inhibitor 3-Isobutyl-1-Methyl Xanthine (IBMX) (Galantino,, 1997). This cAMP regulation of PTP is mediated by protein kinase-A (PKA). The ability of cAMP agonists to enhance, and cAMP antagonists to inhibit, both capacitation and the pattern of protein tyrosine phosphorylation supports the hypothesis that mammalian sperm capacitation is mediated by a signalling pathway that involves cross-talk between cAMP/PK-A and tyrosine kinase/phosphatase signalling pathway (Galantino, *et al.*, 1997; Leclerc *et al.*, 1996)

Capacitation requires the presence of serum albumin and $NaHCO_3$, (Boatman and Robbins 1991; Dow and Bavister 1989; Go and Wolf 1985; Lee and Storey 1986). Serum albumin which serves as an extracellular sink for sperm membrane fatty acids and or cholesterol efflux is the major protein in the female genital tract secretions and is important both during *in vitro* and *in vivo* sperm capacitation (Davis 1980; Dow and Bavister 1989; Go and Wolf 1985). The resultant alteration in membrane fluidity and membrane destabilisation accelerates ionic changes that ultimately result in sperm capacitation and the obsevered protein tyrosine phosphorylation. Although these changes occur *in situ* following sperm residence in the female reproductive tract, and could therefore be modulated by reproductive tract factors such as, pH, pO_2, and pCO_2, *in vitro* incubation of sperm in relatively simple defined media totally supports capacitation. Davis *et al.*,(1979) and Yanagimachi (1994), have suggested that the initiation and completion of capacitation may be regulated by processes intrinsic to the sperm cell itself.

1.2.5.4 Postulated Mechanisms of Acrosome Reaction

The dynamics of the cell surface during the process of capacitation and acrosome reaction have been demonstrated by Topfer *et al.*, (1990). At fertilisation, the sperm releases its acrosomal contents in a calcium-dependent process known as the acrosome reaction. In mammals, this process is slow and therefore amenable to biochemical dissection (Harrison and Roldon 1990). Labelling sperm phospholipids with phosphorus (^{32}P) and subsequent induction of AR with calcium ionophore A23187 has shown that membrane fusion occurs as a result of a sequence of events following Ca^{2+} entry (Harrison and Roldon 1990).

Membrane phospholipids are under the control of dynamic turn over mechanisms whereby newly synthesised phospholipids continuously replace existing molecules. Membrane phospholipids usually undergo a catabolic deacylation reaction catalysed by phospholipase A_2 to give glycerylphosphorylcholine, which in turn is broken into free fatty acids and lysophospholipids under the same enzyme (Selle *et al.*,1993; Tijburg *et al.*,1991). Increasing levels of lysophospholipid can have lytic effect (Sedhis *et al.*,1988). Hence the concentration of these potentially lethal compounds must be tightly regulated.

In spermatozoa, the major pathway to regulate the levels of lysolipids is via their hydrolysis by lysophospholipase (Atreja and Anand 1985). Lysophosphotidylcholine (LPC) has long been known as an inducer of the acrosome reaction in sperm (Flemming and Yanagimachi 1981). The levels of lysophospholipase, (an enzyme that breaks down lysolipids) and the modulation of its activity are important factors in controlling the flux

of lysophospholipids. Immunoflourescent localisation of the enzyme indicated that the enzyme is located on the membrane overlying the acrosome as well as on the inner acrosomal membrane of the sperm head (Nathalie and Roberts, 1995). Lysophospholipids were implicated in the cascade leading to the acrosome reaction, following the observations that increased degradation of these lipids produced by addition of lysophospholipase significantly reduced the acrosome reaction while exogenous lysophosphatidylcholine enhanced acrosomal exocytosis (Roldon and Fragio 1993).

Polyclonal antibodies, against lysophosphatase led to an increase in the AR (Nathalie and Roberts, 1995), suggesting that inhibition of lysophospholipase produces a higher lysophosphstidylcholine concentration and results in level similar to that obtained by calcium and calcium ionophore A23187. A local increase in the concentration of lysophospholipids is conducive or necessary to the membrane fusion seen during acrosome reaction. However, the lysophopholipase studied by Nathalie and Roberts, (1995) was neither stimulated nor inhibited by 1 mM Ca^{2+}. In addition, enzyme activity did not change in the presence of ethylene diamine tetra acetic acid (EDTA), a calcium chelator. Contrary to this, Harrison and Roldon (1990) had shown that the AR is initiated by or preceded by a large scale breakdown of polyphosphoionositides catalyzed by a calcium dependent phospholipase C (PLC) to produce diacylglycerol (DAG). By itself DAG seemed neither to act directly as a fusogen nor acted through a protein kinase C, but was an essential product stimulating a later process. Harrison and colleague (1990), have postulated that DAG appears to simultaneously activate phospholipase A_2 and inhibit lysophosphotide acyl transferase (LPAT) to cause a large scale build up of fusogenic

lysophospholipids in the acrosomal region and calcium may bring about membrane fusion when the levels of these lipids have risen above a necessary threshold.

Another school of thought has proposed that when ZP3 of the zona pellucida binds to the sperm receptor Rzp, it causes an aggregation of these receptors with resultant activation of Rzp (Leyton and Saling 1989). Activated sperm receptors in turn trigger the reaction cascade that leads to the AR. Activated Rzp stimulates PLC in the sperm plasma membrane which in turn cleaves phosphatidylinositol diphosphate (PIP2) into DAG and Inositol triphosphate (IP3); subsequently, IP3 increases intracellular concentration of Ca^{2+} ions from the intracellular stores while DAG activates calcium-dependent protein kinase C (PK-C) which in turn phosphorylates proteins. Part of IP3 is then methylated to become inositol tetraphosphate (IP4) which regulates the opening of voltage-dependent Ca^{2+} channels (Florman *et al.,* 1992), thus allowing a massive influx of extracellular Ca^{2+} ions. Some of the Ca^{2+} acts on the membrane phospholipids directly to facilitate membrane fusion. At the same time the activated G-protein also stimulates phospholipases A_2 (PLA_2) which cleaves phosphotidylcholine (PC) into lysophosphotidyl choline and arachidonic acid both known to be highly fusogenic (Flemming and Yanagimachi 1981:1984). Simultaneously, phospholipase D activated by G-protein cleaves PC to choline and phosphatidic acid (PA), which is also fusogenic. Calcium itself is fusogenic by neutralising a negative charge of membranes, causing phase transition and phase separation of membrane phospholipids (Yanagimachi 1981). The activated G-protein also triggers another chain reaction, by activating adenylate cyclase (AC), which in turn stimulates cAMP production (Brandelli 1997). Protein kinase which is cAMP-dependent

phosphorylates proteins essential for the AR. Some of the cAMP acts on gated Na^+ channels to allow Na^+ influx and H^+ efflux, bringing a rise in intracellular pH. Influx of Ca^{2+}, a rise in the intracellular pH and the production of the fusogenic compounds mentioned above, are all believed to be essential for the AR.

1.2.5.5 Induction of Acrosomal Loss

Because the proportion of sperm that undergo spontaneous acrosomal reaction is relatively low, acrosomal loss has been induced following exposure to biological and physiologic agents such as cumulus cells, follicular fluid, zona pellucidae and physicochemical agents such as calcium ionophores, lysophosphatidylcholine and electro-permeabilisation (Avrech *et al.*,1997; Flemming and Yanagimachi 1981; Wolf 1989). According to Yanagimachi, (1989) the site and cause of the acrosome reaction is not as important. For as long as sperm are alive and acrosome-reacted, they will fuse with the egg plasma membrane. It is the rate and the mechanism of induction at which each of these reagents precisely act to initiate acrosomal loss that is little understood.

1.2.5.6 Assessment of Sperm Capacitation and Acrosome Reaction

Several techniques have been used to study sperm capacitation and acrosomal status at microscopic level including; triple stain technique, indirect immunofluorescence using mono/polyclonal antibodies and labelling sperm with fluorescein probes such as lectins or chlortetracycline (Cross *et al.*, 1986; Lee *et al.*, 1987; Talbot and Chacon 1980:1981; Wolf *et al.*, 1985). In spite of this, Cohen-Deyang and Eisenbach (1995) argue that a definitive

assay to identify capacitated sperm is still at large, because of lack of a clear cut definition of a capacitated spermatozoon.

There are two classes of fluorescent probes for the acrosome: those that detect intra-acrosome-associated material (and therefore require the cell be permeabilized before labelling) and those that are used on living, nonpermeabilised cells. In the first category are lectins and antibodies to intracellular acrosome antigens; in the second category are chlortetracycline, and antibodies to externally exposed antigens (Cross and Meizel 1989). Labelling permeabilized sperm with internally directed probes renders acrosome-intact cells brightly fluorescent over the anterior sperm head and for many of these probes, the equatorial region as well. Acrosome reacted sperm have either no acrosomal labelling, or only an equatorial band of the label. Lectins are the most accessible reagents of this group; they bind to glyco-conjugates of the acrosomal matrix or the outer acrosomal membrane (Cross *et al.*, 1986; Mortimer *et al.*, 1987). Many lectins such as, *Ricinus communis agglutinin, Pisum sativum agglutinin* (PSA) and *Peanut agglutinin* (PNA), conjugated with fluorescein isothiocyanate (FITC) are available for use (Cross *et al.*, 1986; Mortimer *et al.*, 1987; Talbot and Chacon 1980). Permealibised sperm can also be labelled by indirect immunofluorescence with antibodies (polyclonal or monoclonal (mAbs)) directed against intracellular, acrosome-associated antigens (Cross *et al.*, 1986; Kallajoki *et al.*, 1986; Wolf *et al.*, 1985). The other group of fluorescein probes consist of reagents that are used without permeabilizing the cells. There are two types of mAbs that can reveal the acrosome status of unfixed, non-permeabilized sperm described by Saling *et al.*, (1985), and these are; one type which reacts with the equatorial region of reacted, but not intact sperm and the second type which reacts with the plasma membrane overlying the acrosome, so that labelling is

lost after the acrosome reaction. Chlortetracycline (CTC) a fluorescent antibiotic, is also used on nonpermeabilized cells and produces fluorescent patterns that reflect acrosomal status. The nature of the CTC signal is not understood in detail, but is related to the increased fluorescent emission when CTC binds to a divalent cation such as, calcium in a nonpolar environment (Saling and Story 1979). The Ca^{2+}-CTC complex bound to sperm membranes shows greatly enhanced fluorescence; however the nature of the membrane groups that bind the complex and the changes involved are unknown (Lee *et al.*, 1987). Capacitation is generally conceded to result in a change in these membrane groups reflected by a change in fluorescent patterns. Chlortetracycline can be used on unfixed sperm or the sperm can be fixed simultaneously in glutaldehyde (Lee *et al.*, 1987; Saling and Story 1979). Chlortetracycline patterns vary among species, so the use of CTC in a new species requires assignment of the relevant patterns. Chlortetracycline pattern correlates well with transmission electron microscope for mouse sperm and with both the triple stain and mAbs labelling for human sperm (Endo *et al.*, 1988; Lee *et al.*, 1987). After CTC staining the distribution of the fluorescence on the sperm head allows for the identification of capacitated spermatozoa in many species (Das Gupta *et al.*, 1993; Fraser and Herod 1990; Varner *et al.*, 1987).

1.2.5.6.1. Advantages and Disadvantages of Various Assay Systems

Lectins and antibodies produce labelling of greater intensity and contrast than do coloured stains. Antibodies are of particular importance when or where the cells are surrounded by glyco-conjugates to which lectins would bind and obscure the sperm; however indirect immunofluorescence assays require more time to complete (from 0.5 to

3.5 hours) than does lectin labelling. Some fluorescent lectins and antibodies have the advantage that the assay can be delayed until a more convenient time, for example, Cross and Meizel (1989) found that, incubation of sperm with FITC-PSA or monoclonal antibody (mAb) HS21 can be delayed for several days without interference with acrosomal labelling as long as sperm are kept in fixative at 4 ^{o}C until labelled. In order to assess viability, a supravital fluorescent nuclear stain, Bis-benzimide (H258) should be included in the FITC-PSA assay (Cross *et al.*, 1986). However, separate filter pairs are required to view the two probes individually. Alternatively, with a microscope capable of producing sufficiently intense fluorescence, the two fluorochromes can be visualised simultaneously as blue (H258) and yellow (FITC-PSA).

Although staining permeabilized sperm with fluorescein labelled lectins such as PSA is a rapid and simple assay for acrosomal status, fluorescence appears to decrease when acrosome reaction is induced with calcium ionophore (Cross and Watson, 1994). Besides, it does not allow monitoring of capacitation in with time, *Pisum sativum agglutinin,* which binds intensely to anterior and equatorial acrosomal regions and weakly to the other regions of the sperm, is suitably specific for intracellular, acrosome-associated glyco-conjugates (Cross and Watson 1994). In addiction, lectins can not be used in the presence of glyco-conjugates such as those found in cervical mucus or zona pellucida components because they lack specificity, and would therefore obscure the labelling pattern of spermatozoa.

1.2.6 Rationale and Specific Objectives

In the developing countries, human induced pressures associated with land urbanisation and agriculture, mineral resource and timber exploitation are steadily reducing the areas available to wild primates. It is only a matter of time before the numbers of even the most common species become threatened with extinction. In general, intensive destruction of suitable primate habitat has resulted in accelerated process towards extinction of primate species. Habitat loss and degradation, bushmeat hunting, illegal trade and timber have become major threats to African primates. Information is lacking on how many individuals of each species of primate are captured and die, or how many are exported illegally or killed due to crop raiding. In addition there is insufficient quantitative data on the conservation status of wild population and sub-populations of African primate species and subspecies. The size, distribution and main threats to those species suspected to be in most danger of extinction is only slowly accumulating (Batynski 1996). Such information is urgent for assessing conservation status, and thus, prioritising conservation strategies.

Conservation measures have traditionally recommended habitat protection and translocation of species at risk to protected areas, in the face of continuing advance on forest clearance due to expanding human population. This has taken the form of forest conversion of natural habitat to pasture and agricultural fields (Elastrada and Rosamond 1988; Karere *et al.*,1997). In Kenya De Brazza's monkeys and the Tana magabeys are threatened with extinction for similar reasons (Batynski 1996; Brennan 1985; Karere *et al* 1997). Data on gamete physiology or reproduction in general in these species is not

available, work on these species has focused on ecological and behavioural studies, (Sabater, 1984, Horn, 1987) as cited by Ehardt (1988) and (Karere, *et al.*, 1997). Among other strategies, fertility and reproductive success can be improved by application of techniques used in semen collection, analysis, preservation and artificial insemination which have benefited livestock. With the development and application of reproductive technologies that are currently being utilised in livestock and human operations, the same potential exists as a fast reproductive conservation approach to endangered species. It is integration of assisted reproductive techniques (ART) with traditional propagation strategies that is essential to help safeguard the genetic health and future survival of wild life species as discussed by Lasley *et al.*,(1994). Such strategy requires good knowledge on gametes *in vitro* studies which is still lacking for many primate species. Therefore, steps to plan breeding programs are necessary and understanding gamete physiology of the species concerned is the first approach along this line.

1.2.6.1 Specific objectives

(i) To retrieve semen by electro-stimulation per rectum (Rectal probe electro-ejaculation).

(ii) To examine physical parameters of semen specifically; sperm concentration and morphology, sperm motility, pH and volume of semen, coagulum, viscosity, and the round cell population in ejaculates to give baseline data and or identification of possible male factor attributes.

(iii) To compare acrosomal status in spontaneously and chemically induced acrosome reaction of sperm by staining with FITC-PSA.

CHAPTER TWO

2 MATERIALS AND METHODS

2.1 Electro-Ejaculation and Physical Parameters of Semen

2.1.1 Animals of Study

Mature monkeys, Tana Mangabeys (*Cercocebus galeritus* n=4) code named [G15, G20, G24, G27] and De Brazza's monkeys (*Cercopithecus neglectus* n=3) code named [D44, D45, D46] of breeding age were recruited for electro-ejaculation. Acquisition data showed that their age was in the range 9-19 years old, with body weight in the range 8.4-13.8 kg. The Tana Mangabeys and D46 were colony-bred while D44 and D45 were wild-caught and had stayed at the colony for at least 11 years. Among the Tana Mangabeys, only G20 had sired offsprings. All the De Brazza's monkeys had sired at least two offsprings each.

The monkeys were removed from their outdoor family-group cages to indoor co-joined single cages in order to facilitate restraint and handling (anesthesia and food restriction prior to electro-ejaculation). Records showed absence of major health ailments or clinical operation. During captivity, sanitation and health care were maintained according to routine standard conditions, established at the Institute of Primate Research (IPR) (Chai, 1993). Animals were fed on monkey cubes (Unga Feeds, Nakuru, Kenya), and fruits such as bananas and vegetables. Water was available *ad libitum* and artificial lighting with fluorescent tubes was provided for 9.5 hours per day during the period of electro-ejaculation.

2.1.2 Semen collection

2.1.2.1 Anesthesia

Food was with held both the previous evening and the morning of semen collection. Animals were sedated by intramuscular injection of a mixture of ketamine (10 mg/kg), and xylazine (5 mg/kg) in a mixture ratio of 1:20 per volume (that is, 50 mg/ml xylazine, and 100 mg/ml ketamine respectively). The mixed anaesthetic was administered at a single dose of 0.1 ml/kg per animal each time before electro-ejaculation, and this lasted the whole duration of semen collection/ electro-ejacution. The weight of the monkeys were measured by a weighing balance (Avery type 3303-COB. Birmingham, England) every time electro-ejaculation was done.

2.1.2.2 Aseptic procedure

Since clipping prepucial hairs reduces the degree of contamination by 67-98% (Roussel and Austin, 1968) as cited by Wildt (1986), after mild anaesthesia, in order to minimise bacteria contamination, the scrotal-genital and penile areas were clipped and later disinfected with cotton wool soaked in 70% alcohol.

2.1.2.3 Ejaculations

With the animals on lateral recumbence on a surgical table, a three electrode probe 1.7 cm diameter and 7.2 cm in length (Carol 18/2 SV Type UL CSA) initially lubricated with Vaseline jelly (Vaseline, Elida Pond's EA. Industries) was carefully inserted transrectally so as to lie dorsal to the area around the intrapelvic accessory sex glands. Stimulating current was manually applied by means of a rechargeable SPE electro-ejaculator, (Standard Precision Electronic, Inc. USA: Appendix V). One operator controlled the stimulus input and probe positioning while an assistant handled the penis ready for semen collection. Current stimulation was generated in a sinusoidal pattern/manner starting at 0 volts,

increasing to a peak (25 to 50 volts), pausing there, then rapidly decreasing to 0 volts. The duration of each segment in seconds was quite variable depending on animal response seen as muscular contraction, vocalisation and hind limb extension. In no case was the 50 volt peak exceeded. Frequency of stimulation was increased when urethral wetting was observed. Semen was collected by manually massaging the penile urethra to fully express all the ejaculate into sterile corning plastic tubes (Fisher scientific, cat #25331-50 Pittsburgh USA). Electro-stimulation was done to obtain one ejaculate when the animals responded and no further electro-stimulation attempts were made after obtaining the first ejaculate or when the monkeys exhibited signs of exhaustion. Frequency of electro-ejaculation (Appendix IV) was dictated by the physical status of the animals between May and September 1997.

2.1.3 Determination of Semen Physical Parameters, (Baseline Data)

2.1.3.1 Macroscopic Examination

Liquefaction

The semen was allowed to stand for 10 - 15 minutes at room temperature to observe for liquefaction.

Semen Appearance and Consistency/Viscosity

The appearance of the semen sample was noted before and immediately after liquefaction within 20 minutes of ejaculation by simple inspection. The colour and degree of opacity was recorded. Presence or absence of debris or grains was noted. For estimation of viscosity, a sterile tip of an eppendorf pipette P20, was introduced into the sample on a slide and observation for the approximate length of the thread that forms on withdrawal of

the tip recorded. The 2 cm length mark (WHO 1992) for human spermatozoa was used as standard.

pH:

Seminal pH was measured with pH paper (special indicator strips, pH 4.5 to 10.0; Sigma chemical co. St. Louis Mo.) by placing a drop of semen on indicator strips. Reading was done within 20 minutes of ejaculation but while the strip was still moist, approximately 30 seconds.

Weight/Volume Measurements:

Where liquefaction took place, the weight (g) of the coagulum was obtained from the computation below, otherwise the volume (in microlitres) of the semen was measured by an eppendorff pipette, P100 (with a disposable tip) by aspirating and subsequently adjusting the volume settings of the pipette. Because of the difficulty in measuring small volumes in microlitres, of a very viscous material such semen using a pipette, it was additionally considered to weigh the semen collected on weighing balance. This was particularly so with semen obtained from De Brazza's monkeys that comprised of jelly-like coagulum. Weight of empty tube (W_1), Weight of tube, semen and coagulum (W_2), Weight of coagulum and tube (W_3), Weight of semen ($Wt_{sem,}$) were all measured by a digital balance (Sartorius Gotting German). The liquefied portion was aspirated into fresh 15 ml centrifuge plastic tubes (fisher scientific, pittsburgh USA.) while the weight of the coagulum in the collecting tubes was recorded.

2.1.3.2. Microscopic Investigation

Estimates of sperm concentration, and initial sperm motility and the presence of debris from fresh semen were determined under positive phase contrast microscope.

2.1.3.2.1 Initial sperm motility estimation

Sperm motility was estimated on a slide from a drop of semen extended in modified Tyrode's salt (5µl semen: 100µl medium) on phase contrast microscope with a prewarmed stage at 37 ^{0}C. The preparation was then examined at x 32 objective. Sperm motility was graded according to a manual for the examination of Human Semen and Sperm-Cervical Mucus Interaction (WHO 1992).

2.1.3.2.2 Sperm concentration

The concentration of spermatozoa (millions/ml) was determined using an improved Neubauer haemocytometer method. Sperm diluent was made from 25g $NaHCO_3$, 5 ml of a 35%v/v formalin solution and adding distilled water to give a final volume of 500 ml. For use, either 1:100 or 1:200 sperm dilution (that is, 5 µl semen : 495µl or 995 µl diluent) was made from the fresh sample for estimation of sperm concentration in Q-vet glass tubes. This was mixed well by hand shaking several times before loading 5 - 8 µl on both chambers of a haemocytometer, initially covered with a glass cover slip. The haemocytometer was allowed to stand for 2-3 minutes in order to allow sedimentation of sperms before mounting on a microscope stage for counting. Counting was done at × 25 objective on a light microscope following guide lines given by Comhaire and Vermeulen (1995) and the WHO (1992) Laboratory Manual for examination of human spermatozoa.

2.1.3.2.3 Other Cell Types in Semen

The round cell population, (epithelial cell form urethral tract, leukocytes or immature germ cells) were determined from papanicouloau stained smears on slides. By scanning many fields of view, in a battlement pattern at the smear tip the number of these cells encountered per 200 spermatozoa was recorded.

2.1.3.2.4 *Evaluation of Differential Sperm Morphology:*

For morphological analysis of spermatozoon, papanicolaou stain modified for sperm was used. A smear was made from fresh semen (Mangabey) or from the liquefied fraction (De Brazza's monkeys), air dried for 10 to 20 minutes before fixation in equal volumes of 95% ethanol and ether for eight minutes at room temperature. Fixed smears were stored in sealed plastic containers/boxes at room temperature for two weeks before staining. Staining was performed according to the protocol given by the WHO (1992) for examination of human spermatozoa with modifications. At least 200 spermatozoa were scanned for observation on a light microscope under ×100 oil-immersion objective. Only recognisable spermatozoa were considered for differential morphology count. For each sperm encountered, its morphology was recorded either as normal or abnormal and accordingly, the nature of defect on the head, mid piece, or tail was noted. Sperm were considered to be normal if they exhibited an oval shaped-head with a regular outline and an acrosomal zone covering one third of the head surface (WHO 1992).

2.1.4 Data Analysis

The mean values of semen parameters are expressed as standard deviation (SD), standard error (SE), Coefficient of Variation (CV), maximum and minimum values. Comparison between means of the semen parameters were made using unpaired student 't'-test at 5% level of significance. Pearson's coefficient of correlation (r), was computed for paired semen parameters that were considered.

2.2. *Experiment I (Induction of Acrosome Reaction)*

2.2.1 Preparation of Reagents and Sperm Suspension.

2.2.1.1 Preparation of Spermatozoa suspension:

Spermatozoa used in this experiment were obtained by electro-ejaculation from the Tana Mangabeys. A motile sperm fraction was selected by 'direct swim up' from fresh semen by modifying Moohan and Lindsays'method (1995) for human specimen. The semen was carefully transferred to the bottom of 15 ml plastic centrifuge tubes by an eppendorf pipette P100, where it was thoroughly mixed with 30µl of pre-incubated modified Tyrode's salt (TALP-hepes media). Then 1000µl of media, preincubated at 37 ^{0}C in 5% CO_2 : 95% humidified air was carefully overlaid by a pipette without disturbing the bottom sediment. The tubes were then incubated at an inclined angle to the vertical for 45 to 60 minutes at 37 ^{0}C, in 5% CO_2: 95% humidified air in order to allow actively motile sperm to swim to the top surface. At least 500 - 800 µl of the top motile fraction was harvested by a pipette and put into fresh 15 ml centrifuge tubes. To this, 1 ml of fresh medium was added, mixed gently by hand shaking and centrifuged at 500*g* (Beckman model TJ-6R centrifuge) between 18 - 20 ^{0}C for 8 minutes to wash sperm in the motile fraction. The supernatant was discarded, and fresh media, 500 - 800 µl carefully overlaid on the pellet. The tubes were loosely capped and returned to the incubator for another hour. A high proportion of washed motile sperm was then obtained by pipetting 500 µl of the top swim up so obtained to another tube. This was further maintained in the incubator for 1 hour before challenging them to dbcAMP activator and calcium ionophore A23187.

2.2.1.2 *Fluorescein Isothiocyanate-Pissum sativum agglutinin (FITC-PSA)*

Pissum sativum agglutinin conjugated with fluorescein Isothiocyanate (FITC-PSA), containing 2.5 moles FITC per mole of lectin (from Sigma chemical Co. St. Louis USA) was dissolved in Dulbecco's phosphate buffer saline (DPBS) at 2 mg per ml. The stain was stored in the dark at -20 °C in aliquots. For use, the stain was diluted 1: 15 with DPBS and used immediately.

2.2.1.3 *Dibutyryl Cyclic Adenosine Monophosphate (db cAMP).*

Dibutyryl cyclic adenosine monophosphate (N6, 2'-O-Dibutyryl adenosine 3'5-cyclic monophosphate (from Sigma chemicals Co. St. Louis USA) originally stored at -20 °C was used. A 10 mM solution of dbcAMP was prepared by dissolving 0.005g dbcAMP in 1 ml of modified and equilibrated Tyrode's salt [TALP-hepes: (Appendix I)]. This solution was diluted 1:10 with sperm suspension prepared by direct swim up (DSU) to form a final concentration of 1.0 mM dbcAMP activator sperm suspension. (That is; 10 µl of 10 mM dbcAMP : 90 µl of sperm suspension). For the control tubes equal volumes of sperm /TALP-hepes suspension were prepared but without dbcAMP.

2.2.1.4 *Calcium Ionophore A23187.*

Vials in aliquot of 10µl containing 10 mM stock solution of calcium ionophore A23187 (Cal A23187) originally stored in the dark at -20 °C were available. This stock solution had been prepared by dissolving 0.01g of free acid Cal A23187 in a mixture of 100µl of dimethyl sulfoxide (DMSO) and 1.8 ML of 99.7% absolute ethyl alcohol as a solvent. For use, to make a working solution of Cal A23187 for the induction of

acrosome reaction, the stock solution was diluted as follows; The 10 mM stock CaI A23187 was diluted 1:100 in TALP-hepes, thus; (10 µl stock CaI A23187 : 990 µl TALP-hepes = 1000 µl of a 0.1mM or 100µM CaI A23187 solution. The 100µM CaI A23187 solution was then diluted 1:20, with sperm suspension originally mixed with dbcAMP activator above and put in 15ml plastic centrifuge tubes. Thus; (10 µl of 100µM CaI A23187 : 190 µl dbcAMP-activator-sperm suspension = 5 µM CaI A23187). For the negative control, similar procedure using the solvent DMSO but without CaI A23187 was done in separate tubes. The centrifuge tubes were loosely capped, held in a vertical position in a stand and then transferred to the incubator. The sperm suspensions (both control and experimental) were further incubated in 5 % CO_2 : 95 % humidified air at 37 ^{o}C as previously described, and aliquots drawn at 30 minutes time interval over a period of 2.5 hours for preparation of spot slides

2.2.1.5 Spot Slide Preparation and staining

Aliquots (20 microlitres) of sperm suspension were pipetted onto clean spot slide wells in duplicates where sperm motility was checked before drying on a warm microscope stage at 37 ^{o}C. The slides were then fixed in methanol for five minutes. Excess methanol was wiped by touching the edge of the slides with tissue paper, and the slides were immediately dipped once in a beaker of DPBS. The slides were then dried on a warm microscope stage before putting them in a dry clean plastic box which was kept at 4 ^{o}C for overnight storage.

Tissue paper moistened with phosphate buffer saline (DPBS) was overlaid at the bottom of a light proof slide box. The box was then covered with a black polythene paper.

Spot slides were removed from storage, and their wells overlaid with DPBS for two minutes after which excess DPBS was carefully wiped by touching edges of the slides with tissue paper. Slides were then put in a slide box and 20 - 25 µl of FITC-PSA stain introduced per well. The box was then closed and placed in the dark at room temperature for one and a half hours. After this period, excess FITC-PSA was carefully wiped off the spot slides with tissue paper and the slides subsequently rinsed three times by dipping them in three jars containing fresh DPBS each time for 10 minutes, using tissue paper to wipe DPBS between jars. Five microlitres of a thawed PPD antiquench, originally stored at -20 °C was pipetted on each slide well to minimise fading of the stain, before covering the slides with 25 x 25 mm coverslipes. Slides were read immediately by counting 200 spermatozoa for both control and experimental wells at x100 objective oil immersion using a fluorescent microscope (Leitz Dialux 22EB German). For each well, serially arranged at 30 minutes time interval, the number of sperm staining with a given pattern were counted by a manual four-digit tally counter after scanning through many fields.

2.3 Data Analysis

Spermatozoa were counted for each pattern of staining for both control and experimental specimens and the means used for test of significance at five percent level, using student 't' test at every 30 minutes time interval as described by Medhi (1982). Mean percentage sperm counts per pattern were plotted against time.

CHAPTER THREE

3 Electro-Ejaculation and Semen Analysis in Tana Mangabey and De Brazza's monkeys'

3.1 Introduction

Traditionally, the quality of semen is evaluated by examining physical parameters such as, semen volume, the grade and percentage of sperm motility, the prevalence of spermatozoa abnormalities and sperm count to assess male fertility potential (Kumi, and Badtram, 1994; WHO 1992). Although these visibly measurable characteristics have low correlation and low predictive values to male fertility, they remain the most available means of male assessment for potential fertility (Amann, 1989). Semen evaluation also allows male fertility comparison. Semen preservation is of value in insemination of females that can be induced to ovulate in the non breeding season, and for the transport of semen for use in artifitial insemination at distant centres. Preservation of sperm can ensure perpetuation of outstanding sires and or species that are ecologically endangered.

In Kenya, the Tana Mangabey (*Cercocebus galeritus*) and De Brazza's monkeys (*Cercopithecus neglectus Schlegel*) are endangered (Karere *et al.,* 1997; Batynski, 1996). Captive colonies at the Institute of Primate Research have not bred well. There is no data on the spermiogram about these primates yet, this datum is vital should a strategy involving assisted reproductive technique be applied. Studies on semen analysis and assessment of fertility are therefore needed. This study addressed recovery of semen by electroejaculation for evaluation in the two species to establish base line data and to explore male fertility potential from spermatozoa.

3.2 Results

A total of 59 electro-ejaculations between 5 to 10 per monkey (Appenidix III) were performed in order to retrieve semen from Tana Mangabeys and De Brazza's monkeys kept under captivity. In the course of the study, Mangabey code named G27 developed orchitis and was discontinued while De Brazza code named 46 died. The period of time required to electroejaculate De Brazza's monkeys was more 15 minutes while that for Tana Mangabey was less than 10 minutes. Erection was achieved in all attempts although with difficulty and irregularity among De Brazza's monkeys. In all cases ejaculate were non projectile necessitating stripping/ milking the semen from the penile urethra to collecting tube. Of the 33 attempted electro-ejaculations in the Tana Mangabey, 26 (78.7%) resulted in ejaculates (semen), while 7 (21%), produced no ejaculates. Similarly, with the De Brazza's monkeys of the 26 attempts, 17 (65.3%) ejaculated while 8 (30.7%) failed. In all ejaculates, semen from the Tana Mangabey was opaque, milky yellow or creamy in colour and homogenous in consistency without coagulum or liquefaction within 20 minutes of collection at room temperature. Among the Tana Mangabey, twelve out of 17 (70.6%) samples had a viscosity less than 2 cm, while 5 (29.4%) had a viscosity greater than 2 cm. Among the De Brazza's monkeys all ejaculates obtained had high viscosity (> 2cm). Ejaculates from De Brazza's monkeys were characterised by an initial secretion of a highly viscous fluid devoid of sperms followed by a whitish or colourless secretion before emission of a semi-solid jelly-like white mass or sometimes strips of coagulum. This ejaculate underwent liquefaction within 15 minutes of collection to form a clear colourless liquefied fraction leaving coagulum as a small clot. With the exception of animal number G15, all ejaculates were devoid of debris among the Tana Mangabeys while debris was a common finding in 11

(64.7%) out of the 17 ejaculates in De Brazza's monkeys. Other characteristic of semen from the two species are shown (appendix IV). The seminal plasma pH was in the range of 8.0 to 9.0, mean ± s.e.m 8.4 ± 0.07 in De Brazza's monkeys and 8.3 ± 0.05 in the Tana Mangabey monkeys.

Sperm motility recorded as percentages mean ± s.e.m was, grade **a**; 65 ± 3, grade b; 13.5 ± 2.4, grade **c**; 10.2 ± 1.2 and grade **d**; 10.2 ± 0.6 in mangabeys. Meanwhile for De Brazza's monkeys, mean percentage sperm motility was; grade **a**; 50 ± 2 grade **b**; 11.5 ± 1.7 grade **c**; 14.2 ± 1.3 and grade **d**; 23.2 ± 2.6 respectively.

Semen volume and weight

The weight (g) of the fresh semen in both species, plus the weight of liquefied fraction for De Brazza's monkeys was recorded per ejaculate. The mean weight, approximate age and semen parameters of individual monkeys are shown in tables 1 and 2. There was a strong positive correlation $r = 0.94$ ($p < 0.05$) between the weight of the semen and its volume in the Gary Mangabey. However there was no correlation $r = 0.067$ ($p = 0.7$) between sperm concentration and the ejaculate volume. Likewise the weight of fresh ejaculate was not correlated to the sperm concentration $r = 0.1$ ($p = 0.6$). Among De Brazza's monkeys, a correlation of $r = 0.96$ ($p<0.05$) was found between the weight of fresh ejaculate and the volume of the liquefied fraction. The weight of the liquefied semen fraction was similarly positively correlated ($r = 0.92$, $p<0.05$). to the fresh ejaculate. In addition there was a correlation ($r = 0.88$, $p< 0.05$) between the weight of liquefied semen fraction and its volume (Tables 3 and 4).

The mean± s.e.m ejaculate volume in microlitres was 57.4 ± 6.8 and 39.2 ± 3.6 for Mangabeys and De Brazza's monkeys respectively showing significant difference (P <0.05) (P= 0.0263). The volume of semen obtained from De Brazza's monkeys reflects volume of the liquefied fraction as it was directly impossible to measure whole ejaculate volume due to presence of coagulum. Sperm concentration was estimated from fresh ejaculates in the Tana Mangabey and from the liquefied fraction from semen obtained from De Brazza's monkeys. The mean sperm concentration in millions per ml among Mangabeys was 1989 ± 200 while that of De Brazza's monkeys was 64.1 ± 12, showing a significant difference (P< 0.01) between the two species (Tables 5 and 6). Between species (De Brazza and Mangabey) the mean of weight of ejaculate, liquefied fraction, semen volume were compared. There was no significant difference (P>0.05) between the means of the weight of fresh ejaculates obtained in the two species. Additionally, there was no significant difference (P> 0.05) between the means of volume of fresh ejaculate from Mangabeys and the liquefied fraction of the De Brazza's monkeys.

Table 1. Semen Parameters Expressed as Mean ± s.e.m per Monkey in Tana Mangabeys

	G15	G20	G24	G27
Approx age (years)	19	17	13	9
Mean weight of animal (kg)	11.16 ± 0.38	11.60 ± 0.42	12.21 ± 0.24	10.60 ± 0.70
Mean Wt. of semen (g)	0.104 ± 0.02	0.085 ± 0.03	0.112 ± 0.014	0.08 ± 0.008
Mean Volume of semen (µl)	37.87 ± 7.6	48.75 ± 14.8	72.2 ± 1.6	46.0 ± 6.5
Sperm concentration x 10^6/ml	3138 ± 47.4	1661 ± 145	1885 ± 396	1442.3 ± 16
Mean pH	8.5 ± 0.16	8.3 ± 0.11	8.30 ± 0.11	8.5 ± 0.0
Mean ± s.e.m (%) sperm motility				
a: Rapid Progressive	55.7 ± 1.1	56 ± 2.6	72 ± 4.1	76 ± 2.6
b: slow progressive	18.3 ± 4.1	22.3 ± 2.3	8.5±3.7	5 ± 0.5
c: Nonprogressive	11.3 ±2.8	10.5 ± 2.7	8.5±3.4	10.3 ± 2.4
d: Nonmotile	10 ±1.1	11.2 ± 2.3	11±0.9	8.6 ± 0.5
N° of ejaculations	8	10	10	5

Table 2. Semen Parameters, Expressed as Mean ± s.e.m per Animal in De Brazza's monkeys

	D44	D45	D46
Approx. age (years)	11	11	10
Mean Wt of animal (kg)	9.1 ± 0.62	8.33 ± 0.38	10.71 ± 2.0
Mean Wt. of semen (g)	0.108 ± 0.02	0.113 ± 0.03	0.094 ± 0.01
Mean Wt. of liquefied semen (g)	0.082 ± 0.03	0.070 ± 0.02	0.056 ± 0.005
Mean volume of semen (µl)	38.20 ± 6.4	42.85 ± 7.7	35. ± 1.8
Sperm concentration x 10^6/ml	37.76 ± 7.8	43.74 ± 14.3	118.9 ± 20
Wt. of coagulum (g)	0.03 ± 0.008	0.0414 ± 0.02	0.044 ± 0.005
Mean pH	8.5 ± 0.13	8.35 ± 0.08	8.5 ± 0.13
Mean ± s.e.m (%) sperm motility			
a: Rapid Progressive	49.6 ± 3.8	47 ± 3.4	52.8 ± 3.5
b: slow progressive	8.7 ± 1.6	14.1 ± 2.4	11.6 ± 0.5
c: Non-progressive	15.1 ± 3.8	14.6 ± 0.8	13 ± 2.3
d: Nonmotile	26.5 ± 3.1	24.1 ± 1.8	22.5 ± 4.3
Number of ejaculations	9	10	7

Table 3. Coefficient of correlation (r) between ejaculate parameters with corresponding P levels in De Brazza's monkeys

Variables	Coefficient of correlation (r)	p level
Semen volume vs. Weight of semen	0.96	$p<0.05$
Sperm concentration vs. Wt. of semen	-0.051	$p>0.05$
Sperm concentration vs. volume	-0.038	$p>0.05$
Wt. of liquefied semen vs. Wt. of semen	0.927	$p<0.05$
Wt. of liquefied semen vs. volume	0.88	$p<0.05$
Wt of liquefied semen vs. concentration	-0.16	$p>0.05$
Wt. of coagulum vs. Wt. of semen	0.615	$p<0.05$
Wt of coagulum vs. volume	0.625	$p<0.05$
Wt of coagulum vs. concentration	0.2661	$p>0.05$
Wt of coagulum vs. Wt of liquefied semen	0.3018	$p>0.05$

Table 4. Coefficient of correlation (r) between ejaculate parameters and corresponding P levels in Tana mangabey monkeys.

Variables	Coefficient of correlation (r)	p level
Weight of semen vs. semen volume	0.944	$p<0.05$
Sperm concentration vs. semen volume	0.068	$p>0.05$
Sperm concentration vs. Weight of semen	0.108	$p>0.05$

Table 5. Semen Parameters in Tana Mangabey monkeys:Expressed as Mean ±s.e.m, with Minimum and Maximum Values.

Parameter	Mean	s.e.m	max	min
Weight of semen (g)	0.097	0.009	0.25	0.03
Volume of semen (µl)	57.4	6.9	150	20
Sperm concentration (Millions/ml)	1989	200	4100	100

Table 6. Semen Parameters in De Brazza's Monkeys Expressed as Mean±s.e.m with Minimum and Maximum Values.

Semen Parameter	Mean	s.e.m	max	min
Weight of semen (g)	0.105	0.016	0.30	0.03
Sperm concentration (Millions/ml	64.1	12	180	15
Wt. of coagulum (g)	0.038	0.006	0.10	0.01
Wt. of liquefied semen (g)	0.069	0.014	0.22	0.02
Volume of liquefied semen (µl)	39.2	3.6	80	20

Sperm Morphology

Sperm morphology was evaluated using papanicolaou stained smears. The mean prevalence of abnormal sperm forms was 29% among De Brazza's monkeys and 16.2% for Tana mangabeys. Ten and 12 types of sperm defects were identified in Mangabeys and De Brazza's monkeys respectively (Tables 7 and 8). Coiled and folded tails were the predominant abnormalities occurring in total at a mean rate of 10.5% and 15.9% in Mangabey and De Brazza's monkeys' respectively. The total mean percentage of sperm head defects was 4.47% among Mangabeys and 10.1% in De Brazza's monkeys. Amorphous head constituted 0.83 % and 4.0% in the Tana Mangabey and De Brazza's monkeys respectively. Big sperm heads constituted 1.2% and 1.36% in Mangabey and De Brazza's monkeys respectively. Other head defects such as, tapering head, round head, double heads, pin head and small head occurred rarely (plates 1-10).

Cytoplasmic droplet was seen once in sperm from De Brazza's monkeys and not in the Mangabeys. The round cell population constituted of; immature germ cells, 0.95%, polygonal epithelia cells (PEC) 1.2% and white blood cells, (WBCs) 0.91% in the Mangabey. Immature germ cell, PECs and WBCs occurred in the De Brazza at a percentage mean of 0.90%, 1.0% and 0.36% respectively. In general, the percentage mean occurrence of sperm with normal morphology was 83.8% and 71% in the Mangabey and the De Brazza's monkeys' respectively.

Table 7. Prevalence(%) of sperm morphologies by type in Tana Mangabey. monkeys per animal.

Nature of sperm defect	G15	G20	G24	G27	Mean
Amorphous head	1.9	0.0	0.32	1.1	**0.83**
Big head	1.0	1.5	1.4	0.9	**1.20**
Small head	2.2	0.6	0.48	0.0	**0.82**
Round head	0.8	0.0	0.0	0.0	**0.20**
Tapering head	1.4	0.8	1.1	2.1	**1.35**
Coiled tail	6.5	5.8	4.9	7.9	**6.27**
Folded Tail	5.4	4.3	3.2	4.2	**4.27**
Mid piece defect	0.0	0.2	0.0	0.0	**0.0**
Germ cells	1.7	0.6	0.32	1.2	**0.95**
Pin Heads	0.0	0.0	0.0	1.1	**0.27**
(%)Total abnormal sperm morphology	**20.9**	**13.8**	**11.7**	**18.5**	**16.2**

Table 8. Prevalence(%) of sperm morphologies by type in De Brazza's monkeys per monkey

Nature of sperm Defect	D44	D45	D46	Mean
Big head	1.8	0.0	2.3	**1.36**
Small head	2.0	0.0	1.2	**1.0**
Round head	-	0.0	2.3	**0.76**
Tapering head	-	5.3	2.0	**2.4**
Coiled tail	14.0	10.6	8.2	**10.9**
Folded Tail	4.9	3.0	7.2	**5.0**
Double Tail	-	1.1	-	**0.36**
Mid piece defect	-	3.4	0.7	**1.36**
Germ cells	1.2	-	1.5	**0.90**
Pin heads	0.4	1.1	0.25	**0.58**
Cytoplasmic droplet	-	-	0.5	**0.16**
Amorphous head	3.7	5.7	2.8	**4.0**
(%)Total abnormal sperm morphology	**28**	**30.2**	**28.9**	**29**

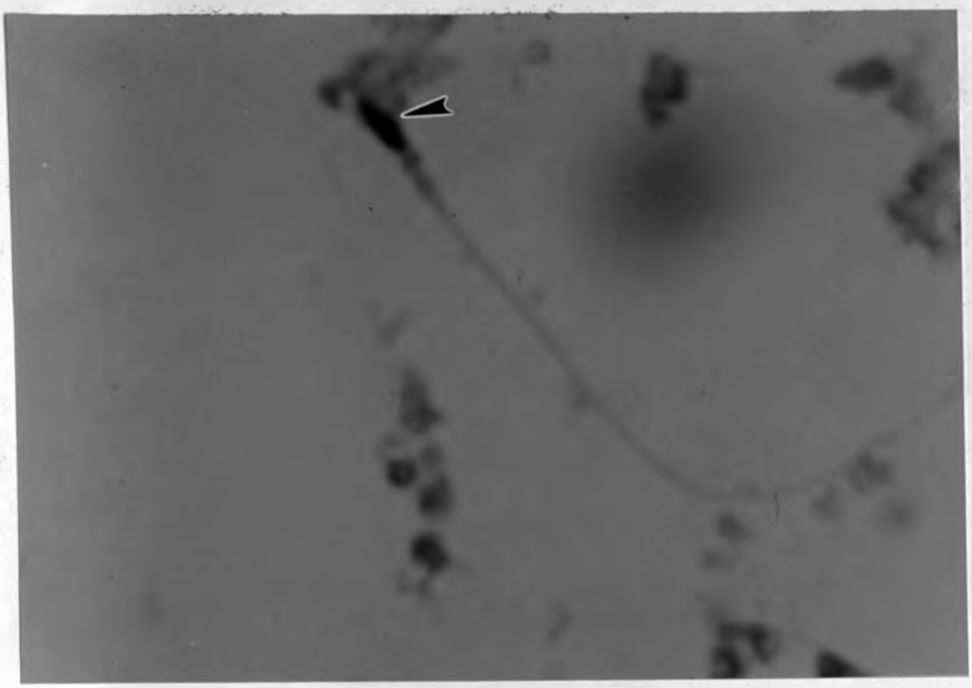

Plate 1. Pin head sperm from De Brazza's monkey ×100. Papanicolaou stain

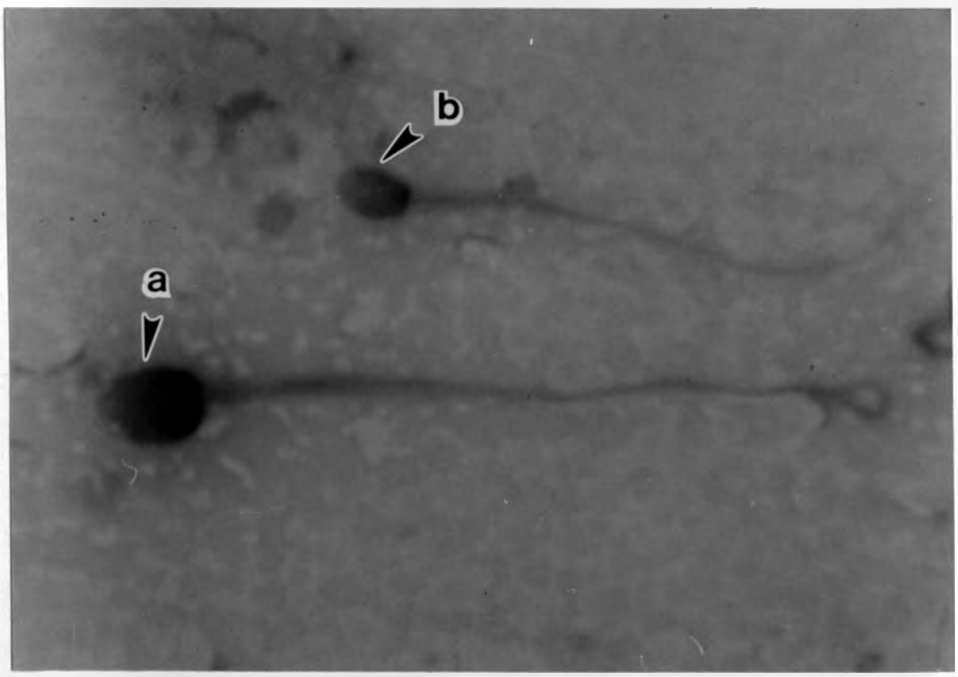

Plate 2. Spermatozoa from Mangabey a:Big head, b:Normal head Papanicolaou stain ×100.

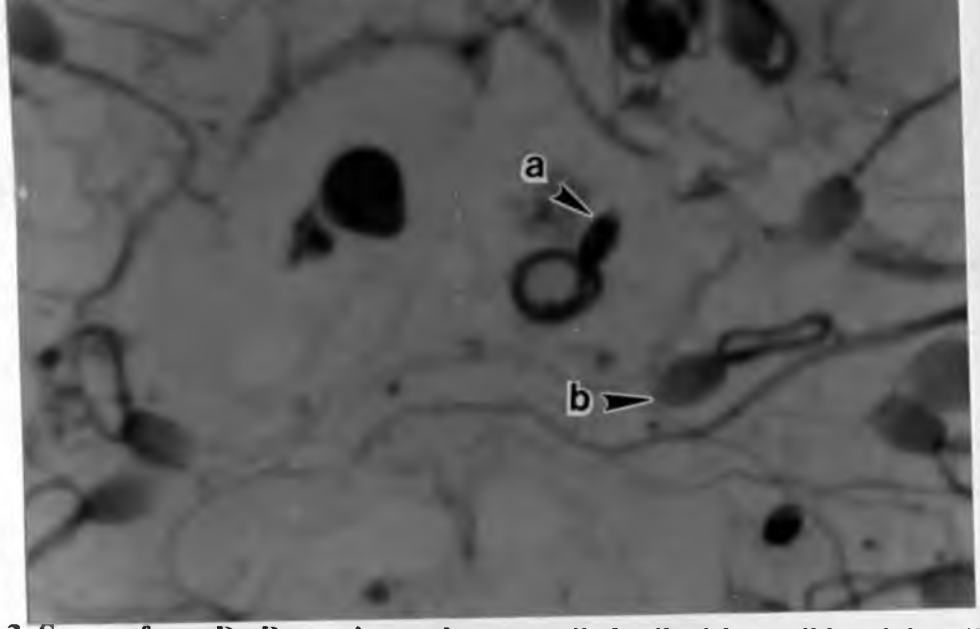

Plate 3. Sperm from De Brazza's monkey; a: coiled tail with small head, b:coiled tail with normal head ×100.
Papanicolaou stain

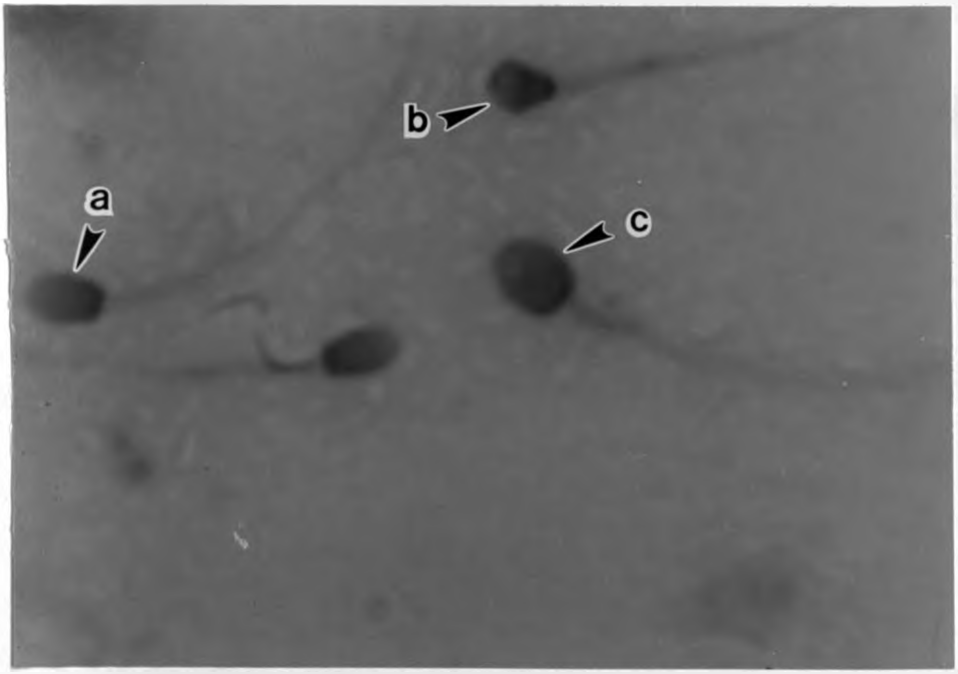

Plate 4. Sperm from De Brazza's monkey showing; a: Normal head, b:Pyriform head, and c: Big round head ×100.
Papanicolaou stain

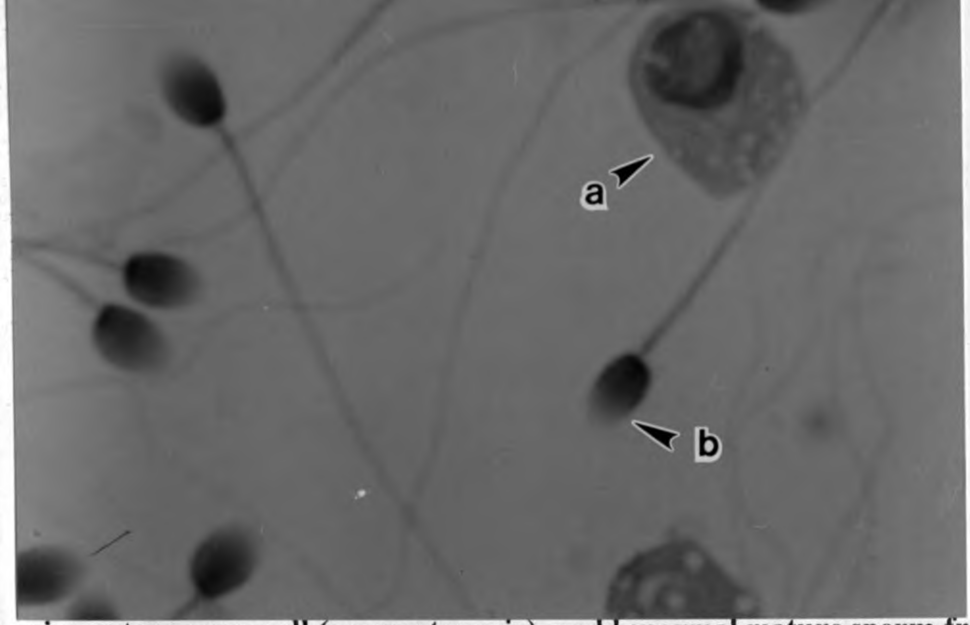

Plate 5. a: immature germ cell (spermatogonia), and b:normal mature sperm from Mangabey ×100.
Papanicolaou stain

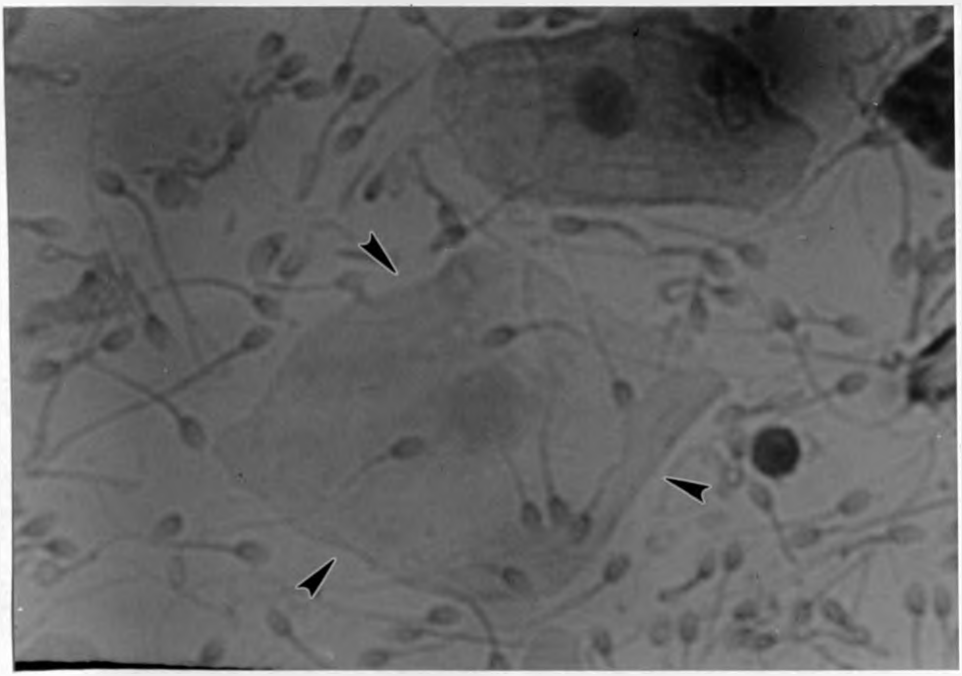

Plate 6. Polygonal epithelial cell (see arrows) overlying spermatozoon from Mangabeys ×40
Papanicolaou stain

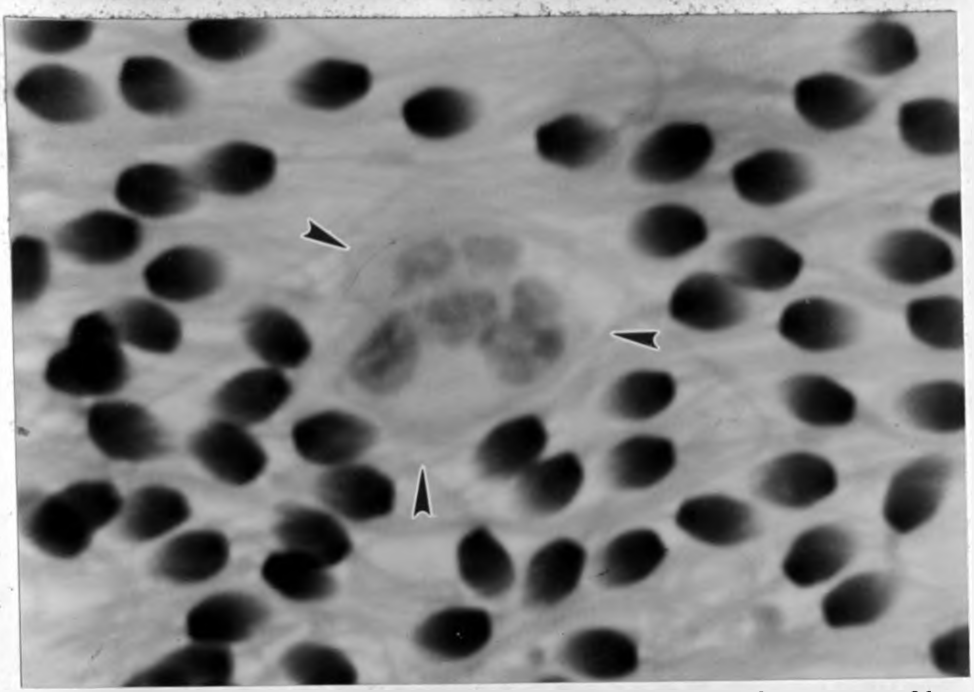

Plate 7. Poly morphonuclear cell (see arrows) from Mangabey semen, Note the segmented granular nucleus ×100.
Papanicolaou stain

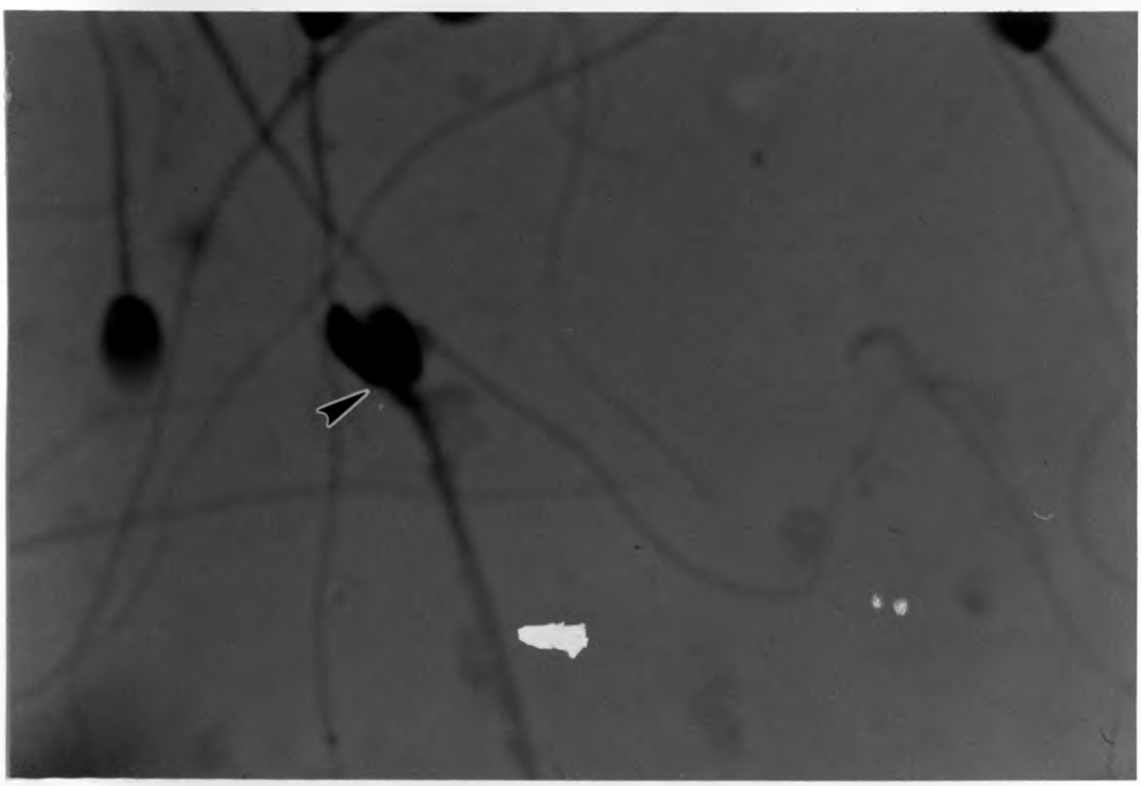

Plate 8. Sperm from Mangabey showing double heads ×100
Papanicolaou stain

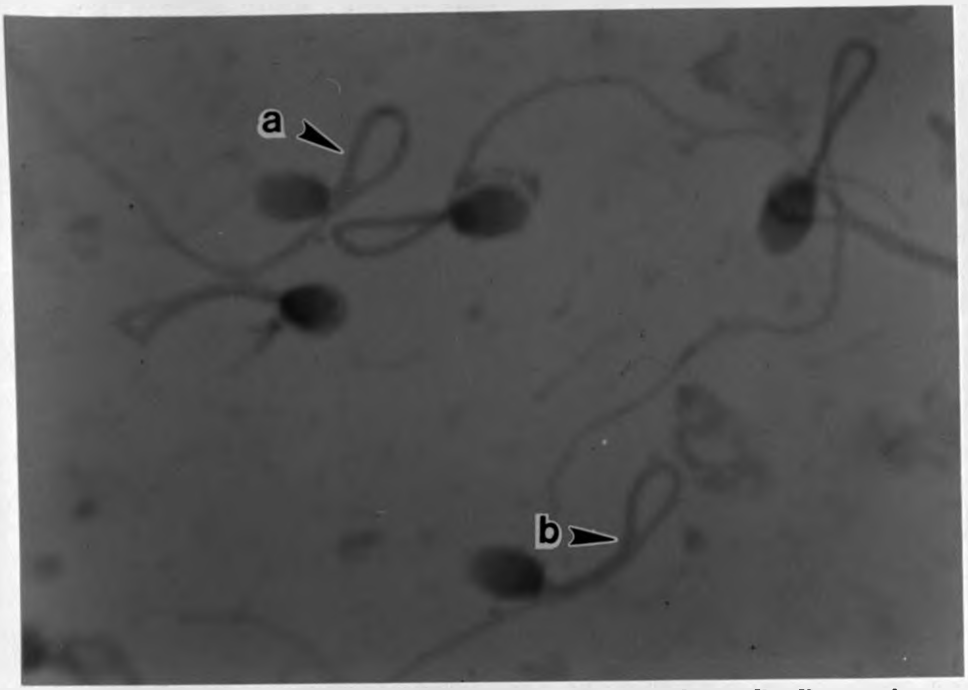

Plate 9. Various degrees of folded sperm tails a: and b: from De Brazza's monkey ×100
Papanicolaou stain

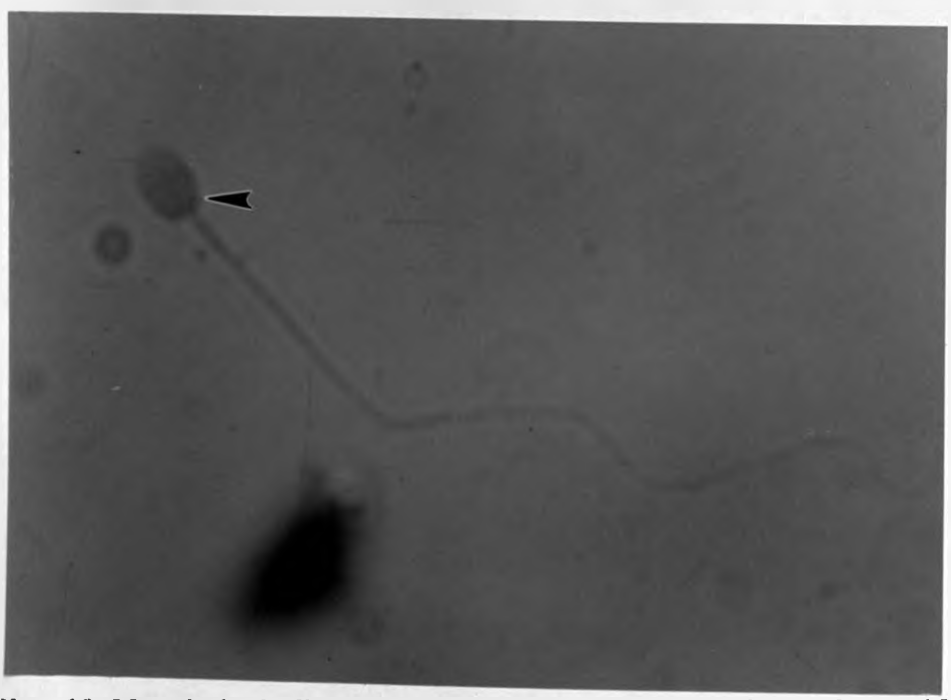

Plate 10. Morphologically normal sperm from De Brazza's monkeys ×100.
Papanicolaou stain

3.3 *Discussion*

Data concerning the physical parameters of semen obtained from captive Tana Mangabey and De Brazza's monkeys is provided for the first time. Rectal probe electro-ejaculation (RPE) is a suitable method for providing ejaculates for evaluation and has been reliable in most attempts in a variety of monkeys species (Harrison 1980; Sarason 1991; Ke-hui Cui *et al.,* 1991; Seier *et al.,* 1996). The rate of success of the procedure, indicated that it is possible to retrieve semen from these species which can be used in reproductive research or insemination. However, ejaculate volumes were very low. The 78.7% and 65.3% success in the Tana Mangabey and De Brazza's monkeys respectively were lower than 96% success rate reported by Harrison (1980) in the chacma baboon, but still demonstrates the feasibility of the technique in this species. Such differences may arise from the operator, in the stimulation technique, the gadget used and species differences in response to stimulation. Although semen obtained by artificial vagina and penile electro-stimulation is considered superior in quality to RPE (Gould and Mann 1988; Gould and Young 1996), our finding show that RPE may be a suitable method of semen retrieval from primates that can not be trained to masturbate or use an artificial vagina.

The assessment of seminal parameters therefore gives a representative clue on the spermiogram for the two species under captive local condition. However this may not be representative of the species in general since captive management may influence the true picture and differs from the animals in the wild due to environmental/habitat factors. For example, seasonality has been detected in semen parameters in rhesus monkeys (Gould and Mann 1988). Seier *et al.*, (1989) had demonstrated controversial results in semen

parameters between singly caged males and breeding male vervet monkeys. This evidence indicates that the environment may modify monkey spermiogram but the extent to which this may occur is unclear. Nevertheless, this clearly shows that fertility potential of an individual is likely to fluctuate in part, due to external factors. The monkeys studied were singly caged males of different fertility and age (range 9-19 years), factors that should be considered when comparing results. In addition, ejaculates obtained by coitus are likely to differ in quality from those obtained artificially.

A short photoperiod has been associated with suppression of spermatogenesis due to diminished or loss of testicular LH, FSH and prolactin receptors and suppression of testicular testosterone in a hamster model (Bartke 1995). In humans seasonal fluctuation have been detected in sperm count, motility, morphology and blood levels of LH and testosterone as well as sexual activity. The latter are believed to be related to effects of photoperiod. For six months during electro-ejaculation, the primates were kept indoors on an 8/16 hours, light/dark length and by itself, this may modify the spermiogram by hormonal mechanisms. It was not the scope of this study to assay hormone profiles although such data is worth having. Temperature, food, especially strictly seasonally available diet components plus chemical messages received from other members of the same species as social and density dependent factors act as stressors and suppress reproductive function (Bartke 1995). Each of these factors is capable of exerting profound suppressive effects on the production of sperm and androgens by the testis. The extent, although still obscure is likely to impart some influence for captive managed animals.

Additionally, although semen was collected many times per animal, (Appendix III), the sample size was small. This was due to limitations of research in endangered species and the availability of reproductively mature males capable of donating semen. Bias associated with small sample sizes should therefore be borne in mind. The differences in the time taken to electroejaculate may result from species difference in the sensitivity of tissues to current (anatomical differences in the two species). But the effect of inconsistent maintenance of a suitable (threshold) current density and stimulation frequency associated with electro-ejaculation as argued by Lanzendorf and colleagues (1990), is a real possibility.

In our study, both coagulum and the process of semen liquefaction were not observed in ejaculates obtained from Mangabeys as opposed to De Brazza's monkeys. Coagulum is a documented finding in semen from primates including man (Kreamer and Cruz, 1969; Bornman *et al.,* 1988; Gould *et al.,* 1988; Thomson *et al.,* 1992; Mandal and Bhattacharyya 1994; Adoyo, P. personal communication). Failure to observe coagulum and liquefaction in the Tana Mangabeys could be in part due to the very small ejaculate volumes so obtained, or the relatively lower secretions from accessory glands might also be explained by the artificial technique of semen collection. Proteinous secretions from the male accessory glands of most primates including man, are responsible for seminal coagulation within seconds after ejaculation process (Greer *et al.,* 1968; Dukelow 1971; Kinzey 1971). *In vivo*, among other functions coagulum is responsible for the formation of the vaginal plug during/after copulation allowing slow release of sperm and reducing the reflux of sperm from the female tract (Kinzey 1971; Coffey 1995). It is possible that

semen from Mangabey may contain coagulum but in amounts not as obviously and readily formed/observed in most primate species. This may be attributable to a species difference regarding type and quantity of secretions that contribute to an ejaculate in these species and therefore an analytical investigation should be conducted. Variation in liquefaction/dissolution of coagulum by natural or experimental means has a profound effect and is responsible for a wide variation in the reported constituents of semen such as sperm concentration (Kinzey 1971; Coffey 1995). This may explain in part the inter- and intra-animal variations in semen parameters observed.

Small ejaculate volumes (mean ± s.e.m), 57.4 ± 6.8 and 39.2 ± 3.6 in microlitres for the Mangabeys and the De Brazza's monkeys respectively were very difficult to measure using the conventional method. Measurement of small volumes of highly viscous semen with an eppendorf pipette with a disposable tip has inherent inaccuracies, therefore all volumes should be taken as approximations. In part, this could have under-estimated the real ejaculate volume due to the viscous nature of semen. Such small volumes have also been reported in *Macaque fuscicuralis, M. mulatta,* and the common marmoset (*Callithrix jachus*) ejaculates (Sarason *et al.,* 1991; Ke-Hui Cui *et al.,* 1991). Semen volumes obtained from our primates of study were substantially less than the range 0.1 to 2 ml reported in baboons, great apes, and *Cercopithecus aethiops.* (Kreamer and Cruz 1969; Young and Smithwick 1995; Adoyo, P. personal communication). Such a big difference could emanate from differences in size of the animals. The monkeys we studied and those studied by Kreamer and Cruz (1969), are very small in size compared to the baboon. The size of the animal is likely to influence testicular diameter which in

turn may affect the volume of the ejaculate. Secondly a species difference as regards secretions from the accessory glands upon stimulation or even epididymal capacity is another possibility. Therefore such differences can influence an animals response to electrostimulation that may result in varying ejaculate volumes. In addition, the real technique and collection procedure such as, type of electroejaculator, size of probe, current density, electrode dimension and the frequency of stimulation which most workers do not give details about may contribute to this. For example, Roussel and Austin (1968) as cited by Wildt (1986) reported mean semen volume of 1.3 ml among *Cercocebus galeritus* using RPE , a volume much higher that our finding.

It is recognised that collection of semen from primates by electro-ejaculation can produce variable results because of inconsistent stimulation conditions (Lanzendorf *et al.*, 1990). This can also result from incomplete collection of the ejaculate. Electro-ejaculation can also result in both antegrade and retrograde flow of semen so that large numbers of viable sperm may be lost in the urinary bladder. The extent to which this may occur varies from one attempt to the next within the same animal or different species due to slight differences in stimulation. Higher total sperm numbers in the bladder infusate than in the collected ejaculates have been reported in lion tailed macaque (Schaffer *et al.*, 1989). On the other hand, Thomson *et al.*, (1992) has reported no significant finding between the bladder infusate and the collected ejaculate in *Macaca nigra*. Although our study did not retrieve semen from the urinary bladder because of lack of appropriate instruments, a possibility of retrograde ejaculation can not be ruled out.

In general, among other factors, it is known that the relative contribution of the epididymis and accessory sex glands to the seminal plasma of a given ejaculum are dependent on the interval of sexual abstinence, the duration of stimulation, and the species-response (Amann *et al.,* 1995). We collected semen at irregular frequencies (Appendix IV) a fact that was dictated by the physical status of the animals as evaluated by the clinician following development of orchitis in one Mangabey and death of one De Brazza's monkey. Additionally, stimulation differs with each attempt due to inconsistencies in maintaining a uniform current density. The extent to which each of these factors may contribute to differences in ejaculates is unclear. This suggests that the outcome may also be attributable to operator differences. It must be noted that electro-ejaculation is only meant to simulate the normal coital process and large inter-animal and intra-animal variation in semen parameters may be due to expected biological variability of animals in population studies.

The coefficient of correlation (r= 0.96) and (r = 0.94) between the weight of semen and its volume in the De Brazza's monkeys and the Tana Mangabeys respectively suggests that semen weight just like volume may be adopted as a routine parameter for assessing semen quality for highly viscous specimen. The coefficient of correlation (r = 0.93) between the weight of ejaculate and the weight of liquefied fraction in the De Brazza's monkeys suggests that the ejaculate weight can be used as a predictive value for liquefied fraction. A correlation (r = 0.88) between weight of liquefied fraction and its volume in De Brazzas suggests that any of the two parameters can be used in assessing semen volume.

Although liquefaction time must be considered in measuring semen parameters as suggested by Young and Smithwick (1995), there is no standard time recommended regarding non-human primate species. Twenty minutes liquefaction time was chosen after preliminary observations from a pilot study. Longer liquefaction time ≥ 30 minutes was associated with drying of semen in view of highly viscous and small ejaculate volumes. A pH range of 8.0-8.5 was a common finding comparable to that recorded for baboon ejaculates in our laboratory (Adoyo, P. personal communication) and occasionally pH 9 suggesting slightly higher alkaline secretion from accessory glands. Therefore pH of between 8.0 to 8.5 appears to be the normal range. Other workers have recorded pH in the range of 7.0 to 8.1 in the marmmoset monkey and mean pH 7.8 in singly caged vervet monkeys and mean pH of 9.0 in breeding males (Seier *et al.,* 1989; Ke-hui cui *et al.,* 1991). Semen pH is a result of secretions from male accessory glands. It can be seen that the range falls between pH 7.0 to 9.0 in most reports. It is possible that the final pH may result from the gland secreting the bigger proportion of seminal plasma. This may depend of the relative size and response to current stimulation of the accessory glands. The pH of semen obtained by artificial vagina or masturbation would reflect the true physiological status. Otherwise it is difficult to explain such differences even within the same animal. On the other hand the type of indicator used may also attribute to this in the species reported.

The very high sperm concentration in Mangabey semen as compared to the De Brazza's monkeys suggests a higher spermatogenetic potential in the latter. This could also be due to either a higher concentration efficiency of their epididymis or a higher

epididymal capacity. This high sperm concentration compares well with the mean 1638 ± 115 in millions per ml reported in *Macaque fascicularis* (Sarason *et al.*, 1991). However such big difference between De Brazza's monkeys and Mangabeys could also have been amplified because of using two different fractions of semen for estimation of sperm concentration. While the liquefied semen fraction was used for De Brazza's monkeys, fresh ejaculates were directly used in Mangabeys since their semen contained no observable coagulum and did not undergone liquefaction. Therefore it is possible that some spermatozoa could have remained entrapped in coagulum of semen from De Brazza's monkeys. The sperm concentration retained in coagulum mass was not investigated in this study but Kreamer and Cruz (1969), showed such retention in the baboon indicating that such possibility existed wherever coagulum is formed. However, the suggestion that De Brazzas testis have a low spermatogenic potential cannot be wholly ignored. This together with a high incidence of abnormal sperm forms may in part explain, as a male factor, why the De Brazzas have not bred well under captive conditions. The extremely high sperm concentration in the Mangabey clearly reveals the potential for cryopreserving sperm that can later be used for research, insemination or any assisted reproductive techniques as a breeding strategy.The absence of correlation ($r = -0.038$) between volume of liquefied fraction and sperm concentration in De Brazzas and ($r = 0.067$) between semen volume and its concentration in Mangabey suggests that accessory glandular secretion is independent of epididymal sperm out put.

Although computer assisted motion analysis (CAMA) of sperm motion provides comprehensive objective and reproducible analysis, the technique is rarely available and is therefore not a routine procedure for semen analysis (Comhaire and Vermeulen 1995.

Accordingly, this and similar techniques were not available for our study, consequently, assessment of sperm motility was subjectively estimated as is recommended in routine semen analysis (WHO 1992). The generally better initial progressive sperm motility in Mangabeys compared to De Brazza's monkeys suggests higher fertilization potential in the former since initial sperm motility has been associated with fertilisation potential in many species (Hafez 1987; Yanagimachi 1994). This finding is controversial because fertility history shows that the De Brazza although with low sperm motility all have sired offsprings unlike the Mangabeys where, only G20 has sired an offspring. This is possible because fertilisation does not only depend on sperm motility.

For the first time sperm morphology and the prevalence of abnormal forms together with the population of round cells in semen is described in De Brazza and Mangabey monkeys kept under captivity at the Institute colony. Description of sperm morphology is an essential part of defining the sperm characteristics of any mammal not only because different abnormalities may predominate in different species, but because baseline data can provide information in the diagnosis of pathology of the reproductive system and is also a useful tool in reproductive research. In the vervet monkey, Seier *et al.*, (1989, 1996) first reported morphological sperm defects and then later calculated their prevalence indicating that the rate of occurrence of abnormalities gives better descriptive information.

It has been demonstrated that sperm morphology is correlated with fertility. In humans, normal sperm morphology (a vital aspect of function) is associated with a variety of sperm functions such as, spontaneous acrosome reaction, hyalurodinase

activity, and sperm motility parameters such as, swimming velocity and flagella beat frequency (Fukuda *et al.*, 1989; Mayada *et al.*, 1995; Katz *et al.*, 1982; Morales *et al.*, 1988b). This structure-function relationship of sperm also exist in other mammalian species. Coiled and folded tails were the predominant abnormalities, a finding similar to that reported in vervets (Seier *et al.*, 1996). It is known that tail coiling and possibly tail folding are multifactorial in origin, being a result in part, of drying, cooling or contamination of samples and hyposmotic shock (Harrison 1980; WHO 1992). Therefore such high prevalence is a possible combination of processing (artefact) and defective spermatogenesis. Use of wet smears for analysis of sperm morphology were not performed because of the extremely high concentration of motile sperm in the Mangabey and the persistent presence of debris in De Brazza semen, factors that combined to obscure visibility of individual spermatozoa under phase contrast microscopy. Cytoplasmic droplet is an indication of premature spermiation or immature spermatozoa (Peters and Ball 1987). Cytoplasmic droplets were seen only in one sample of the De Brazza and non in the Mangabey, suggesting that the defect is uncommon a finding similar to that reported in vervet monkeys (Seier *et al.*, 1996). However according to Harrison (1980), cytoplasmic droplets are rather more difficult to see from stained smears than from fresh wet ones under phase contrast. Other abnormal sperm morphologies encountered were big heads, tapering heads, pyriform head, and pin heads. The prevalence being higher in De Brazza's monkeys than in Mangabeys. This suggests that the process of spermatogenesis produces more malformed spermatozoa in De Brazza's monkeys as compared to the Mangabeys.

The high prevalence of abnormal sperm in the De Brazza's monkey (29%) and the Tana Mangabey (16.2%) contradicts the concept that monkeys, unlike men, have sperm of remarkably uniform structure with relatively few morphologic abnormalities. Both results are higher than the documented < 5% in other monkeys (Harrison 1980; Harrison and Wolf 1985; Seier *et al.,* 1989; Zamboni *et al.,* 1971;). Our finding is similar in range to that reported in vervet monkeys (Seier *et al.,* 1996). The higher occurrence of abnormal forms among De Brazza's monkeys suggests that the species is more prone/susceptibility to sperm malformation compared to the Mangabeys since all the animals were under similar management. Nevertheless, a high occurrence of abnormal forms in our study as compared to other workers may originate from different conditions of managing captive animals. It is known that abnormal sperm are produced mainly as a result of defective spermatogenesis or adverse environmental conditions including temperature (Peters and Ball 1987). It is difficult to attribute captive management to this spermiogram since a wild caught comparative group was not available for study. However, it is possible that different stress factors of capture such as nutrition and photoperiod may influence the process of spermatogenesis. In a study on sperm morphology by Seier *et al.,* (1996), there were no significant differences between wild caught and captive bred groups except for one abnormality, the nipple acrosome which our study did not come across. There is no doubt that management of captive primates at different research institutes can vary greatly. In another observation, Van Der Calf *et al.,* (1991), recorded considerable variation in gross normal morphology in ejaculates from the same individual suggesting that a single or even two ejaculates may not be representative of an individual's reproductive potential.

CHAPTER FOUR

4. Induction Of Acrosome Reaction In Tana Mangabey Spermatozoa

4.1 Introduction

The fertilisation potential of mammalian sperm depends upon the ability of sperm to undergo capacitation and the acrosome reaction. The acrosome reaction can occur spontaneous *in vitro* or can be induced by various substances under defined set of conditions. Bioassays for the assessment of capacitation in human sperm such as penetration of zona free hamster eggs or penetration of zona of non living oocytes are available (Boldt and Wolf 1984; Overstreet, *et al.,* 1980). However, these bioassays are cumbersome and do not adequately represent the physiologic events of fertilisation.

Techniques that evaluate sperm changes during capacitation or at intermediate stages during the acrosome reaction are of significance in diagnostic application of acrosomal status quantitation. However, there is no general consent as to the assay(s) that can distinguish capacitated from non-capacitated sperm because of lack of a clear cut definition of a capacitated sperm (Cohen-Deyang and Eisenbach 1995). Secondly, capacitation is usually induced under conditions that also stimulate the acrosome reaction, and no distinction between these physiological stages has been made, leading to the confusion with the extent and characteristics of sperm capacitation. Transmission electron microscopy has been recommended for the quantitation of acrosomal status because of its ability to define intermediate stages during the acrosome reaction. Other techniques that have been used include staining with fluorescent conjugated probes such

as, lectins, chlortetracycline and indirect immunofluorescent assays that employ polyclonal or monoclonal antibodies (Lee *et al.,* 1987, Wolf 1989). Although these are considered acceptable methods for rapid quantitation of acrosome status, it is urged (Wolf 1989) that the ideal assay, (Simultaneous assessment of sperm motility and acrosomal status on the same living cells) is still lacking. Additionally these methods have only gained wide application in human and livestock spermatozoa. The purpose of this experiment was to demonstrate the acrosome in the Mangabey by staining with FITC-PSA and to compare the acrosome status in spontaneous and chemically induced AR using calcium ionophore A23187 and dibutyryl cyclic adenosine monophosphate.

4.2 Results

Spermatozoa selected from seminal plasma by direct swim up technique were used. Spermatozoa concentration as estimated by a hemocytometer was in the range of 5 to 20 million per ml. Three distinct fluorescence patterns were seen namely; Pattern IA, with fluorescence uniformly distributed over the anterior 2/3 of the sperm head, Pattern PA, with fluorescence as above, but in a patchy manner and Pattern AR, with a fluorescence band across the sperm head along the equatorial segment (plate 11). Pattern IA represented sperm with intact acrosomes while Pattern PA represented sperm with patchy or partial acrosomal loss and Pattern AR represented complete acrosome reacted spermatozoa

The mean percentage of sperm with pattern IA from time, t = 0 to time t = 150 minutes, decreased from 99% to 67.1% in control compared with 100% to 34% in experimental while sperm with pattern PA increased from 1% to 19.4% in control compared with 0% to 29% in experimental. The mean percentage of sperm with pattern

AR increased from 0 % to 13.4% in control compared with 0% to 37% in experimental (Fig. 1). Significant differences ($P<0.05$) between control and experimental tubes for the three sperm patterns where noticed throughout except at 120 minutes ($P=0.0504$) incubation. The maximal inducible complete acrosome reaction in spermatozoa was 37%.

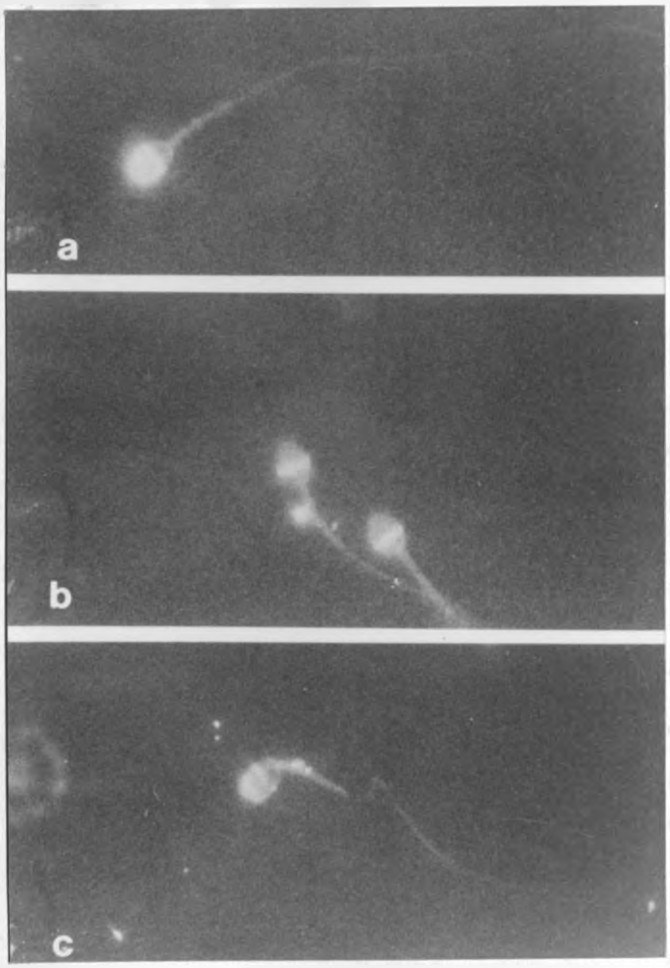

Plate 11. a: Sperm with intact acrosome, c: patchy acrosome and b:complete acrosome reacted spermatoza. Note the fluorescence patterns over the head. covering anterior 2/3 in a:, as a band in b: and panctate/patchy anterior head in c: (FITC-PSA stain M×100)

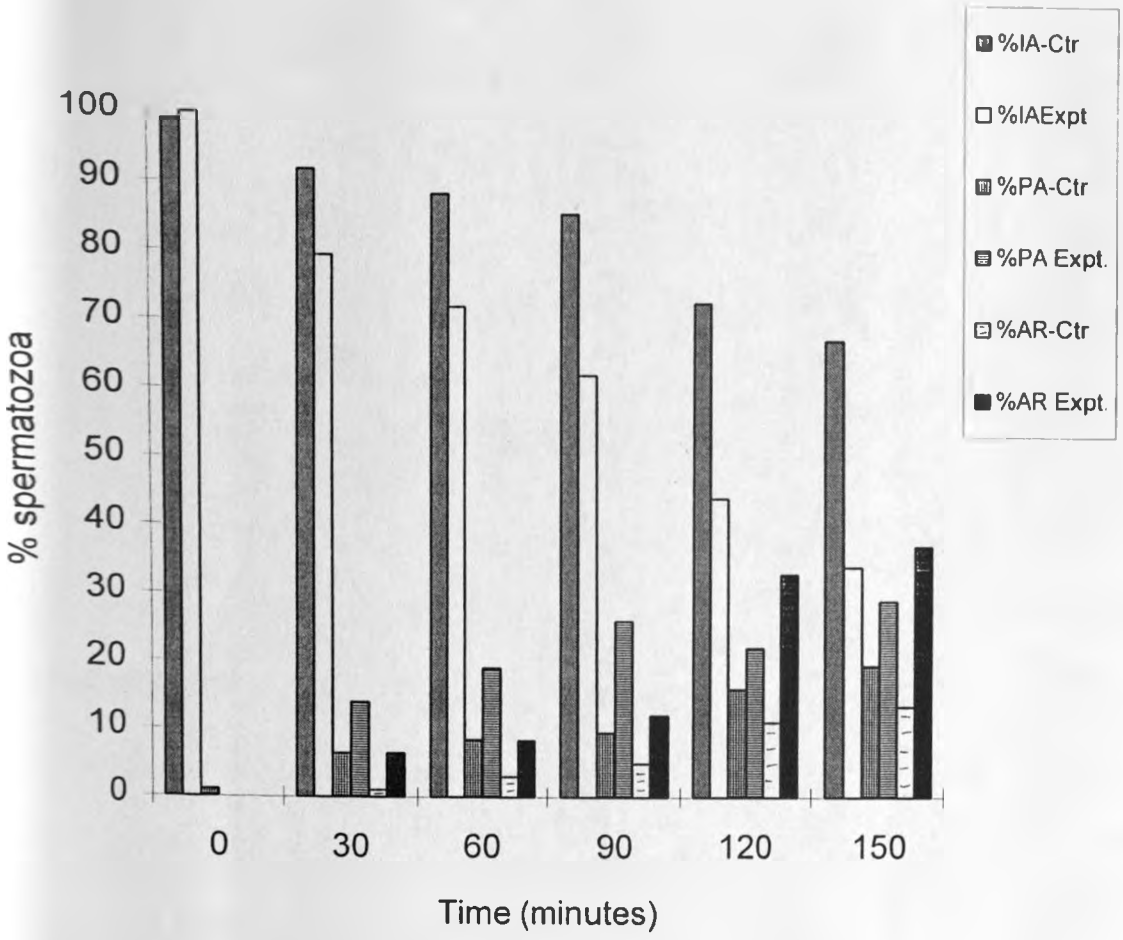

Fig. 1. Percentage Variation of Acrosome status of Mangabey spermatozoa in experimental (Expt) and Control (Ctr) tubes with time Monitored with FITC-PSA stain. IA = Intact Acrosomes, PA = Patchy acrosomes and AR = Acrosome Reacted spermatozoa.

4.3 *Discussion*

Three distinct fluorescence patterns were observed after staining sperm obtained from the Mangabey with FITC-PSA (Plates 11). Pattern AI, represents sperm with intact acrosome membrane while pattern PA, represents sperm that have started to undergo acrosomal membrane loss. Pattern AR represents sperm that have undergone complete acrosomal loss. The presence of fluorescence only on the sperm head and not elsewhere indicates that the stain, FITC-PSA binds specifically to glyco-conjugates localised on the sperm head. This confirms earlier work in other species that the glyco-conjugates of the acrosomal matrix or outer acrosomal membrane that bind lectins are largely localised on the sperm head (Cross *et al.*, 1986; Mortimer *et al.*, 1987). We detected three distinct fluorescence patterns on the sperm head contrary to Cummins *et al.*, (1991) who demonstrated four patterns in human sperm using FITC-PSA. As argued by Cummins *et al.*, (1991) the fourth pattern (light fluorescence on head and tail) is not usually considered as part of the physiologically normal acrosome reaction or loss, because it appears to be non-specific (artefactual). It is known that *Pissum sativum* agglutinin (PSA) is lectin specific for intracellular, or acrosome associated glyco-conjugates and our finding confirms earlier observations (Cross and Watson 1994)

At every 30 minutes time intervals there was a significant difference ($p<0.05$) between sperm with different acrosomal status shown by three fluorescence patterns between control and experimental tubes (Appendix IV). This indicates that addition of dbcAMP and calcium ionophore A23187 in the sperm suspension produced a higher proportion of sperm undergoing acrosome changes. It is known that at fertilisation sperm

release acrosomal contents in a calcium dependent process called acrosome reaction and the influx of calcium across the sperm membrane is considered to be the first step of the acrosome reaction (Yanagimachi 1981). Calcium ionophore A23187, a divalent permeating agent is known to fasten this entry. Cyclic adenosine monophosphate or its analogues such as dbcAMP are known to induce protein tyrosine phosphorylation a prerequisite for the completion of sperm capacitation. Therefore the two reagents by fastening calcium entry and capacitaton respectively were responsible for the higher proportion of spermatozoa showing acrosome loss in the experimental tubes. The faster decrease in the percentage of sperm with pattern IA with time in the experimental compared to the control implies that more spermatozoa in the experimental tubes underwent membrane changes towards the acrosome reaction. Likewise the appearance of higher percentages of sperm with patterns PA and AR, indicates loss of binding sites for FITC-PSA on the sperm heads, confirming the redistribution and or release of membrane glyco-conjugates and other membrane molecules seen during sperm capacitation and the acrosome reaction

The differences in staining pattern, show that the lectin PSA is able to bind to conjugates at some foci on the sperm head that are dependent on membrane changes of capacitating spermatozoa on a time basis. The staining pattern can be explained by two facts; FITC-PSA is specific for sugar related molecules such as, glycoproteins found on the sperm head (Cross and Watson 1994). During capacitation, marked changes in the level and arrangement of redistribution and release of these sugar related molecules occurs (Cohen-Dayag and Eisenbach 1995). Therefore pattern IA sperm whose membranes are still intact bind intensely and over the entire sperm head while pattern PA

and AR spermatozoa that have undergone membrane remodelling with some loss of foci bind less intensely and at only some regions of the sperm head. A high initial percentage of sperm with pattern AI, shows abundant binding sites for FITC-PSA and therefore intact acrosome membranes in the first few hours of incubation. Meanwhile, a low percentage of sperm with patterns PA and AR indicated that relatively fewer cells have undergone membrane changes.

The proportion of sperm undergoing acrosome reaction is higher in the experimental than in the control tubes showing that spontaneous acrosome reaction proceeds very slowly. This finding is similar to studies conducted by Kholkute *et al.*, (1990) following *in vitro* incubation. The maximum inducable sperm acrosomal loss (pattern AR) was 37% after two and a half hours of activation indicating that not all the sperm responded identically, (some proportion of the sperm population failed to respond). This might be due to some defects such as defective acrosome or chemical mechanisms. For example, it is known that round sperm heads with no acrosome may not undergo acrosome reaction (Lalonde *et al.*, 1988).

This study measured the main end point of sperm capacitation, the acrosome reaction and is not able to state the time course of sperm capacitation. The work described in this experiment is a simple way of evaluating the course of acrosomal status in a sperm population and did not include the supravital stain Hoesch 33258 (H258), for distinguishing live sperm from dead ones as is currently recommended (Cross *et al.*, 1986; Cross and Meizel 1989). Therefore the sperm counts can not be distinguished into

those with true and false acrosome reaction. Although we used sperm with more than 70% progressive motility after selection from seminal plasma, it is possible that during the period of incubation some sperm could have died and undergone the AR. Failure to use a vital stain was complicated by two factors; lack of a powerful fluorescent optical source with filters capable of distinguishing optically the two fluorochromes, H258 and FITC-PSA simultaneously. Secondly, H258 was not available in the laboratory

Our results demonstrate that spermatozoa from the Mangabey incubated under capacitating conditions do not readily undergo acrosomal loss spontaneously but are responsive to the stimulants; dbcAMP and calcium ionophore A23187. This is similarly reported for sperm obtained from the squirrel monkey but contrasts with human spermatozoa (Kholkute *et al.,* 1990;1992). Although the acrosome reaction can be equated with fertilisation potential a 37% inducable acrosome reaction in a population of sperm where vitality is missing, is quite low. It must be pointed out that capacitating conditions by various workers can differ. Various grades of BSA, calcium, calcium ionophore A23187 and other agents induce sperm AR in a concentration dependent manner (Kholkute *et al.*,1990; Lee *et al.*,1987; Uto and Yamaha 1996). Therefore this might explain the differences in the onset and the proportion of sperm responding in the various species.

CHAPTER FIVE

5.1 General Discussion and Future Work

There is a controversy over the fertility status based on history of the monkeys and our finding based on semen analysis; Among Tana Mangabeys, only G20 had sired offsprings while all De Brazza's monkeys had sired at least two offsprings each. Our results in general show that it is possible to retrieve semen from the two species of monkeys by RPE. but with more difficulty among De Brazza's monkeys. Sperm from De Brazza's monkeys had more abnormal forms as compared to that from Mangabeys yet among the later, only one monkey had sired an offspring contrary to all De Brazza's monkeys which had each sired offsprings. This might be expected since male fertility could have fluctuated from the time the animals sired offsprings to the time we performed semen analysis. Secondly, the process of fertilisation does not entirely depend on the sperm factors or a single parameter in semen. Thirdly a species difference is another possibility. We observed coagulum in semen obtained from DeBrazza's unlike the Tana Mangabeys suggesting a gross difference in the contribution of accessory glands to seminal plasma in the two species in response to electrostimulation.

Two most commonly used methods for collecting semen from monkeys are penile electro-stimulation and rectal probe electrostimulation. Although the former produces superior semen, it requires use of trained non-anaesthetised animals (Harrison 1980). This was not practically possible in this study. We have demonstrated that ejaculatory semen may be routinely collected by RPE to provide semen both in the Mangabey and

De Brazza's monkeys. Mangabey code named G27 developed orchitis and was discontinued, while D46 died in the course of the study. Necropsy revealed purulent orchitis in G27 and adhension of the rectum and the colon in D46. There is hardly any data on the adverse effects of rectal probe electro-ejaculation or male fertility thereafter. However it is documented that, current density in excess of 0.75 mA/mm^2 causes local damage with subsequent pain, irritation and rectal mucosal heating (Gould *et al.*, 1978).

The study reveals baseline data on the spermiogram of Tana Mangabeys and De Brazza's monkeys. While this shows the fertility potential under captive management, it must be emphasised that other factors besides semen parameters are involved in the ability to produce offsprings. Social factors, age, experience, season and rearing conditions are all significant, let alone the female factors (Harrison 1980; Elizabeth *et al.*, 1991). In addition the sample size was dictated by unavailability of mature males in an endangered species population. There was great difficulty in measuring small ejaculate volumes of highly viscous semen, the correlations r = 0.94 and r = 0.96 between weight of semen and volume in Mangabeys and De Brazza's respectively suggests that weight as a parameter may be adopted where ejaculate volume are too small.

5.2 Conclusion and Recommendation

5.2.1 Semen Analysis

This study has shown that RPE can be used to retrieve semen from Mangabeys and De Brazza's monkeys although with greater difficulty in the latter. Ejaculate volumes were very low at any one time to permit assessment of all parameters at once as is routinely

done. Measurement of semen volume by a pipette may have inherent errors especially when dealing with a viscous substance. Therefore the volumes should be taken as approximation. Low sperm concentration coupled with high abnormal sperm forms in De Brazza's monkeys suggest poor spermatogenesis and therefore a low fertility potential. This may explain in part, the poor breeding results in this species. The results give only a clue on the spermiogram (Baseline data) of these species under captivity over a period from May to September. However, because fertility may fluctuates with season, it is suggested that studies that recover sperm in all seasons should be undertaken. Additionally, wild caught animals should be electro-ejaculated for comparison.

While the ejaculate volume is low among Mangabeys a very high sperm concentration together with a lower prevalence of abnormal sperm reveals a high spermatogenetic potential. It is difficult to explain the lack of natural propagation from semen parameters studied in this species. Semen retrieval in these species reveals a potential for studies that address insemination using fresh semen, in vitro fertilisation, semen extension and cryopreservation for long term storage. This is because assisted reproductive techniques hold considerable promise of producing offsprings and preserving genetic diversity indefinitely especially where mating may be severely restricted due to social and behavioural factors or where gamete interaction necessitates artificial help. Sperm functional studies such as sperm penetration assay using sperm so obtained are strongly suggested since by themselves semen parameters do not entirely determine fertility.

5.2.2 *Acrosome Reaction*

This study has also demonstrated that the lectin PSA-FITC binds specifically to conjugates on the anterior sperm head and can therefore be used to monitor sperm membrane dynamics, specifically the acrosome reaction. Our results show that a finite number of fluorescence patterns occur and changes in these patterns occur over a time course where capacitation and AR might occur in Mangabeys as is reported in the mouse, human and squirrel monkey spermatozoa (Kholkute *et al.*,1990; Ward and Story 1984). Although we were unable to differentiate true from false acrosome reactions in spermatozoa, the study shows that spontaneous AR is slower than that artificially induced by calcium ionophore and dbcAMP confirming earlier hypothesis that a good sperm sample should display elevated levels of acrosome reacted sperm following an appropriate stimuli and low level of spontaneous AR. In the absence of supravital fluorochomes such as, H258 an *in vitro* study designed to assay rates of oocyte penetration by spermatozoa following induction of acrosome reaction would be more conclusive. Therefore a sperm penetration assay would have been more conclusive.

Attempts to study acrosomal status of sperm from De Brazza's monkeys using the same procedure were a failure due to loss of spermatozoa in the course of preparation. This was due the very low sperm concentration of their ejaculates.

CHAPTER SIX

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APPENDIX I

COMPOSITION AND PREPARATION OF TALP-HEPES MEDIA

TALP-HEPES (Tyrode's salt, Albumin, Lactate Pyruvate)

0.97 g Tyrode's stock powder (Sigma chemicals co. St. Louis, USA) was dissolved in 100 ml distilled water and stirred before adding 0.1g $NaHCO_3$. The following supplements were added

0.003 g Sodium pyruvate

0.3 g Bovine serum albumin (BSA)

370 microlitres Sodium lactate

1 ml Pen/Streptomycin

HEPES buffer (= N-(2-hydroxyethyl) piperazine- N'- (ethanosulfonic acid)) added at concentration of 2 ml/100 ml of media

One drop of phenol red

pH was adjusted to 7.4 and the media was filtered through 20 micrometer nalgene filters before storage at 4 °C within a period of 3 weeks. Media used from above stock was incubated at 5% : 95% CO_2 at 37 °C overnight.

TYRODE'S SALT, stock powder (from Sigma chemicals)

Component	g/L
Sodium chloride	8.0
Potassium chloride	0.2
Calcium chloride (dihydrate)	0.265
Magnesium chloride (hexahydrate)	0.214
D-Glucose	1.0
Sodium phosphate monobasic (anhydrous)	0.05
Grams/Litre of powder required per 1L liquid media	9.7

APPENDIX II

TABLES 1-7, ELECTRO-EJACULATION RESULTS PER ANIMAL AMONG TANA MANGABEYS AND DEBRAZZA'S MONKEYS.

Table 1. (G15)

Date of electroejaculation 1997	*wt. of semen (g)*	*vol. of semen (µl)*	*sperm concentration x 10^6/ML*	*pH*	*viscosity*	*Semen appearence*
12/6	0.12	48	3500	8.0	> 2	cream yellow
24/6	NE-	-	-	-		
08/7	NE					
07/8	0.07	30	2190	8.5	< 2	cream yellow
14/8	0.18	120	3990	8.5	> 2	cream yellow
28/8	0.06	30	4100	8.5	< 2	
04/9	0.09	40	1830	8.0	< 2	cream yellow
21/9	NE	-	-	-	-	-

Table 2. (G20)

Date of electroejaculation 1997	*wt. of semen (g)*	*vol. of semen (µl)*	*sperm concentration x 10^6/ML*	*pH*	*viscosity*	*Semen appearence*
29/5	0.25	150	2400	8.5	> 2	cream yellow
12/6	0.10	50	1800	8.5	> 2	cream yellow
24/6	0.03	30	1310	8.5	< 2	cream yellow
08/7	0.03	20	1635	8.0	>2	cream yellow
29/7	0.06	30	1500	9.0	<2	cream yellow
05/8	0.06	30	1930	8.0	<2	cream yellow
12/8	0.07	35	1675	8.5	>2	cream yellow
28/8	0.08	45	1040	8.0	<2	cream yellow
04/9	NE					
12/9	NE	-	-	-	-	-

Table 3. (G24)

Date of electroejaculation 1997	*wt. of semen (g)*	*vol. of semen (µl)*	*sperm concentration x 10^6/ML*	*pH*	*viscosity*	*Semen appearence*
22/5	0.15	80	1280	8.0	<2	cream yellow
04/6	0.12	70	1500	8.0	>2	cream yellow
18/6	0.20	150	1000	9.0	<2	cream yellow
01/7	0.12	60	1125	8.5	<2	cream yellow
08/7	NE	-	-	-	-	
29/7	0.10	60	2000	8.0	>2	cream yellow
05/8	0.08	40	2710	8.5	>2	cream yellow
12/8	0.07	40	1200	8.0	<2	cream yellow
01/9	0.17	100	4070	8.5	>2	cream yellow
15/9	0.08	50	2950	8.5	>2	cream yellow

Table 4. (G 27)

Date of electroejaculation 1997	wt. of semen (g)	vol. of semen (µl)	sperm concentration x 10^6 /ML	pH	viscosity	Semen appearence
21/5	0.12	62	1140	8.0		cream yellow
05/6	NE	-	-	-	-	-
12/6	0.06	32	1195	8.5		cream yellow
19/6	0.10	50	1674	8.5		cream yellow
01/7	0.08	40	1760	8.5		cream yellow

Table 5. (D44)

Date of electroejaculation 1997	wt. of semen (g)	vol. of semen (µl)	sperm concentration x 10^6 /ML	pH	viscosity (cm)	wt. of coagulum
14/5	NE	-	-	-	-	-
05/6	NE	-	-	-	-	-
19/6	0.02	35	32	8.5	>2	0.06
01/7	NE	-	-	-	-	-
07/8	0.08	40	65.5	8.5	>2	0.03
13/8	NE	-	-	-	-	-
19/8	0.07	36	42.8	9.0	>2	0.04
01/9	0.15	60	28.5	8.5	>2	0.02
15/9	0.04	20	20	8.5	>2	0.03

Table 6. (D45)

Date of electroejaculation 1997	wt. of semen (g)	vol. of semen (µl)	sperm concentration x 10^6 /ML	pH	viscosity	wt. of coagulum (g)
21/5	0.03	30	23.8	8.5	>2	0.01
04/6	0.03	35	32	8.5	>2	0.03
10/6	NE	-	-	-		-
18/6	0.02	20	24	8.5	>2	0.01
01/7	0.06	40	15	8.5	>2	0.04
13/8	0.08	60	120	8.5	>2	0.10
19/8	NE	-	-	-		-
02/9	0.22	80	22.4	8.0	>2	0.04
17/9	0.05	35	69.0	8.0	>2	0.02
25/9	NE	-	-	-	-	-

Table 7. (D46)

Date of electroejaculation 1997	*wt. of semen (g)*	*vol. of semen (µl)*	*sperm concentration x 10^6 /ML*	*pH*	*viscosity*	*wt of coagulum (g)*
27/5	0.07	38	120	8.5		0.05
10/6	0.06	35	90	8.5		0.04
24/6	NE	-	-	-	-	-
08/7	NE	-	-	-	-	-
14/8	0.04	30	140	8.0		0.03
19/8	0.08	40	64.5	9.0		0.06
02/9	0.05	33	180	8.5		0.04

Grade and Percentage of Sperm Motility in Mangabey

Date 1997	Animal	Percentage grade of sperm motility			
		a	b	c	d
	G15				
12-6		54	26	12	8
14-8		58	12	6	10
04-9		55	17	16	12
	G20				
29-5		57	19	10	14
12-6		60	21	6	13
28-8		51	27	15.5	6.5
	G24				
24-6		66	8.5	15.5	10
29-7		70	15	5	10
01-9		80	2	5	13
21-5	**G27**	72	5	14	9
19-6		75	6	11.5	7.5
01-7		81	4	5.5	9.5

Grade and Percentage of Sperm Motility in De Brazza's monkeys

Date 1997	Animal	Percentage grade of sperm motility			
		a	b	c	d
	D44				
19-6		54	5.6	7.8	32.5
7-8		42	11.5	21	25.5
1-9		53	9	16.5	21.5
	D45				
1-7		41	18.5	13	27.5
13-8		47	14	15	24
2-9		53	10	16	21
	D46				
27-5		48.5	12.5	9	30
14-8		60	12	13	15
19-8		50	10.5	17	22.5

key:

Grades a: percentage Rapid progressive sperm motility

b: percentage slow progressive sperm motility

c: percentage Nonprogressive sperm motility

d: percentage Nonmotile sperm

NE = No Ejaculate

APPENDIX III

Table 1 Characteristics of ejaculates obtained from Mangabeys and De Brazza's monkeys (Mean±s.e.m)

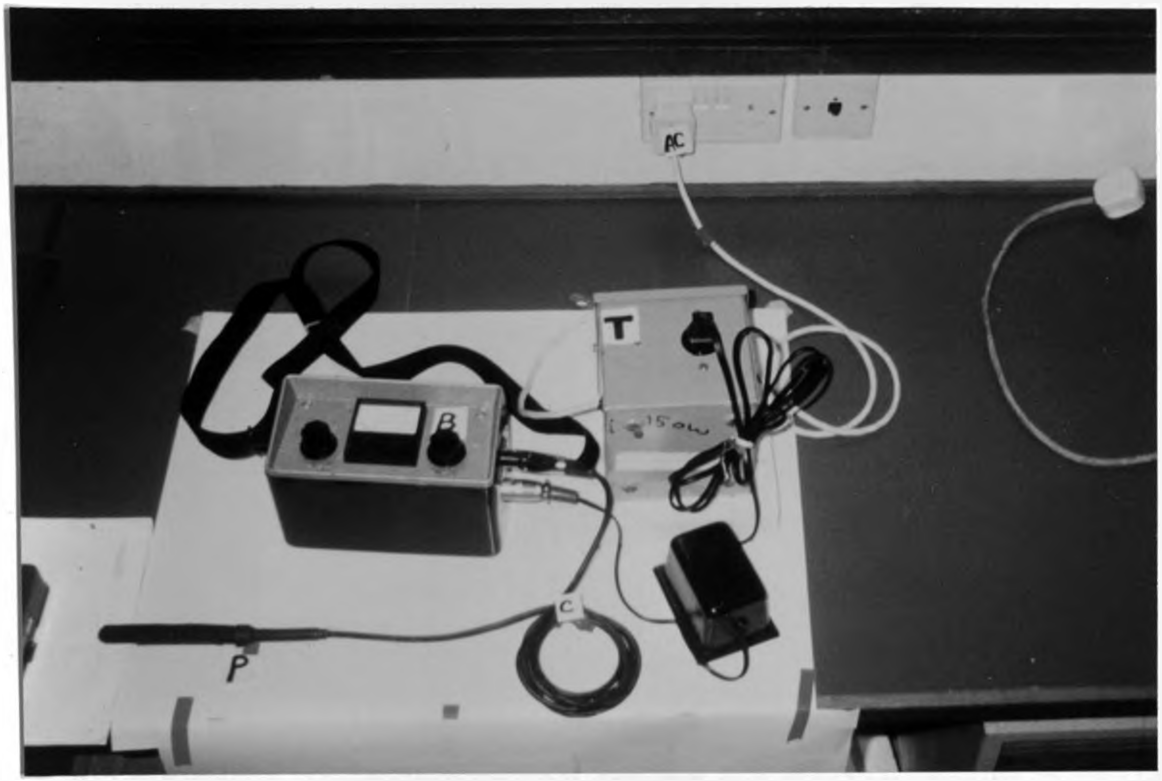
	Mangabeys (n=4)	De Brazza's monkeys (n=3
Mean time (minutes) for ejaculation	7.5	18.5
Number of successful ejaculations	26 (78.7%)	17 (65.3%)
Appearance of semen	opaque creamy yellow	turbid, colorless with coagulum
Ejaculates with viscosity >2 cm.	5 (29.4%)	17 (100%)
Semen pH	8.3 ± 0.05	8.4 ± 0.07
Mean ejaculate volume (µl)	57.4 ± 6.8	39.2 ± 3.6
Mean sperm concentration ($\times 10^6$/ ml)	1989 ± 200	64.1 ± 12
(%) Mean ±s.e.m Initial sperm motility	grade a: 65 ± 3 grade b: 13.5 ± 2.4 grade c: 10.2 ± 1.2 grade d: 10.2 ± 0.6	grade a: 50 ± 2 grade b: 11.5 ±1.7 grade c: 14.2 ± 1.3 grade d: 23.2 ± 2.6
Mean total sperm count/ejaculate	113.96 × 10^9/ml	2.513 × 10^9/ml

APPENDIX IV

Mean Counts of Fluorescent Acrosome Pattern in Mangabey Spermatozoa (Mean±SD)

Time (minutes)		Pattern IA	Pattern PA	Pattern AR
0.0	control	198 ± 0	2 ± 0	0.0
30	control	184 ± 4	13.3 ± 5.7	2.0 ±3.4
	experimental	158.6 ± 6.1	28.3 ± 9.2	13 ± 3.6
	P value	0.002	0.038	0.009
60	control	177.1 ± 5.5	17.3 ± 5.7	6 ± 5.2
	experimental	147 ± 14.4	40.3 ± 5.5	17 ± 2.3
	P value	0.015	0.004	0.0014
90	control	171.3 ± 3	18.6 ±5.8	10 ±3.6
	experimental	123.6 ± 3.5	52 ± 5.20	24 ± 5.5
	P value	0.0001	0.0009	0.0098
120	control	145.3 ± 5	32 ±5.20	22.6 ±1.1
	experimental	82.6 ± 25	43.3 ±7.5	62 ± 9.1
	P value	0.006	0.050	0.0009
150	control	134.6 ± 5	38.6 ± 4.1	26.6 ± 3.
	experimental	67.6 ± 7.6	58.3 ± 9.7	74.4 ± 4
	P value	0.0001	0.016	0.0001

APPENDIX V



B: A rechargeable Standard Precision Electronic electro-ejaculator (SPE), **P**: Probe, **T:** transformer, **C**: probe cable and **AC**: alternating current source for charging.