ASPECTS OF THE REPRODUCTIVE PHYSIOLOGY OF MALE VERVET MONKEYS MAINTAINED IN A LABORATORY ENVIRONMENT

by

Jürgen Victor Seier

Submitted in partial fulfilment of

the requirements for the degree of

Master of Science

in the Department of Physiology

University of the Western Cape

Promoter: Professor Gerhard van der Horst

Joint Promoter: Dr. Roelof Menkveld

Submitted: November 1995

I, Jürgen Victor Seier, declare that the "ASPECTS OF THE REPRODUCTIVE PHYSIOLOGY OF MALE VERVET MONKEYS MAINTAINED IN A LABORATORY ENVIRONMENT" is my own work and that all sources I have used or quoted have been indicated and acknowledged by means of complete references.

Signature: J. V. Seier Date: November 1995

Acknowledgments

I would like to thank the following persons and institutions for making this thesis possible:

Professor Gerhard van der Horst for his support, advice and guidance

Dr. Roelof Menkveld for his support and advice

Dr. Sonia Wolfe-Coote for her support

Mr. Abraham Davids and Mr. Timothy Collop for expert animal care and technical assistance throughout the study

Mrs. Jacoba Laubscher for her help with the statistical analyses

Mrs. Elmarie Conradie for her technical advice

Dr. Edmund Oettlé for advice on cryopreservation

The Medical Research Council for their support of this study.

Dedicated to my wife Sally

and my parents

CONTENTS

List of tables

Abstract

In biomedical research limited use has been made internationally of African non-human primates. As a result, their reproductive physiology has been less well defined, when compared to the more commonly used macaque species. There have also been no extensive developments in the associated field of assisted reproduction for African non-human primates.

To contribute to the knowledge of the reproductive physiology of African non-human primates, this study presents semen characteristics from the vervet monkey. The focus is on abnormal sperm morphology which has not been described in detail. All individuals utilized were either wild caught or colony bred and maintained in an indoor laboratory breeding colony.

A detailed description of the frequency and prevalence of specific morphologically abnormal forms of sperm from vervet monkeys and illustrations of the different types of abnormalities is provided for the first time. Most features, such as the prevalence of tail abnormalities, particularly coiled and bent tails, were similar to what has been reported for other Old World cercopithecines. A total of 28 types of morphologically abnormal forms were found including 13 head abnormalities, seven midpiece abnormalities and eight principal- and endpiece abnormalities.

Sperm head abnormalities were rare, occurring at a rate of less than 2% in each group. Except for the nipple defect, there was no difference between colony bred and wild caught individuals in the rate at which defective forms occurred. On the other hand not all types of abnormalities were found in each group.

A relationship between morphology and fertility could not be established because some individuals with the highest rate of abnormal morphology were successful breeders. Evaluation of consecutive ejaculates revealed highly variable semen characteristics both within and amongst different individuals. This observation extends to other sperm characteristics such as the concentration, vitality, speed of forward progression (FP) and motility. The evaluation of reproductive potential based on one or two ejaculates is therefore not possible.

The pH of vervet monkey semen was similar to that of humans. Out of two indicator papers tested to determine this variable, only one was found to be reliable in terms of reference values obtained with a pH meter.

Another aim of the study was to develop a cryopreservation method which yields a satisfactory post-thaw recover of progressively motile sperm. No specialized equipment other than a liquid nitrogen storage container was needed. Semen samples were diluted with a Tes-Tris extender containing 5% glycerol before being cooled to 5° over 30 minutes.

This was followed by aspiration of the samples into paillettes and freezing to a plunge temperature of -139°C for 20 minutes in liquid nitrogen vapour. The paillettes were finally stored in liquid nitrogen. Conspicuous features of this method were the addition of glycerol at 32°C, a short glycerol equilibration time, a fast freezing rate, a low plunge temperature and a fast thawing rate.

Opsomming

In biomediese navorsing op internasionale gebied, is die gebruik van Afrika-nie-menslike-primate beperk. Derhalwe is hul voortplantingsfisiologie minder deeglik gedefinieer as dit vergelyk word met die mees algemeen gebruikte *Macacca* spesies. Daar is ook geen uitgebreide ontwikkelings in die geassosieerde veld van geassisteerde reproduksie vir Afrika-nie-menslike-primate nie.

Om by te dra tot die kennis van die voortplantingsfisiologie van die Afrika-nie-menslike-primaatspesies, verstrek hierdie studie die semeneienskappe van die blouaap. Die fokus is op abnormale spermmorfologie, wat tot nog toe baie min aandag geniet het. Die diere vir die studie is onderhou in 'n binnenshuise laboratorium-teelkolonie en bestaan uit individue wat in die natuur gebore is, sowel as diere wat in gevangeskap gebore is.

Die voorkomsyfer en frekwensie van spesifieke morfologies abnormale vorms van blouaap sperme, word beskryf en geïllustreer vir die eerste keer. Die meerderheid van kenmerke soos die voorkoms van stertabnormaliteite, veral gekronkelde en geboë sterte, was soortgelyk aan die wat gerapporteer is vir ander ou-wêreld *Cercopithecidae*. 'n Totaal van 28 morfologies abnormale vormtipes is gevind en bestaan uit 13 kopabnormaliteite, 7 middelstukabnormaliteite en 8 hoof- en

endstukabnormaliteite.
Spermkopafwykings was skaars, met 'n voorkomsyfer van minder as 2% in elke groep. Met die tepelafwyking buite rekening gelaat, was daar geen verskille in die tempo waarteen abnormale vorme voorgekom het tussen koloniegeteelde en natuurgebore individue nie. Nie alle tipe abnormaliteite het egter by elke groep voorgekom nie.

'n Verwantskap tussen morfologie en vrugbaarheid kon nie gevind word nie, as gevolg van die feit dat van die individue met die hoogste abnormale tellings, goed geteel het. Ontleding van opeenvolgende ejakulate het groot variasie in semeneienskappe tussen monsters van dieselfde individue en ook tussen verskillende individue getoon. Hierdie bevinding geld ook vir ander spermeienskappe soos konsentrasie, lewenskragtigheid, spoed en voorwaartse beweging en beweeglikheid. Dit is daarom nie moontlik om voortplantingspotensiaal te evalueer op een of twee ejakulate nie. Die pH van die blouaap semen, was soortgelyk aan die van die mens en slegs een van die twee tipes toetsstrokies, wat vir hierdie doel gebruik is, het waardes weerspieël wat vergelyk het met pH-meterlesings.

Nog 'n rede vir hierdie studie was om 'n vriesbewaringsmetode vir blouaapsperme te ontwikkel, wat 'n aanvaarbare na-ontdooiingsopbrengs van sperme met 'n voorwaartse beweeglikheid sou gee. Die enigste spesiale apparaat wat hiervoor benodig

was, is 'n bergingshouer met vloeibare stikstof. Semenmonsters is verdun met 'n Tes-Tris verdunningsvloeistof wat 5% gliserol bevat, voordat hulle afgekoel is tot 5°C, oor 'n periode van 30 min. Hierna is die monsters opgetrek in strooitjies en afgekoel vir 20 min in vloeibare stikstofdamp tot 'n voor-onderdompelingstemperatuur van -139°C. Na onderdompeling word die strooitjies so bewaar. Uitstaande kenmerke van hierdie metode is die byvoeging van gliserol by 32°C, 'n kort gliserol-balanseringstyd,'n vinnige vriestempo, 'n lae dompeltemperatuur en 'n vinnige ontdooiingstempo.

CHAPTER I

1. General introduction and background

The mammalian order of primates is regarded as the highest evolved in terms of brain size and complexity and cognitive abilities (Ankel-Simmons 1983 a, Fobes and King 1982). Both human and non-human primates belong to this order and many behavioural, genetic, anatomical and physiological similarities reflect this close phylogenetic relationship (Hendrickx and Binkerd 1990, King *et al.* 1988, Müntzing *et al.* 1975, Smith and Williams 1974). As a result, non-human primate species are often regarded as the most relevant and important animal model in biomedical research (Hendrickx and Binkerd 1990, King *et al.* 1988, Lapin 1982, Müntzing *et al.* 1975, Smith and Williams 1974).

The similarities are particularly conspicuous when comparing the reproductive physiology and anatomy of human and non-human primates (Ankel-Simmons 1983 b, Hendrickx and Binkerd 1990, King *et al.* 1988, Müntzing *et al.* 1975). These include obvious features such as the menstrual cycle, a simplex uterus and a pendulous penis (Ankel-Simmons 1983 b). But there are many other similarities such as aspects of the endocrinological control of the reproductive cycle and pregnancy, implantation, placentation and embryogenesis, male accessory gland function, spermatogenesis and spermiogenesis (Ankel-Simmons 1983 b, Hendrickx and Binkerd 1990, King *et al.* 1988, Müntzing *et al.* 1975, Tanimura and Tanioka 1975).

Detailed definition and understanding of the reproductive physiology of primates, which is only possible by maintaining captive populations, is necessary for three main reasons:

1. To support the management of wild populations in their natural habitats and for a complete understanding of their reproductive biology and phylogenetic position.

2. It is essential for the effective breeding management of captive non-human primates. Effective breeding is supported by the development of associated techniques for assisted reproduction such as cryopreservation of semen. Captive propagation is regarded as the only ethical alternative to ensure the future supply of primates for biomedical research and to preserve the gene pool of rare and or endangered species.

3. Reproductive research utilizing primates needs to be based on defined models and detailed baseline data.

The primate species most commonly utilized in biomedical research are from the genus *Macacca*, all of which except for one species occur in Asia (Held and Wolfle 1994, King *et al.* 1988). The reasons for this preference are historical rather than biological or scientific. Consequently the reproductive physiology of members of this genus has been extensively studied and is well defined (Austin 1975, Barnes *et al.* 1978, Goodman *et al* 1977, Gulyas *et al.* 1976, Hein *et al.* 1989, Mahoney 1975, Monfort *et al.* 1986, Moudgal 1984, Shackleton and

Mitchell 1975, Tanimura and Tanioka 1975, Wehrenberg *et al.* 1979).

Limited use has been made internationally of African primate species, resulting in a particular lack of data of aspects of male reproductive physiology. The reason is that the study and definition of the reproduction of non-human primates has often been a by-product of contraceptive research. This frequently targets the female and uses the male only as a sperm donor. Since the vervet monkey is indigenous to Southern Africa, it is one of the two primate species of choice in local biomedical research. Because of it's size it is, however, the only species with the potential of complementing or substituting the internationally more commonly used macaques. Yet little contribution has been made locally to the study and definition of vervet reproduction or the development of techniques for assisted reproduction.

The following is an introduction to the vervet monkey's natural history and breeding biology and a review of vervet monkey and general non-human primate spermatology. Since the focus will be on sperm morphology and the cyropreservation of semen some basic principles are reviewed and discussed in both fields.

1.1 The vervet monkey

1.1.1 Natural history

The vervet monkey (*Cercopithecus aethiops*), also called African green monkey, is one of four primate species indigenous to Southern Africa (Napier and Napier 1967). Taxonomically it is one of about 20 species and over 60 subspecies of the genus *Cercopithecus* (Fobes and King 1982, Napier and Napier 1967). This genus together with previously mentioned *Macacca* and *Papio* species belong to the same family, the *Cercopithecidae* (Fobes and King 1982, Napier and Napier 1967). All are categorized into the infraorder Old World primates (*Catarrhini*) and the mammalian order of primates (Fobes and King 1982).

Being widely distributed throughout sub-Saharan Africa and often abundant where it occurs, it's habitat include most areas except deserts, open grasslands, dense forests and mountains (Eley 1992, Napier and Napier 1967). The vervet monkey is an omnivorous feeder and lives in small multi male multi female groups of up to 50 to 60 individuals. Groups are socially highly organised with a linear dominance relationship amongst the males and a matriarchal kin group relationship amongst the females (Eley 1992). The body weight of an adult male ranges from 4 to 7 kg and that of a female from 3 to 5 kg. Vervets can lead an arboreal and terrestrial existence which has made them so successful in terms of distribution.

1.1.2 General breeding biology

The vervet monkey has been described as a marginal seasonal breeder but breeds all year round in captivity (Eley 1992, Seier 1986). The females experience no estrogen stimulated cyclic perineal swelling which is the hallmark of cyclicity of many other primate species (Seier *et al.* 1991). Males and females mate throughout the cycle and not just pre-or periovulatory which, according to one hypothesis, is typical for species without cyclic perineal swelling. Normally one infant per year is produced which stays for about 12 months with it's mother.

1.2 Review of Vervet male reproductive physiology with a focus on spermatology and cryopreservation of semen

1.2.1 *Spermatogenesis, spermiogenesis and semen biochemistry*

The cellular associations and morphological features during spermatogenesis and spermiogenesis have been described for vervet monkeys by Barr and Clermont (1973, 1969). The former author also defined the duration of spermatogenesis and the length of one seminiferous cycle (Barr 1973). The structure of Leydig cells is reported to follow the general pattern found in other mammals (Camatini *et al.* 1981). Defective spermatozoa are removed by phagocytosis from the epididymis (Roussel *et al.* 1967).

No seasonal changes in seminiferous tubule diameter nor changes in spermatogenic activity suggesting seasonality were reported from captive vervets from East Africa (Eley *et al.* 1986).

A number of biochemical parameters studied in vervet semen including fructose, lactic acid and citric acid were within the range of many other non-human primate species (Ackermann and Roussel 1968). The same authors also investigated the citric acid, lactic acid and oxygen metabolism of fresh and frozen vervet semen (Ackerman and Roussel 1971).

Testosterone concentrations in adult male vervets are similar to those in rhesus monkeys (Eley *et al.* 1986) and no seasonal changes were recorded. However diurnal fluctuations of androgen and estradiol concentrations were recorded in adult male vervets (Beattie and Bullock 1978).

1.2.2 *Spermatology of the vervet monkey*

Most studies on vervet semen were confined to investigating sperm concentrations and motility. Roussel and Austin (1967) studied, amongst others, the motility of sperm from five non-human primate species. A mean sperm motility of 53% was recorded for eight samples from three vervet monkeys. This was similar in the other four non-human primate species investigated in the same study. A more detailed spermiogram of 11 primate species was provided by Ackermann and Roussel (1968). Six ejaculates from two individual vervet monkeys were

evaluated according to four parameters. The sperm concentration was very variable ranging from 57 million/ml to 288 million/ml and the motility varied from 34 to 38%. The percentage of vital spermatozoa was 45% and 50% as determined with eosin staining. The spermatozoa with abnormal morphology were reported to be 26% and 40% but the different abnormal forms, their rate and prevalence were not investigated. In another study the sperm concentration and motility of seven semen samples from two vervet monkeys were determined (Ackermann and Roussel 1971). The concentration of 1428 million/ml and extremely low motility of 1.1% was not in agreement with any of the other values reported previously.

Another more complete spermiogram was provided by Valerio and Dalgard (1975). This included for the first time the ejaculated volume which was 1.24ml and was made up of a liquid portion of 1ml and a coagulum of 0.24ml. The sperm concentration was found to be 144 million/ml, the motility 58% and spermatozoa with abnormal morphology 24%. Again no specific defects, their prevalence or rates at which they occur were reported. The ratio of dead sperm was 13%, which was lower than reported previously for vervet monkeys. The sample size for this study was not mentioned. Hendrickx *et al.* (1978) reported some semen characteristics of 23 ejaculates from four vervet monkeys. The average ejaculate volume was reported to be 0.9 ml, the mean sperm concentration 440 million/ml and the motility was 39%. These results reflected again the big variability in the reported semen characteristics of vervet

monkeys. In a study to investigate the influence of ethanol on the semen characteristics of six adult males, the sperm concentration and gross normal morphology were determined (Van der Colf *et al.* 1991). The sperm concentration agreed mostly with what was reported in above mentioned studies. Few defective spermatozoa were found and the gross normal morphology varied mostly between about 67% and 100%. No reference was made to specific defects.

The only mention of a specific morphological defect in ejaculated semen in vervet monkeys was made by Conradie *et al.* (1994). The acrosomal integrity was studied in fresh spermatozoa and it was found that an average of 61.2% were intact, 18.4% mildly damaged, 16.8% severely damaged and 4.6% lost or reacted acrosomes. The work was done on 10 semen samples from 10 different monkeys.

Despite the big variation of results, vervet monkey sperm characteristics were similar to those of other *Cercopithecines*. However, nobody has provided a detailed description and or illustration of the type of abnormal forms, the rate at which they occur and their prevalence. The pH is another semen characteristic that has not been investigated.

Although the influence of abnormal morphology on fertility has not been established in non-human primates, a definition is necessary for a complete understanding of vervet spermatology and as baseline data from which to evaluate changes in the semen profile. This will assist in the investigation of

reproductive potential, disease states, and the influence of compounds on the male reproductive system during pharmakodynamic and general toxicological studies.

1.3 General primate spermatology

The viability of ejaculated spermatozoa are usually evaluated by establishing a spermiogram using standard techniques and parameters (World Health Organisation 1992). Spermiograms have been established for a number of primate species and the most frequently reported parameters are: sperm concentration, motility, vitality and the proportion of total abnormal or normal morphology (Ackermann and Roussel 1968, Bornman *et al.* 1988, Bush 1975, Gould and Mann 1988, Harrison *et al.* 1986, Harrison and Wolf 1985, Harrison 1980, Hendrickx *et al.* 1978, Sarason *et al.* 1991, Schaffer *et al.* 1992, Thomson *et al.* 1992 Valerio and Dalgard 1975, Lang 1967).

The different morphologically abnormal forms which occur in most ejaculates and the rate at which they occur appears not to have been studied in detail and most investigators report only the total normal or abnormal morphology (Ackermann and Roussel 1968, Bornman *et al.* 1988, Bush *et al.* 1975, Gould and Mann 1988, Harrison *et al.* 1986, Harrison and Wolf 1985, Harrison 1980, Hendrickx *et al.* 1978, Lang 1967, Sarason *et al.* 1991, Schaffer *et al.* 1992, Valerio and Dalgard 1975).

In a study to evaluate semen from capuchins monkeys it was found that the most common abnormalities were detached heads and bent and coiled tails (Bush *et al*. 1975). Although no correlation with motility could be demonstrated, it appeared that abnormalities were most commonly found in dead spermatozoa as determined by supra-vital staining. Tail defects were again the most common abnormality in marmoset spermatozoa with a median of 50% (Cui *et al*. 1991). Head defects were on the other hand rare with a median of 4.5%. The same applied to tamarins where the most frequent abnormalities were midpiece defects (Harrison and Wolf 1985). Coiled tails, cytoplasmic droplets and detached heads were also observed but in very small numbers. The overall rate of defective spermatozoa was low and more than 5% was considered abnormal for this species.

Abnormal forms were found to be also rare in the spermatozoa of rhesus monkeys and none of the ejaculates of 100 males investigated contained more than 5% abnormal forms (Harrison 1980). The most common defect found was abnormal midpieces. Others were amorphous heads, cytoplasmic droplets, small heads, detached heads and coiled tails but none of these occurred more frequently than 2-3%. Abnormal spermatozoa were relatively rare in cynomolgus monkeys and less than 10% of all ejaculates contained more than 30% abnormal forms including 15% bent and 8% kinked tails (Mohamed *et al*. 1987). Only tail abnormalities appear to have been observed during this study.

The trend towards the prevalence of tail defects was also

observed in Sulawesi macaque sperm (Thomson *et al.* 1992). Coiled, kinked and short tails were the main defects found in four ejaculates from four males. Only few head defects occurred in three individuals but the type of defects were not reported. The overall rate of morphologically normal sperm was high (96.8%).

Ejaculated spermatozoa from cynomolgus monkeys were morphologically similar to those aspirated from various regions of the epididymis except for the location and occurrence of the cytoplasmic droplet (Mahony *et al.* 1993). The ultrastructure of bonnet monkey sperm was similar to that of other non-human primate species (Kalla *et al.* 1986).

It appears that in most non-human primate species the most common abnormalities are confined to the tail while head defects are rare.

1.4 Classification of abnormal morphology

1.4.1 *The normal sperm:*

The point of reference for the study and description of abnormal morphology is normal morphology. Both normal and abnormal morphology have been well defined in the human and a number of domestic animal species (Freund 1966, Garner and Hafez 1985, Hafez 1985 a, Hofmann and Haider 1985, Menkveld *et al*. 1990, Menkveld *et al*. 1991, MacLeod 1964, Oettlé *et al*. 1991, Oettlé and Soley 1988, World Health Organisation 1992). Relatively few and limited data exist on the normal and abnormal morphology of sperm from non-human primates (Cui *et al*. 1991, Bush *et al*. 1975, Mohamed *et al*. 1987, Thomson *et al*. 1992)

Sperm morphology includes the following features:

1.4.1.1 *The sperm head.*

This is subdivided into four sections

A) and B) The apical and principal segment consisting of the acrosomal cap and apical ridge. The acrosome contains enzymes such as acrosin and hyaluronidase which are liberated during the acrosome reaction and are necessary for penetrating the zona pellucida (Garner and Hafez 1985).

C) The equatorial segment, which is a narrow band at the

posterior border of the acrosome and is the area of first contact and fusion with the oocyte membrane (Garner and Hafez 1985).

D) The post acrosomal region which is the remaining portion of the nucleus protruding from under the acrosome (Garner and Hafez 1985). The nucleus is composed of dense chromatin containing DNA (Garner and Hafez 1985).

According to WHO criteria for human spermatozoa the head length to width ratio must be 1.50 to 1.75 (World Health Organisation 1992). The head must have a smooth oval configuration with a regular outline. The acrosome should cover 40-70% of the head area. The length of the head in the human spermatozoa should be 4.0-5.5 micrometer and the width 2.5-3.5 micrometer. The sperm head dimensions can however vary with the type of sperm preparation and processing (Davis and Gravance 1993).

1.4.1.2 *The sperm tail*

This is subdivided into four sections.

A) The neck forms the baseplate which should be axially attached to the nucleus. The baseplate is continuous with nine coarse fibers which are part of the tail (Garner and Hafez 1985)

B) The midpiece is the portion of the tail from the neck to the annulus. It has to be slender, free of bends or kinks,

with a regular outline and must be less than one third the width of the head (Menkveld *et al*. 1991). The axoneme, which consists of two central filaments surrounded by nine pairs of microtubules and nine course fibers is the part of the midpiece continuous with the tail (Garner and Hafez 1985). This is covered in a sheath of coils of mitochondria which generate energy for motility (Garner and Hafez 1985). Cytoplasmic droplets which are usually attached to the midpiece must not be larger than one third of the head area (World Health Organisation 1992). The length of the midpiece in the human spermatozoa should be approximately 7-8 micrometers or one and a half times the head length (Menkveld *et al*. 1991, Menkveld *et al*. 1990).

C) The principal piece is made up of the axoneme surrounded by nine course fibers all covered in a fibrous sheath (Garner and Hafez 1985). It has to be slightly thinner than the midpiece, uncoiled, free of kinks and bends and with a regular outline (Menkveld *et al*. 1990, Menkveld *et al*. 1991). The flagellar movements of the tail provides propulsion.

D) The endpiece consists of the axoneme covered by a plasma membrane (Garner and Hafez 1985). The length of the principal and endpiece in human sperm should be about 45 micrometers (Menkveld *et al*. 1991, Menkveld *et al*. 1990).

1.4.2 *The abnormal sperm*

During spermatogenesis, spermiogenesis and the passage of sperm through the epididymis a certain number of spermatozoa are produced which will deviate to any degree from the majority of the "standard" or normal forms. The incidence varies from species to species (Bush *et al*. 1975, Cui *et al*. 1991, Hafez 1985 a, Oettlé *et al*. 1991, Oettlé and Soley 1988). The influence of these forms on fertility depends on their proportion in an ejaculate and whether they are major or minor abnormalities (Hafez 1985 a, Oettlé *et al*. 1991, Oettlé and Soley 1988, Oettle and Soley 1985). Some defects responsible for lowered fertility can also be inherited such as the nuclear pouch or diadem defect, the knobbed sperm defect, Dag defect and decapitate defect which all occur in the bull (Hafez 1985 a). In humans the influence of abnormal sperm morphology on fertility has been better elucidated through techniques such as oocyte penetration tests and *in vitro* fertilization (Menkveld *et al*. 1991). In many mammals, particularly wild species, this relationship is however still uncertain, if it at all exists.

Recent trends have been to classify abnormalities according to their origin in spermatogenesis, the construction principle of spermatid differentiation during spermiogenesis and Sertoli or Leydig cell dysfunction (Hofmann *et al*. 1982, Hofmann and Haider 1985, Hofmann and Freundl 1986). However the identification of the basic types of abnormal forms has not changed (Hafez 1985 a, Hofmann and Haider 1985, MacLeod 1966,

Oettlé *et al*. 1991, World Health Organisation 1992).

Aberrant forms are broadly classified into head and tail abnormalities. Either of these can be subdivided into minor or major abnormalities (Freund 1966, Hafez 1985 a, MacLeod 1964, Oettlé *et al*. 1991, Oettlé and Soley 1988,).

1.4.2.1 **Common abnormal forms**

1.4.2.1.1 *Head abnormalities.*

Elongation and or tapering: This may affect the whole head or either the acrosome or post acrosomal region individually (Cui *et al*. 1991, Hofmann and Haider 1985, MacLeod 1964, Oettlé *et al*. 1991). This defect can occur to varying degrees and is often associated with abnormal acrosome development (Hofmann and Haider 1985, Oettlé *et al*. 1991). It is believed that the cause may be Sertoli cell dysfunction since their cytoplasmic filaments are probably responsible for the elongation and flattening of the sperm head (Hofmann and Haider 1985, Oettlé *et al*. 1991). Acrosome defects are believed to be caused by disturbed steroid synthesis due to Leydig cell dysfunction. The pyriform or pear shaped and pointed head also belong to the elongated and tapering group (Oettlé *et al*. 1991).

Round heads: In the human this is often accompanied by a reduced or absent acrosome (Hofmann and Haider 1985).

Macro-and Microcephalus: This is rare in humans and some non-

human primate species (Cui *et al.* 1991, Hofmann and Haider 1985). The small forms are often associated with various acrosome abnormalities (Hofmann and Haider 1985). "Too small" has been quantified as being less than 60% of the mean, and "too large" as more than 140% of the mean (Freund 1966).

Duplicates: Two or more heads are usually contained in a plasma membrane. This is due to a mitosis defect and such heads can also be microcephalic (Hofmann *et al.* 1982, Oettlé *et al.* 1991).

Amorphous: This is a catch all group for bizarre shapes and forms which are difficult to fit into any above mentioned categories (Cui *et al.* 1991, MacLeod 1964).

Vacuoles, cysts and invaginations: The diadem defect in the bull and human is caused by single or multiple invaginations into the nucleus (Hafez 1985 a, Oettlé *et al.* 1991). Multiple invaginations often occur in a row in the equatorial region, giving the appearance of a diadem around the head. A number of vacuoles can occur in the acrosome and post acrosomal region (Oettlé *et al.* 1991, Oettlé and Soley 1988,). This occurs in normal human sperm and is often only registered when more than two vacuoles are present or the vacuole is of unusually large size (Hofmann and Haider 1985).

Detached Head: The head is separated from the tail (Bush *et al.* 1975, Hafez 1985 a).

1.4.2.1.2 *Neck and tail abnormalities*

An abaxial implantation of the neck is considered abnormal (Oettlé *et al*. 1991).

A retained cytoplasmic droplet of residual cytoplasm can be attached distal or proximal to the midpiece and is associated with sperm immaturity (Garner and Hafez 1985, Oettlé and Soley 1988). It is considered abnormal in most species (Garner and Hafez 1985) but in the human it is only abnormal if larger than one third the head size (World Health Organisation 1992).

Further abnormalities of the midpiece include kinks and bends and thickening of the midpiece to more than one third of the head width (Menkveld *et al*. 1991, Oettlé and Soley 1985, World Health Organisation 1992). Some of these can lead to infertility if they occur in large numbers (Oettlé and Soley 1985). A number of midpiece abnormalities involve partial loss of the mitochondrial sheath (Hafez 1985 a, Oettlé *et al*. 1991, or loosening of the mitochondrial sheath and displacement of the mitochondria distally or proximally or both (Oettlé *et al*. 1991). This exposes the axoneme and is sometimes called pseudodroplet defect (Oettlé *et al*. 1991, Oettlé and Soley 1988).

The corkscrew defect is, as it's name implies, a corkscrew like deformation of the midpiece and can occur in the bull (Hafez 1985 a).

As with the other segments a midpiece can also be duplicated in

conjunction with duplication of the rest of the tail (Cui *et al*. 1991, Hafez 1985 a).

Principal - and endpiece: Some of the abnormalities in these regions are thought to have their origin in the epididymis (Oettlé *et al*. 1991). A common abnormality is coiling and folding of the tail to varying degrees (Bush *et al*. 1975, Cui *et al*. 1991, Hafez 1985 a, Hofmann and Haider 1985, Oettlé *et al*. 1991, Oettlé and Soley 1988, Thomson et al. 1992, World Health Organisation 1992). An extreme form is the Dag defect, which was first noted in Danish Jersey bulls and is characterized by tight coiling of the principal piece over the midpiece or around the head (Hafez 1985 a). Simple folding of the tail is also sometimes considered a minor Dag defect (Oettlé *et al*. 1991). The endpiece can be involved in a specific type of coiling called terminal coiling (Oettlé *et al*. 1991). As with the other segments duplication can occur (Hafez 1985 a, Oettlé and Soley 1988).

Although spermatozoa can vary in shape and size in different animal species, the morphology of spermatozoa in species with an oval sperm head and division into distinct regions is universal (Freund 1966, Garner and Hafez 1985, Hafez 1985 a, Kalla *et al*. 1986, MacLeod 1964, Oettlé and Soley 1988). This also applies to the classification of the types of abnormal spermatozoa (Bush *et al*. 1975, Cui *et al*. 1991, Hafez 1985 a, Harrison 1980, MacLeod 1964, Oettlé and Soley 1988, World Health Organisation 1992).

Therefore the majority of the classification and terminology used in this thesis will be based on work done in the human and domestic animal species.

1.5 Cryopreservation of semen

For many years it has been possible to preserve viable spermatozoa from humans and a number of domestic animals by freezing (Bamba and Adams 1990, Hafez 1985 b, Wolf and Patton 1989). Cryopreservation has since become an indispensable tool in human and animal assisted reproduction. Obtaining semen from domestic animals is relatively easy, since they are habituated to human handling. However most wild animal species including non-human primates, regardless of whether they are wild caught or bred in captivity, will not permit the type of handling necessary for the collection of semen. Manual restraint is stressful to wild animals and not commensurate with electrostimulation. This means sedation or anaesthesia is necessary for every semen collection. A method for the cryopreservation of semen is therefore particularly relevant for wild species.

The type of extender, freezing and thawing rates and equilibration times necessary to achieve the best post-thaw recovery varies among species (De Leeuw *et al.* 1990, Hammerstedt *et al.* 1990, Healey 1969, Tollner *et al.* 1990). Also specific freezing protocols have to be matched with

specific extenders (Tollner *et al*. 1990).

Most basic cryopreservation protocols include the dilution of semen in an extender containing egg yolk or skim milk, a buffer and a cryoprotectant which is usually glycerol (Ackermann and Roussel 1971, De Leeuw *et al*. 1990, Hafez 1985 b, Healey 1969, Tollner *et al*. 1990, Wolf and Patton 1989). The temperature reduction to -196°C is carried out stepwise and between steps the extended semen is maintained for different lengths of time at certain temperatures which is called equilibration (Denis *et al*. 1976, Hafez 1985 b, Tollner *et al*. 1990, Wolf and Patton 1989). The published cryopreservation methods are often difficult to compare because of the different combinations of extenders, buffers, cryprotectants, cooling and freezing methods used. The cryobiological principles involved will be discussed in detail in a following chapter.

Despite the availability of electrostimulation methods for the collection of semen from non-human primates, cryopreservation of semen has not received much attention (Ackermann and Roussel 1971, Denis *et al*. 1976, Mahone and Dukelow 1978, Kraemer and Vera Cruz 1969, Roussel and Austin 1967, Tollner *et al*. 1990). However spermatozoa from a number of species have since been frozen with mostly good success (Denis *et al*. 1976, Kraemer and Vera Cruz 1969, Roussel and Austin 1967, Tollner *et al*. 1990). Again most knowledge in this field has been gained using *Macacca* species (Mahone and Dukelow 1978, Roussel and Austin 1967, Tollner *et al*. 1990).

1.5.1 *Cryopreservation of semen from vervet monkeys.*

There are only few studies on the cryopreservation of vervet monkey semen but, like many studies with other non-human primate species, the work is outdated.

Eight semen samples from three adult males were collected by electroejaculation and the coagulum subjected to trypsin digestion (Roussel and Austin 1967). The semen was then extended in a ratio of 1:10 with 20% egg yolk, 64% of a 3% w/v sodium glutamate solution in double glass distilled water and 14% glycerol as cryoprotectant. This was followed by equilibration at room temperature for 30 minutes and sealing in glass ampoules. The ampoules were lowered into the neck of a liquid nitrogen refrigerator about five centimetre above the liquid nitrogen for five minutes. Finally they were plunged into liquid nitrogen and stored for three days. Thawing was accomplished by placing the ampoules in a water bath at 5°C for about three minutes. The survival rate of 44-56% was similar to that of four other primate species investigated in the same study (Roussel and Austin 1967). It was also found that good quality semen samples had the best chance of survival in terms of post-thaw motility and that there were differences in survival rates among individuals within a species. In another study seven semen samples from two males were frozen according to the protocol mentioned above but maintained for 6-12 months (Ackermann and Roussel 1971). The poor post-thaw motility of 0.4% was attributed to long term storage.

1.5.2 *Cryopreservation of semen from non-human primates other than vervet monkeys*

In the above mentioned studies on vervet monkeys, semen from other primate species were also frozen according to the same protocols. Roussel and Austin (1967) cryopreserved semen from rhesus and stumptail macaques, patas and chimpanzees and achieved similar recoveries as with vervet semen. Long term storage for 6-12 months was attempted semen from rhesus, stumptail and patas with similarly poor results than in vervet monkeys. The recovery of 1-1.7% was however slightly higher than in the vervet monkey (Ackermann and Roussel 1971). The suitability of spontaneous - and electroejaculates from baboons for cryopreservation was compared by Kraemer and Vera Cruz (1969). Semen was mixed with a sodium glutamate-egg yolk extender and the samples were frozen only in liquid nitrogen vapour. The survival rate was 63.1% and 64.5% for spontaneous and electro-ejaculates respectively.

A more elaborate method for the freezing of squirrel monkey semen involved two pre-extension equilibration periods of 45 minutes at 37°C and 10 minutes at room temperature (Denis *et al.* 1976). This was followed by an initial extension with 11% (w/v) lactose, 20% (v/v) egg yolk and 4% (v/v) glycerol using half of the final volume. The temperature was then reduced to 5°C and maintained for 20 minutes after which the final dilution took place with the second half of the extender. After another equilibration time of 25 minutes at 5°C, the

extended semen was dropped onto dry ice for 3-10 minutes. The pellets formed in this way were immersed in liquid nitrogen. Thawing took place in 0.9% saline at 37°C. A total of 11 and 13 samples were frozen from two individuals respectively. The mean recovery rate for one male was 87.9% ranging from 14 to 150% and for the other 83.4% ranging from 31 to 140%. No correlation between the dilution factor and the post-thaw motility could be found. There was an improvement in sperm motility after freezing.

The effect of extender pH, glycerol and egg yolk concentrations and different equilibration times on cryosurvival of spermatozoa was investigated using semen from four adult cynomolgus monkeys (Mahone and Dukelow 1978). The basic protocol included an extender composed of 4% glycerol, 20% egg yolk and 11% lactose in distilled water. The pH was adjusted if necessary with a 4% Tris buffer. Following an equilibration at 37°C the semen was maintained at room temperature for 10 minutes. The temperature was reduced to 4°C and 45 microliter of extender was added to a semen aliquot at that temperature. This was followed by the addition of another 90 microliter of extender and a second equilibration period of 25 minutes at 4°C. Finally the extended semen was pellet frozen on dry ice for 35 minutes and then thawed at 37°C for 15 minutes. The egg yolk concentrations during the freezing trials were 10, 20, 30 and 40%, the pH 6, 6.5, 7.2, 8 and 8.7 and the glycerol concentrations were 0, 4, 7, 10 and 20%. The glycerol equilibration times were 1, 25 and 45 minutes. The extender in

the pH range of 7.2 to 8 produced the best post thaw motility. Glycerol levels of 7-10% were most effective but glycerol concentrations of 20% were detrimental. The best post-thaw motility was obtained with short glycerol equilibration times. There was no correlation between the different egg yolk concentrations and post-thaw motility.

Three extenders were compared using 12 ejaculates from six cynomolgus monkeys (Tollner *et al.* 1990). Two of these extenders were egg yolk based and one was buffered with sodium citrate and the other with Tes-Tris. The egg yolk concentration for both was 30%. The third extender contained 20% skim milk based and was buffered with Tes-Tris. The pH was adjusted to 7.35-7.40 and the osmolality was 320mmol/kg. The concentration of glycerol was 3 and 5%. The extended semen was cooled from room temperature to 5°C for 120 minutes, which was followed by freezing in liquid nitrogen vapour at a controlled rate of -60°C/min. The samples were finally submerged in liquid nitrogen at -196°C. After at least one week of storage the semen was thawed in a water bath at 37°C for 5 minutes. Post-thaw motility was investigated by computer assisted sperm analysis. A post-thaw motility of 12, 56 and 67% was achieved with the citrate buffered, the Tes-Tris buffered and the skim milk based extender respectively. Tes-tris buffered extenders were therefore superior to the citrate buffered one which is often used in the cryopreservation of human spermatozoa. The Tes-Tris buffered extenders also preserved acrosomal integrity better. A glycerol concentration of 3% was found to be more

effective than one of 5% with a post thaw motility of 65% and 58% respectively. During this study artificial insemination was performed with frozen semen on three females. One became pregnant and delivered a healthy infant. This appears to be the only reported case in non-human primates of a successful insemination with cryopreserved semen (Tollner *et al*. 1990).

Protocols for freezing human semen have been well established for some time (Beck and Silverstein 1975, Wolf and Patton 1989). Extenders vary from citrate buffered egg yolk glycerol combinations to Tes-Tris buffered egg yolk glycerol combinations (Wolf and Patton 1989). Programmable freezers have recently been used enabling tightly controlled freezing rates (Wolf and Patton *al*. 1989). Samples are often typically cooled to an intermediate holding temperature of around 4°C before freezing to -196°C.

1.6 Principles of cryobiology

Much of the early development and progress of cryopreservation methods has been empirical (Hammerstedt *et al*. 1990). However the knowledge of the effect of temperature reduction and freezing on cells, their membranes and transport of solutes across these membranes has increased and cryopreservation is now based on sound cryobiological principles.

1.6.1 *The plasma membrane*

The organelle which plays the key role during cooling, freezing and thawing is the plasma membrane (De Leeuw *et al.* 1990, Hammerstedt *et al.* 1990, Mazur 1970). The latter is composed of proteins and lipids and regulates the passage of solutes and water (Hammerstedt *et al.* 1990, Mazur 1970). The membrane composition varies in animal species and herein lies the reason for the frequent need of species specific freezing protocols (De Leeuw *et al.* 1990, Hafez 1985 b, Hammerstedt *et al.* 1990, Tollner *et al.* 1990).

The plasma membrane is the prime target for cryodamage since cooling and freezing alters it's physical properties and function by reorganizing and redistributing membrane components (De Leeuw *et al.* 1990, Hammerstedt *et al.* 1990, Mazur 1970). In fact physical properties of the membrane are already considerably altered at 15-20°C (Hammerstedt *et al.* 1990). Damage to spermatozoa due to temperature fluctuations above the freezing point is termed cold shock which is species specific (Mahony *et al.* 1990, Mayer and Lanzendorf 1986). For example human spermatozoa are quite resistant to cold shock whereas boar spermatozoa are highly sensitive (Mayer and Lanzendorf 1986). These changes in protein lipid interaction and composition influence water and cryoprotectant transport which occurs during freezing and thawing (De Leeuw *et al.* 1990, Hammerstedt *et al.* 1990).

It is believed however, that structural alterations to the plasma membrane are not just caused by freezing and thawing. The cryoprotectant glycerol may also have a direct effect on the membranes by altering their structure (Hammerstedt *et al.* 1990). This again influences water and ion movement during freezing and thawing (Hammerstedt *et al.* 1990).

The plasma membrane blocks the passage of ice crystals from the extracellular fluid, which prevents the seeding of ice and intracellular ice formation at temperatures of about -10 and/or -15°C (Mazur 1977, Mazur 1970).

1.6.2 *Volume changes*

As previously mentioned, freezing and thawing causes water and solute movement across the sperm membrane (Hammerstedt *et al.* 1990, Mazur 1970, McGann 1978). During the freezing process water is removed from the extracellular fluid in the form of ice which results in increased osmolarity (Hammerstedt *et al.* 1990, Mazur 1970, Meryman *et al.* 1977). Most cells remain unfrozen until -10 or -15°C whereas ice already starts forming at -5°C in the extracellular fluid (Mazur 1977, Mazur 1970). This results in higher solute concentration in the extracellular fluid which creates an osmotic gradient causing an efflux of water from the cell and cell shrinkage (Hammerstedt *et al.* 1990, Mazur 1970, Meryman *et al.* 1977). The reverse happens during thawing. These volume changes are

central to the freezing process.

Osmotic dehydration prevents the formation of intracellular ice which damages the organelles (Mazur 1970, McGann 1978). To achieve osmotic dehydration an appropriate freezing rate is necessary, which depends on the ratio of cell volume to surface, the cell's water content and it's permeability to water (Hammerstedt *et al.* 1990, Mayer and Lanzendorf 1986, Mazur 1977, Mazur 1970). For example ova have a smaller surface area per unit volume and high water content and have to be frozen at much lower rates than spermatozoa (Mayer and Lanzendorf 1986). A freezing rate that is too fast will result in intracellular ice formation (Hammerstedt *et al.* 1990, Mazur 1977, Mazur 1970). The crystals formed in this way are small and also tend to recrystalize to large ones during thawing (Mazur 1970). Too slow freezing is believed to expose the cells for too long to high concentrations of extracellular solutes which is called the solution effect (Mazur 1970, McGann 1978, McLaughlin *et al.* 1992, Meryman *et al.* 1977). The optimum freezing rate for mammalian spermatozoa is reported to be between 10° and 100°C /minute (Mayer and Lanzendorf 1986).

For optimum cellular post-thaw survival the freezing rate must be matched with an appropriate thawing rate (Hammerstedt *et al.* 1990, Mazur 1970). Fast cooled cells which contain some intracellular ice must be thawed fast to prevent recrystalization of smaller into larger ice crystals (Mayer and Lanzendorf 1986). Slow cooled cells which have dehydrated must

be thawed slowly to allow reverse osmosis (Mayer and Lanzendorf 1986).

1.6.3 *Cryoprotectants*

The balance between a freezing rate that is too slow and too fast can be so delicate that without the addition of cryoprotectants few cells survive freezing (Mayer and Lanzendorf 1986, Mazur 1970, McGann 1978). A milestone in the development of cryopreservation methods was the discovery of the cryoprotectant effect of glycerol (Mazur 1970, Wolf and Patton 1989). Glycerol has since been the most successful and popular cryoprotectant (Mahony *et al.* 1990, Wolf and Patton 1989). Other cryoprotectants, such as dimethyl sulfoxide, have been used for particular applications such as the freezing of ova and embryos (Mayer and Lanzendorf 1986).

There are two groups of cyroprotectants: Those which penetrate cells and those which do not (Hammerstedt *et al.* 1990, McGann 1978) and these act in three basic ways:

1. Penetration of the cell and osmotic removal of water, which helps to prevent intracellular ice formation (Williams and Harris 1977).

2. Depression of the freezing point (Mahony et al. 1990, Mayer and Lanzendorf 1986, Meryman *et al.* 1977). This provides more unfrozen extracellular fluid at any temperature.

3. Diluting and reducing the high concentration of extracellular solutes during freezing which helps to prevent damage due to the solution effect (Mayer and Lanzendorf 1986, Mazur 1970, McGann 1978, McLaughlin *et al*. 1992, Meryman *et al*. 1977).

Changes in the cell membrane at different temperatures influences their permeability to glycerol which is believed to be low at 0°C and higher at 20°C (Hammerstedt *et al*. 1990, McGann 1978). This means glycerol can be used as a penetrating or non-penetrating cryoprotectant. It is therefore important at which temperature glycerol is added to semen.

The use of cryoprotectants is not without pitfalls since most are themselves toxic to the cell (McLaughlin *et al*. 1992). Some of this is due to the osmotic stress imposed on the cell and some due to direct action on the cell membrane. At high concentrations glycerol inhibits energy metabolism (Hammerstedt *et al*. 1990, McLaughlin *et al*. 1992). For the purpose of cryopreservation glycerol concentrations of between 3 and 10% are most commonly applied, with lower concentrations of glycerol for faster freezing rates (McLaughlin *et al*. 1992, Tollner *et al*. 1990, Wolf and Patton 1989). It has also been suggested that spermatozoa become dependent on glycerol so that once it has been removed such as during the passage through the cervix motility declines (Jeyendran *et al*. 1984).

1.6.4 *Extenders and buffers*

For use in artificial insemination semen is collected into an extender which is composed of a variety of ingredients. One function of the extender is to increase the volume of the ejaculate for multiple inseminations. However it must also provide nutrients as a source of energy for sustained motility, maintain osmotic pressure and electrolyte balance, prevent shifts in pH and inhibit bacterial growth (Hafez 1985 b).

Extenders are commonly based on egg yolk or milk, typically in concentrations of 20% (Hafez 1985 b), Tollner *et al*. 1990, Wolf and Patton 1989). Both can protect spermatozoa against cold shock probably by the stabilizing effect of their lipoproteins on the cell membrane (Hafez 1985 b, Mayer and Lanzendorf 1986). A number of different buffers are used in extenders and include citrate buffers, phosphate buffers, glutamate buffers and organic buffers such as HEPES, MES and Tes with Tris as a buffer component (De Leeuw *et al*. 1990, Hafez 1985 b, Mahone and Dukelow 1978, McLaughlin *et al*. 1992, Roussel and Austin 1967, Tollner *et al*. 1990). The type of buffer needed for successful cryopreservation can be highly species specific (Hafez 1985). For example citrate buffers produce a poor post-thaw motility in cynomolgus monkeys compared to Tes-Tris buffers (Tollner *et al*. 1990). To inhibit bacterial growth antibiotics such as streptomycin and penicillin are included in the extender composition (Hafez 1985 b). Finally many extender compositions include glucose or fructose as a source of energy

for spermatozoa (De Leuww *et al.* 1990, Hafez 1985 b, McLaughlin *et al.* 1992, Tollner *et al.* 1990).

Despite common cryobiological principles there is a huge variety of freezing protocols and material and methods in cryopreservation often have to be highly species specific.

1.7 Semen collection

1.7.1 *Obtaining semen from non-human primates*

The preferred method of obtaining semen from various domestic animal species is by the use of an artificial vagina and occasionally by electrostimulation. Laboratory bred and wild caught non-human primates will however not permit the type of handling necessary to obtain semen. Although laboratory housed primates frequently masturbate and some can be trained to produce ejaculates on demand, it is difficult to train them to use artificial vaginas (Gould *et al.* 1978, Hendrickx *et al.* 1978, Seier pers. obs.). Sedation or anaesthesia and electrostimulation is therefore often the most practical and humane way for the collection of semen (Gould and Mann 1988, Gould *et al.* 1978, Harrison 1980, Hendrickx *et al.* 1978).

1.7.2 *Anaesthesia during electrostimulation*

Ketamine hydrochloride (Centaur Lab) is the most commonly used anaesthetic for the chemical immobilization of non-human primates, usually administered at a dose of 10mg/kg bodyweight (Gould *et al.* 1978, Martin *et al.* 1972, Seier *et al.* 1991). It is a dissociative anaesthetic which is reported to maintain autonomic reflexes better than others. For this reason it is often also the anaesthetic of choice for the purpose of chemical restraint of non-human primates during electrostimulation (Bornman *et al.* 1988, Gould and Mann 1988, Gould *et al.* 1978, Harrison 1980).

1.7.3. *Electrostimulation methods and equipment*

A number of electroejaculation methods have been developed which have proven safe and effective in a wide variety of non-human primate species (Gould and Mann 1988, Gould *et al.* 1978, Harrison 1980, Hendrickx *et al.* 1978, Lang 1967, Sarason *et al.* 1991, Thomson *et al.* 1992). The equipment usually consists of a variable voltage transformer and rheostat, which connects to a rectal probe with vertically or horizontally arranged electrode areas or contacts as in the case of penile stimulation (Gould and Mann 1988, Gould *et al.* 1978). The current is often applied in a rhythmic way i.e. a few seconds of decreased voltage, desired voltage and increased voltage, followed by a few seconds of rest period (Bornman *et al.* 1988,

Gould and Mann 1988, Harrison 1980, Thomson *et al.* 1992).

1.7.4 *Electrical parameters*

The frequencies during electrostimulation range from 20-60Hz and usually no more than 13V are necessary to achieve ejaculation in most species (Bornman *et al.* 1988, Gould and Mann 1988, Harrison 1980, Hendrickx *et al.* 1978, Schaeffer *et al.* 1992, Thomson *et al.* 1992). Sine wave stimuli and frequencies low enough to permit repeated contractions and relaxations are best for rectal stimulations, which is the method most frequently applied to non-human primates (Bornman *et al.* 1988, Gould and Mann 1988, Harrison 1980, Hendrickx *et al.* 1978, Thomson *et al.* 1992). A square wave is used with penile electrostimulation (Hendrickx *et al.* 1978, Sarason *et al.* 1991). Here afferent nerve endings in the penis are stimulated, which transmit the stimuli to the efferent pathways causing erection and ejaculation (Gould and Mann 1988). This is achieved by attaching electrodes directly to the penis

Penile electrostimulation is sometimes carried out without anaesthesia on subjects immobilized in a restraining chair and is also reported to yield a higher concentration of spermatozoa per ml of semen when compared to rectal stimulation (Gould and Mann 1988, Sarason *et al.* 1991).

Ejaculation occurs often within 10 minutes and at 4-7V in most *Macacca* species and at 8-10V in baboons (Hendrickx *et al.*

1978). A current density of less than 0.75mA/mm^2 electrode area is believed to be safe and will not cause damage or irritation to the rectal mucosa (Gould *et al*. 1978).

1.8 Purpose of the study

The purpose of this thesis is to contribute to the knowledge of vervet monkey male reproductive physiology in a captive laboratory environment. The goal is to study aspects of spermatology in areas where either no data are available or where baseline data are limited and outdated or carried out with outdated technology. This is particularly relevant in the case of cryopreservation of semen. The focus will be on the description and illustration of the types of morphologically abnormal forms of spermatozoa, the rate of their occurrence and the development of a method for the cryopreservation of vervet monkey semen.

Specific aims are:

1. Establishment of a basic spermiogram from individuals of wild caught and colony bred origin.

2. Description and illustration of abnormal forms of spermatozoa. Quantify their prevalence and rate of occurrence in ejaculates from wild caught and colony bred individuals.

3. Development of a method for the extension and cryopreservation of vervet monkey sperm.

4. To achieve aims 1-3, utilize, adapt or develop techniques which require a minimum amount of specialized equipment. This should enable the staff of most laboratory animal and/or primate facilities to carry out such investigations or procedures.

Parts of the work used for this dissertation have already been published in the international literature by the author. These are not included in the literature survey or references since they will be combined with a substantial amount of new data.

Chapter II

Materials and Methods

Note: The study was approved by the Ethics Committee of the Medical Research Council. The project was carried out in the Primate Unit of the Experimental Biology Program, Medical Research Council, Parowvalley.

2.1 Semen collection

Semen was obtained by peri-prostatic electrostimulation applied per rectum. The electroejaculator consisted of 175.0mm long and 15.4mm diameter homemade rectal probe made from epoxy resin with two brass bands embedded in the tip. A probe diameter of about 14.0mm is recommended for most medium sized primates (Gould *et al.* 1978). To prevent trauma to the rectal mucosa, the probe surface was perfectly smooth and sufficiently long to reach the prostatic area. The bands were 4.2 and 4.0mm wide respectively, 10.0mm apart and the distal band was located 5.0mm from the tip. Both bands were wired internally to contacts at the base of the probe.

The two contacts were connected to a transformer with five taps which controlled voltage output form 2.5-5.5V by 1.0V increments and had one additional 8.0V output. The stimulus was provided by an alternating current with a frequency of 50Hz and a sinusoidal wave form.

The current applied and current densities at increasing voltages are shown in Table 2.1.

Table 2.1. Measured current (mA) and current densities (mA/mm^2) at increasing voltages

Voltage	Measured Current	Current Densities
2.5	5.5	0.01
3.5	10.0	0.03
4.5	10.0	0.03
5.5	19.7	0.05
8.0	38.0	0.10

To achieve ejaculation a male was placed in dorsal recumbency after having been anaesthetized with ketamine hydrochloride at 10 mg/kg bodyweight intramuscularly. The probe was lubricated with a gel containing a disinfectant (Betadine, Adcock Ingram Pharmaceuticals, Johannesburg) and inserted into the rectum so that the brass bands lay approximately at the level of the prostate gland. In this way peripheral neuromuscular junctions were stimulated which caused contraction and relaxation of the relevant reproductive organs and ejaculation (Gould and Mann

1988). The position could be confirmed by transabdominal palpation.

Electrostimulation was started at 2.5V and the full current was applied by switching the transformer on. The electrostimulation was response orientated and did not follow a set pattern as described in chapter I under 1.7.3 *Electrostimulation methods and equipment*. The probe was inserted, activated and held steady at a point until erection was achieved. The erection would often cease after 15-30 seconds and the probe had to be gently withdrawn and re-positioned to re-establish erection. This had to be done repeatedly throughout the procedure in a gentle massaging movement. The current was applied for 1-2 minutes followed by a 15-20 second rest period. Voltages were increased by connecting the leads of the probe onto the tap for the next higher voltage until ejaculation was achieved.

Loss of erection and leg muscle contractions during stimulation indicated the need to change to the next higher voltage.

All ejaculates were collected into 5ml screw topped plastic tubes.

2.2 The choice of subjects for this project

The subjects were chosen randomly but availability at the time of sampling determined and often limited the choice. The criteria for inclusion were sexual maturity and the absence of overt pathology of or lesions on the genitalia.

Two distinct groups in terms of origin reside in the colony in which this study was carried out. One group was bred and raised in the colony and the other has been captured from the wild. All individuals in the latter group had spent at least five years in captivity at the time of sampling. The study did not intend to examine differences between these two groups, however, their existence had to be recognized. Therefore, wherever sufficiently balanced numbers could be obtained from both groups the results were presented separately and compared.

2.2.1 *Number of semen characteristics, males and ejaculates*

The number of semen characteristics and the respective number of males and ejaculates investigated are provided in table 2.2.

Table 2.2 Semen characteristics and the respective number of males and ejaculates investigated in this study

Semen characteristic	No. of males	No. of ejaculates
pH	20	20
Concentration	46	121
Forward Progression (FP)	52	121
Motility	58	138
Vitality	49	110
Abnormal morphology	28	28

Three additional ejaculates were taken at two week intervals from a number of males (in brackets) and evaluated for the following characteristics: motility (23-33), FP (20-31), concentration (22-33) and vitality (20-27). Another two ejaculates were taken separately at two weeks interval from 10 males to evaluate abnormal morphology. These serial samples were needed to evaluate:

a) whether the results are reproducible when ejaculates were obtained with above mentioned methods

b) whether the characteristics of semen, obtained by above mentioned methods, are actually representative of an individuals reproductive potential.

2.3 Housing, environmental conditions and nutrition

All individuals were housed singly and permanently indoors in 0.6 x 0.6 x 0.8m and 1.2 x 0.6 x 0.8m wall mounted stainless steel home cages. Access to 0.6 x 0.6 x 2.0m exercise cages was provided every six days for 24 hours. Each exercise cage also contained a female social partner. To further promote psychological well-being the home cages were fitted with perches, foraging containers and sterilized cattle femurs for manipulations. Each windowless animal room received about 15 air changes per hour and there was a 12 hour photoperiod. The temperature was maintained at 25°C and the humidity at 40%.

The diet consisted of pre-cooked maize meal which was mixed to a stiff porridge with water, blended with a vitamin, mineral and protein supplement and fed in the morning. Apples were fed at noon and in the afternoon again pre-cooked maize meal which, this time, contained only milled sunflower seeds and maize kernels to stimulate more foraging. The diet has supported good reproductive performance through two generations. Drinking water was supplied *ad libitum* via an automatic device.

2.4 Semen evaluation

2.4.1 *pH*

The pH of human semen is frequently measured by using indicator paper (Hellinga 1976 a), World Health Organisation 1992)

whereas the pH of semen from non-human primates, according to available literature, has not been reported and there are no standardized methods.

In this study ejaculates were tested with Merck universal indicator paper and Panpeha paper (Riedel de Hahn). This was achieved by aspirating semen with a Pasteur pipette and placing a drop on each square of the indicator paper. Both values obtained in this way were compared with those obtained by electrometry with a Schott model CG 820 pH meter (Schott Geräte, Hofheim. Germany) using the same ejaculate. The probe of the meter was designed for small volumes as can be expected from medium sized non-human primates. The instrument was calibrated at ph 7 and 10, using standard solutions (Beckmann instruments). The range of both indicator papers was from 0-14 and all semen samples were tested immediately after ejaculation.

2.4.2 *Concentration*

The concentration of sperm in this study was determined using a Neubauer haemocytometer. This is one of the most commonly used methods (Acosta *et al.* 1986, Harrison 1980, Sarason *et al.* 1991).

The semen was diluted with the aid of a white or red blood cell pipette. The diluting fluid consisted of 16g sodium bicarbonate and 4g phenol in 400ml of distilled water (Freund

1968). The phenol is spermicidal and immobilizes the spermatozoa to enable counting. Dilution ratios ranged from 1:10 to 1:200, depending on the initial estimated concentration which was determined with a microscope under bright field illumination at 100 x magnification. For the dilution, a rubber pipe with mouthpiece was attached to the red or white blood cell pipette and semen was aspirated up to the first or second mark depending on the desired dilution. This was followed by aspiration of the diluting fluid up to the third mark which is above the bulb containing the mixing bead.

After mixing the semen and diluting fluid in the pipettes for about one minute, the first few drops were discarded and a small drop was allowed to spread under the cover slip of the haemocytometer. The four large corner blocks containing 16 squares each were visualized and the spermatozoa in all four blocks of each chamber were counted with the aid of a microscope under bright field illumination at 400 x magnification. The concentration of spermatozoa per ml of semen was calculated according to the formula:

$$\text{concentration /ml} = \frac{\text{number of cells counted x dilution x 10 000}}{\text{number of blocks counted}}$$

2.4.3 *Motility*

Two motility parameters were evaluated subjectively:

1. The percentage of progressively motile spermatozoa in each ejaculate.

2. The level of forward progression of the motile spermatozoa (FP).

Semen was aspirated with a Pasteur pipette within five minutes of collection and a drop was placed on a glass microscope slide. After placing a glass cover slip on the sample it was examined under a microscope with bright field illumination and 400 x magnification. The progressive motility was estimated in 10% units of motile spermatozoa and the speed of forward progression rated on a scale from 0-4 according to the following classification:

0 = no movement

1 = movement in place without progress

2 = slow forward progression

3 = fast forward progression

4 = swimming at maximum speed

2.4.4 *Vitality*

To determine the proportion of live and dead sperm one drop of semen was mixed with one drop of 1% aqueous eosin solution for 15 seconds which was then mixed with 2 drops of a 10% aqueous nigrosin solution. A thin smear was made on a glass slide, air dried and 100 or 200 spermatozoa were counted using a microscope with bright field illumination and a 1000 x magnification (Eliasson 1977). Spermatozoa which were alive at the time of staining appeared white and those which were dead appeared pink against a dark background.

A consideration of supra-vital staining is that a number of factors such as certain diluents and pH can influence the staining characteristics and can make the results artifactual (Hellinga 1976 c).

2.4.5 *Abnormal morphology*

The first step in the determination of abnormal morphology was to examine a wet preparation of fresh semen microscopically under bright field illumination at 400 x magnification. The presence of bent midpieces and principal pieces and coiled and folded tails was determined qualitatively. This was later compared with the results obtained from stained specimens. Abnormal spermatozoa in the wet preparations were rated as absent or present in small, moderate and large numbers. It was anticipated that certain defects such as coiled and bent tails

and midpieces would be microscopically visible in the fresh semen. This would help to ascertain that these defects were not artifacts of processing. Tail coiling, for example can be caused by drying, cooling or contamination of the sample (Harrison 1980).

For the actual quantitative determination of all defective forms, a thin semen smear was made on a glass slide and air dried for approximately five minutes. This was followed by staining with Spermac stain (Stain Enterprises, Wellington 7655, Republic of South Africa) according to the manufacturer's instructions. The staining procedure included the following steps:

1. Immersion of the smear into a formaldehyde based fixative for 30 minutes.
2. Rinsing slides by gently dipping in tap water.
3. Staining for 1 minute in stain A and rinsing in tap water.
4. Staining for 45 seconds in stain B and rinsing in tap water.
5. Staining for 1 minute in stain C and rinsing in tap water.

Spermac stain has been tested in a number of species including dogs, vervet monkeys and humans and allowed differentiation of the acrosome, the post-acrosomal region and tail (Conradie *et al*. 1994, Oettlé *et al*. 1991, Oettlé and Soley 1988). The post acrosomal region and nuclear portion of the head stains red, and the acrosome midpiece and tail green.

To illustrate defective forms, the entire smear from each of the 28 vervet monkeys was scanned. Photographs were taken from all representative abnormalities, selected from suitable areas of the smear. For the determination of the abnormal morphology 200 spermatozoa were rated from each smear according to the following categories:

2.4.5.1 *Head defects*

Any deviations from the normal oval shape, size and duplications were recorded: particularly, but not restricted to: micro-and macrocephalic, asymmetrical, round, elongated, narrow, amorphous, tapering, pyriform and detached heads, vacuoles, cysts and invaginations (World Health Organisation 1992). Where possible, the defects were evaluated within the separate regions of the head including the acrosome, equatorial segment and post-acrosomal region. The classification system is essentially the same as described in chapter I under 1.4.2. *The abnormal sperm*.

2.4.5.2 *Tail defects*

Defects were, where possible, recorded within the distinct regions of the tail and included the following categories (World Health Organisation 1992):

Neck: abaxial implantations, broken, detached heads.

Midpiece: bends, kinks, thickening, mitochondrial stripping or displacement, detachment, duplication, vacuoles and cysts.

Principal-and endpiece: coiling, folding, bends, kinks, thickening, detachment and duplication.

All results were expressed as percentage of counted spermatozoa. Morphologically abnormal forms were photographed with an Olympus BH 2 microscope with automatic micrographic system model PM-10 ADS and a PM-CTR colour temperature module. The objective was a PLAN Apochromatic 100-1.3 oil, the eyepiece a 3.3 NFK photo and the film used a 50 ASA FUJI daylight colour reversal film. A blue filter was placed over the light source and the exposure was one second. The processed film was mounted and the slide projected to locate defective sperm. The slide was then placed into a transparent sleeve and the defective spermatozoa encircled with a marker pen on the sleeve. A 13 x 8 cm print was made from the section of the slide containing the abnormal sperm.

2.5 Cryopreservation

2.5.1 *Semen collection*

Twenty five ejaculates were collected from 25 males by electroejaculation as described under 2.1. *Semen collection*. The semen was collected at room temperature into 5ml plastic screw capped tubes with a diameter of 12.0mm, a length of

85.0mm and a wall thickness of 1.0mm.

2.5.2 *Extension and extender composition*

Unglycerolated extender at 32°C was added to the semen immediately after collection in the ratio of 1:1 and the specimen was kept at this temperature until cooling commenced. An extender buffered with a combination of Tes (N-TRIS [hydroxymethyl] methylaminomethane sulfonic acid, Sigma Chemical Company, St. Louis USA) and Tris ([hydroxymethyl] aminomethane, Merck, Darmstadt, Germany) was used for all methods described and is presented in Table 2.3.

Table 2.3 Extender composition, pH and osmolality

Tes	4.325g
Tris	1.027g
Dextrose	1.000g
Egg yolk	30% (v/v)
Glycerol	3 and 5% (final concentrations)
Procaine pencillin	0.015g
Streptomycin	0.025g
pH	7.10-7.18
Osmolality	330-345mOsmol/L

The buffer, dextrose and antibiotics were made up to 100ml in double distilled deionized water. Egg yolk was next added to a concentration of 30%. Egg yolk was extracted by cracking a fresh chicken egg into a sterile Petri dish and the yolk was aspirated with a sterile 10ml syringe and 16g needle. The extender was divided into three equal volumes and 6 and 10% (v/v) glyerol was added to two of these.

The extender composition was adapted from Tollner (Tollner *et al.* 1990) and the choice was based on:

a) it has been used to successfully cryopreserve semen from cynomolgus monkeys including fertilization by artificial insemination.

b) the local availability of the ingredients

c) a pilot trial using a citrate buffered extender yielded a poor pre - and post-thaw motility of vervet monkey spermatozoa.

Before the addition of glycerol, the pH and osmolality of every new batch of extender was determined with a pH meter (Beckman 70 pH meter) and with a osmometer (Knauer, Semi Micro Osmometer, Balzers Union) by measuring the depression of the freezing point. Extenders were stored in a deep freeze at -20°C.

2.5.3 *Evaluation of semen*

One of the hallmarks of a successful cryopreservation method is a good post-thaw progressive motility (Beck and Silverstein 1975, McLaughlin *et al.* 1992, Taylor *et al.* 1982, Wolf and Patton 1989) Together with the pre-freeze and post-thaw motility, this was evaluated subjectively by light microscopy as described under 2.4.3. *Motility*. The pre-freeze and post-thaw motilities were compared and the percentage recovery of progressively motile spermatozoa was calculated from these two figures according to the formula:

$$\text{Post-thaw recovery (\%)} = \frac{\text{post-thaw motility (\%) x 100}}{\text{pre-freeze motility (\%)}}$$

All samples were evaluated after 24 hours storage in liquid nitrogen.

2.5.4 *Cooling and feezing*

Many primate facilities do not have specialized equipment and personnel for the freezing of gametes. The aim was therefore not only to develop a technique which is effective for vervet monkey semen but also one which is practical and does not depend on specialized apparatus.

Different techniques defined under methods 1-4 were tested. Cooling is defined as temperature reduction from 32°C to 5°C and freezing from 5°C to plunge temperature before immersion in

liquid nitrogen.

2.5.5 *Method 1*

Step 1: Extended semen was cooled from 32 to 5°C over 85 minutes by submerging the collection tube containing unglycerolated extended semen in a glass beaker filled with 400 ml of water at 32°C. The diameter of the beaker was 85.0mm, the height was 115.0mm and the wall thickness was 2mm. This beaker was placed into a freezer at -15°C until extended semen reached 5°C.

Step 2: The separate extender containing 10% glycerol was pre-cooled to 5°C and added drop by drop to the unglycerolated extender containing the semen until a ratio of 1:1 and a final glycerol concentration of 5% (v/v) was achieved. To maintain a temperature of 5°C throughout this and all other subsequent steps in all methods, they were carried out in a temperature controlled room at 5°C. The Pasteur pipettes which were used to add glycerol as well as the glycerolated extender and the paillettes were also cooled to this temperature.

Step 3: The extended glycerolated semen was drawn into the pre-cooled plastic paillettes with a length of 130mm, a diameter of 3mm and a volume of 0.5ml. These were sealed at one end with polyvinyl alcohol powder and supported four centimetre above the liquid nitrogen. To achieve this a 25 x 17 x 14cm polystyrene box with a wall thickness of 1.5cm and

containing 1.3L of liquid nitrogen was marked inside to indicate two and four centimetre above the liquid nitrogen surface. The supports consisted of two suitably shortened hacksaw blades which were positioned 10cm apart and forced into the sides of the box, supporting the paillettes above the liquid nitrogen surface at the desired height (Figure 2.1).

Figure 2.1 Polystyrene container with paillettes suspended four centimetre above liquid nitrogen.

Step 4: After 20 minutes the paillettes were plunged into the liquid nitrogen at the bottom of the polystyrene box, transferred to a 30L flask and stored. Fast transfer avoided excessive temperature fluctuations.

2.5.6 *Method 2*

Step 1: Extender containing 10% glycerol and at 32°C was added drop by drop at room temperature to extended semen without glycerol in the ratio of 1:1. This produced a final concentration of 5% (v/v) glycerol. The sample was then cooled to 5°C over 30 minutes by submerging the collection tube into a glass beaker filled with 400ml of water pre-cooled to 5°C. The beaker dimensions were the same in step 1 of method 1 and the beaker was kept in the same temperature controlled room.

Step 2: The same as method 1 step 3.

Step 3: The same as method 1 step 4.

2.5.7 *Method 3*

This was identical to method 2 except that at step 2 the paillettes were supported two centimeter above liquid nitrogen.

2.5.8 *Method 4*

This was identical to method 3 except the final glycerol concentration was 3% (v/v). This was achieved by using the extender which initially contained 6% glycerol.

2.5.9 *Thawing*

Cryopreserved semen was thawed to 32°C by placing paillettes for one minute into a beaker containing 400ml of water at 32°C. The breaker was of the same dimensions as the one used in method 1 step 1. The end of the paillette which was plugged with cotton wool extended beyond the surface to prevent water from entering.

2.5.10 *Determination of cooling and freezing rates*

To determine the cooling rate, a collection tube containing the extended semen was closed with a rubber stopper which was holed through the centre. The thermal probe of a Bailey thermometer model BAT - 12 (Bailey Instruments Inc., Saddlebrook, NJ USA) was inserted through this hole in the stopper to be immersed in the extended semen. To measure the freezing rates the probe was inserted through the sealed end of a paillette containing the extended semen. This was followed by cooling or freezing according to the protocol under investigation.

The cooling rates for method 1 were measured at five minute intervals and all other cooling and all freezing rates at one minute intervals. The cooling and freezing rates were determined five times for every method.

2.6 Statistics

i) The difference in the pH between the ejaculates was determined by a two-tailed student's t-test.

ii) The difference of specific types of abnormal sperm between colony bred and wild caught individuals was determined by Wilcoxon's 2-sample test.

iii) The prevalence of sperm abnormalities (Figure 3.35) was calculated according to the following formula:

$$\text{Prevalence (\%)} = \frac{c \times 100}{a \times b}$$

a = 14 (the total number of males in each group)

b = Number of different types of abnormalities per morphological structure

c = sum of males per group with specific defects

Chapter III

Results

3. Electroejaculation

The method of electroejaculation appeared to be ineffective in only three (2%) out of 150 males and no ejaculates were obtained from the former. During electrostimulation the vervets responded with strong contractions of the thigh muscles and testicular and tail movements. Weakening of this response and cessation of erection was an indication to select the next higher voltage. Most erections with a good glans response occurred at 2.5-3.5V in all males and most ejaculations occurred after about five minutes. Some males occasionally ejaculated before erections or during rest periods. Urination frequently occurred if the males were stimulated after ejaculation and at 5.5-8V.

3.1 Semen analysis

3.1.1 *pH*

The results of the pH determinations are illustrated in Table 3.1. The mean pH value obtained with the pH meter was 7.67 and was identical for both colony bred and wild caught primates. This value was similar to the pH of 7.75 obtained with Panpeha paper and which was also identical in both groups. The mean value obtained with the Merck indicator paper fell into a more alkaline range with 8.75 and 8.88 for wild caught and colony bred males respectively. These results did not correlate well with those obtained by the other two methods.

According to a two-tailed student's t-test the difference between the results obtained with a pH meter and the Merck paper was highly significant ($p < 0.0001$). The difference between the pH meter and the Panpeha paper was not significant ($p > 0.05$).

There was no difference in the pH of semen from captive bred or wild caught individuals (Table 3.1).

Table 3.1 Mean pH values for 20 ejaculates by method and group

Origin	pH meter	Merck paper	Panpeha paper
Wild caught			
mean	7.67	8.75	7.75
± SD	0.20	0.45	0.50
n	12.00	12.00	12.00
Colony bred			
mean	7.67	8.88	7.75
± SD	0.34	0.35	0.46
n	8.00	8.00	8.00
Combined			
mean	7.67	8.80	7.75
± SD	0.26	0.41	0.47
n	20.00	20.00	20.00

3.1.2 *Concentration, motility, forward progression (FP) and vitality*

The results of all four parameters are combined and compared in Table 3.2. Despite differences in the number of males and ejaculates, the two groups were similar for all parameters. The most variable parameter was the sperm concentration, which was reflected by large standard deviations. A number of males were electroejaculated three times and Table 3.3 summarizes the semen characteristics of these ejaculates. The mean values of the semen characteristics of three consecutive ejaculates appeared quite constant (Table 3.3). There were however large variations within individuals. Table 3.4 illustrates some examples. The motility differed by 100% and more in at least two consecutive ejaculates from eight (38.1%) males. The sperm concentration differed by 100% and more in at least two consecutive ejaculates from 13 (61.9%) males. Both males with a consistent sperm concentration of <40 x 10^6/ml were successful breeders. No males were found with consistently low values for any of the other parameters.

Considerable differences in consecutive ejaculates from the same individual were also found for the forward progression (FP) and vitality. Table 3.5 summarizes some examples. Here at least two consecutive ejaculates from eight (42.1%) males differed by 100% and more from each other in terms of FP. At least two consecutive ejaculates from six (31.6%) males differed by at least 100% from the next in terms of vitality.

Table 3.2 Concentration (x10^6/ml), motility (%), FP (0-4) and vitality (%) of vervet monkey spermatozoa.

	Concentration	Motility	FP	Vitality
Wild caught				
mean	231.72	51.78	2.75	57.34
± SD	268.22	17.81	0.90	17.85
(ejaculates) n	101.00	112.00	94.00	92.00
(males) n	39.00	45.00	28.00	42.00
Colony bred				
mean	273.67	52.88	2.77	50.44
± SD	399.54	18.50	1.03	18.03
(ejaculates) n	20.00	26.00	26.00	18.00
(males) n	7.00	12.00	13.00	7.00

Table 3.3 Characteristics of consecutive ejaculates

	1st ejaculate	2nd ejaculate	3rd ejaculate
Motility (%)	52.88	52.27	50.00
± SD	14.79	17.37	17.77
n	33.00	33.00	23.00
FP	2.55	2.74	2.50
± SD	0.93	0.73	1.00
n	31.00	31.00	20.00
Concentration/ml ($x10^6$)	205.12	266.33	262.46
± SD	167.03	392.53	377.17
n	33.00	33.00	22.00
Vitality (%)	53.78	55.96	59.70
± SD	14.01	19.91	20.56
n	27.00	27.00	20.00

Table 3.4 Examples of the large variation in sperm motility (%) and concentration (x10^6/ml) in consecutive ejaculates from the same individual

	Motility			Concentration		
Number	**1**	**2**	**3**	**1**	**2**	**3**
801	45	15	65	160	86	134
632	20	45	10	28	122	93
854	35	55	10	453	258	162
502	60	85	40	74	16	10
564	60	55	60	192	146	274
574	65	50	40	126	192	558
589	65	55	65	106	1181	226
606	60	50	65	333	230	858
607	70	45	50	640	117	94
462	30	40	65	202	180	260
857	55	55	30	284	334	206
519	70	80	60	494	109	94
559	30	50	85	160	134	259
490	50	50	50	208	169	202
639	70	40	40	32	71	44
585	50	40	80	375	427	398
722	40	20	10	512	112	4
678	60	75	40	21	32	11
667	60	50	50	443	296	387
668	45	45	55	230	648	1728
678	60	75	40	21	32	11

Table 3.5 Examples of the large variation in sperm FP (0-4) and percentage of live sperm in consecutive ejaculates from the same individual.

	FP			Vitality		
Number	**1**	**2**	**3**	**1**	**2**	**3**
801	3	1	3	67	24	45
632	2	3	1	45	61	10
854	1	3	1	62	40	59
502	2	3	3	74	78	56
564	3	3	3	58	77	44
574	3	3	3	39	61	47
589	3	3	3	80	65	35
606	3	3	3	45	70	69
607	1	2	2	55	55	45
462	3	3	1	48	37	78
857	3	3	1	61	60	63
519	4	4	3	67	77	85
490	2	3	3	48	60	90
585	3	3	4	52	64	91
722	2	1	1	46	64	64
678	3	3	3	72	34	44
667	1	2	3	61	59	73
668	3	3	3	62	47	71
678	3	3	3	72	34	44

3.1.3 *Morphology*

A total of 28 different sperm abnormalities were found amongst the ejaculates of 28 vervet monkeys. From a total of 13 head abnormalities, three types were only found in colony bred individuals. From a total of seven midpiece abnormalities three types were only found in wild caught individuals but all eight types of principal - and endpiece abnormalities occurred in both groups. Table 3.6 lists all defective forms found per morphological area and according to the vervet monkey's origin.

Part 1 of this chapter illustrates and describes the defective forms found in all individuals. The magnifications of the photomicrographs are as follows: 0.67 micrometer/mm for Figures 3.1, 3.2 and 3.27; 0.40 micrometer/mm for Figure 3.28 and for all others 0.33 micrometer/mm. The bar in Figure 3.3 represents 3.3 micrometer and applies to all Figures with the same magnifications. The above mentioned Figures with different magnifications have separate bars. The four normal heads in Figure 3.28 are each approximately 5.60 micrometers long and 2.80 micrometers wide, which is a ratio of 2:1.

Part 2 will provide a detailed description of the prevalence and rate of occurrence of morphologically abnormal forms.

Table 3.6 Abnormal forms of sperm from vervet monkeys

Head: macrocephalic (c+w), microcephalic (c+w), round no acrosome (c), narrow (c+w), acrosomal cysts (c+w), equatorial cysts (c+w), nipple acrosome (c+w), pointed (c+w), asymmetrical (c), duplication (c+w), pyriform (c+w), tapered (c+w), amorphous (c).

Midpiece: bent (c+w), thickened (c+w), abaxial implantations (c+w), cytoplasmic droplets (c+w), bent neck (w), pseudodroplet defect (w), duplication (w).

Principal and endpiece: Coiled (c+w), folded (c+w), detached at midpiece (c+w), detached endpiece (c+w), duplication (c+w), terminal coiling (c+w), detached (c+w), bent (c+w).

key to letters in brackets: c = occurred in colony bred individuals, w = occurred in wild caught individuals

3.1.3.1 Morphology part 1: Illustrations

3.1.3.1.1 Normal Sperm

Figure 3.1 **Figure 3.2**

Figure 3.1 The bar at the bottom represents 6.7 micrometer and applies to both Figures. The head of the sperm in the centre is about 5.4 micrometer long and 3.0 micrometer wide, which is a ratio of 1.8:1. The midpiece is about 12.1 micrometer long and 0.7 micrometer wide. The principal piece length is about 60.3 micrometer. The head is oval and the outline of the entire sperm is smooth. The tail has no kinks, bends, coils or folds. **Figure 3.2** The length of the head of the sperm in the centre is about 5.4 micrometer and the width about 2.3 micrometer. The midpiece is about 11.0 micrometer long and about 0.7 micrometer wide. The principal piece is about 60.0 micrometer long. Other features are as Figure 3.1.

3.1.3.1 Morphology part 1: Illustrations

3.1.3.1.2 *Head Abnormalities*

Figure 3.3 **Figure 3.4**

Figure 3.3 The bar at the bottom represents 3.3 micrometer and applies to all Figures except where indicated otherwise. The entire sperm head is stretched or elongated. The post acrosomal region is tapered. Note the coiled tail and thickened midpiece. **Figure 3.4** The length of this head is normal but it is narrow and compressed at the equatorial segment. The acrosome is patchy and most acrosomal material is absent. The midpiece appears duplicated and fused and the tail detached near or at the annulus.

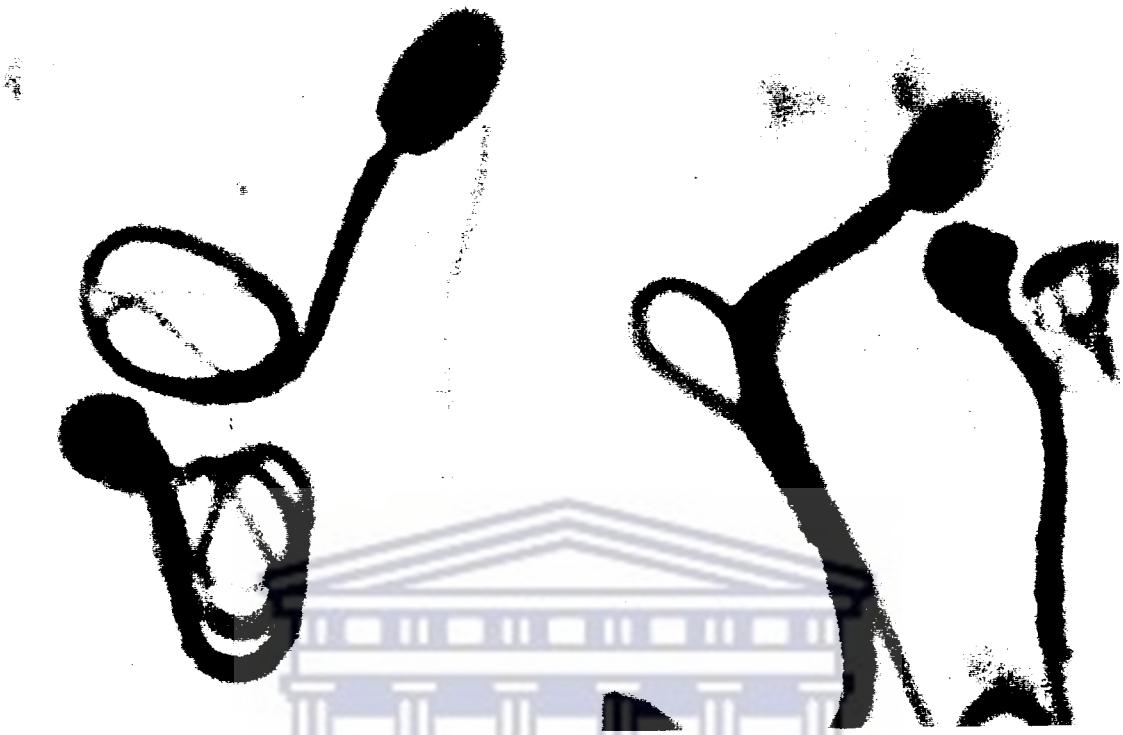


Figure 3.5 **Figure 3.6**

Figure 3.5 This microcephalic head has bilateral equatorial protrusions. There appears to be a vacuole in the centre of the equatorial segment. The acrosomal region is disproportionately small in relation to the entire head. Note the coiled tail. Compare with the normal head in the top right corner of the figure. **Figure 3.6** This head is more microcephalic than Figure 3.5 and a large equatorial protrusion makes this head amorphous. There is a large acrosomal cyst or vacuole and the entire head stains denser than Figure 3.5. Note: the spermatozoon above has no acrosome and has a bent midpiece.

Figure 3.7 Figure 3.8

Figure 3.7 This severely microcephalic head is even smaller than those in Figures 3.5 and 3.6. The post acrosomal region stains very dense and the small acrosome is densely aggregated at the tip. Compare with the normal spermatozoon above and to the left. **Figure 3.8** A cyst or vacuole appears in the centre of the equatorial segment. Notice the hairpin folding of the tail.

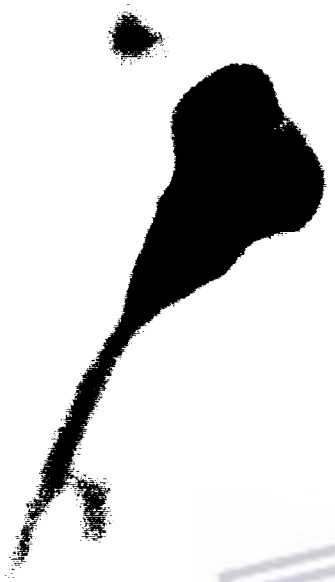
Figure 3.9

Figure 3.10

Figure 3.9 A large vacuole can be seen in the equatorial segment and there appears to be some linear vacuolation on the opposite side of the head as well. This could possibly be a diadem defect. There is no acrosome, just two patches of acrosomal material aggregated in the top of the head. Note also the coiled tail. **Figure 3.10** A large cyst occupies the entire top third of the head. No acrosomal material is evident. The head is also asymmetrically shaped and more convex on the right hand side. Note the head is detached from the neck and the midpiece is bent.

Figure 3.11 **Figure 3.12**

Figure 3.11 Apical aggregation of acrosomal material forms a nipple defect. Note also the coiled tail. **Figure 3.12** The spermatozoon on the bottom left has a nipple defect. The acrosome of the spermatozoon on the top right is small and the acrosomal material has a patchy distribution. Note the head of the spermatozoon on the left is detached.



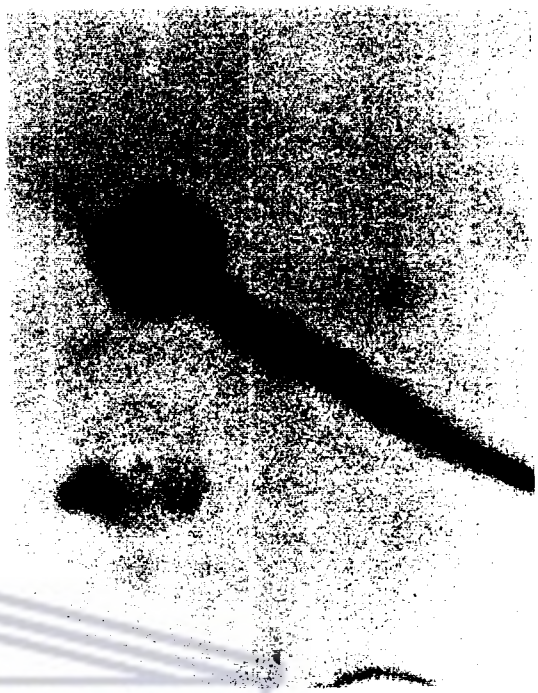


Figure 3.13 **Figure 3.14**

Figure 3.13 These duplicate heads are also round, microcephalic and have no acrosomes. The plasma membrane can be faintly seen at the top of the right duplicate head. Notice also the short, thick triangular shaped midpiece. This probably consists of two fused midpieces. **Figure 3.14** The bottom fused head is normally shaped while the top one is slightly asymmetrical. Both heads are microcephalic. Note also the fused duplicate midpiece and duplicate principal piece.

3.1.3.1 Mophology part 1: Illustrations

3.1.3.1.3 Tail Abnormalities: midpiece

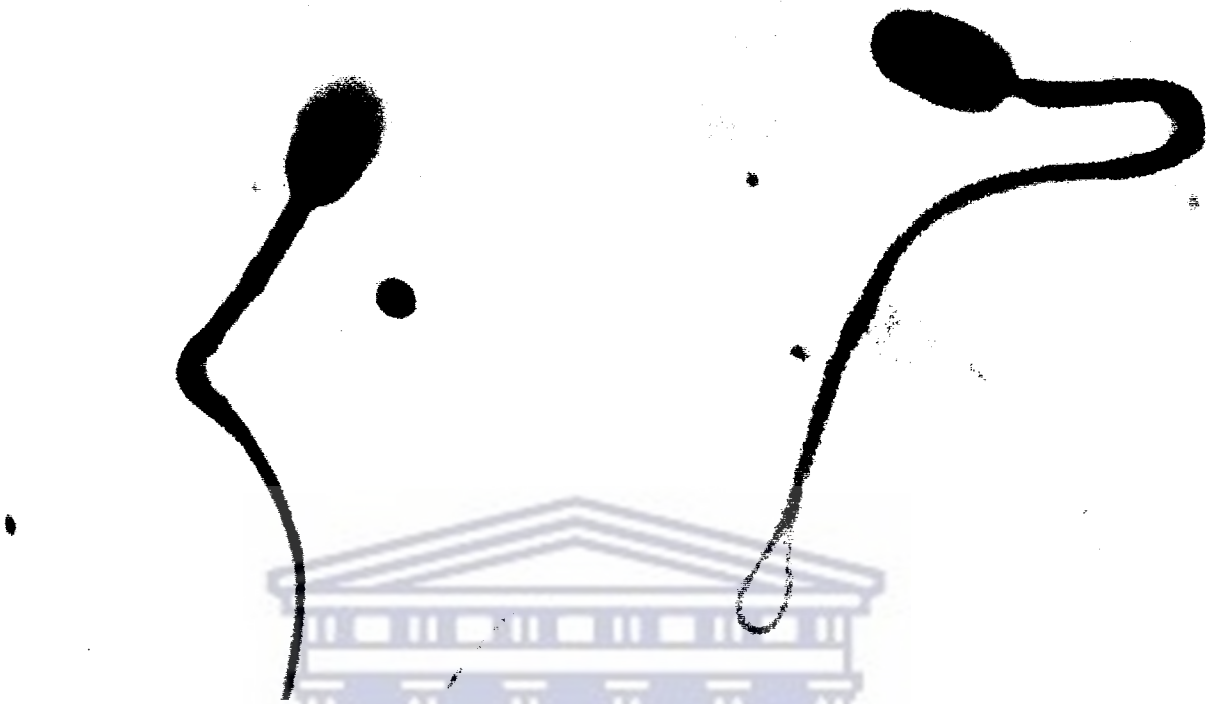


Figure 3.15 **Figure 3.16**

Figure 3.15 Bent midpieces like these often impede normal motility in fresh semen. Note the disintegrated acrosome.
Figure 3.16 Spermatozoa with bent midpiece and hair pin tail.

Figure 3.17 **Figure 3.18**

Figure 3.17 The neck is bent and the mitochondria are stripped from the proximal section of the midpiece. The rest of the midpiece is thickened. The principal-and endpiece are either detached or covered by mucus or cytoplasm at the bottom of the figure. **Figure 3.18** Only a small proximal section of the mitochondria remains while the rest of the midpiece is completely denuded of mitochondria. Note also the patchy acrosome (compare with the acrosome of Figure 3.16). The acrosomal area seems smaller than in Figure 3.16.

Figure 3.19 **Figure 3.20**

Figure 3.19 The midpiece is thickened to almost half the head width. The acrosome is only faintly visible. **Figure 3.20** The midpiece is short and thick. This could be due to the mitochondria having been displaced proximally. Note the kink in the principal piece and the absent acrosome.

Figure 3.21

Figure 3.22

Figure 3.21 There is distal thickening of the midpiece. The dense staining thickened area is split in two. **Figure 3.22** A proximal cytoplasmic droplet. The spermatozoon on the left has a bent midpiece.

Figure 3.23 **Figure 3.24**

Figure 3.23 A single head with a duplicated midpiece. The principal piece is thick and probably consists of two fused principal pieces. **Figure 3.24** The tails of these spermatozoa have broken off from the head at the midpieces. Various lengths of midpieces are the only remains of the tail.

Figure 3.25

Figure 3.26

Figure 3.25 The midpiece is abaxially implanted on the left side of the basal plate. **Figure 3.26** Detached tails.

3.1.3.1 Morphology part 1: Illustrations

3.1.3.1.4 Tail Abnormalities: principal - and endpiece

Figure 3.27 **Figure 3.28**

Figure 3.27 The bar at the bottom represents 6.7 micrometer. Ejaculate containing a large number of coiled and folded tails. These are the most common abnormalities found in vervet monkey semen. The folding often shapes the tail like a hairpin. There are also a few bent midpieces. **Figure 3.28** The bar at the bottom represents 4.0 micrometer. More folding, hairpin folding and coiling. Notice at the bottom of the Figure the amorphous head with a small acrosome, a cyst in the equatorial region and constriction of the post acrosomal segment.

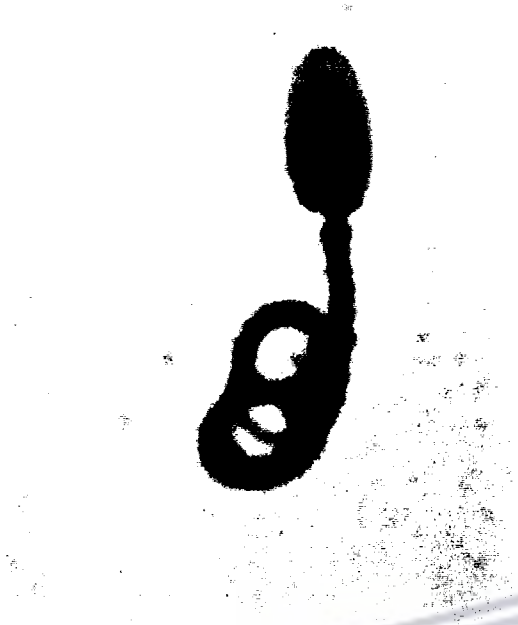


Figure 3.29 **Figure 3.30**

Figure 3.29 Coiling occurs to varying degrees. Here about four fifths of the tail is involved and a figure of eight is formed. **Figure 3.30** More coiling variations. About two thirds of the tail of the top spermatozoon is coiled whereas the entire tail of the bottom spermatozoon is involved. Note the faint staining acrosome of the top and the absent acrosome of the bottom spermatozoon.

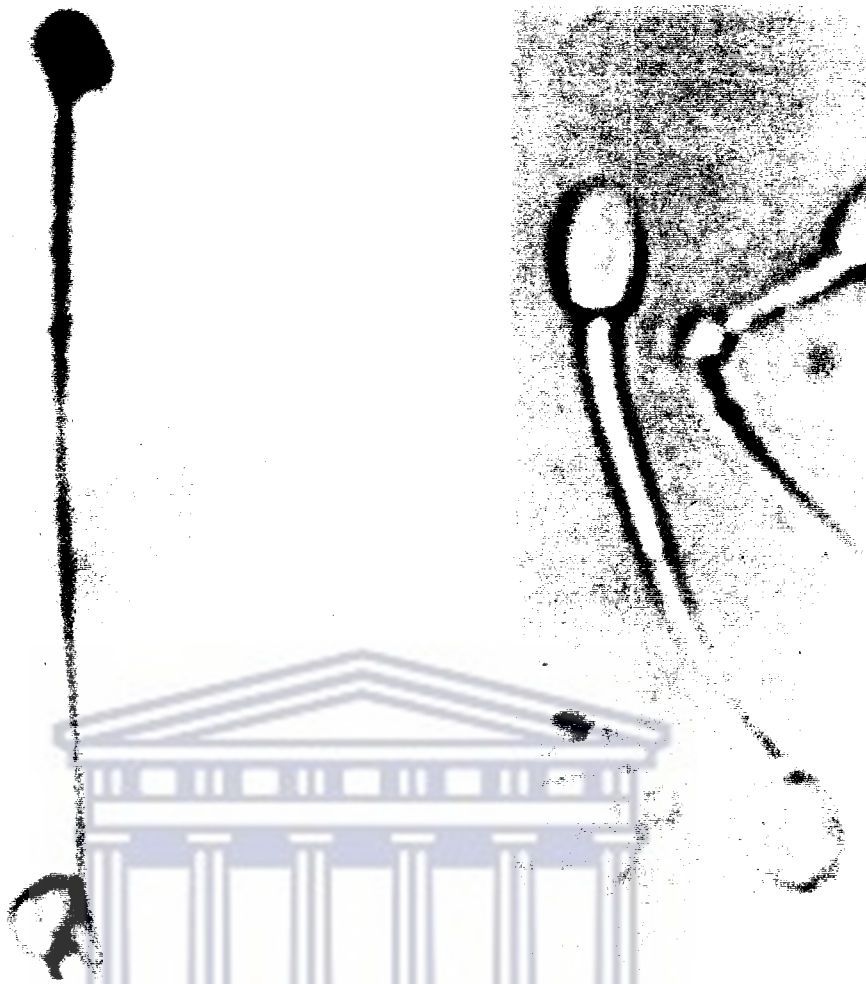


Figure 3.31 **Figure 3.32**

Figure 3.31 A terminally coiled tail. Note the amorphous microcephalic head. **Figure 3.32** A coiled tail and bent midpiece in a fresh unstained specimen.

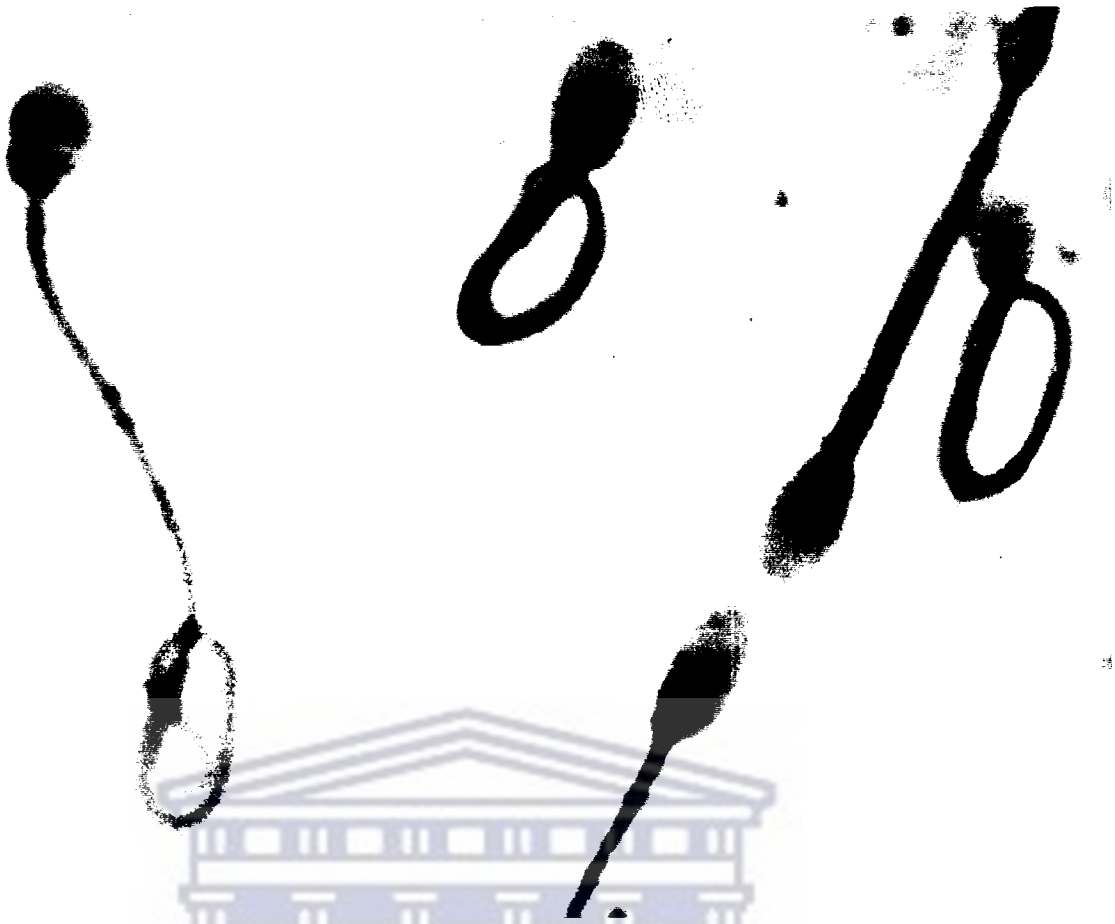


Figure 3.33 **Figure 3.34**

Figure 3.33 Coiling involving the distal two thirds of the tail. Note the small acrosome, the post-acrosomal nuclear invagination and possible diadem defect. **Figure 3.34** The entire tail is involved in the coiling in these two spermatozoa. Note the spermatozoon with the thickened midpiece.

Figure 3.35

Figure 3.36

Figure 3.35 The principal - and endpiece are duplicated. The midpiece appears thickened but this could be due to two midpieces being fused. Note the dense staining microcephalic head without acrosome. **Figure 3.36** Another example of tail duplication. Here the midpiece is also thickened. Note the small acrosomal area and the loss of acrosomal material. The head also appears to be macrocephalic.

3.1.3.2 Morphology part 2: Tables

3.1.3.2.1 *Head abnormalities*

a) Colony bred males

Out of a total of 13 types of head abnormalities found, the nipple defect (Figure 3.11, 3.12) was the most prevalent occurring in 7 (50%) males. None of the other defects were found in more than 4 (28.6%) males. Nipple defects occurred at one of the highest mean rates of 0.29% . All abnormalities are summarized in Table 3.7 which shows, that none occurred at a rate of more than 1%. The same applies to the mean values of the individuals with specific defects with one exception: the asymmetrical head (Figure 3.10) occurred at the highest rate. It could however only be found in 21.4% of all males.

3.1.3.2.1 *b) Wild caught males*

A total of 10 abnormalities were found of which the narrow head (Figure 3.4), the acrosomal cyst (Figure 3.6) and the microcephalus (Figure 3.5, 3.6, 3.7) were the most prevalent, each found in 4 (28.6%) males. All three of these defects also occurred at the highest overall mean rates, including the mean rate generated only from amongst the males with the defect. The results are summarized in Table 3.8 which shows that all of the defects occurred at a mean rate of less than 1%.

Table 3.7 Prevalence and percentage of head abnormalities found in 14 ejaculates from 14 colony bred vervet monkeys

Defect	No. of males with defect	(%)	Mean 1 (%)	± SD	Mean 2 (%)
Macrocephalic	4	28.6	0.21	0.38	0.75
Microcephalic	3	21.4	0.11	0.21	0.50
Round no acrosome	2	13.3	0.07	0.18	0.50
Narrow	4	28.6	0.18	0.32	0.63
Acrosomal cysts	2	14.3	0.11	0.29	0.75
Equatorial cysts	1	7.1	0.04	0.13	0.50
Nipple defect	7	50.0	0.29	0.32	0.57
Pointed	2	14.3	0.07	0.18	0.50
Asymmetrical	3	21.4	0.29	0.80	1.33
Duplication	3	21.4	0.11	0.21	0.50
Pyriform	1	7.1	0.04	0.13	0.50
Tapered	2	14.3	0.07	0.18	0.50
Amorphous	2	14.3	0.07	0.18	0.50
Total			**1.64**	**1.39**	

Footnote: Mean 1 = generated from entire group

Mean 2 = generated from males with specific defect

Table 3.8 Prevalence and percentage of head abnormalities found in 14 ejaculates from 14 wild caught vervet monkeys.

Defect	No. of males with defect	(%)	Mean 1 (%)	± SD	Mean 2 (%)
Macrocephalic	1	7.1	0.01	0.05	0.20
Microcephalic	4	28.6	0.21	0.37	0.73
Narrow	4	28.6	0.21	0.38	0.75
Acrosomal cysts	4	28.6	0.21	0.38	0.75
Equatorial Cysts	2	14.3	0.11	0.29	0.75
Nipple defect	2	14.3	0.07	0.18	0.50
Pointed	1	7.1	0.07	0.27	0.75
Duplicate	2	14.3	0.11	0.29	0.75
Pyriform	2	14.3	0.07	0.18	0.50
Tapered	2	14.3	0.07	0.18	0.50
Total			**1.15**	**1.02**	

Footnote: Mean 1 = generated from entire group

Mean 2 = generated from males with specific defect

3.1.3.2.2 *Midpiece abnormalities*

a) Colony bred males

Out of four abnormalities identified, the most prevalent were bent midpieces (Figure 3.15, 3.16), which occurred in 9 (64.3%) of all males. This defect also occurred at the highest mean rate. The rate and prevalence of all other abnormalities was considerably lower and all results are summarized in Table 3.9.

3.1.3.2.2 *b) Wild caught males*

A total of seven midpiece abnormalities were identified of which bent midpieces (Figure 3.15, 3.16) occurred in 10 (71.4%) males. This defect also occurred at the highest mean rate of 8.57% and 12.00% generated from amongst the males with the defect. Table 3.10 summarizes all results. Abaxial implantations (Figure 3.25) and thickened midpieces (Figure 3.19) were also common occurring in 50.0 and 57.1% of all males however these and the other defects occurred at a rate of less than 2%.

Table 3.9 Prevalence and percentage of midpiece abnormalities found in 14 ejaculates from 14 colony bred vervet monkeys

Defect	No. of males with defect	(%)	Mean 1 (%)	± SD	Mean 2 (%)
Bent	9	64.3	5.75	6.71	8.94
Thickened	7	50.0	0.68	1.32	1.44
Abaxial implantations	5	35.7	1.54	2.89	4.50
Cytoplasmic droplets	2	14.3	0.04	0.13	0.50
Total			**8.00**	**7.29**	

Footnote: Mean 1 = generated from entire group

Mean 2 = generated from males with specific defect

Table 3.10 Prevalence and percentage of midpiece abnormalities found in 14 ejaculates from 14 wild caught vervet monkeys

Defect	No. of males with defect	(%)	Mean 1 (%)	± SD	Mean 2 (%)
Bent	10	71.4	8.57	10.55	12.00
Head bent at neck	2	14.3	0.07	0.18	0.50
Thickened	8	57.1	0.82	1.23	1.44
Abaxial implantations	7	50.0	0.32	0.37	0.64
Pseudodroplet defect	1	7.1	0.07	0.27	1.00
Cytoplasmic droplet	2	14.3	0.11	0.29	0.75
Duplicate	1	7.1	0.04	0.13	0.50
Total			**10.00**	**11.11**	

Footnote: Mean 1 = generated from entire group

Mean 2 = generated from males with specific defect

3.1.3.2.3 *Principal-and endpiece abnormalities*

a) Colony bred males

Out of a total of eight abnormalities, coiled (Figure 3.27-3.32) and folded tails (Figure 3.27, 3.28) were the most prevalent occurring in 13 (92.9%) and 14 (100%) males respectively. Coiled tails also occurred at the highest mean rate with 17.18% whereas folded tails occurred at a mean rate of 7.00%, which is the second highest rate. Table 3.11 summarizes all results.

3.1.3.2.3 *b) Wild caught males*

Out of a total of eight abnormalities coiled and folded tails (Figure 3.27-3.32) were the most prevalent and occurred in 12 (85.7%) and 13 (92.9%) males respectively. Coiled tails occurred at the highest mean rate followed by detached and folded tails in that order. Table 3.12 summarizes all results.

Table 3.11 Prevalence and percentage of tail abnormalities found in 14 ejaculates from 14 colony bred vervet monkeys

Defect	No. of males with defect	(%)	Mean 1 (%)	± SD	Mean 2 (%)
Coiled	13	92.9	17.18	19.98	18.50
Folded	14	100.0	7.00	9.02	7.00
Detached at midpiece	2	14.3	0.07	0.18	0.50
Detached at endpiece	2	14.3	0.14	0.41	1.00
Duplication	1	7.1	0.07	0.27	1.00
Terminal coiling	5	35.7	0.68	1.62	1.90
Bent	1	7.1	0.29	1.07	4.00
Detached	14	100.0	3.89	3.91	3.89
Total			**29.32**	**27.42**	

Footnote: Mean 1 = generated from entire group

Mean 2 = generated from males with specific defect

Table 3.12 Prevalence and percentage of tail abnormalities found in 14 ejaculates from 14 wild caught vervet monkeys.

Defect	No. of males with defect	(%)	Mean 1 (%)	± SD	Mean 2 (%)
Coiled	12	85.7	16.54	15.33	21.63
Folded	13	92.9	3.79	2.12	4.62
Detached at midpiece	4	28.6	0.71	1.55	2.50
Detached at endpiece	2	14.3	0.21	0.58	1.50
Duplication	4	28.6	0.29	0.58	1.00
Terminal coiling	5	35.7	0.79	1.41	1.92
Bent	1	7.1	0.04	0.13	0.50
Detached	11	78.6	7.00	5.72	7.32
Total			**29.36**	**17.20**	

Footnote: Mean 1 = generated from entire group

Mean 2 = generated from males with specific defect

3.1.3.3 *Summary of tables*

Principal piece abnormalities were found to be the most prevalent and to occur at the highest mean rate amongst both wild caught and colony bred monkeys (Figure 3.35 and 3.36). Midpiece abnormalities occurred at a slightly lower prevalence in colony bred individuals but at a considerably lower prevalence in the wild caught group (Figure 3.35). The rate at which midpiece abnormalities occurred in both groups was considerably lower than principal piece defects (Figure 3.36). Head abnormalities occurred at the lowest prevalence and rate in both groups (Figure 3.35 and 3.36). Most types of defects were found in the head region (Figure 3.37). This was followed by the principal and midpiece region in that order (Figure 3.37). Large standard deviations from the means of all defects amongst all males, reflect the large individual differences.

The values for Figures 3.35-3.37 were calculated or derived from Tables 3.7-3.12.

Figure 3.35: Prevalence Of Defects Per Morphological Structure

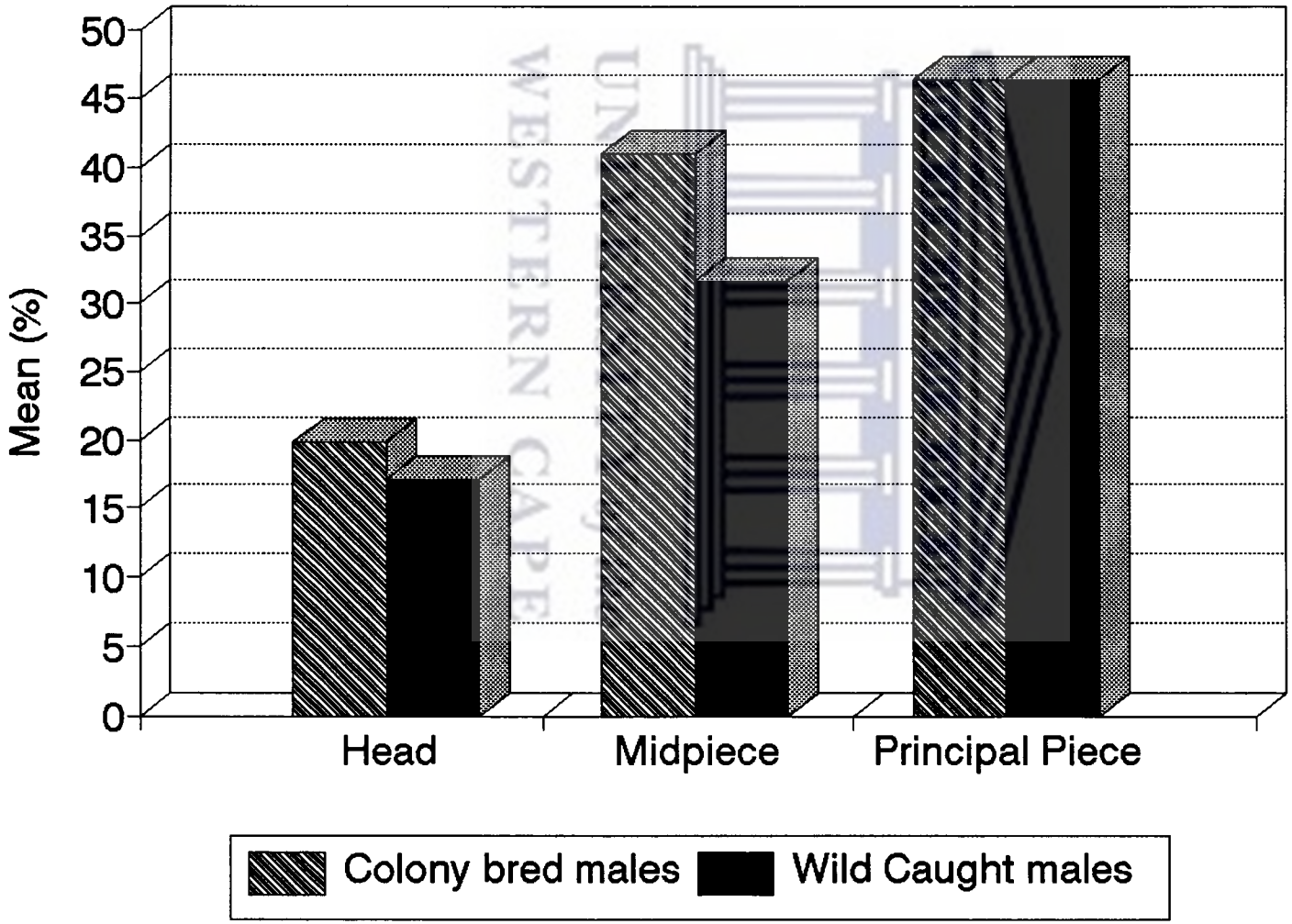


Figure 3.36: Average Rate Of Defects Per Morphological Structure

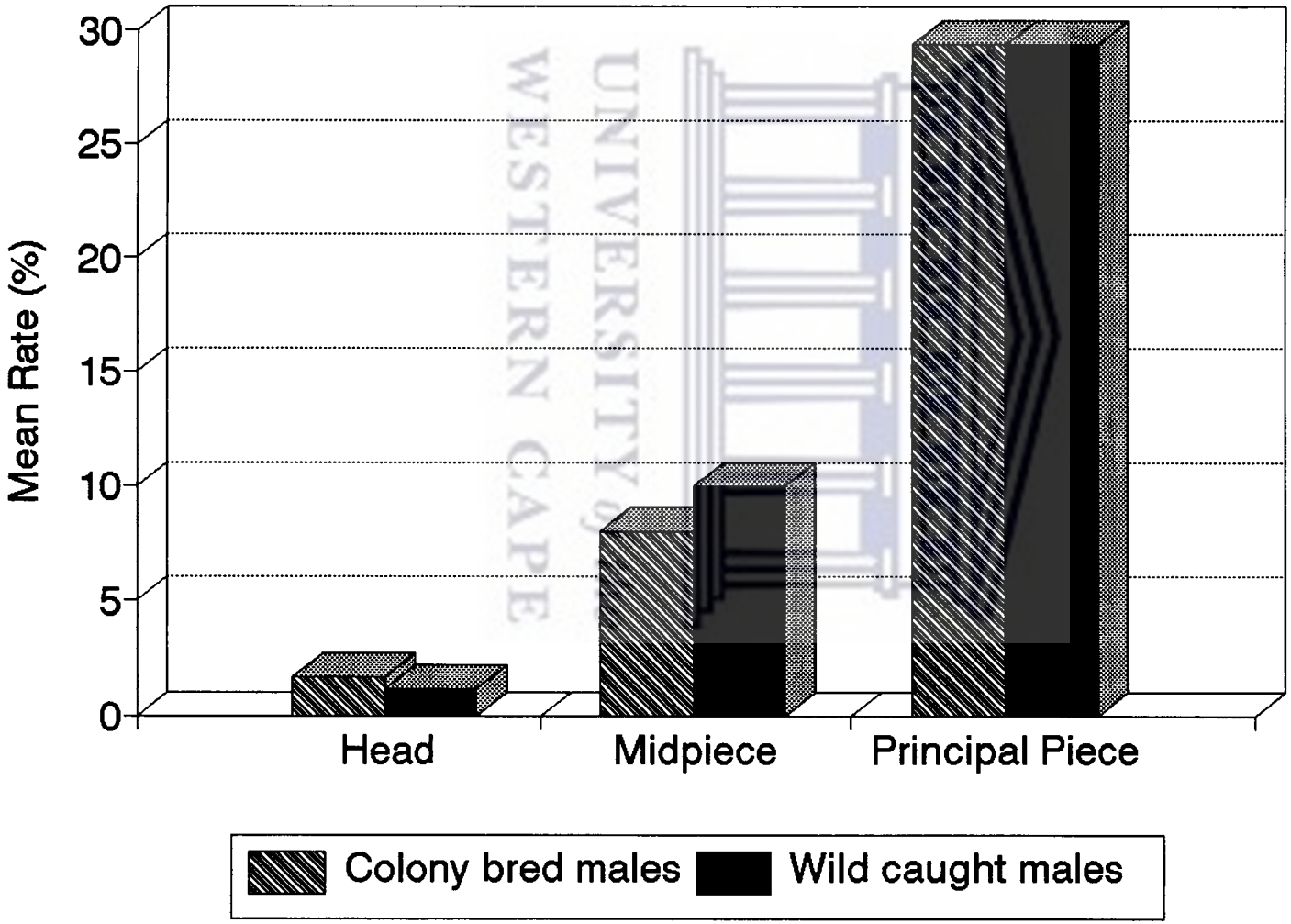
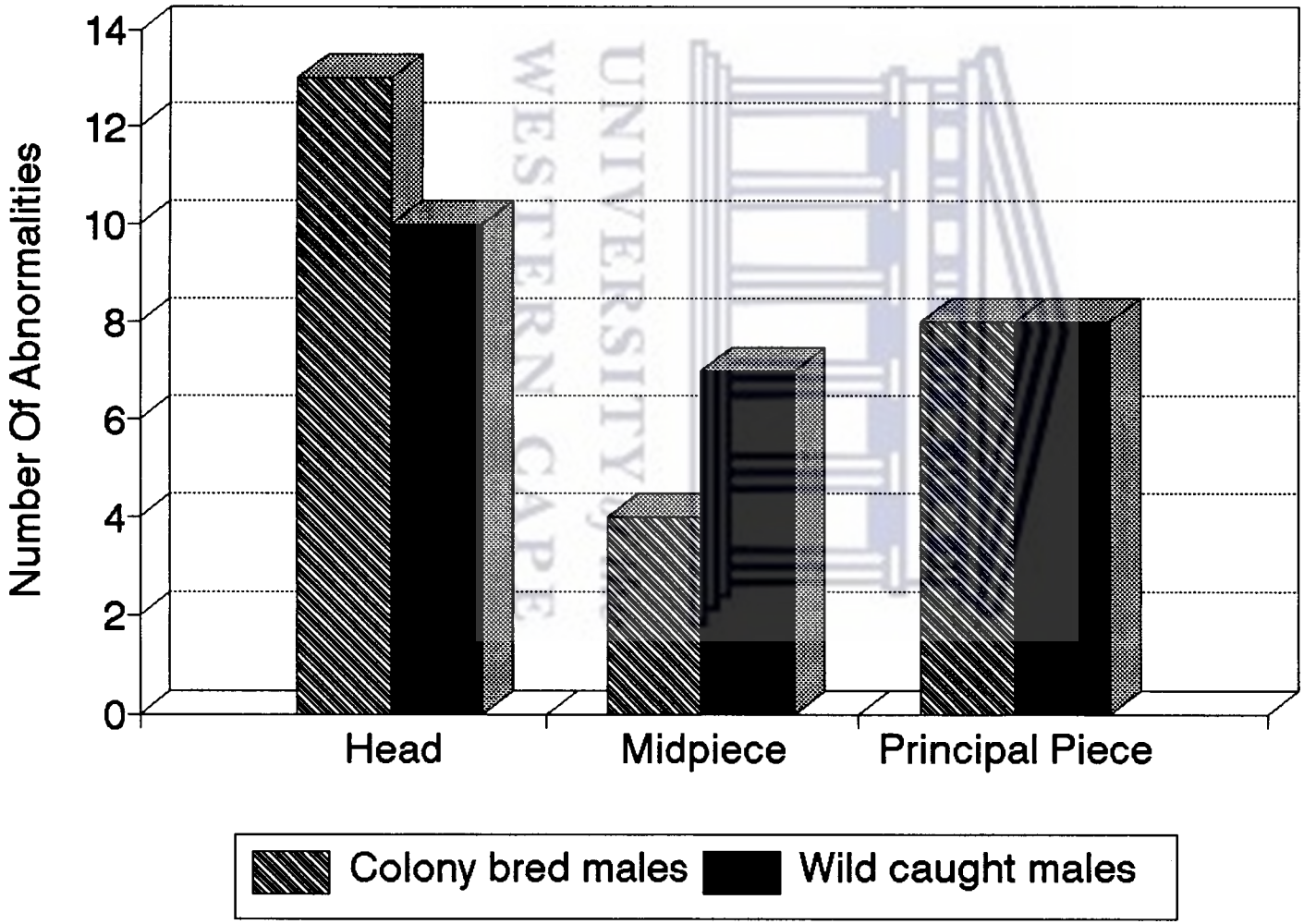


Figure 3.37: Number Of Different Defects Found



3.1.3.4 *Serial Morphology*

The results of the morphological evaluation of 10 ejaculates taken twice from 10 males at an interval of two weeks are summarized in Table 3.13 and 3.14 Considerable differences could be observed between consecutive ejaculates of most individuals, even reflecting the mean values of three parameters (Table 3.13 and 3.14).

In 6 (60)% males, differences of over 100% were recorded in consecutive ejaculates for coiled tails. Folded tails differed by more than 100% in 5 (50)% males.

Detached tails differed by more than 100% in consecutive ejaculates of 9 (90%) males (Table 3.14) and bent midpieces by more than 100% in consecutive ejaculates of 7 (70%) males (Table 3.14).

Out of four males with the consistently highest number of coiled tails (20-66%) two were successful breeders. The male with the highest number of folded tails is a successful breeder. Out of two males with the highest number of detached tails (17-45%) one male is a successful breeder, while both males with the highest number of bent midpieces (Table 3.14) are successful breeders.

Table 3.13 Coiled and folded tails in 10 ejaculates from 10 males taken twice at an interval of two weeks

	Coiled Tails (%)		Folded Tails (%)	
	Ejaculate		Ejaculate	
Number	1	2	1	2
46	52	20	5	5
879	1	4	2	3
889	10	19	0	5
660	0	18	0	29
940	5	45	17	3
934	43	35	8	3
777	66	63	6	8
563	28	10	5	4
884	18	33	4	8
754	50	20	12	11
Mean	**27.30**	**26.65**	**5.90**	**7.90**
± SD	**24.01**	**17.65**	**5.32**	**7.88**

Table 3.14 Detached tails and bent midpieces in 10 ejaculates from 10 males taken twice at an interval of two weeks

	Detached Tails (%)		Bent Midpieces (%)	
	Ejaculate		Ejaculate	
Number	1	2	1	2
46	10	0	6	3
879	22	8	6	9
889	32	17	0	8
660	45	1	2	14
940	8	10	0	15
934	3	7	2	16
777	1	13	1	12
563	6	14	20	13
884	0	1	35	26
754	14	1	3	18
Mean	**14.10**	**7.10**	**7.45**	**13.40**
± SD	**14.72**	**6.36**	**11.35**	**6.22**

3.1.3.5 *Statistics*

Table 3.15 P-values determined by Wilcoxon's 2-sample test for the difference in sperm head abnormalities between colony bred and wild caught vervet monkeys

Defect	p-value	Defect	p-value
Macrocephalus	0.1214	Nipple acrosome	0.0455
Microcephalus	0.6280	Pointed	0.6378
Pyriform	0.5774	Tapered	0.9999
Duplicate	0.7301	Narrow	0.9308
Acrosomal cyst	0.3865	Equatorial cyst	0.5491

A statistical difference between the two groups exist only for the nipple acrosome defect ($p < 0.05$).

Table 3.16 P-values determined by Wilcoxon's 2-sample test for the difference in sperm midpiece abnormalities between colony bred and wild caught vervet monkeys

Defect	p-value
Bent	0.5909
Thickened	0.5908
Abaxial implantation	0.9796
Cytoplasmic droplet	0.5491

Since $p > 0.05$ for all values, no statistical difference exists between any of the above mentioned parameters.

Table 3.17 P-values determined by Wilcoxon's 2-sample test for the difference in sperm tail abnormalities between colony bred and wild caught vervet monkeys

Defect	p-value	Defect	p-value
Coiled	0.8180	Folded	0.9632
Detached midpiece	0.2904	Detached endpiece	0.9699
Duplication	0.1686	Terminal coiling	0.9143
Bent	0.9999	Detached	0.2308

No difference exists between the two groups for any of the abovementioned parameters since p >0.05 in all cases.

3.2 Cryopreservation

Figures 3.38-3.40 illustrate the cooling and freezing rates for all methods. The cooling rate for method 1 was constant, with no equilibration at 5°C. The cooling and freezing rates in all other methods decreased with decreasing temperatures. For methods 2-4 it took about eight minutes of cooling until 5°C was reached. This means, since the total cooling time was 30 minutes, there was an equilibration time of 22 minutes at 5°C. It took approximately five to six minutes to freeze the samples from 5°C to plunge temperature in all methods. Because the paillettes were suspended for a total of 20 minutes in liquid nitrogen vapour, an equilibration time of 14-15 minutes remained at plunge temperature. Table 3.18 compares and illustrates methods 1-4. Method 3 is the most successful, with a recovery rate of 63.60%. This method includes a high temperature at which glycerol was added, a glycerol concentration of 5%, a fast freezing rate and a low plunge temperature (Figure 3.4).

Table 3.18 shows that changing the temperature at which glycerol was added from 5°C in method 1 to 32°C in method 2 improved the recovery by more than 100%. The change from method 2 to method 3 was lowering of the plunge temperature from -105.80 to -139.00 (Table 3.18) and thereby increasing the freezing rate (Figure 3.40). This again improved the recovery by about 60%. Lowering the glycerol concentration to 3% in method 4 produced a 19% lower recovery than method 3.

Table 3.18 Recovery of progressively motile spermatozoa and summary of some conspicuous features of methods 1-4

Method	1	2	3	4
Mean recovery (%)	16.60	38.30	63.60	51.20
± SD	17.20	26.10	27.80	32.50
Glycerol concentration (%)	5.00	5.00	5.00	3.00
Temperature at which glycerol was added (°C)	5.00	32.00	32.00	32.00
n	25	25	25	25
Plunge Temperature (°C)	-105.80	-105.80	-139.00	-139.00
n	5	5	5	5

Figure 3.38
Cooling rate: Method 1

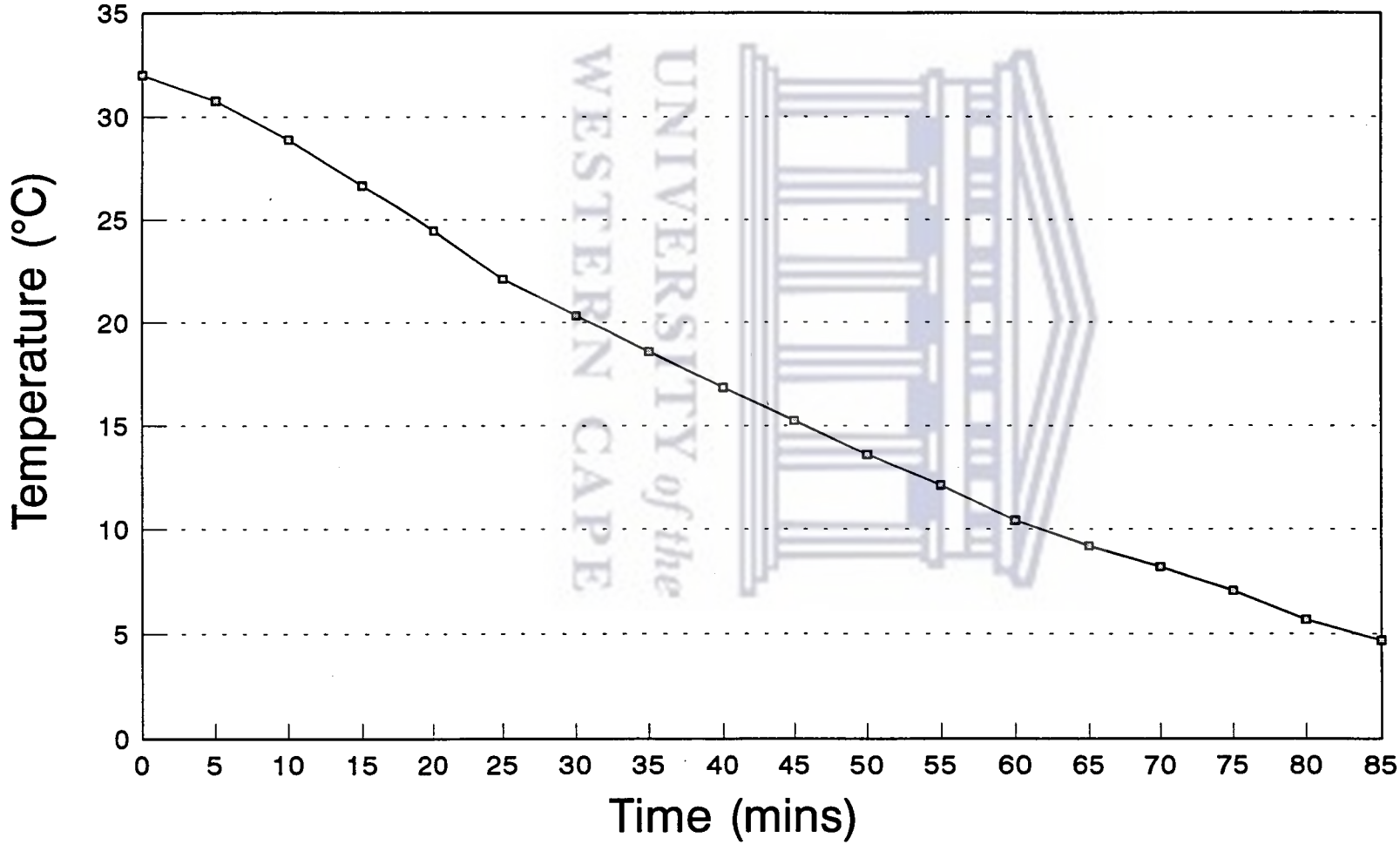


Figure 3.39
Cooling rates: methods 2-4

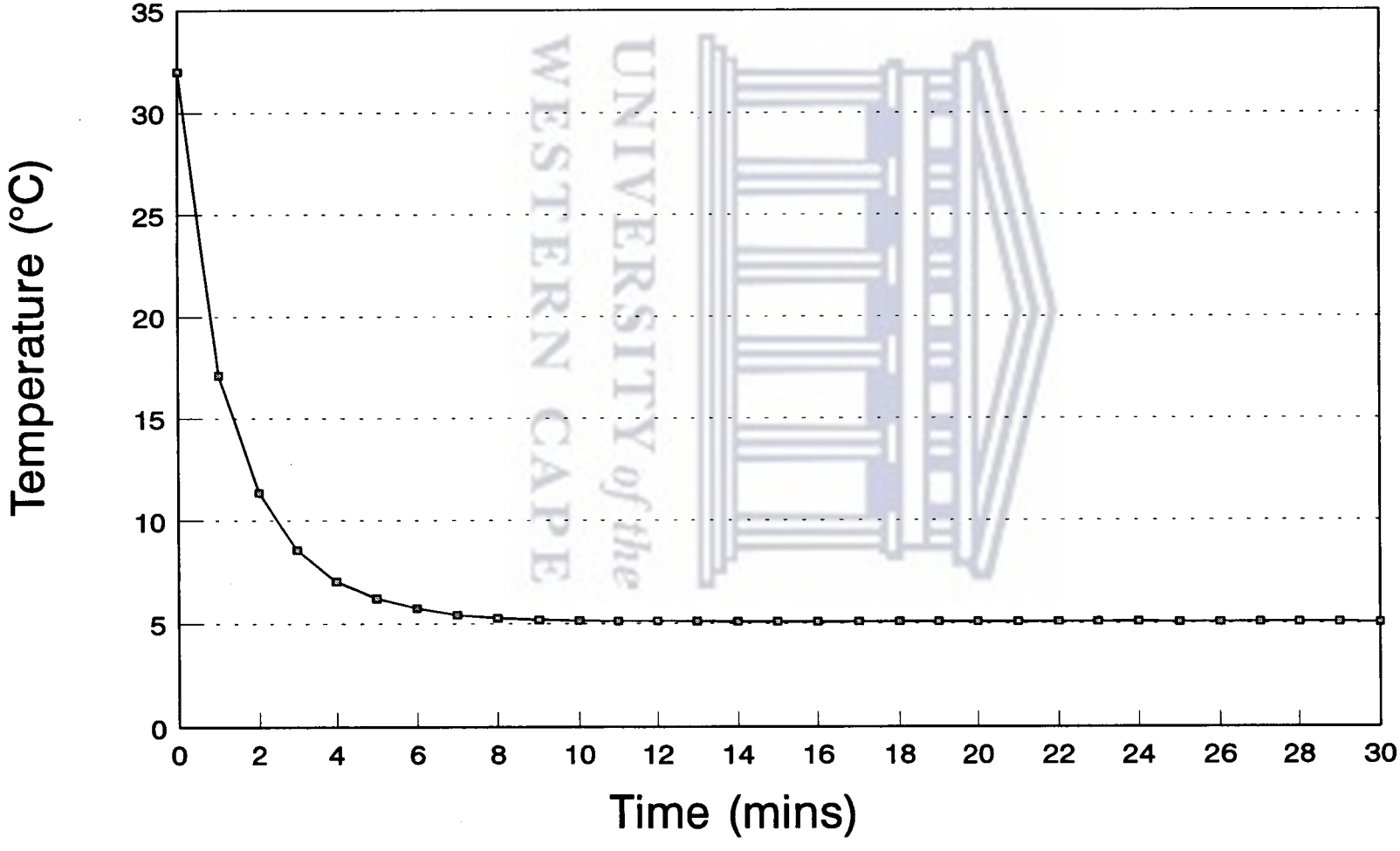
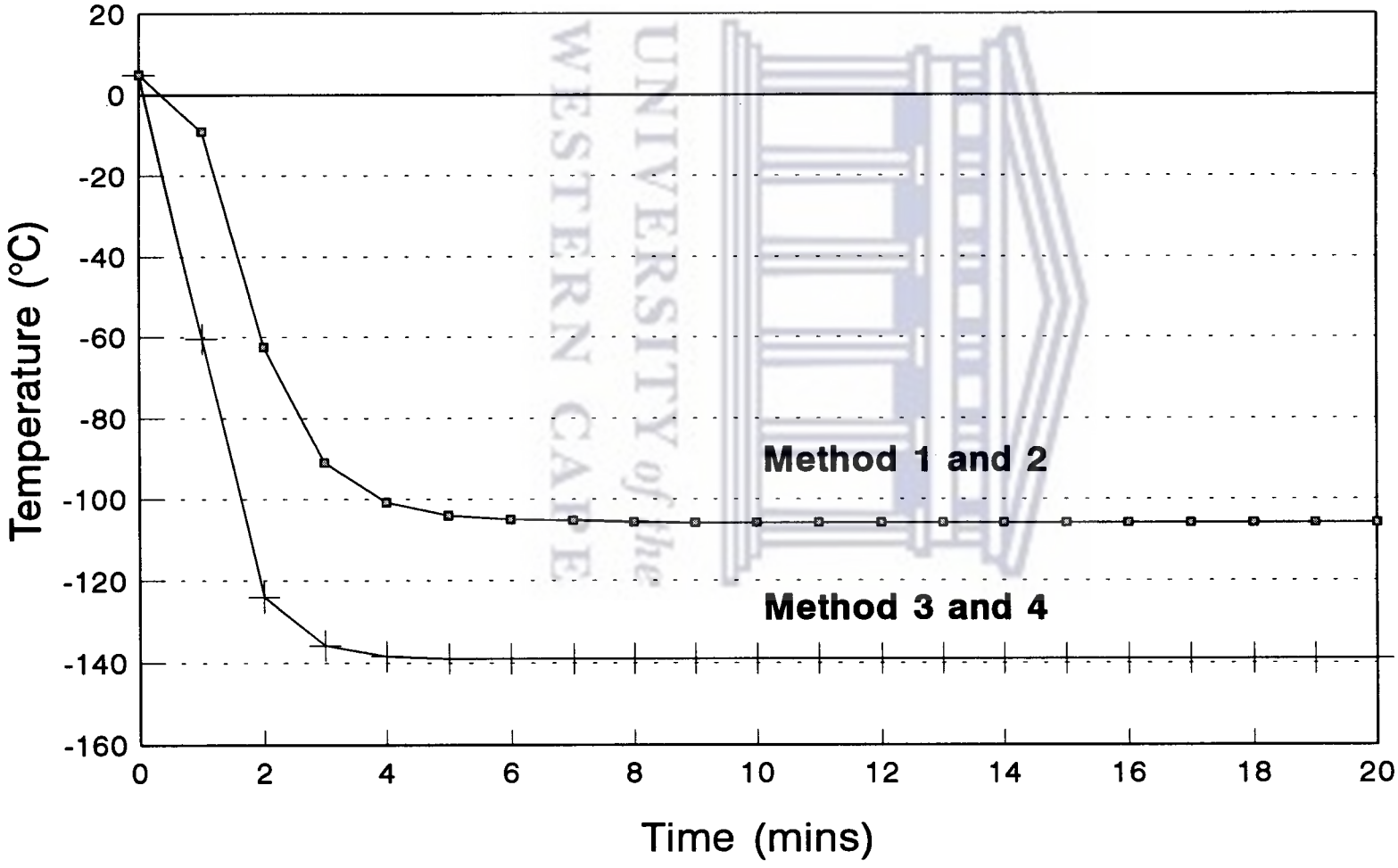


Figure 3.40
Freezing rates: methods 1-4



Chapter IV

Discussion and Conclusions

4.1 Electroejaculation

The technique and equipment used in this study enabled consistent, safe semen sampling from vervet monkey males. The stimulation technique did not follow the pattern used by a number of other workers (Bornman *et al.* 1988, Gould and Mann 1988, Harrison 1980, Thomson *et al.* 1992). The reasons are partially because obtaining semen from vervet monkeys has previously been described as technically unsatisfactory (Valerio and Dalgard 1975) and partially because pilot trials suggested that the commonly used rhythmic application of the current appeared to be unnecessary in our situation. Changing the stimulation technique also enabled the use of equipment which was much simplified from that used by others (Gould *et al.* 1978, Harrison 1980, Thomson *et al.* 1992).

The equipment used in this project functioned within the safe electrical parameters established by Gould *et al.* (1978). Even the maximum current density at the highest voltage was considerably below the 0.75mA/mm^2 safe limit at which damage to the rectal mucosa can occur (Gould *et al.* 1978). Ejaculations occurred within the same range of voltages as reported for other non-human primates but on average sooner (Hendrickx *et al.* 1978).

The anaesthetic agent, Ketamine Hydrochloride, did not appear to have an influence on the success of the electrostimulation,

which is in agreement with other reports (Gould *et al.* 1978). However, recently a Tiletamine-Zolazepam combination has been used with good success and producing ejaculates of comparable quality to Ketamine (Thomson *et al.* 1992). Better muscle relaxation and analgesia was reported during the use of the above mentioned drug combination.

The semen which was collected during this study by using this method and equipment appeared to be of comparable quality and characteristics than that reported by other workers for a number of non-human primate species including vervet monkeys. This will be discussed in detail below. There is some evidence that in humans electrostimulation produces semen of different quality than self-masturbation, particularly in terms of certain movement characteristic evaluated by computer assisted sperm analysis (Suresh *et al.* 1994). The electric current has, on the other hand, no effect on the sperm morphology (Suresh *et al.* 1994). It cannot be speculated to what extent some sperm characteristics reported here or elsewhere are artifacts of electrostimulation. Apart from above mentioned data, nothing else appears to have been published on the effect of electrostimulation on other semen characteristics. Only a comparison with semen obtained by manual masturbation can provide an answer. Semen has been collected by manual masturbation from vervet monkeys but a large number do not respond to this treatment (Hiyaoka and Cho 1990).

It is reported that penile stimulation of non-human primates

produces semen with a higher spermatozoa concentration when compared to rectal stimulation (Gould and Mann 1988). Because penile stimulation is often carried out without anaesthetic, this method was regarded to be unsuitable for this study.

4.2 pH

The seminal plasma is composed of secretions from the accessory glands. These include citric acid, acid phosphatase and zinc from the prostate, glycerolphosphorylcholine and carnitine from the epididymis and fructose, proteins and prostaglandins from the seminal vesicles (Acosta *et al.* 1986, Hafez 1985 a). Although any of these can be determined individually, the necessary technology for such analyses is seldom routinely available. Measuring the pH is therefore still part of the complete spermiogram because it is the most practical, easy and economical way to monitor accessory gland function (World Health Organisation 1992). Despite this, the pH appears not to be included in spermiograms from non-human primates (Ackerman and Roussel 1968, Bornman *et al.* 1988, Bush *et al.* 1975, Cui *et al.* 1991, Harrison and Wolf 1985, Harrison 1980, Hendrickx *et al.* 1978, Sarason *et al.* 1991, Thomson *et al.* 1992, Valerio and Dalgard 1975). According to available literature, this study presents for the first time data on the pH of vervet monkey semen.

A standard method for measuring the pH of human semen is the

use of indicator paper (World Health Organisation 1992). In the absence of standardized methods for non-human primate semen, the performance of two indicator papers was compared with the results obtained with a pH meter for this study. The pH of semen from both colony bred and wild caught individuals was found to be similar to that of human semen (World Health Organisation 1992). Of the two indicator papers, the one manufactured by Merck was found to differ significantly from the results obtained with the pH meter. According to the Merck paper the semen from both colony bred and wild caught individuals was more alkaline. This was the case for both group means and was consistent for all individuals (raw data). The values were also outside the range of 7.2-8.0 recommended by the WHO for normal human semen (World Health Organisation 1992). Reliable results were obtained with Panpeha paper and the group mean obtained with this paper did not differ significantly from that obtained with the pH meter. There was no difference in the pH of seminal plasma between wild caught and colony bred individuals.

4.3 Concentration, motility, vitality and forward progression

Except for the forward progression (FP), these are the most often and frequently exclusively reported variables of non-human primate sperm characteristics. All three characteristics were within the range which has been reported for non-human primates including vervet monkeys (Bornman *et al.* 1988,

Bush *et al.* 1975, Cui *et al.* 1991, Harrison and Wolf 1985, Harrison 1980, Hendrickx *et al.* 1978) In Table 4.1 the results from this study are compared with what others have reported for vervet monkey semen. The sperm concentration was highly variable among different individuals which is reflected in large standard deviations and confirmed by many other reports (Ackerman and Roussel 1968, Bush *et al.* 1975, Cui *et al.* 1991, Hendrickx *et al.* 1978, Schaffer *et al.* 1992, Van der Colf *et al.* 1991). There were also large variations within individuals when consecutive ejaculates were evaluated and sperm concentrations differed by more than 100% in most males in this study. This has also been recognized by Van der Colf *et al.* (1991) in the vervet monkey. The ejaculates were taken at long intervals to exclude reduction in sperm concentrations due to frequent sampling (Hendrickxs *et al.* 1978).

Although the motility was less variable than the concentration there were still large differences among and within individuals which is in agreement to what has been reported for other primate species (Ackerman and Roussel 1968, Bush *et al.* 1975, Cui *et al.* 1991, Harrison 1980, Roussel and Austin 1967) The motility appeared to be lower than sometimes reported for macaque sperm (Hendrickx *et al.* 1978, Sarason *et al.* 1991).

The percentage of live sperm determined by supra-vital staining was similar to that of the motility estimation. This implies that most non-motile sperm were dead. The number of live sperm was in agreement to that reported by Ackerman and Roussel

(1968) for vervets but was considerably lower than that reported by Valerio and Dalgard (1975) also for vervets. These differences could have been due to the labile nature of supra-vital staining with eosin nigrosin. A number of factors such as certain diluents and pH can influence the staining characteristics (Hellinga 1976 c). Differences among and within individuals were noted again and agreed with what was found in other primate species (Ackerman and Roussel 1968, Valerio and Dalgard 1975).

There were no differences in any of the above mentioned parameters between colony bred and wild caught individuals.

None of the above mentioned characteristics could be related to fertility since males with a consistently low sperm concentration were successful breeders. Additionally no consistently low sperm motility, vitality or FP could be found in any male.

Table 4.1 Comparison of semen characteristics of vervet monkeys reported by different workers.

No.	Concentration (mill/ml)	Motility (%)	Live (%)	Source
6	145	58	87	1
6	57-288	34-38	45-50	2
2 (7)	1428	1.1	-	3
23 (4)	440	39	-	4
14 (14) W	232.72	51.78	57.34	5
14 (14) C	273.67	52.88	52.88	5
3 (8)	-	40-55	-	6

Key to source numbers: 1 = Valerio and Dalgard 1975, 2 = Ackerman and Roussel 1968, 3 = Ackerman and Roussel 1971, 4 = Hendrickx *et al.* 1978, 5 = results from work for this thesis, 6 = Roussel and Austin 1967.

Footnotes: 1. the number of ejaculates is in brackets where available. 2. No comparable data exist for the FP. 3. key to abbreviations: W = wild caught, C = colony bred.

4.4 Morphology

4.4.1 *Specific morphologically abnormal forms*

The prevalence and rate of specific morphological abnormalities of sperm from wild caught and colony bred vervet monkeys, as well as illustrations of these abnormalities are provided for the first time. Reports on the morphology of vervet monkey sperm have been confined to reporting gross abnormal morphology without quantifying specific defects (Ackerman and Roussel 1968, Valerio and Dalgard 1975, Van der Colf *et al.* 1991). It is emphasized that the reference is to individuals which are wild caught but not living in the wild during sampling. The sperm characteristics of individuals actually living in the wild is apparently not known.

Most features, including the overall rate of abnormal morphology and the prevalence of tail defects particularly coiled and bent tails, agree with what has been reported for other Old World ceropithecines (Harrison 1980, Mohamed *et al.* 1987, Thomson *et al.* 1992). Tail defects also predominate in sperm from New World primates of the *cebidae* and *callitrichidae* families including capuchins, marmosets and tamarins, (Bush *et al.* 1975, Cui *et al.* 1991, Harrison and Wolf 1985). Head defects appeared to be rare in all taxa investigated, which concurs with what has been found in this study for vervet monkey sperm. The high rate of coiled and folded tails as well as bent midpieces in the vervet could be confirmed qualitatively in fresh unstained specimens. This was important

since some tail abnormalities can be artifacts of drying, cooling and contamination or the results of hypo-osmotic stress (Harrison, 1980, World Health Organisation 1992).

The different types of morphologically abnormal forms recorded in vervet monkeys were of the same types than described for various other mammals including humans and to a limited extent other non-human primate species (Cui *et al*. 1991, Hafez 1985 a, Harrison 1980, Harrison and Wolf 1985, Mohamed *et al*. 1987, Oettlé and Soley 1988, Oettlé *et al*. 1991). However, few investigators quantify in detail specific sperm defects of non-human primates. Most descriptions are limited to reporting gross abnormal or normal morphology and some differentiate only between head and tail regions (Ackerman and Roussel 1968, Bornman *et al*. 1988, Bush *et al*. 1975, Harrison and Wolf 1985, Sarason *et al*. 1991, Valerio and Dalgard 1975).

Semen from rhesus monkeys contained spermatozoa with amorphous heads, cytoplasmic droplets, small heads, large heads, detached heads and coiled tails. No specific rate or prevalence is mentioned other than that no semen sample from any individual contained more than 5% abnormal forms (Harrison 1980). In contrast, the semen from many individuals in this study contained substantially more abnormal forms. On the other hand, as in the rhesus monkey, head abnormalities were rare and the same type of abnormalities were found. The predominance of tail abnormalities is also confirmed in cynomolgus monkeys but it appears that head abnormalities were not studied in detail

(Mohamed *et al.* 1987). A low rate of abnormal morphology was found in sperm of crested macaques (Thomson *et al.* 1992). The rate of head abnormalities was 1-2% amongst four individuals which is similar to a range of 1.15-1.64% found in this study in vervet monkeys. Tail abnormalities also occurred at a higher rate than head abnormalities in the crested macaques. This is similar to vervet monkeys but coiled tails occurred at a higher rate and bent tails at a lower one in this species. Specific head abnormalities are not mentioned for the crested macaques. These are provided by Cui *et al.* (1991) for marmoset sperm. Three types of head abnormalities were found in the semen from 16 males including macrocephalic, microcephalic, and amorphous forms. The latter included narrow, tapering, round and "misshapen". The number and type of tail abnormalities is similar to what was found in this study in the vervet monkey. They included eccentric tail insertions (abaxial implantations), disrupted head-tail junctions, kinked tails, hairpin tails, looped (folded) tails, club tails, coiled tails, double tails, and "eroded" midpieces (pseudodroplet defect). Head abnormalities were also found to be rare in marmosets occurring at a median of 4.5% with the most common being the microcephalic and amorphous head. The most common head abnormalities of vervet monkey sperm were the nipple acrosome and microcephalus in colony bred and the microcephalus, narrow head and acrosomal cysts in wild caught individuals. As in the vervet, one of the most common abnormalities in marmosets was folded tails, followed by hairpin tails and neck defects but all occurring at a low rate. Bent and coiled tails were,

together with detached heads, also common in capuchin monkeys another New World species (Bush *et al.* 1975).

Only one investigator mentions four specific midpiece abnormalities (Cui *et al.* 1991) and four and seven distinct midpiece abnormalities were also found in this study in colony bred and wild caught vervet monkeys respectively.

4.4.2 *Gross Morphology*

The gross abnormal morphology of sperm from various non-human primate species including vervet monkeys varies considerably (Ackerman and Roussel 1968, Bornman *et al.* 1988, Harrison 1980, Valerio and Dalgard 1975). This includes the gross sperm morphology of another South African cercopithecine, the chacma baboon. The gross abnormal morphology determined in this study for colony bred and wild caught vervet monkeys is at the higher end of the above mentioned range. Table 4.2 compares the figures of gross abnormal morphology of vervet monkey sperm as reported by various authors.

From the very limited amount of measurements done in this study it appears, that vervet monkey sperm is slightly longer than human sperm (Menkveld *et al.* 1990, World Health Organisation 1992).

Table 4.2 Comparison of gross abnormal morphology of vervet monkey sperm reported by different authors.

Abnormal morphology (%)	No. of males	Source
0-32	4	Van der Colf *et al.* 1991
26-40	6	Ackerman and Roussel 1968
24	6	Valerio and Dalgard 1975
38.96 (Captive bred)	14	Seier, 1995 (this thesis)
40.51 (Wild caught)	14	Seier, 1995 (this thesis)

4.4.3 *Abnormal morphology of consecutive ejaculates*

Only four of the most common abnormalities, all affecting the tail, were investigated in the consecutive ejaculates. The rates at which head defects occurred were too low to produce meaningful comparisons and results. All four abnormalities, which included: coiled tails, folded tails, detached tails and bent midpieces were highly variable within all individuals. Coiled and folded tails can be artifacts of processing and the large variability within individuals in this study might support this conclusion (Harrison 1980, World Health Organisation 1992). Detached heads and bent midpieces, were, however, also highly variable and are not reported to be

produced artifactually. Considerable variations in the gross normal morphology of consecutive ejaculates from the same individuals have also been observed by Van der Colf *et al.* (1991) in vervet monkeys.

The results of this study demonstrate, that a single or even two ejaculates can not be representative of an individual's reproductive potential. This is also reflected by the variability of other consecutive sperm characteristics examined in this study.

4.4.4 *Speculation on the relation to fertility, pathology and general remarks*

Abnormal morphology had no effect on fertilization in the vervet monkey. The main reason is that most individuals with the highest abnormal morphology were highly successful breeders. Additionally no data appear to exist on the influence of abnormal sperm morphology on fertility in non-human primates. In contrast to the human and some domestic animal species, it is also not defined what proportion of sperm in an ejaculate can be abnormal to be classified as fertile or infertile (Acosta *et al.* 1986, Hafez 1985 a, Kruger *et al.* 1991). Apart from economical reasons, this could be partially due to the fact that the "classic" defects of impaired spermatogenesis and spermiogenesis are those of the sperm head (Hofmann and Freundl 1986, Hofmann and Haider 1985, Hofmann *et*

al. 1982.) These occur at a very low rate in many non-human primate species (Cui *et al*. 1991, Harrison and Wolf 1985, Harrison 1980, Thomson *et al*. 1992). The mean overall rate of head abnormalities, as determined in this study for vervet monkeys, was 1.64% in colony bred and 1.15% in wild caught individuals. Such low rates would make the determination of threshold figures for fertility very difficult if not impossible.

The low rate of head abnormalities in non-human primate sperm is agreed upon by all authors whereas the data on gross abnormal morphology are conflicting. Some found non-human primate sperm to be relatively free from morphological abnormalities, with a range of 0-20% defective forms (Bush *et al*. 1975, Harrison 1980, Harrison and Wolf 1985, Van der Colf et al. 1991). Others report higher rates for many species of up to and over 50% abnormal (Bornman *et al*. 1988, Cui *et al*. 1991, Ackermann and Roussel 1968). This is likely to be due to species specific differences in some cases and a lack of standardized methods and systems in others. But there are also conflicting reports involving the same species such as in the capuchin (Bush *et al*. 1975, Ackermann and Roussel 1968).

The results of this study support a higher overall rate of abnormal morphology. But tail abnormalities, particularly coiled and folded tails contribute over 70% to this rate. The potential for error when evaluating these has been recognized, since they can be artifacts of processing (Harrison 1980, World

Health Organisation 1992). In this study the presence of these abnormalities was confirmed in fresh samples but only qualitatively. Care was taken to eliminate the factors which support the production of these variables as artifacts.

Except for one defect, there was no difference between captive bred and wild caught individuals. Although this was not expected, differences might nevertheless have been produced by different stress levels, nutrition and exposure. The nipple defect was the only abnormality found in a larger number of colony bred males and at a considerably higher rate than in wild caught individuals. This abnormal form consists of an apical aggregation of acrosomal material sometimes forming a cyst. A fault during the cap formation phase of spermiogenesis might be the reason for such defects.

4.4.5 *Comparison with other species including humans*

Apart from other non-human primates, the types of abnormalities found in vervet monkey sperm during this study were also found in a variety of other mammalian species including humans (Hafez 1985 a, Hofmann and Freundl 1986, Menkveld *et al.* 1990, Oettlé and Soley 1988, Oettlé *et al.* 1991). The location of these abnormalities and their rate and prevalence was however quite different. In dogs the largest number of sperm abnormalities were found in the midpiece (Oettlé and Soley 1988). At a rate of 11% head abnormalities were also relatively common but neck

and tail abnormalities were found at a low (7%) rate. Although the significance of many sperm abnormalities of dogs is not known (Oettlé and Soley 1988), some could be related to infertility (Oettlé and Soley 1985). Sterilizing defects such as knobbed sperm, decapitate, and tail stumps are also found in all morphological regions of bull sperm (Hafez 1985 a). The literature on all aspects of abnormal morphology of human sperm is considerable and specific defects can be linked to infertility and grouped according to their origin in spermatogenesis, spermiogenesis or in the epididymis (Freund 1966, Hofmann *et al*. 1982, Hofmann and Haider 1985, Hofmann and Freundl 1986, MacLeod 1964, Oettlé *et al*. 1991, Menkveld *et al*. 1990, Oehninger *et al*. 1991). Attempts at universal standardization of the morphological evaluation of human sperm are fairly recent (Menkveld *et al*. 1990, World Health Organisation 1992). Many of the above mentioned studies were carried out to study pathomorphological spermatozoa and to investigate their basis in testicular pathology. Most abnormalities found in these studies occurred in large numbers in infertile individuals. Therefore it is not possible to speculate whether the type of abnormal forms found occasionally in "normal" ejaculates of fertile individuals have the same basis in testicular dysfunction or extrapolate to different species such as the vervet monkey.

4.4.6 *Comments on the suitability of the vervet monkey as a model for humans*

Tail abnormalities predominate in the sperm of vervet monkeys and many other Old and New World non-human primate species (4.4.1 in this chapter). These are believed to have their origin in the epididymis (Oettlé *et al.* 1991). Head abnormalities in contrast, which predominate in man, have their origin in the testes (Hofmann and Freundl 1986, Hofmann and Haider 1985, Hofmann *et al.* 1982). Head abnormalities in return occur at a very low rate in non-human primates (4.4.1 in this chapter). One might therefore be led to conclude that non-human primates, including the vervet monkey, are poor models for man. On the other hand it has been established that the administration of certain substances causes the same type of sperm defects in man and in non-human primates (Kalla *et al.* 1986, Mohamed *et al.* 1987). It cannot be assumed that the sperm characteristics of modern man reflect the natural physiological condition of our species. Many factors such as stress, consumption of harmful or toxic substances, environmental pollution and clothing can influence sperm quality including morphology.

4.4.7 *The staining method*

The Spermac stain enabled good general microscopic visualization of the entire sperm. This has been confirmed by

other workers while evaluating the sperm from a number of mammalian species including vervet monkeys (Conradie *et al.* 1994, Oettlé *et al.* 1991, Oettlé and Soley 1988). While the morphological regions could always be differentiated, the quality of the staining reaction was not consistent in all cases. Both strong staining reaction with definite demarcation of the regions and more faint staining reactions occurred. The type of staining reaction was sometimes characteristic of certain individuals. The advantage that Spermac stain has over some others is that it is quick, easy to use and yet effective.

4.5 Cryopreservation

A cryopreservation technique for vervet monkey semen which yields a satisfactory post-thaw recovery of progressively motile spermatozoa (Wolf and Patton 1989) has been developed with method 3. The important features of this method were a 5% glycerol concentration, addition of glycerol at 32°C, cooling to 5°C over 30 minutes and a low plunge temperature of -139°C resulting in a fast freezing rate. Although no glycerol equilibration time was planned, a short equilibration would have taken place while slowly adding glycerol to the extended semen. A short glycerol equilibration time was also most effective when freezing semen from *Macacca fascicularis* (Mahone and Dukelow 1978). Many features of the method developed in this study relate to freezing protocols formulated for humans, including an intermediate hold at or near 5°C and a plunge

temperature at or near -140°C. However, human sperm survival is not dependent on the temperature at which glycerol is added (Mahony *et al*. 1990). In contrast, changing the temperature at which glycerol was added from 5°C in method 1 to to 32°C in method 2 in this study, improved the post-thaw recovery by more than 100%. Glycerol affects every zone of the cell membrane and any change in their organisation can influence water and cryoprotectant transport across these membranes (Hammerstedt *et al*. 1990, Mazur 1970). Efflux of water from the cell is central to the freezing process, since it influences the ability of the cell to survive the volume changes that happen during freezing (Hammerstedt *et al*. 1990, Mazur 1970). There is evidence that although glycerol is generally classified as a penetrating cyroprotectant, the permeability changes at different temperatures. It is low at 0°C and higher at 20°C (McGann, 1978). Therefore by adding glycerol at the higher temperature used in this study, it is possible that the sperm cell was penetrated quicker and more completely. Since glycerol alters cell membrane structures, this might help to protect the vervet monkey sperm from cold shock, notwithstanding the toxicity of glycerol itself. The question at what temperature to add glycerol can therefore be quite fundamental to the success of a cryopreservation method (Hammerstedt *et al*. 1990).

Another change, which improved the post-thaw recovery by about 60% was decreasing the plunge temperature from -105.80°C in method 2 to -139.00°C in method 3. This inreased the freezing

rate (Fig 3.40), which is believed to be more effective when combined with a low glycerol concentration (McLaughlin *et al.* 1992). However, no further improvement in post-thaw recovery could be recorded when reducing the glycerol concentration from 5% in method 3 to 3% in method 4 and there was a reduction in recovery. A concentration of 3% was found to be more successful when freezing cynomolgus monkey semen, but the results cannot be compared since slower cooling and faster freezing rates throughout appeared to have been used (Tollner *et al.* 1990). However, species differences in sperm membrane composition may necessitate other freezing methods (Hammerstedt *et al.* 1990). The freezing rates of method 3 were about -65°C/min after one minute and -64°C/min after two minutes (Figure 3.40). This is similar to the rate of -60°C/min used to successfully freeze cynomolgus semen (Tollner *et al.* 1990). After three minutes the freezing rate in method 3 decreased to about 12°C/min (Figure 3.40). However by this time the temperature in the sample had already reduced to -136°C (Figure 3.40 page).

A final glycerol concentration of five percent was the most effective in this study but higher concentrations are still used in many species (De Leeuw *et al.* 1990, Hafez 1985, Mahony *et al.* 1990). It is generally difficult to compare methods since many variations of different extender-freezing rate combinations are used by various investigators (Hendrickx *et al.* 1978, Denis *et al.* 1976, Mahone and Dukelow 1978, Roussel and Austin 1967, Tollner *et al.* 1990, Wolf and Patton 1989).

The mean recovery of 63.60% is in agreement with that reported for various non-human primate species and man, in which cryopreservation methods are well established (Kraemer and Vera Cruz 1969, Mahone and Dukelow 1978, Roussel and Austin 1967, Tollner *et al*. 1990, Wolf and Patton 1989).

No specialized equipment other than a liquid nitrogen storage cylinder is needed and the method is practical and can be carried out in any facility.

4.6 Conclusions

4.6.1 Vervet monkey sperm characteristics are similar to those of other Old World *Cercopithecoidae* and New World *Ceboidae*.

4.6.2 Sperm characteristics are highly variable among and within individuals.

4.6.3 Because of 4.6.2, a spermiogram, which is established by using the methods described in this study, cannot be related to fertility and or breeding success. Even two consecutive ejaculates cannot determine an individuals breeding potential.

4.6.4 Sperm from vervet monkeys can be successfully cryopreserved in terms of post-thaw motility. The equipment and material needed is minimal.

References

1. Acosta AA, Van Uem J, Ackerman SB, Mayer JF, Stecker JF, Swanson RJ, Pleban P, Yuan J, Chillik C and Brugo S: Estimation of male fertility by examination and testing of spermatozoa. In, Jones HW, Jones GS, Hodgen GD and Rosenwaks Z (eds): "In Vitro Fertilization". Williams and Wilkins, Baltimore, 1986, 126-152.

2. Ackermann DR and Roussel JD: Fructose, lactic acid and citric acid content of the semen of eleven subhuman primate species and of man. J Reprod Fertil 17: 563-566, 1968.

3. Ackermann DR and Roussel JD: Citric acid, lactic acid and oxygen metabolism of frozen-thawed semen from four subhuman primate species. J Reprod Fertil 27: 441-443, 1971.

4. Ankel-Simons F: The brain. In Ankel-Simons F (ed): "A survey of living primates and their anatomy". Macmillan Publishing Co. New York, 1983 a, 136-150.

5. Ankel-Simons F: Placentation, reproduction, and growth. In Ankel-Simons F (ed): "A survey of living primates and their anatomy. Macmillan Publishing Co. New York, 1983 b, 228-243.

6. Austin CR: Ovulation induction in primates. In Perkins FT and O'Donoghue PN (eds) Laboratory Animal Handbook 6: "Breeding simians for developmental biology". Laboratory Animals LTD, London, 1975, 121-126.

7. Bamba K and Adams CE: Freezing of rabbit semen by the use of BF5 diluent. Lab Anim 24: 172-175, 1990.

8. Barnes RD, Lasley BL and Hendrickx AG: Midcycle ovarian histology of the bonnet monkey, *Macacca radiata*. Biol Reprod 18: 537-553, 1978.

9. Barr AB: Timing of spermatogenesis in four non-human primate species. Fertil Steril 24: 381-389, 1973.

10. Beattie CW and Bullock BC: Diurnal variation of serum androgen and Estradiol 17 Beta in the adult male green monkey (*Cercopithecus* sp). Biol Reprod 19: 36-39, 1978.

11. Beck WW and Silverstein I: Variable motility recovery of spermatozoa following freeze preservation. Fertil Steril 26: 863-867, 1975.

12. Bornman MS, van Vuuren M, Meltzer DGA, van der Merwe CA and Rensburg SJv: Quality of semen obtained by electroejaculation from chacma baboons (*Papion ursinus*). J Med Primatol: 17, 57-61, 1988.

13. Bush DF, Russel LH, Flowers AI and Sorensen AM: Semen evaluation in capuchin monkeys (*Cebus apella*). Lab Anim Sci 25: 588-593, 1975.

14. Camatini M, Franchi E and Curtis ID: Ultrastructure of Leydig cells in the African green monkey. J Ultrastruct Res 76: 224-234, 1981.

15. Clermont Y: Two classes of spermatogonial stem cells in the monkey (*Cercopithecus aethiops*). Am J Anat 126: 57-72, 1969.

16. Conradie E, Oettlé EE and Seier JV: Assessment of acrosomal integrity of vervet monkey spermatozoa after cryopreservation. J Med Primatol 23: 315-316, 1994.

17. Cui KH, Flaherty SP, Newble CD, Guerin MV, Napier AJ and Matthews CD: Collection and analysis of semen from the common marmoset (*Callithrix jacchus*). J Androl 12: 214-220, 1991.

18. Davis RO and Gravance CG: Standardization of specimen preparation, staining and sampling methods improves automated sperm-head morphometry analysis. Fertil Steril 59: 412-417, 1993.

19. Denis LT, Poindexter AN, Ritter MB, Seager SWJ and Deter RL: Freeze preservation of squirrel monkey sperm for use in timed fertilization study. Fertil Steril 27: 723-729, 1976.

20 De Leeuw FE, Chen HC, Colenbrander B and Verkleij AJ: Cold induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiol 27: 171-183, 1990.

21. Eley RM, Else JG, Gulamhusein N and Lequin RM: Reproduction in the vervet monkey (*Cercopithecus aethiops*): 1. Testicular volume, testosterone and seasonality. Am J Primatol 10: 229-235, 1986.

22. Eley RM: Reproductive biology of the vervet monkey (*Cercopithecus athiops*): a review. Utafiti 4: 1-33, 1992.

23. Eliasson R: Supravital staining of human spermatozoa. Fertil Steril 28: 1257, 1977.

24. Fobes JL and King JE: Measuring primate learning abilities. In Fobes JL and King JE (eds): "Primate behavior". Academic Press, New York, 1982, 289-326.

25. Freund M: Standards for the rating of human sperm morphology. Int J Fertil 11: 97-179, 1966.

26. Freund M: Semen analysis. In: Behrman SJ and Kistner RW (eds): "Progress in Infertility". Little, Brown & Co, Boston, 1968, 593-627.

27. Garner DL and Hafez ESE: Spermatozoa and seminal plasma. In Hafez ESE (ed): "Reproduction in farm animals" Fifth edition. Lea and Febiger, 1985, 189-209.

28. Goodman AL, Nixon WE, Johnson DK and Hodgen GD: Regulation of folliculogenesis in the cycling rhesus monkey: selection of the dominant follicle. Endocrinol 100: 155-161, 1977.

29. Gould KG, Warner H and Martin DE: Rectal probe electroejaculation of primates. J Med Primatol 7: 213-222, 1978.

30. Gould KG and Mann DR: Comparison of electrostimulation methods for semen recovery in the rhesus monkey (*Macacca Mulatta*). J Med Primatol 17: 95-103, 1988.

31. Gulyas BJ, Yuan L, Tullner WW and Hodgen GD: The fine structure of corpus luteum from intact, hypophysectomized and fetectomized pregnant monkeys (*Macacca mulatta*) at term. Biol Reprod 14: 613-626, 1976.

32. Hafez ESE: Semen evaluation. In Hafez ESE (ed): "Reproduction in farm animals" Fifth edition. Lea and Febiger, 1985 a, 455-480.

33. Hafez ESE: Preservation and cryopreservation of gametes and embryos. In Hafez ESE (ed): "Reproduction in farm animals" Fifth edition. Lea and Feibiger, 1985 b, 571-600.

34. Hammerstedt RH, Graham JK and Nolan JP: Cryopreservation of mammalian sperm: what we ask them to survive. J Androl 11: 73-88, 1990.

35. Harrison RM: Semen parameters in *Macacca mulatta*: ejaculates from random and selected monkeys. J Med Primatol 9: 265-273, 1980.

36. Harrison RM and Wolf RH: Sperm parameters and testicular volume in *Saguinus mystax*. J Med Primatol 14: 281-284, 1985.

37. Harrison RM, Lewis RW and Roberts JA: Pathophysiology of varicocele in nonhuman primates: long term seminal and testicular changes. Fertil Steril 46: 500-510, 1986.

38. Healey P: Effect of freezing on the ultrastructure of the spermatozoon of some domestic animals. J Reprod Fertil 18: 21-27, 1969.

39. Hein PR, Schatorjé JSJO, Frencken HJAAM, Segers MFG and Thomas CMG: Serum hormone levels in pregnant cynomolgus monkeys. J Med Primatol 18: 133-142, 1989.

40. Held JR and Wolfle TL: Imports: Current trends and usage. Am J Primatol 34: 85-96, 1994.

41. Hellinga G: Investigation of the semen. In Hellinga G (ed):" Clinical Andrology". William Heinemann Medical Books LTD, London, 1976 a, 15-29.

42. Hellinga G: The seminal plasma. In Hellinga G (ed):" Clinical Andrology". William Heinemann Medical Books LTD, London, 1976 b, 87-93.

43. Hellinga G: The spermatozoa. In Hellinga G (ed):" Clinical Andrology". William Heinemann Medical Books LTD, London, 1976 c, 93-105.

44. Hendrickx AG, Thompson RS, Hess DL and Prahalada S: Artificial insemination and a note on pregnancy detection in the non-human primate. Symp Zool. Soc. Lond. 43: 219-240, 1978.

45. Hendrickx AG and Binkerd PE: Nonhuman primates and teratological research. J Med Primatol 19: 81-108, 1990.

46. Hiyaoka A. and Cho F: A method for collecting semen by fingers in the African green monkey (*Cercopithecus aethiops*) and properties of the semen collected. Exp Anim 39: 121-124, 1990.

47. Hofmann N, Freundl B and Hilscher BW: Über die Zuordnung der pathomorphologischen Spermabefunde zu testiculären Erkrankungen. Aktuelle Dermatologie 8: 191-197, 1982.

48. Hofmann N and Freundl G: Die mikrosokopische Spermaanalyse. Fertilität 2: 135-143, 1986.

49. Hofmann N and Haider SG: New results on the morphological diagnosis of disorders concerning spermatogenesis. Gynäkologe 18: 70-80, 1985.

50. Jeyendran RS, van der Ven HH, Kennedy W, Perez-Pelaez M and Zaneveld LJD: Comparison of glycerol and a zwitteron buffer system as cryoprotective media for human spermatozoa. J Androl 5: 1984.

51. Kalla NR, Steiner M, Weinbauer G, Rovan E, Foo J, Hurkadli KS, Sheth AR and Frick J: Ultrastructure of monkey (*Macacca radiata*) spermatozoa: effect of gossypol *in vivo*. Urol Res 14: 247-252, 1986.

52. King FA, Yarbrough CJ, Anderson DC, Gordon TP and Gould KG: Primates. Science 240: 1475-1482, 1988.

53. Kraemer DC and Vera Cruz NC: Collection, gross characteristics and freezing of baboon semen. J Reprod Fertil 20: 345-348, 1969.

54. Kruger TF, Menkveld R, Oettlé EE, Swanson RJ, Acosta AA and Oehninger S: Clinical application. In Menkveld R, Oettlé EE, Kruger TF, Swanson RJ, Acosta AA and Oehninger S (eds): "Atlas of human sperm morphology". Williams and Wilkins, Baltimore, 1991, 7-14.

55. Lang MC: A technique for the collection of semen from squirrel monkeys (*Saimiri sciureus*) by electro-ejaculation. Lab Anim Care 17: 218-221, 1967.

56. Lapin BA: Use of nonhuman primates in cancer research. J Med Primatol 11: 327-341, 1982.

57. MacLeod J: Human seminal cytology as a sensitive indicator of the germinal epithelium. Int J Fertil 9: 281-295, 1964.

58. Mahone JP and Dukelow RW: Semen preservation in *Macacca fascicularis*. Lab Anim Sci 28: 556-561, 1978.

59. Mahoney CJ: The accuracy of bimanual rectal palpation for determining the time of ovulation and conception in the rhesus monkey (*Macacca Mulatta*). In Perkins and O'Donoghue PN: "Breeding Simians For Developmental Biology, Laboratory Animal Handbook 6. Laboratory Animals LTD, London, 1975, 127-140.

60. Mahony MC, Morshedi M, Scott RT, De Villiers A and Erasmus E: Role of spermatozoa cryopreservation in assisted reproduction. In Acosta AA, Swanson RJ, Ackerman SB, Kruger TF, van Zyl J and Menkveld R (eds): "Human spermatozoa in assisted reproduction. Williams and Wilkins, Baltimore, 1990, 310-318.

61. Mahony MC, Oehninger S, Doncel G, Morshedi M, Acosta A and Hodgen GD: Functional and morphological features of spermatozoa microaspirated from the epididymal regions of cynomolgus monkeys (*Macacca fascicularis*). Biol Reprod 48: 613-620, 1993.

62. Martin DP, Darrow CC, Valerio DA and Leiseca SA: Methods of anaesthesia in nonhuman primates. Lab Anim Sci 22: 837-843, 1972.

63. Mazur P: Cryobiology: The freezing of biological systems. Science 168: 939-949, 1970.

64. Mazur P: The role of intracellular freezing in the death of cells cooled at supraoptimal rates. Cryobiol 14: 251-272, 1977.

65. Mayer JM and Lanzendorf SE: Cryopreservation of gametes and pre-embryos. In: Jones HW, Jones GS, Hodgen GD and Rosenwaks Z (eds): "In Vitro Fertilizaton". Williams and Wilkins, Baltimore, 1986, 260-269.

66. McGann LE: Differing actions of penetrating and nonpenetrating cryoprotective agents. Cryobiol 15: 382-390, 1978.

67. McLaughlin EA, Ford WCL and Hull MGR: The contribution of the toxicity of a glycerol-egg yolk-citrate cryopreservative to the decline in human sperm motility during cryopreservation. J Reprod Fertil 95: 749-754, 1992.

68. Menkveld R, Stander FSH, Kotze TJvW, Kruger TF and van Zyl JA: The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. Hum Reprod 5: 586-592, 1990.

69. Menkveld R, Oettlé EE, Kruger TF, Swanson RJ, Acosta AA and Oehninger S: Appendices. In Menkveld R, Oettle EE, Kruger TF, Swanson RJ, Acosta AA and Oehninger S (eds): Atlas of human sperm morphology". Williams and Wilkins, Baltimore, 1991, 115-118.

70. Meryman HT, Williams RJ and Douglas MST J: Freezing injury from solution effects and its prevention by natural or artificial cryoprotection. Cryobiol 14: 287-302, 1977.

71. Mohamed MK, Burbacher TM and Mottet NK: Effects of methyl mercury on testicular functions in *Macacca fascicularis* monkeys. Pharmacol & Toxicol 60: 29-36, 1987.

72. Montfort SL, Jayaraman S, Shideler SE, Lasley BL and Hendrickx AG: Monitoring ovulation and implantation in the cynomolgus macaque (*Macacca fascicularis*) through evaluations of urinary estrone conjugates and progesterone metabolites: a technique for the routine evaluation of reproductive parameters. J Med Primatol. 15: 17-26, 1986.

73. Moudgal NR: Corpus luteum of the nonhuman primate. Advances in veterinary science and comparative medicine 28: 343- 366, 1984.

74. Müntzing J, Varkarakis MJ, Saroff J and Murphy GP: Comparison and significance of respiration and glycolysis of prostatic tissue from various species. J Med Primatol 4: 245-251, 1975.

75. Napier JR and Napier PH: Cercopithecus. In Napier JR and Napier PH (eds): "A handbook of living primates". Academic Press, London, 1967, 100-111.

76. Oehninger S, Swanson RJ, Acosta AA, Kruger TF, Menkveld R and Oettlé EE: Case studies. In Menkveld R, Oettlé EE, Kruger TF, Swanson RJ, Acosta AA and Oehninger S (eds): "Atlas of human sperm morphology". Williams and Wilkins, Baltimore, 1991, 97-111.

77. Oettlé EE and Soley JT: Infertility in a maltese poodle as a result of a sperm midpiece defect. J SA Vet Assoc 56: 103-106, 1985.

78. Oettlé EE and Soley JT: Sperm abnormalities in the dog: a light and electron microscopic study. Vet Med Review 1: 28-70, 1988.

79. Oettlé EE, Menkveld R, Swanson RJ, Oehninger S, Kruger TF and Acosta AA: Photomicrographs with interpretations. In Menkveld R, Oettlé EE, Kruger TF, Swanson RJ, Acosta AA and Oehninger S (eds): "Atlas of human sperm morphology". Williams and Wilkins, Baltimore, 1991, 15-96.

80. Roussel JD and Austin CR: Preservation of primate spermatozoa by freezing. J Reprod Fertil 13: 333-335, 1967.

81. Roussel JD, Stallcup OT and Austin CR: Selective phagocytosis of spermatozoa in the epididymis of bulls, rabbits and monkeys. Fertil Steril 18: 509-516, 1967.

82. Sarason RL, VandeVoort CA, Mader DR and Overstreet JW: The use of nonmetal electrodes in electroejaculation of restrained but unanaesthetized macaques. J Med Primatol 20: 122-125, 1991.

83. Schaffer NE, McCarthy TJ, Fazleabas AT and Jeyendran RS: Assessment of semen quality in a baboon (*Papio anubis*) breeding colony. J Med Primatol 21: 47-48, 1992.

84. Shackleton CHL and Mitchell FL: The comparison of perinatal steroid endocrinology in simians with a view to finding a suitable animal model to study human problems. In Perkins FT and O'Donoghue PN (eds) "Breeding simians for developmental biology", Laboratory Animal Handbook 6. Laboratory Animal LTD, London, 1975, 159-181.

85. Seier JV: Breeding vervet monkeys in a closed environment. J Med Primatol 15: 339-349, 1986.

86. Seier JV, Venter FS, Fincham JF and Taljaard JJF: Hormonal vaginal cytology of vervet monkeys. J Med Primatol 20: 1-5, 1991.

87. Smith RL and Williams RT: Comparative metabolism of drugs in man and monkeys. J Med Primatol 3: 138-152, 1974.

88. Suresh CS, Wang R, Kukuy E, Walker CF and Hellstrom WJG: The detrimental effects of electric current on normal human sperm. J Androl 15: 145-150, 1994.

89. Tanimura T and Tanioka Y: Comparison of embryonic and foetal development in man and rhesus monkey. In Perkins FT and O'Donoghue PN (eds): "Breeding simians for developmental biology", Laboratory Animal Handbook 6. Laboratory Animal LTD, London, 1975, 205-233.

90. Taylor PJ, Wilson J, Laycock R and Weger J: A comparison of freezing and thawing methods for the cryopreservation of human semen. Fertil Steril 37: 100-103, 1982.

91. Thomson JA, Iliff-Sizemore SA, Gliessman PM and Wolf DP: Collection and fertilization potential of sperm from the Sulawesi crested black macaque (*Macaca nigra*). Am J Primatol 28: 289-297, 1992.

92. Tollner TL, VandeVoort CA, Overstreet JW and Drobnis EZ: Cryopreservation of spermatozoa from cynomolgus monkeys (*Macacca fascicularis*). J Reprod Fertil 90: 347-352, 1990.

93. Valerio DA and Dalgard DW: Experiences in the laboratory breeding of non-human primates. In: Perkins FT, O'Donoghue PN (eds): "Breeding Simians for Developmental Biology." Laboratory Animal Handbook 6. Laboratory Animals Ltd, London, 1975, 49-62.

94. Van der Colf AP, Kruger TF, Menkveld R, Seier JV, Fincham JE and Swart Y: Effect of ethanol on semen characteristics of vervet monkeys. Arch Androl 26: 25-29, 1991.

95. Wehrenberg WB, Dierschke DJ and Wolf RC: Uteroovarian pathways and maintainance of early pregnancy in rhesus monkeys. Biol Reprod 20: 601-605, 1979.

96. World Health Organisation laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Third Edition: 6-18, 1992.

97. Williams RJ and Harris D: The distribution of cryoprotective agents into lipid interfaces. Cryobio 14: 670-680, 1977.

98. Wolf DP and Patton PE: Sperm cryopreservation state of the art. J in Vitro Fertil and Embryo Trans 6: 325-327, 1989.