GENDER SELECTION: SEPARATION TECHNIQUES FOR X- AND Y-CHROMOSOME BEARING HUMAN SPERMATOZOA

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Thesis presented in fulfilment of the requirements for the degree of

Masters of Science in Medical Science (MMedSc) at the University of

Stellenbosch.

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December 2013

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Michelle van der Linde 2 September 2013

ABSTRACT

Preconceptual sex selection is an ethically justifiable process whereby X- and Y-chromosome bearing spermatozoa are isolated prior to fertilization of the oocyte in order to generate either a male or a female offspring. Although various separation techniques are available, none can guarantee 100% accuracy. There are various physiological differences between X- and Y-chromosome bearing spermatozoa which can be used to separate these two populations of sperm.

For the purpose of this study, X- and Y-chromosome bearing spermatozoa were separated based on (1) their respective abilities to remain viable when subjected to adverse environments, including extreme pH values, increased temperatures and various hydrogen peroxide (H_2O_2) concentrations; (2) the ability of Y-chromosome bearing spermatozoa to swim faster and/or more progressively than X-chromosome bearing spermatozoa; and (3) the X-chromosome bearing spermatozoa's increased size and weight when compared to the Y-chromosome bearing spermatozoa.

The efficacy of live and dead cell separation through (i) Magnetic Antibody Cell Separation (MACS) and (ii) a modified swim-up technique was also assessed and compared. Changes in the sex-chromosome ratio of samples were established by double-label fluorescent in situ hybridization (FISH) before and after processing.

Sperm motility (CASA) and viability (eosin/nigrosin) was assessed before and after each intervention. Ethical clearance for this study was granted by the Health Research Ethics Committee 1 (Ethics #: S13/04/068).

The results indicated successful enrichment of X-chromosome bearing spermatozoa upon incubation in acidic media, increased temperatures, and H_2O_2. In contrast, Y-chromosome bearing spermatozoa were successfully enriched through a direct swim-up method as well as discontinuous gradient centrifugation.

In conclusion, this study demonstrated the potential role for physiological differences between X- and Y-chromosome bearing spermatozoa in the development of preconceptual gender selection through sperm sorting.

Keywords

gender selection, sperm separation, sex-chromosomes, sex-chromosome linked diseases, MACS

August 2013.

OPSOMMING

Prekonsepsie geslagselektering is 'n eties regverdigbare proses waardeur X- en Y-chromosoom draende spermatosoë geïsoleer word voordat bevrugting van die oösiet plaasvind, om óf 'n manlike óf 'n vroulike nageslag te genereer. Alhoewel verskeie skeidingstegnieke beskikbaar is, kan geeneen 100% akkuraatheid waarborg nie. Daar bestaan verskeie fisiologiese verskille tussen X- en Y-chromosoom draende spermatosoë wat skeiding van hierdie twee groepe spermatosoë moontlik kan maak.

Vir die doel van hierdie studie is skeidingsmetodes vir die X- en Y- chromosoom draede spermatosoë gebaseer op (1) hul onderskeie vermoëns om lewensvatbaar te bly tydens blootstelling aan ‘n ongunstige milieu, insluitend ekstreme pH waardes, verhoogde temperature en verskeie waterstofperoksied (H_2O_2) konsentrasies; (2) die vermoë van die Y-chromosoom draende spermatosoon om vinniger en/of meer progressief as X-chromosoom draende spermatosoë te swem; en (3) die X-chromosoom draende spermatosoon se verhoogde grootte en gewig in vergelyking met die Y- chromosoom draende spermatosoon.

Die effektiwiteit van die (i) Magnetiese Anti-liggaam Sel Skeidingstegniek (MACS) en (ii) 'n aangepaste weergawe van die op-swem tegniek om lewendige en dooie selle

te skei is ook bepaal en vergelyk. Veranderinge in die geslagschromosoom verhouding van die monsters is bepaal deur dubbel-etiket fluoresensie *in situ* hibridisering (FISH) voor en na verwerking. Spermmotiliteit (CASA) en lewensvatbaarheid (eosien/nigrosin) is bepaal voor en na elke intervensie. Etiese goedkeuring vir hierdie studie is verleen deur die Gesondheids-Navorsingsetiekkomitee 1 (Etiese # : S13/04/068).

Die resultate dui suksesvolle verryking van X-chromosoom draende spermatosoë deur inkubasie in suur media, verhoogde temperature, en H_2O_2. Y-chromosoom draende spermatosoë is verryk deur middel van 'n direkte op-swem metode sowel as diskontinue gradiënt sentrifugering .

Ten slotte, hierdie studie toon die potensiële rol vir fisiologiese verskille tussen X- en Y- chromosoom draende spermatosoë in die ontwikkeling van prekonsepsie geslagselektering metodes deur skeiding van X- en Y-chromosoom draende sperme.

Sleutelwooorde

geslag seleksie, sperm skeiding, geslags chromosome, geslagschromosoome-gekoppelde siektes, MACS

Augustus 2013.

This dissertation is dedicated to

Madelein Harris,

my sister and my person.

ACKNOWLEDGEMENTS

I would like to thank the following people who contributed to the completion of this dissertation:

- Prof. Stefan du Plessis, my supervisor and mentor, for his continued support and patience, and for always being able to make the work environment exciting.

- My parents, who have always encouraged me in all of my endeavours and who has supported me unconditionally.

- My brother, for all intents and purposes, Juan Harris, for patience and humour when needed, as well as Divan Harris, ultimately the inspiration behind this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ALPHABETICAL LIST OF ABBREVIATIONS

AI	Artificial Insemination
ART	Assisted Reproduction Technique
BBT	Basal Body Temperature
BSA	Bovine Serum Albumin
CASA	Computer Assisted Sperm Analysis
CVS	Chorionic Villus Sampling
DNA	Deoxyribonucleic Acid
FISH	Fluorescent In Situ Hybridization
H_2O_2	Hydrogen Peroxide
HFEA	Human Fertilization and Embryology Authority
GIFT	Gamete Intrafallopian Transfer
ICSI	Intracytoplasmic Sperm Injection
IMSI	Intracytoplasmic Morphologically Selected Sperm Injection
IUI	Intrauterine Insemination
IVF	In Vitro Fertilization
LIN	Linearity
MACS	Magnetic Antibody Cell Separation

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGD	Preimplantation Genetic Diagnosis
PS	Phosphatidylserine
QA	Quinacrine
SCA	Sperm Class Analyser
STR	Straightness
SURGG	Stellenbosch University Reproductive Research Group
VAP	Velocity of Average Path
VCL	Curvilinear Velocity
VSL	Straight Line Velocity
WHO	World Health Organization
WOB	Wobble

CHAPTER 1: INTRODUCTION TO STUDY

1.1 INTRODUCTION

The practice of gender selection is an extremely controversial topic in the scientific community, as it has both ethical and legal aspects that need to be considered. Legally, both pre- and post-conceptual gender selection can be justified, as according to the Law of Persons in South Africa[1], one is only deemed a "natural person" with the right to live and not be discriminated against, from birth. Therefore, neither a fetus nor an embryo is protected from gender discrimination in South Africa. Ethically, however, discarding healthy embryos and/or the abortion of a fetus to achieve gender selection is not tolerated by the general or scientific community.

Circumventing the ethical issues implies that gender selection has to be practiced prior to fertilization. Successful separation of X- and Y-chromosome bearing spermatozoa could have great potential, as it could drastically lower the abortion, infanticide and abandonment statistics of many countries.

1.2 PROBLEM STATEMENT

A need exists for the development of ethical, cost effective and successful methods of gender selection. Currently, it appears that gender selection *before* fertilization is the only method that can be ethically rationalized, as once fertilization has occurred, the personhood of the embryo has to be considered and it becomes unethical to do

anything to harm or discriminate against the unborn baby. It is believed then, that if separation of X- and Y-chromosome bearing spermatozoa can be combined with Shettle's or Whelan's methods[2] of timing of fertilization with regard to ovulation, the chances of successful preconceptual gender selection are very high.

1.3 HYPOTHESIS

Although various studies have reported several morphological and functional differences between X- and Y-chromosome bearing spermatozoa, the differences have not yet been consistently proven to be significant in the separation of these two populations of spermatozoa. For the present study, it is hypothesized that X- and Y-chromosome bearing spermatozoa can be enriched in samples by using methods that are based on some of these basic physiological differences.

As some methods are based on the ability of the spermatozoa to remain viable despite being subjected to hostile environments, there is also a need to develop a simple, cost-effective method to separate the viable spermatozoa from the non-viable spermatozoa. It is therefore hypothesized that a modified version of the direct swim-up, as defined by the World Health Organization (WHO)[3] will be successful in separating live and dead spermatozoa to a degree that is comparable to the results obtained by the more sophisticated Magnetic Antibody Cell Separation (MACS) technique[4].

1.4 RESEARCH STRATEGY

1.4.1 RESEARCH AIMS

The primary research goal of this study was to isolate X- and Y-chromosome bearing spermatozoa by using methods that are based on three of the physiological differences between these sperm populations.

Research Aim 1: Separation of X- and Y-chromosome bearing spermatozoa based on viability.

- Aim 1a: Separation of X- and Y-chromosome bearing spermatozoa according to their respective abilities to remain viable upon exposure to hostile environments.
- Aim 1b: Comparison of the effectiveness of MACS and modified Swim-up techniques in separating live and dead spermatozoa.

Research Aim 2: Separation of X- and Y-chromosome bearing spermatozoa based on their particular motility capacities.

Research Aim 3: Separation of X- and Y-chromosome bearing spermatozoa based on differences in size/weight.

1.4.2 Research Objectives

The main objective during the separation of the X- and Y-chromosome bearing spermatozoa was determining whether there was a change in the sex-chromosome ratio of the sample before and after processing. Other parameters such as motility and viability were also assessed and compared to the sex-chromosome ratios of the spermatozoa. In the comparative assessment of the MACS and modified Swim-up techniques, both motility and viability parameters were used as objectives to evaluate the success of the separations.

1.5 Outline of the Study

1.5.1 Research Aim 1: Separation of X- and Y-chromosome bearing spermatozoa based on viability.

1.5.1.1 Research Aim 1a: Separation of X- and Y-chromosome bearing spermatozoa according to their respective abilities to remain viable upon exposure to hostile environments.

In the first part of the study, separation of X- and Y-chromosome bearing spermatozoa based on their respective abilities to survive exposure to hostile environments was attempted in 3 ways. Spermatozoa were separated from the seminal plasma and directly exposed to (i) pH values ranging from 5.5 to 9.5, (ii) hydrogen peroxide (H_2O_2) concentrations from 50µM to 1000µM, and (iii) increased temperatures up to 45°C. After the exposure, viable cells were isolated from dead

cells by MACS[5] as well as a modified version of the WHO manual's direct swim-up[3]. Sex-chromosome ratios were determined before and after the experiment with fluorescent in situ hybridization (FISH), while motility parameters and viability percentages were recorded throughout the experiment.

1.5.1.2 *Research Aim 1b: Comparison of the effectiveness of MACS and modified Swim-up techniques in separating live and dead spermatozoa.*

This research aim was carried out simultaneously with Research Aim 1a. After incubation of the cells in the respective media, the samples were divided into 2 fractions and live and dead cells were separated by the MACS and modified swim-up methods, respectively. The motility and viability of the live cell fractions were analysed and compared.

1.5.2 *Research Aim 2: Separation of X- and Y-chromosome bearing spermatozoa based on their particular motility capacities.*

During this part of the study, spermatozoa were separated based on their motility parameters, specifically in terms of progressive movement and velocity. The WHO lists the direct swim-up as a standard method for preparation of spermatozoa, selecting the most motile cells in a given sample. In the first phase of this part of the study, a direct swim-up as defined by the WHO manual was performed and sex-chromosome ratios were determined for the different resulting fractions. During the

second phase, culture medium was injected into a capillary tube, followed by semen. According to an article by Joe Kita (1996)[6] spermatozoa have been reported to swim at average velocities of 1-4mm/min, and after 15 minutes of incubation different sections of the tube were analysed for sex-chromosome ratios and motility parameters.

1.5.3 Research Aim 3: Separation of X- and Y-chromosome bearing spermatozoa based on differences in size/weight.

Centrifugation-based protocols, as set out in the WHO manual[3], were followed during this part of the study in an effort to separate the spermatozoa based on their different sizes and/or molecular weights. Heavier cells are reported to sediment faster when centrifuged, although in the presence of a discontinuous gradient the size, molecular density and even motility of the spermatozoa may also play a role. Discontinuous gradient and double wash centrifugation procedures were performed, after which the sex-chromosome ratios and other sperm parameters were assessed.

CHAPTER 2: LITERATURE REVIEW

2.1 GENDER DETERMINATION

2.1.1 INTRODUCTION

Chromosomes are the essential units for cellular division and must be replicated, divided, and passed successfully to the next generation of cells to ensure genetic diversity and, ultimately, survival of the species[7]. Humans have 2 pairs (diploid) of 22 different types of somatic chromosomes and one pair of sex-chromosomes, totalling 46 chromosomes. In the case of females, the two sex-chromosomes are both X-chromosomes, while males have one X-chromosome and one Y-chromosome.

Gametes (oocytes and spermatozoa) are haploid cells, carrying only one set of the 22 somatic chromosomes and one sex-chromosome, equalling 23 chromosomes. Somatic cells multiply by mitosis, which is division of the cell to form 2 identical replicas (daughter cells) of the original (parent) cell. Gametes also undergo mitosis, after which gametogenesis takes place via meiosis, resulting, in the case of males, in formation of 16 spermatozoa (see Figure 2-1). Segregation of the sex-chromosomes during the final stages of meiosis leads to the haploid spermatozoa carrying either the X- or the Y-chromosome in a 1:1 ratio[8].

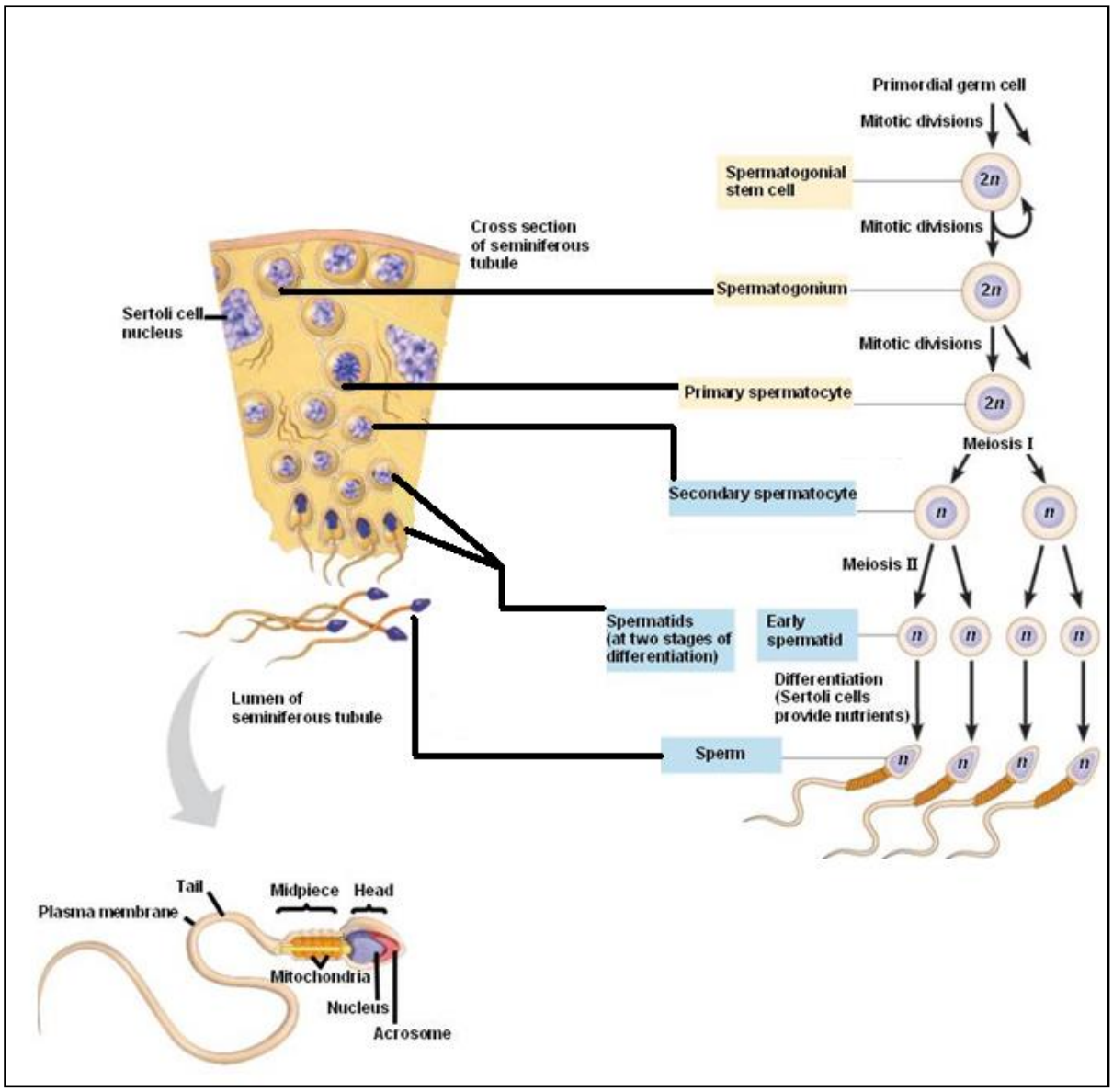

Figure 2-1: Diagram of spermatogenesis and meiosis. Adapted from http://bio1152.nicerweb.com/Locked/media/ch46/spermatogenesis.html.

Gender determination takes place at the moment of fertilization. Since the oocyte always contributes an X-chromosome, it is the X- or Y-chromosome bearing spermatozoon that determines the sex of the resultant embryo. The presence of the Y-chromosome leads to the male karyotype, which results in testicular formation and the male phenotype. Many believe that an unequal ratio of X- and Y-chromosome

bearing spermatozoa in the ejaculate contributes to this imbalance, but segregation during meiosis in males should equalise the number of X- and Y-chromosome bearing sperm, theoretically leading to a 50-50 chance of having either a boy or a girl naturally.

The global ratio of male:female births has been reported to be slightly in favour of males (see Figure 2-2). In America, there are 105 males born for every 100 females[9], while in South Africa, 102 male births are recorded for every 100 females[10].

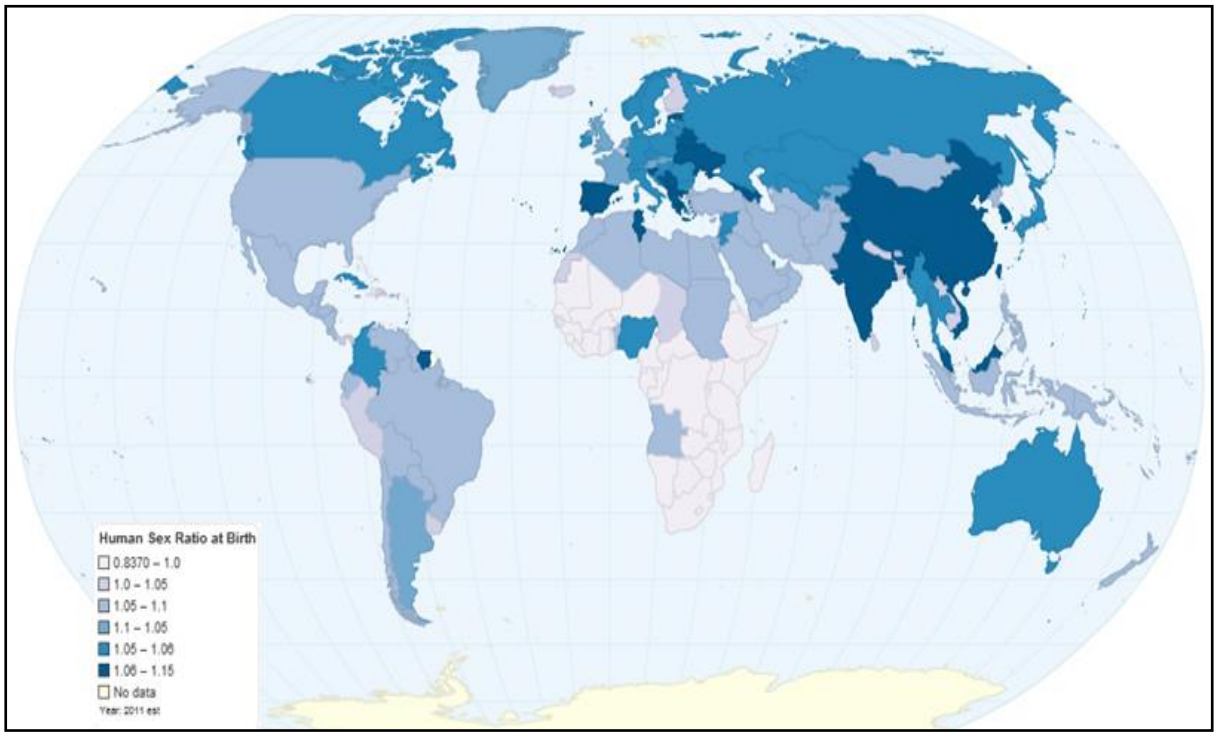

Figure 2-2: Global sex ratios (male:female) of live births. Adapted from ChartsBin statistics collector team 2011, Worldwide Human Sex Ratio at Birth, ChartsBin.com, viewed 20th August, 2013, <http://chartsbin.com/view/2332>.

2.2 GENDER SELECTION

2.2.1 INTRODUCTION

The notion of being able to choose the gender of a child has intrigued many generations of parents. Gender selection can be defined as "any attempt to control the sex of one's offspring to achieve a desired sex"[11]. It can be accomplished in several different ways, either with assisted reproduction techniques (ARTs) or through natural conception, by influencing the timing of fertilization with regard to ovulation[12]. In the case of assisted reproduction, the sex of the embryos is determined by Preimplantation Genetic Diagnosis (PGD) and embryos of the desired sex are selectively implanted[13], or as a more radical method, the gender of a fetus can be determined during early gestation, which is followed by selective abortion of the foetuses of the 'wrong' gender. Gender selection through timely intercourse is based on the respective characteristics of the X- and Y-chromosome bearing spermatozoa, favouring one or the other to reach and fertilize the egg[14].

2.2.2 FACTORS THAT INFLUENCE PARENTS' DECISIONS REGARDING GENDER SELECTION

There are various reasons why parents may choose to practice gender selection, the most common of which will be discussed subsequently.

2.2.2.1 Avoiding sex-linked diseases

There are numerous known sex-chromosome linked diseases. Depending on the nature of the disease, it can be passed on to the next generation in different ways. X-linked disorders are caused by mutations on the X-chromosome. The male offspring of a man with an X-linked disorder will be unaffected (since they receive their father's Y-chromosome) while his daughters will all inherit the condition (since they will receive his only X-chromosome, which is affected)[15]. A woman with an X-linked disorder has a 50% chance of affecting a fetus of either gender[16]. Y-linked disorders are caused by mutations on the Y-chromosome. Because males always inherit a Y-chromosome from their fathers, every son of an affected father will be affected[17] while female offspring will remain unaffected. Therefore, couples in which either parent presents with a sex-chromosome linked disorder may want to plan the gender of their offspring accordingly, in order to minimize the risk of the offspring inheriting the disorder[18]. Although gender selection for medical reasons is currently being practiced in a few countries, it still raises many ethical concerns, as embryos/fetuses presenting with genetic disorders are generally discarded or pregnancies terminated[19].

Although the ethicality of sex selection remains an unsettled and controversial topic, there are countries which allow gender selection for medical purposes, including the USA, Australia and India[20]. In the United Kingdom, sex selection for medical purposes is allowed and regulated by the Human Fertilization and Embryology

Authority (HFEA). Although preconceptual gender selection for non-medical reasons is considered unethical, separation of X- and Y-chromosome bearing spermatozoa is performed in a number of non-HFEA regulated centres in the UK. China prohibits any form of sex selection, whether for social or medical reasons[21], although it is the country with the highest gender preference and imbalance.

2.2.2.2 Cultural influences

In some cultures, producing a male heir is an extremely important act[22]. According to these cultures, a male can carry on the family name and eventually provide support for his parents, as is believed by many African and Middle-Eastern cultures. Gender preference is often in favour of males (see Figure 2-3).

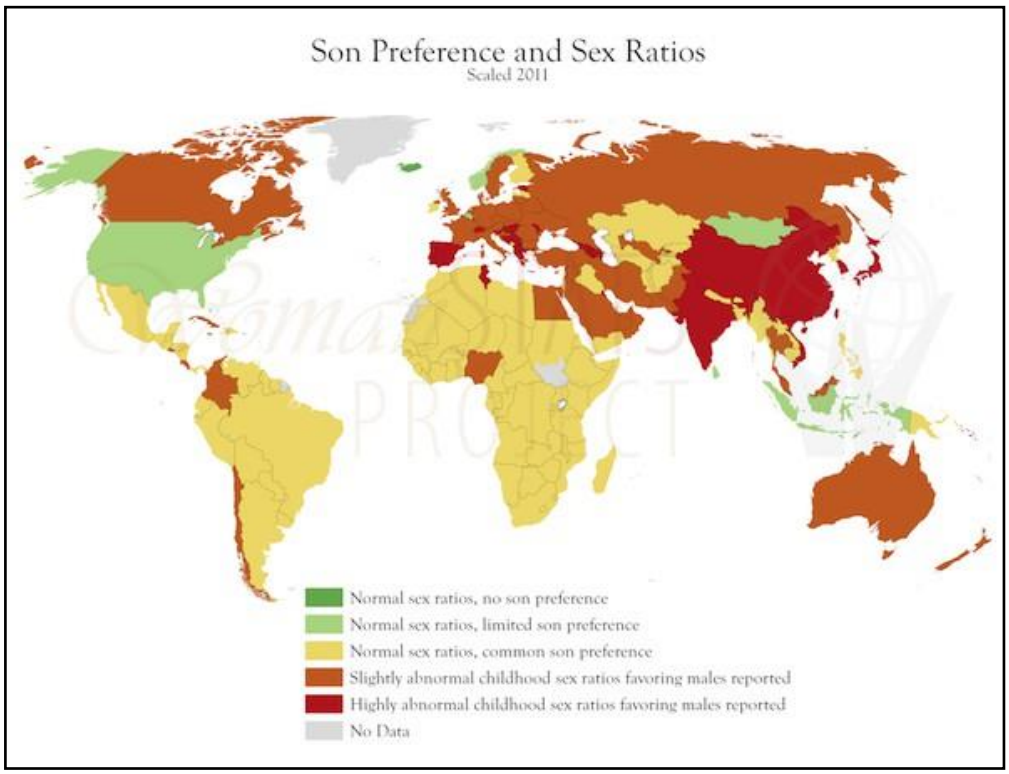

Figure 2-3: Overview of global sex ratios depicting preferences for male offspring. Adapted from *Male Gender Preference Globally* by Claudia Soria. Posted on March 8, 2013. Retrieved from http://www.indexmundi.com/blog/index.php/category/countries/new-zealand/

However, when a society exhibits this kind of prejudice towards a specific gender, it can lead to an unnaturally high male-to-female ratio, as is present in countries such as China and India (see Figure 2-4). China's gender imbalance is further increased by the so-called 'One Child Policy'[23].

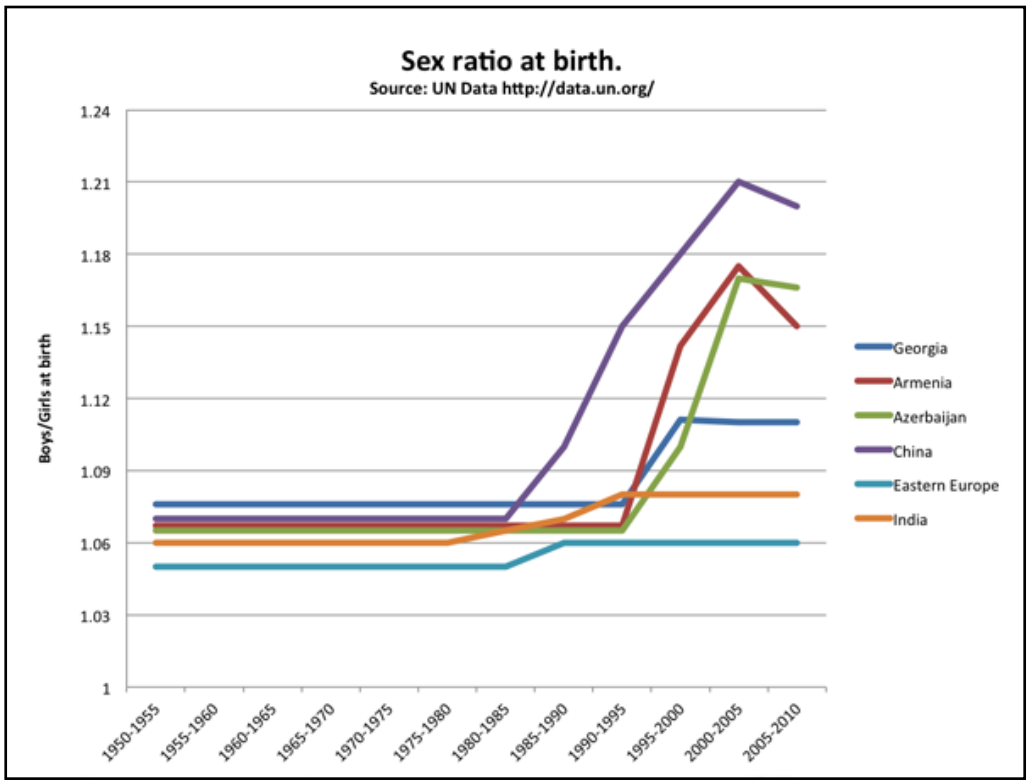

Figure 2-4: Sex ratio at birth (male:female) Adapted from *Sex Ratio at Birth: is the South Caucasus Heading the Way of China?* By Yaroslava Babych. ISET Economist (2011).

It is also believed in some countries that having sisters while growing up – as opposed to having brothers – can enhance the quality of life of an adult (BBC News, 22 August 2009)[24]. Therefore, families who share this belief may be more inclined to want female children.

2.2.2.3 Religious views

According to Jewish law[25], a man is required to have a minimum of 2 children, at least one of each sex. Islamic viewpoints regarding gender selection are that a couple may make use of any means available to them to have the desired boy or girl, providing the couple is married[26]. Christian beliefs, specifically those of the Catholic Church, forbid any form of gender selection, even for medical reasons[26].

2.2.2.4 Family balancing

Many families, regardless of their culture or religion, may prefer to practice gender selection to balance the family – therefore, if they already have one child (or more) of a particular sex, they might want to influence subsequent pregnancies in favour of having a child of the opposite sex[27].

2.2.3 METHODS OF GENDER SELECTION

Gender selection can be divided into different groups based on the timing with regard to fertilization and/or gestation. Gender selection can be achieved in the following ways:

2.2.3.1 Post conceptual gender selection

Post-gestational

Although illegal, it is practiced in some countries that babies of the "undesired" sex are killed (infanticide) or abandoned[28]. Adoption, although not socially viewed as a

form of gender selection, provides parents with a legal, humane means of gender control.

Gestational

A maternal blood test for prenatal sex discernment can be performed from the 6th week of pregnancy, as small amounts of fetal DNA are found in the mother's blood plasma[29]. Alternatively, more invasive and expensive methods of sex determination can be done. Chorionic Villus Sampling (CVS) or an amniocentesis can be performed between weeks 10-12 or weeks 9-18 of gestation, respectively. This involves collecting fetal DNA directly from the placenta (in the case of the CVS) or from the amniotic fluid (amniocentesis). Although these methods are usually employed to determine fetal abnormalities, the sex of the fetus can also be distinguished. With regard to gender selection, these processes are generally followed by selective abortion.

Pre-gestational

When in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) is being performed and the oocyte has been successfully fertilized, PGD [30] can be implemented to screen the embryo for genetic abnormalities as well as for detecting the presence or absence of a Y-chromosome. Embryos of the desired sex are then selectively implanted, while those that remain are discarded.

Currently, because of the ethical problems that surround abortion and discarding of embryos, post-conceptual gender selection with medical warrant is only legal in certain countries.

2.2.3.2 Pre-conceptual gender selection

It is believed that gender selection before conception circumvents most – if not all – of the ethical issues. However, no method in this category can guarantee 100% accuracy. The Shettle's method (and the less-known Whelan method) is aimed at natural conception[2], based on the characteristics of the X- and Y-bearing spermatozoa and the environment in the female genital tract. Both methods are associated mainly with the timing of fertilization with regard to ovulation. According to the Shettle's method, couples can affect the probability of having a child of a desired gender by timing sexual intercourse in relation to ovulation. The theory is based on the Y-chromosome bearing spermatozoa being able to swim faster than the X-chromosome bearing spermatozoa, although they are also more fragile when exposed to acidic environments[2]. While there have been claims of success – with rates as high as 75-90% - there has also been studies disregarding the method, as was published in the New England Journal of Medicine, where it was concluded that "…for practical purposes, the timing of sexual intercourse in relation to ovulation has no influence on the sex of the baby"[14].

The Whelan method is essentially based on timing sexual intercourse with regard to ovulation, taking into account certain changes in the female body – specifically the basal body temperature (BBT). According to this theory, X-chromosome bearing spermatozoa are more likely to fertilize the oocyte when the BBT spikes shortly after ovulation.

However, since these 2 methods contradict one another, it is important that another method of preconceptual gender selection is developed – especially one that might be combined with either one of these timing-related methods.

Sperm sorting

Sperm sorting[31] is a method where the focus is on creating a sample that is rich (if not pure) in spermatozoa carrying the desired sex-chromosome. An advantage of this method is that fertilization can be achieved via less invasive techniques, such as Artificial Insemination (AI), Intrauterine Insemination (IUI) or Gamete Intrafallopian Transfer (GIFT). Depending on the nature of the couple's fertility and the quality of the spermatozoa in the sample after manipulation, IVF or ICSI can also be performed with the sorted spermatozoa.

There is currently no legislation in South Africa regarding sorting of X- and Y-chromosome bearing spermatozoa. Pre-conceptual gender selection has been the target of much controversy for many years, and there have been numerous studies with varying and often conflicting results of spermatozoa separation.

2.3 DIFFERENCES BETWEEN X- AND Y-CHROMOSOME BEARING SPERMATOZOA

Many differences exist between X- and Y-chromosome bearing spermatozoa, including DNA content[32,33] size and density[32], resilience and motility[34] and surface protein properties[34]. Due to the difference in chromosome constitution, X-chromosome bearing spermatozoa have been shown to contain 2.9% more DNA than Y-chromosome bearing spermatozoa[35]. Current methods of sperm separation are based on the presumption of the existence of fundamental, physiological differences between X- and Y-chromosome bearing spermatozoa as well as the assumption that these differences are significant enough to enable separation.

2.3.1 VIABILITY

The X-chromosome bearing spermatozoa are believed to be generally more resilient than the Y-chromosome bearing sperm. There are many reports that X-chromosome bearing spermatozoa have relatively longer lifespans and are able to withstand hostile circumstances such as acidity, variations in temperature and even oxidative stress better than the Y-chromosome bearing spermatozoa[32]. Y-chromosome bearing spermatozoa are generally considered to be the more fragile of the spermatozoa[2].

2.3.2 MOTILITY

One of the major suppositions for separation of spermatozoa is the difference in motility parameters. Ericsson invoked the hypothesis that Y-chromosome bearing spermatozoa swim faster than X-chromosome bearing spermatozoa, which led to development of the so-called Ericsson-method. This method is designed to enrich a sample with Y-chromosome bearing spermatozoa by allowing them to swim through increasingly dense albumin layers[36]. According to his theory, only the most progressively motile spermatozoa – the Y-chromosome bearing spermatozoa – should be able to reach the bottom. Studies on the Ericsson method have generated varying results, but many have reported success in alteration of X:Y sperm ratios as well as clinical pregnancies and live births. Overall, Y-chromosome bearing spermatozoa have been reported to swim both significantly faster and more progressively than the X-chromosome bearing spermatozoa[2].

There are various techniques that can be used to isolate spermatozoa based on their motility parameters. Direct swim-ups, double wash centrifugation, and multi-ZSC system swim-up are a few of the more common techniques, in which only the most motile and/or the fastest swimming spermatozoa are isolated. When using motility as separation objective, the Y-chromosome bearing spermatozoa are most often enriched in the sample, although it stands to reason that spermatozoa left behind should be predominantly X-chromosome bearing. According to a literature review conducted by Flaherty & Matthews (1996)[8], neither discontinuous albumin gradients

(the Ericsson-method) nor modified versions of the WHO's swim-up protocol were capable of clinically significant Y-sperm enrichment. However, they found 12-step Percoll gradients able to produce slight but significant enrichment of X-chromosome bearing spermatozoa[8].

2.3.3 SIZE AND/OR WEIGHT

After sex determination with the aid of Polymerase Chain Reaction (PCR), Cui and Matthews studied the morphological characteristics of individual spermatozoa[33, 37]. With the PCR technique, the presence of primers from the presumed sex-determining gene of the Y-chromosome (SRY) is used to denote a male chromosome bearing spermatozoa. Their results indicated that the length, perimeter and area of the spermatozoa's heads, as well as the lengths of the neck regions and tails were significantly larger and longer in X-chromosome bearing spermatozoa. This study demonstrated for the first time that X-chromosome bearing spermatozoa are statistically bigger than Y-chromosome bearing spermatozoa[37].

There are only a few available methods to separate sperm according to their size and/or weight. The bigger spermatozoa have distinctively different surface charges and therefore the X-chromosome bearing spermatozoa can be isolated by electrophoresis[38] and by the zeta potential method[39]. Live sperm morphology is a selection technique used during Intracytoplasmic Morphologically selected Sperm

Injection (IMSI), which is a variation of the classical ICSI. With this technique a single sperm is selected at a magnification of over 6000×, and therefore the size differences can be seen by the technician[40]. Flow cytometry is currently used to separate spermatozoa according to the amount of DNA in the nucleus, which can be associated with the size and weight of the spermatozoa.

2.4 COMPARISON STUDIES W.R.T. SORTING OF SPERMATOZOA

2.4.1 SWIM-UP METHODS

Success of a swim-up method in enrichment of Y-chromosome bearing spermatozoa was described by Check and Katsoff[41] in 1993. They reported 81% male births after the women were inseminated with spermatozoa that were prepared for Y-chromosome enrichment by modified swim-up. Furthermore, upon staining the cells with quinacrine (QA) they found that the incidence of Y-chromosome bearing spermatozoa in the prepared samples was 83.6%. De Jonge and Flaherty also reported slight but significant enrichment of Y-chromosome bearing spermatozoa following processing by direct swim-up procedure[8]. These studies suggest that isolation of spermatozoa based on their ability to swim faster or more progressively has potential to be useful in male sex selection.

In contrast, in a study carried out by Han et al. (1993), in which spermatozoa were processed by a routine swim-up method and analysed by double-label FISH, it was

reported that there was no enrichment of either population of spermatozoa[42]. According to a review (Flaherty and Matthews, 1996) these conflicting results can be accounted for by the differences in protocols that were followed, including the lengths of incubation and centrifugation[8].

2.4.2 DISCONTINUOUS GRADIENT METHODS

Ericsson et al. (1973)[43] was the first to report successful enrichment of Y-chromosome bearing spermatozoa by discontinuous albumin gradient incubation, where spermatozoa are allowed to swim down out of the seminal plasma and into increasingly dense layers of albumin. The method does not involve centrifugation. Since the method is patented and its use therefore limited to centres that are licenced to use it, there has not been many studies that were able to replicate the exact method to either prove or disprove it. Claassens et al. (1995) were able to increase the incidence of Y-chromosome bearing spermatozoa in a sample from 50.3% to 53.4%[44], while a study by Beernink et al. (1993) claimed 75% success in male birth rates when spermatozoa were prepared by the Ericsson method[45].

X-chromosome bearing spermatozoa were purportedly enriched with Sephadex and 12-step Percoll columns by Steeno et al[46] (1975) and Iizuka et al[47] (1987), respectively. Upon staining with QA, Iizuka et al. (1987) reported 94% X-chromosome spermatozoa enrichment in the 80% Percoll Fraction, as well as a

100% success in female birth rates when spermatozoa were prepared by the 12-step Percoll gradient[47]. Wang et al. (1994) evaluated the Percoll gradient using double-label FISH to assess chromosome ratio, establishing a 6% increase of the X-chromosome bearing spermatozoa in the sample[48]. The discrepancy in these results could possibly be attributed to the efficacy and accuracy of the staining methods – QA has been reported to give false results in various studies[49.50].

2.4.3 Flow cytometry

Flow cytometry, based on the 2.9% difference in DNA content between X- and Y-chromosome bearing spermatozoa, is the one method that has provided consistent, clinically significant results throughout the literature. It was first employed to enrich both X- and Y-chromosome bearing sperm to clinically significant degrees in 1993 by Johnson et al[51]. This method has been thoroughly validated in a variety of different species, and can be applied directly to nuclei of spermatozoa, or to live, intact spermatozoa. Flow cytometry is currently being applied in some developed countries, and is especially useful in the sorting of spermatozoa for breeding purposes, as is done in animal husbandry, where livestock are selectively bred and raised to promote desirable traits with regard to sport, utility or research[52].

However, flow cytometry is an extremely expensive and sophisticated procedure, and therefore impractical, as the use of this method is limited to specialists.

Developing countries have neither the equipment nor the infrastructure to employ this technique.

2.4.4 SUMMARY

Gledhill and Edwards (1993)[34] conducted a literature review and concluded that many sperm separation methods are highly endorsed by the inventers, but that none have been independently confirmed nor the results recreated. Thus none have gained true acceptance in the scientific community due to the mostly inconsistent results[34]. Therefore, there is still a need for the development of clinically significant and recognized techniques for successful sorting of X- and Y-chromosome bearing spermatozoa.

2.5 MACS VS. MODIFIED SWIM-UP SEPARATION TECHNIQUES

Apoptosis of spermatozoa is considered to be a major contributing factor in failed ART and the consequential low fertilization and implantation rates. Externalization of phosphatidylserine (PS) residues is one of the characteristics of apoptosis. MACS is based on magnetically labelling the dead or apoptotic spermatozoa through the binding of the externalized PS to Annexin V, which is conjugated with colloidal super-paramagnetic microbeads. The magnetically labelled sample is then passed through a magnetic column, and the dead cells are retained in the column while live cells with intact membranes are allowed to filter through. Said et al. (2008) found that non-

apoptotic spermatozoa that were prepared by MACS showed higher sperm quality in terms of motility parameters and apoptotic markers[53]. Furthermore, the increased sperm quality was reflected by increased oocyte penetration and cryopreservation survival rates.

MACS, although generally successful, has a few drawbacks. It is a relatively time-consuming process and non-specific binding of the microbeads has been reported to occur, leading to false results. Osmolarity of the binding solution is not regulated for use on spermatozoa, which means that the technique in itself could also be detrimental to the spermatozoa. Therefore, alternative methods for separation of live and dead spermatozoa could be beneficial.

Viable spermatozoa can be isolated form dead cells by a variation of the WHO's swim-up method[3], where spermatozoa swim out of the seminal plasma and into a culture medium that is hospitable to healthy sperm. The method, as defined by the WHO, is modified by increasing the incubation time, so as not to favour fast motile cells, but to include as many viable cells as possible in the live fraction.

2.6 IDENTIFICATION OF X- AND Y-CHROMOSOME BEARING SPERMATOZOA

Spermatozoa can be stained by various fluorescent methods that distinguish between X-and Y-chromosome bearing spermatozoa.

2.6.1 QUINACRINE (QA) STAINING

QA is a flourochrome that stains the Y-chromosome. It binds exclusively to the Y-body at the distal end of the Y-chromosome's long arm. After a smear of the sample is made, the slide is stained with the QA and visualized by means of fluorescent microscopy. Therefore, fluorescing Y-chromosome bearing spermatozoa and non-fluorescing X-chromosome bearing spermatozoa are distinguished.

2.6.2 POLYMERASE CHAIN REACTION (PCR)

PCR is a technique that can amplify particular genes between specific primers that are exclusive to certain chromosomes. A primer of the human spermatozoa receptor gene (ZP3) is used as a control to establish the number of cells in the sample, and a primer for the testis-determining gene (SRY) which is located only on the Y-chromosome, is used to indicate the presence of the a Y-chromosome. After employment of gel electrophoresis the X:Y chromosome ratio can subsequently be calculated.

2.6.3 FLUORESCENT IN SITU HYBRIDIZATION (FISH)

Currently, FISH is the most preferred method for the establishment of the X:Y chromosome ratio in semen or prepared sperm samples. It is the method of choice due to its accuracy in identifying the sex-chromosomes of individual spermatozoa by means of a double-label detection system[8], employing specific probes for the X- and

Y-chromosomes respectively (see Figure 2-5). This method has the added advantage of being able to screen large amounts of spermatozoa in a short period of time. The FISH protocol entails decondensation and denaturation of sperm nuclear DNA to single-stranded DNA. The single-stranded DNA is then probed with short fluorescence-tagged oligonucleotides that are complementary to regions that are specific to the X- or Y-chromosome.

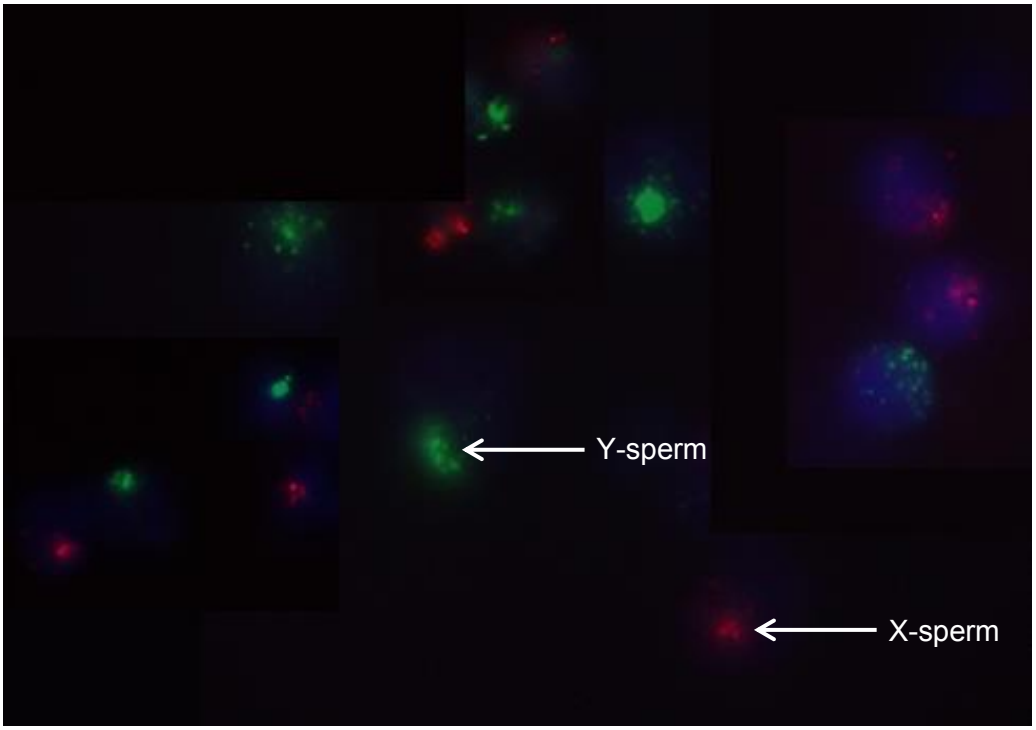

Figure 2-5: Double-label FISH: X- and Y-chromosomes fluorescing orange and green, respectively.

2.7 CONCLUSION

Since sperm sorting is the most ethically sound method of gender selection, there is great value in finding clinically significant methods of isolating healthy, viable X- and Y-chromosome bearing spermatozoa.

CHAPTER 3: MATERIALS AND METHODS

INTRODUCTION

This chapter is divided into two parts and will provide details of all the materials used in both the preliminary investigations and experimental study, as well as comprehensive protocols of all the methods employed. Part A consists of the protocol followed during the preliminary investigations, as well as the results of those particular experiments. Part B describes the protocols followed during the experimental study, the results of which are set out and discussed in Chapters 4 and 5, respectively.

3.1 PART A: PRELIMINARY INVESTIGATIONS

The preliminary studies comprised of:

- a temperature and time curve to establish the best incubation temperatures and period of time for the investigation of the effect of temperature on the sex-chromosome ratio spermatozoa in a given sample.
- a H_2O_2 concentration and time curve to determine the concentrations and incubation times which had the optimal desired effect on the spermatozoa for the investigation of the effect of H_2O_2 on the sex-chromosome ratio spermatozoa in a given sample.

3.1.1 TEMPERATURE CURVE

3.1.1.1 Protocol

Upon collection, the semen of 3 donors was allowed to liquefy for 30 minutes at 37°C. The seminal plasma was then removed from the samples by centrifugation for 10 minutes and the spermatozoa-containing pellets were resuspended in 3% HAMS-BSA. The samples were incubated for 30 minutes at various temperatures (37°C, 40°C, 42.5°C, 45°C and 47°C), with motility parameters being assessed at 10 minute intervals. Results were interpreted in terms of the percentage of static cells in the samples (see Figures 3-1 to 3-3).

3.1.1.2 Results

During the preliminary studies it was determined that 47°C (and any exceeding temperature) had too much of a detrimental effect on the spermatozoa, as illustrated in Figures 3-1 and 3-2. The effect at 45°C could be seen distinctly, while the effects at temperatures between 37°C, 40°C and 42.5°C were overlapping. However, since the standard temperature for laboratory processing of spermatozoa, as prescribed by the WHO, is 37°C, this temperature was chosen to act as a control. Therefore, the final temperatures that were chosen were 37°C and 45°C, as well as the median, 41°C.

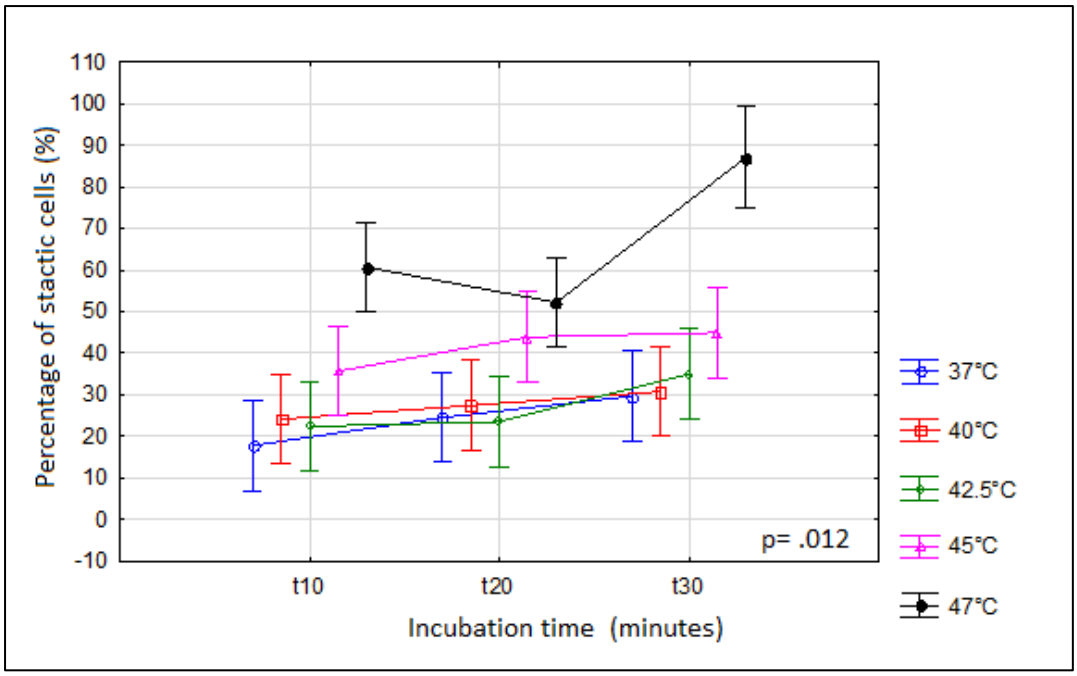

Figure 3-1: Effect of different temperatures and incubation times on the percentage of static cells

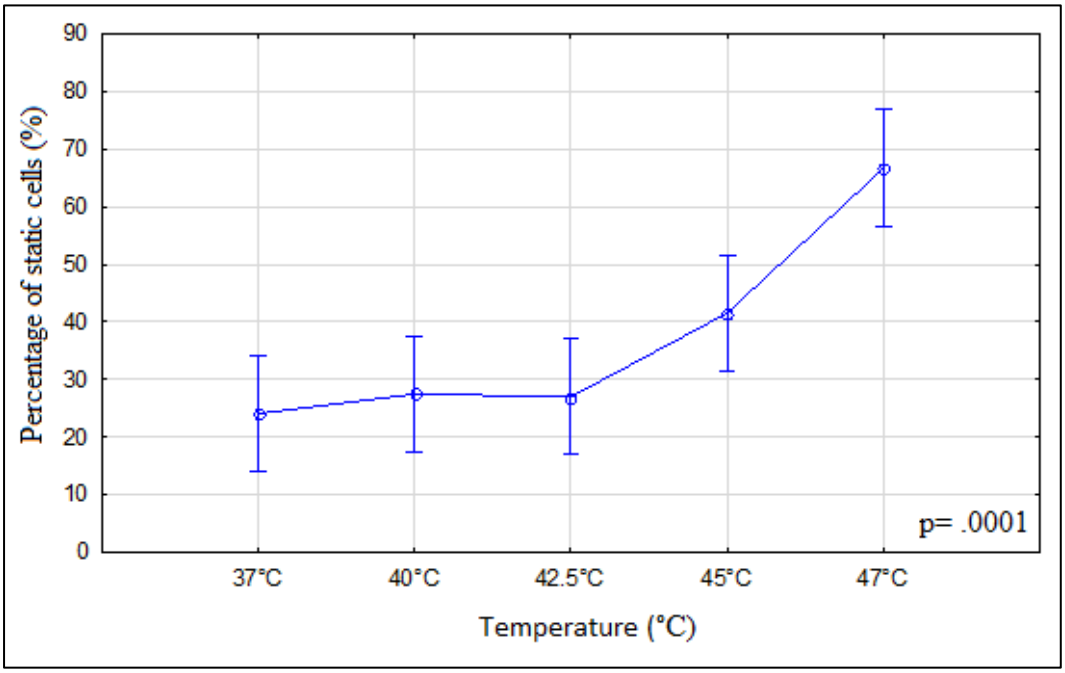

Figure 3-2: The effect of temperature on the average percentage of static cells

For the determination of the optimal incubation period, (see Figure 3-3) a significant increase in the amount of static cells was seen at 30 minutes. In an effort to prevent damaging too many spermatozoa, the optimal incubation time period was set as 25 minutes.

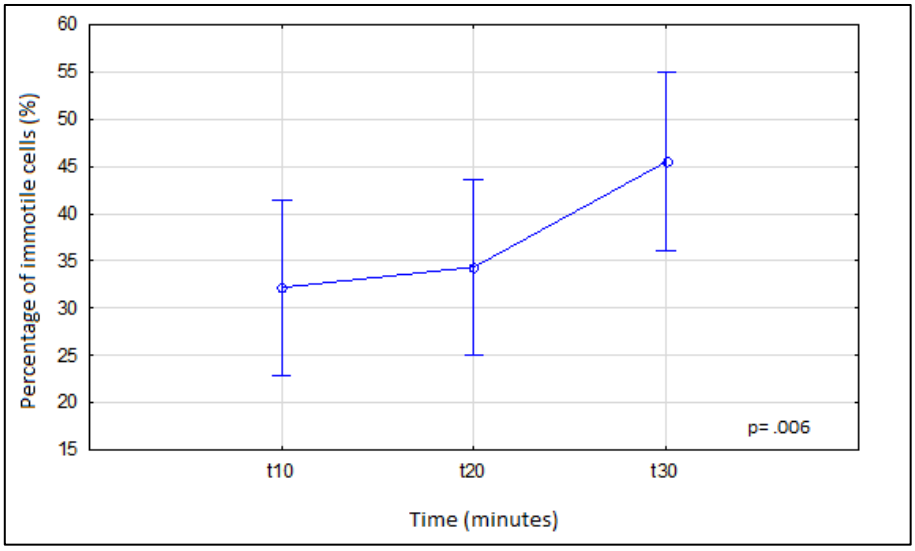

Figure 3-3: Effect of incubation time on percentage of static cells

3.1.2 HYDROGEN PEROXIDE CURVE

3.1.2.1 Protocol

After removal from the seminal plasma, spermatozoa from 8 donors were incubated for 90 minutes in various H_2O_2 concentrations (50µM, 100µM, 200µM, 300µM, 400µM, 500µM, 600µM, 750µM, 8000µM and 1000µM). Motility parameters were assessed at time points 0', 15', 30', 45', 50' 60', 70' and 90'. Results were interpreted in terms of the percentage of static cells in the samples.

3.1.2.2 Results

While establishing the ideal H_2O_2 concentration, the results indicated a decrease in the percentage of static cells at 50µM, and two significant increases ('spikes') at 750µM and 1000µM (see Figure 3-4). Therefore, in addition to the control, which was Phosphate Buffered Saline (PBS) (Gibco, Scotland, UK), the chosen concentrations of H_2O_2 were 50µM, 750µM and 1000µM.

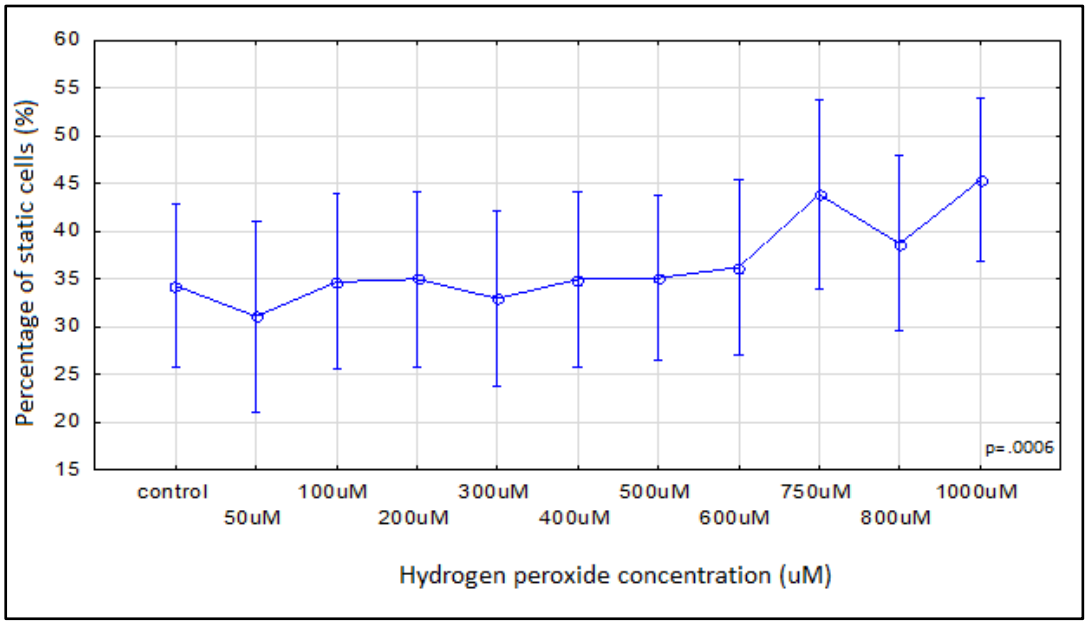

Figure 3-4: Effect of hydrogen peroxide exposure on the percentage of static cells

Incubation time period was set as 25 minutes, as this is the time-point at which spermatozoa are considered to have reached maximum reactive species (ROS) production[54].

3.2 PART B: EXPERIMENTAL STUDY

A simplified overview of the experimental procedure followed in this study is shown in Figure 3-5.

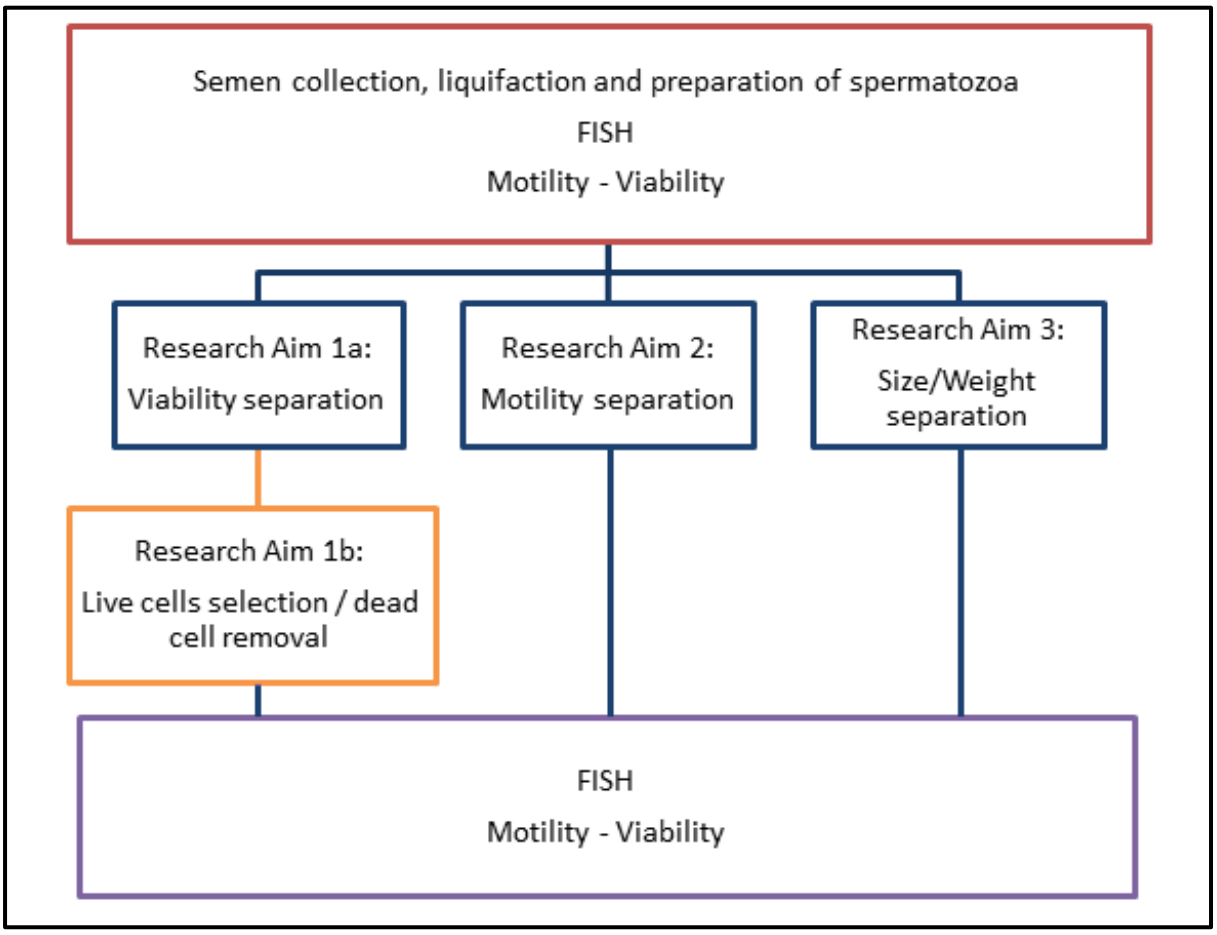

Figure 3-5: Overview of the present study

3.2.1 SEMEN SAMPLING

A total of 45 semen samples were obtained from healthy volunteers taking part in the Stellenbosch University Reproductive Research Group (SURRG) donor program. All the donors were informed that their spermatozoa would be used exclusively for research purposes and discarded in an appropriate fashion, after which they gave

their consent. Ethical clearance for this study was granted by the Health Research Ethics Committee 1 (Ethics #: S13/04/068).

3.2.2 Semen Collection

Semen was collected from healthy donors according to the WHO criteria[3]. During the investigation of Research Aim 1a (Separation of X- and Y-chromosome bearing spermatozoa according to their respective abilities to remain viable upon exposure to hostile environments, as set out in Section 1.5.1.1 of Chapter 1) 18 semen samples were used. For Research Aim 2 (Separation of X- and Y-chromosome bearing spermatozoa based on their particular motility capacities, as set out in Section 1.5.2 of Chapter 1) 14 semen samples were used. Lastly, for Research Aim 3 (Separation of X- and Y-chromosome bearing spermatozoa based on differences in size/weight, as set out in Section 1.5.3 of Chapter 1) 13 samples were used. During Research Aim 1b (Comparison of the effectiveness of MACS and modified Swim-up techniques in separating live and dead spermatozoa, as set out in Section 1.5.1.2 of Chapter 1) the same samples from Research Aim 1a were used. In each instance the semen was allowed 30 minutes to undergo liquefaction in an incubator at 37°C, 95% humidity and 5% CO_2.

All semen samples were analysed for normality according to the WHO standards before they were included in the study. Samples that did not comply were excluded from the study.

3.2.3 SEMEN ANALYSIS

3.2.3.1 Motility

Sperm concentration and motility of each sample was measured prior to the experiment to establish normality of the sample, and thereafter at various time points during each experiment. Sperm concentration and motility was assessed by means of Computer Aided Sperm Analysis (CASA) using the Sperm Class Analyzer (SCA) (Microptics, Barcelona, Spain). The settings of the analyser were as follows: optics: ph+; contrast: 435; brightness: 100; scale: 10x; chamber: Leja 20; capture: 50 images per second; curvilinear velocity (VCL): 10µm/s<slow<15µm/s, 15µm/s<medium<35µm/s, rapid>35µm/s; progressivity>80% of straightness (STR); linearity (LIN): circular<50%; connectivity: 12; average path velocity (VAP): 5 points; temperature: 37°C.

Several motility parameters were assessed, including:

- Total motility (%) (percentage of motile spermatozoa)
- Progressive motility (%) (percentage of progressively motile cells)
- Non-progressive motility (%) (percentage of non-progressively moving cells)
- Rapid cells (%) (the percentage of rapidly moving cells)
- Static cells (%) the percentage of motionless cells)

Velocity parameters (see Figure 3-6) that were measured included:

- Curvilinear velocity (VCL) (µm/s) (the time average velocity of the sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope)
- Straight line velocity (VSL) (µm/s) (the time average velocity of the sperm head along the straight line between its first and last detected position)
- Average path velocity (VAP) (µm/s) (the time average velocity of the sperm head along its average path)
- Linearity (LIN) (%) (the linearity of the curvilinear path)
- Straightness (STR) (%) (the linearity of the average path)

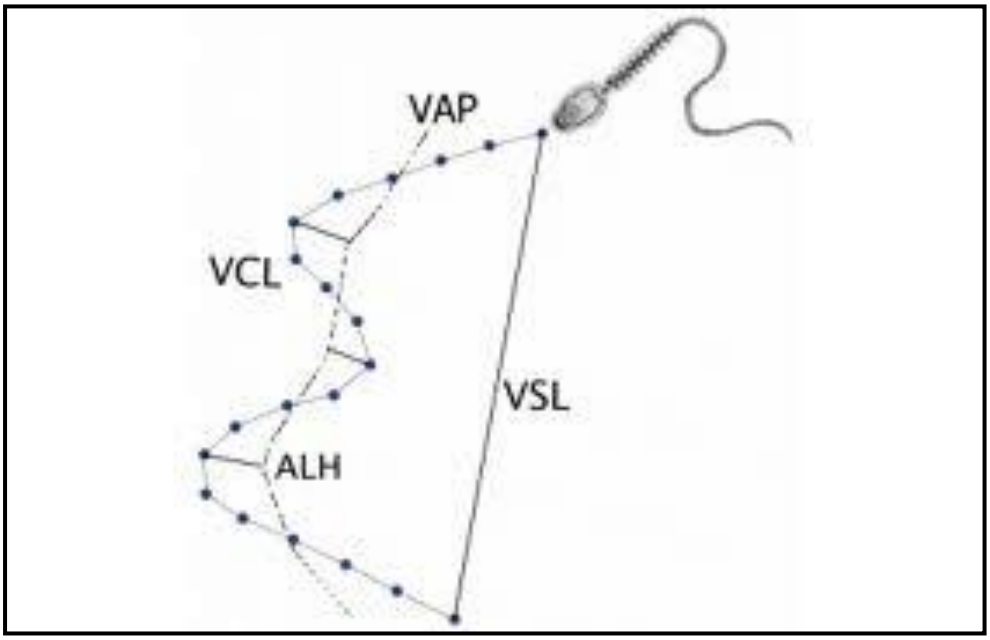

Figure 3-6: Diagram illustrating velocity parameters measured by the SCA. Adapted from SCA® Motility and Concentration, by Microptic Automatic Diagnostic Systems. Available online at http://www.micropticsl.com/eng/products/sperm_analysis_sca_motility_concentration.html.

3.2.3.2 Viability

Viability smears were made of the samples at various stages during each experimental protocol. The sample was mixed with Eosin® and Nigrosin® in a 1:1:1 ratio, smeared across the length of a microscope slide and allowed to air dry overnight. The slide was then mounted with DPX mounting medium (Merck Chemicals®) and a coverslip and manually analysed with light microscopy at 100× magnification.

3.2.3.3 Fluorescent *in situ* Hybridization (FISH)

The ratio of X:Y chromosome bearing spermatozoa was determined with 2 colour FISH. Because of the high cost of this process, samples were pooled for this assessment. The FISH protocol was used as specified by the manufacturer's instructions.

DNA was decondensed and denatured into single strands and slides prepared. The single-stranded DNA was probed with short fluorescence-tagged oligonucleotides that were complementary to regions that are specific to the X- or Y-chromosome. The slides were incubated in the dye overnight, mounted and viewed by fluorescent microscopy. Manual assessment was done and at least 200 cells counted.

3.2.4 *Research Aim 1a: Separation of X- and Y-chromosome bearing spermatozoa according to their respective abilities to remain viable upon exposure to hostile environments.*

The first aim of the present study was to isolate spermatozoa based on their ability to withstand/survive what the literature describes as hostile environments. Figure 3-7 presents an outline of this part of the study. The motility and viability data from the MACS and modified Swim-up techniques were used to answer Research Aim 1b.

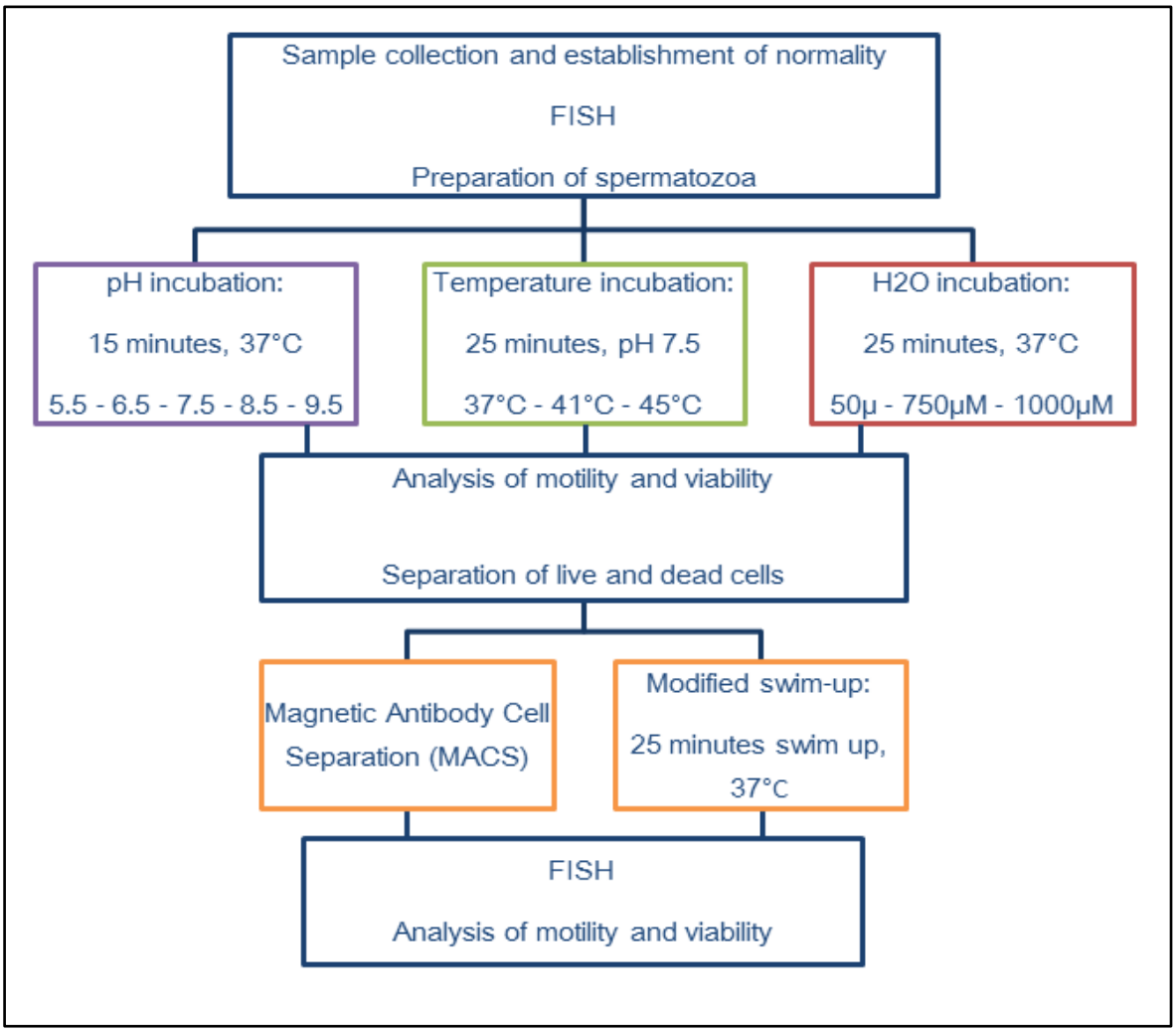

Figure 3-7: Outline for the experimental protocol for Research Aim 1

3.2.4.1 SPERM PREPARATION

A 3% HAMS-BSA solution was made up by adding 0.3g of Bovine Serum Albumin (BSA) (Sigma, SA) to 10ml of HAMS-F10 (Sigma, SA). After 200µl was removed for neat-sample sex-chromosome ratio determination, the remaining semen was transferred to a conical tube, and 2ml of the HAMS-BSA solution added. The use of the HAMS-BSA during the preparation phase is to provide the spermatozoa with the necessary nutrients for cell metabolism. Centrifugation commenced for 10 minutes at 300×g after which the supernatant was discarded and the pellet resuspended in an appropriate amount of 3% HAMS-BSA. The concentration, motility parameters and viability percentages of the spermatozoa were determined after the preparation step.

3.2.4.2 PH INCUBATION

3.2.4.2.1 Preparation of pH media

PBS was used as the incubation medium, and the pH was adjusted to the required values – 5.5, 6.5, 7.5, 8.5, and 9.5 – with 1M NaCl or 1M HCl. A pH meter was used to determine the pH of the solutions, and fresh solutions were prepared daily to ensure the integrity of the incubation media. After processing, the pellet was resuspended in 1.2ml of HAMS-BSA solution. A volume of 200µl of the prepared spermatozoa was added to each of the pH solutions and after ensuring pH remained unchanged, tubes were placed in the incubator for 15 minutes (longer than was done by Hassan, who used 10minutes exposure in an effort to select for motility[55]). The remaining 200µl were used to determine motility and viability at time point 0.

3.2.4.2.2 Incubation

Samples were incubated for 15 minutes at 37°C, 5% CO_2 and 95% humidity, after which motility and viability parameters were assessed. Live cell fractions were isolated by MACS or modified swim-up and stored in liquid nitrogen.

3.2.4.3 TEMPERATURE INCUBATION

3.2.4.3.1 Incubation

The prepared spermatozoa (as described in Section 3.2.4.1) were divided into 3 aliquots and incubated at 37°C, 41°C and 45°C for 25 minutes. Concentration, motility and viability was established at time point 0. After the incubation, the live cell fractions were isolated with the MACS and modified swim-up protocol and stored in liquid nitrogen.

3.2.4.4 HYDROGEN PEROXIDE INCUBATION

3.2.4.4.1 Preparation of H_2O_2 media

H_2O_2 was made up to concentrations of 2000µM, 1500µM and 1000µM by the addition of PBS. Fresh solutions were prepared daily in order to maintain the integrity of the incubation medium. After processing (as set out in Section 3.2.4.1) the pellet was resuspended in 1.2ml HAMS-BSA. A volume of 250µl of the prepared spermatozoa was added to 250µl of each of the H_2O_2 solutions, so that final stimulation concentrations were 1000µM, 750µM and 50µM, respectively. A control

solution of 250µl of spermatozoa and 250µl PBS was created. The remaining 200µl was used to establish the concentration of the spermatozoa after the preparation step, as well as motility parameters and viability percentage at time 0.

3.2.4.4.2 Incubation

The solutions were placed in the incubator for 25 minutes at 37°C, 95% humidity and 5% CO_2, after which live cell fractions were isolated by both the MACS and modified swim-up protocols and stored in liquid nitrogen.

3.2.4.5 Research Aim 1b: Comparison of the effectiveness of MACS and modified Swim-up techniques in separating live and dead spermatozoa Magnetic Antibody Cell Separation

3.2.4.5.1 Magnetic Antibody Cell Separation

This protocol was carried out according to the manufacturer's instructions. Spermatozoa were incubated with magnetically labelled Annexin V beads at room temperature for 15 minutes. These beads are designed to bind to the dead and apoptotic spermatozoa. The sample was then passed through a column which was placed in a magnetic field. The magnetically-labelled cells were retained inside the column while the viable cells were allowed to pass through to be collected at the bottom. These live spermatozoa were then assessed again for motility and viability parameters and stored in liquid nitrogen.

3.2.4.5.2 Modified Swim-up

After the respective incubations, each spermatozoa fraction was transferred into a new conical tube. HAMS-BSA (1ml) was layered carefully on top of the sample, preventing mixture of the solutions. The tube was placed in the incubator at a 45° angle for 25 minutes, after which the top 500µl was carefully removed and the rest discarded. Motility, and viability were assessed and the removed fractions stored in liquid nitrogen.

3.2.4.6 STORAGE

After MACS and modified swim-up processing, all samples were frozen in liquid nitrogen and stored until all the samples for Research Aim 1 were processed. Samples were subsequently thawed and pooled for the final step in the experiment, sex-chromosome determination via FISH.

3.2.4 *Research Aim 2: Separation of X- and Y-chromosome bearing spermatozoa based on their particular motility capacities.*

The second aim of the present study was to isolate spermatozoa based on their motility characteristics. Figure 3-8 presents an outline of this part of the study.

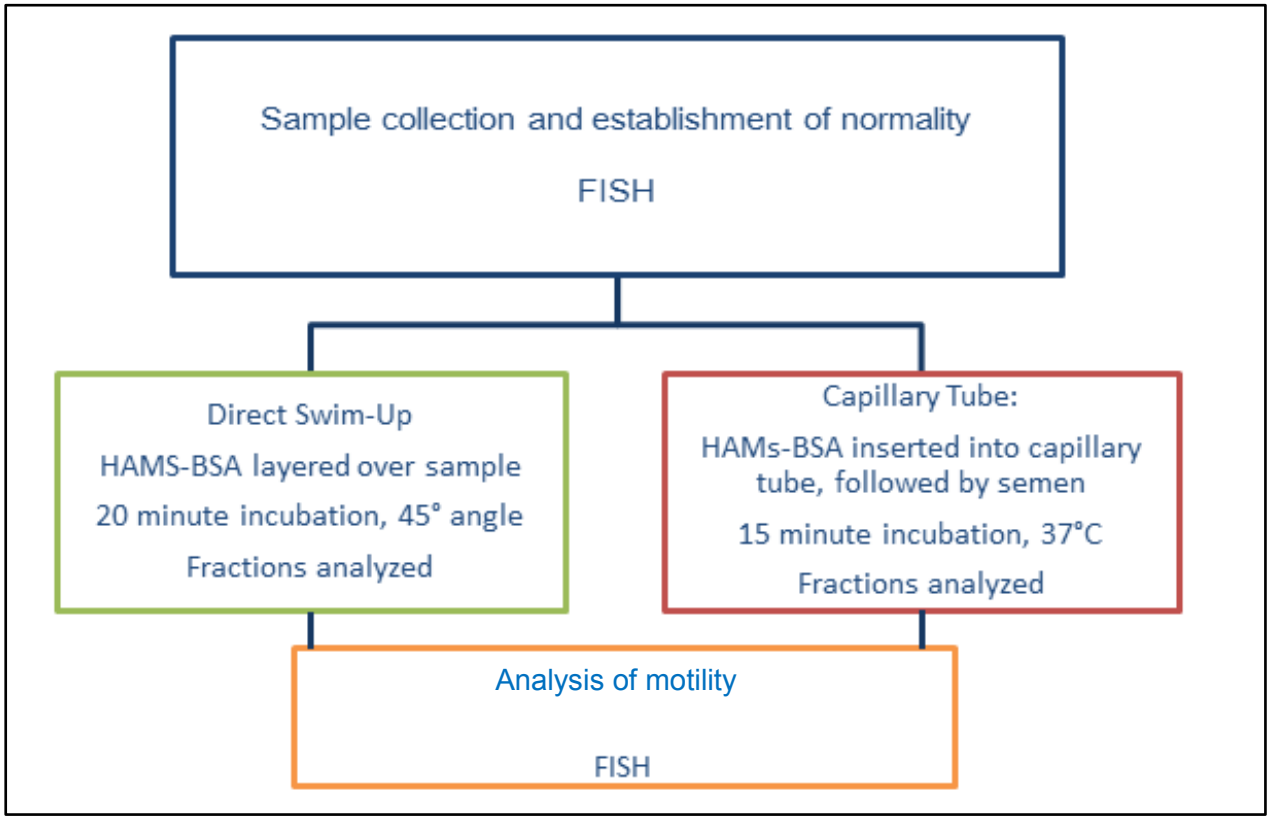

Figure 3-8: Outline of the experimental protocol for Research Aim 2

3.2.5.1 *Direct Swim-up (WHO)*

The direct swim-up was performed according to the WHO protocol[3]. A volume of 1ml of the neat, unprocessed semen sample was placed in a sterile conical tube, after which 1.5ml of 3% HAMS-BSA was layered on top of it, preventing mixture of the

media. The tube was inclined at a 45° angle and incubated at 37°C, 95% humidity and 5% CO_2 for 20 minutes (see Figure 3-9). The uppermost 1ml (Fraction A) as well as the next 1ml (Fraction B) of the medium was removed and used for assessment of sex-chromosome ratios and motility.

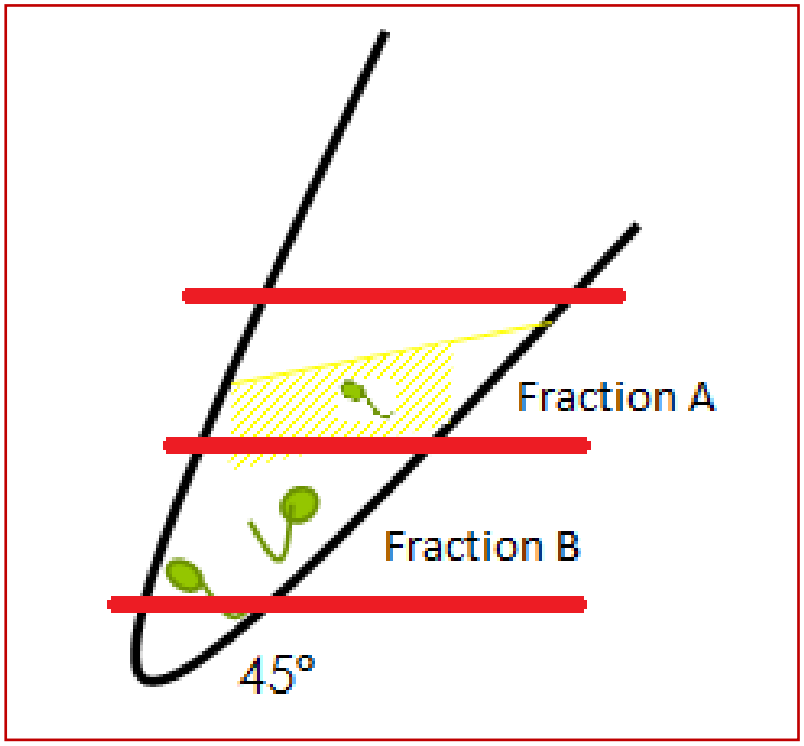

Figure 3-9: Illustration of the direct swim-up fractions after incubation

3.2.5.2 Capillary Tube

Culture medium (3% HAMS-BSA) was pipetted into a long capillary tube to fill 9cm, followed by neat, unprocessed semen (3cm), preventing mixture of the media. The tube was incubated horizontally for 15 minutes at 37°C, 95% humidity and 5% CO_2,

after which three 3cm segments (A, B, C) of the tube were analysed for sex-chromosome ratio and motility parameters (see Figure 3-10).

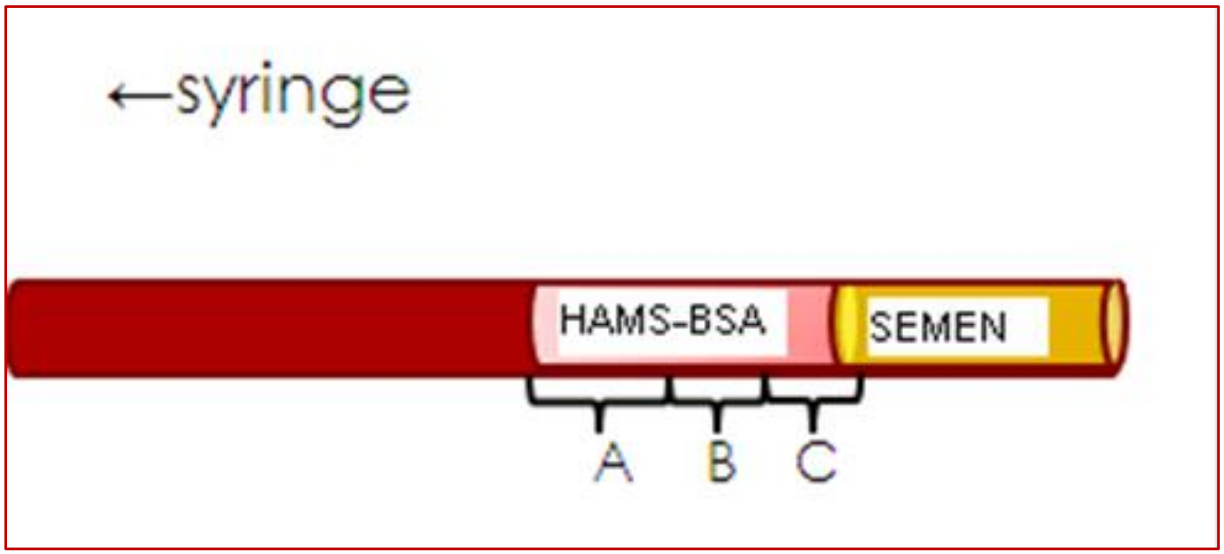

Figure 3-10: Illustration of the capillary tube set-up

3.2.6 *Research Aim 3: Separation of X- and Y-chromosome bearing spermatozoa based on differences in size/weight.*

The third aim of the present study was to isolate spermatozoa based on their different sizes and/or molecular weights. Figure 3-11 presents an outline of this part of the study.

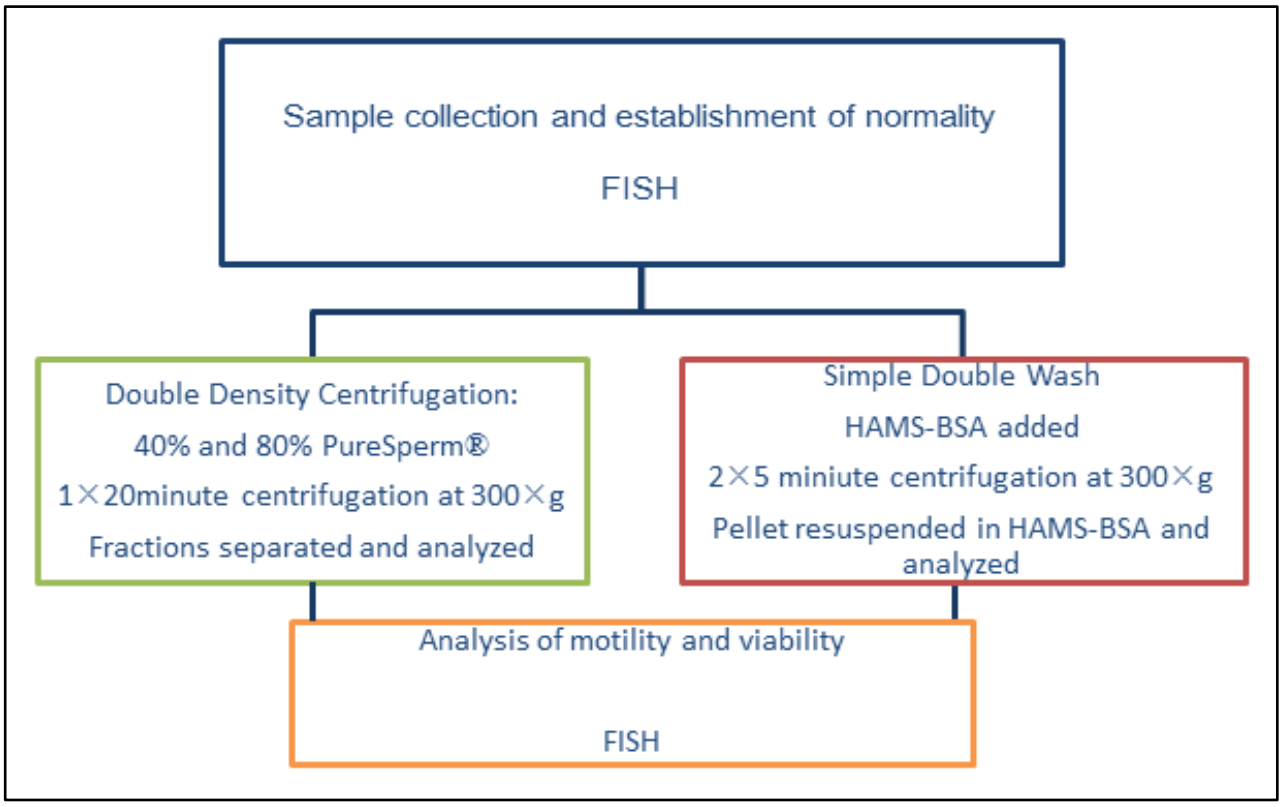

Figure 3-11: Outline of the experimental protocol for Research Aim 3

3.2.6.1 *Double Density Gradient Centrifugation - WHO*

A 40% and 80% PureSperm® (Nidacon, Hunter Scientific Limited, Saffron Walden, Essex, U.K) discontinuous density gradient was used. To prepare the gradient column, 2ml of the 80% PureSperm® gradient solution was placed in the bottom of a

conical tube, followed by 2ml of the 40% gradient carefully layered over it, preventing mixing of the layers. Finally, 2ml of neat, unprocessed semen was layered on top of the 40% layer.

The tube was centrifuged at 300×g for 20 minutes, resulting in different layers and separating the spermatozoa, seminal plasma and other cells accordingly. After centrifugation the different layers (A, B and C) were carefully separated to prevent any disturbances and mixing (see Figure 3-12). The pellet (Fraction C) was resuspended in 1ml HAMS-F10 (Sigma, SA) and also counted as a layer. Each layer was assessed for concentration, motility, viability and sex-chromosome ratio.

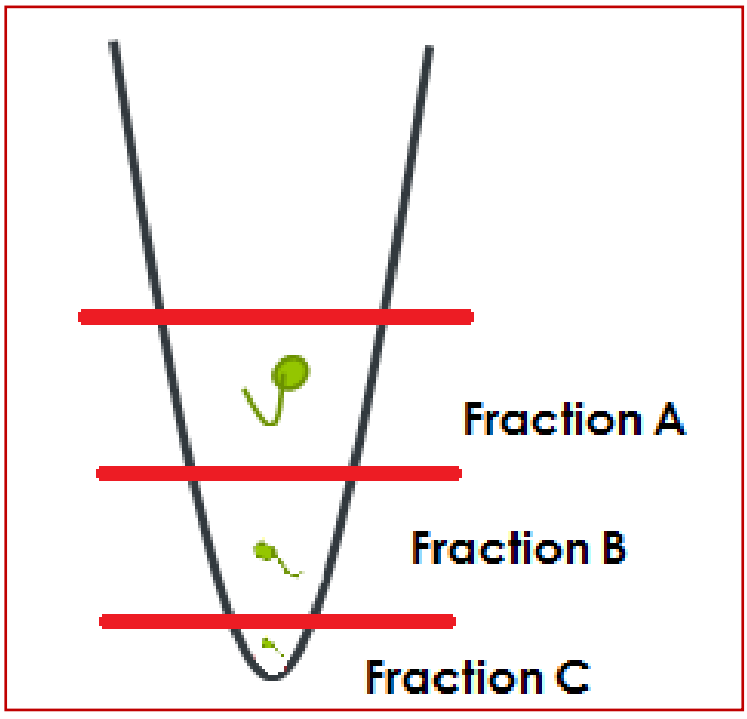

Figure 3-12: Illustration of the double density gradient method after centrifugation

3.2.6.2 DOUBLE WASH

The double wash is a procedure often used in laboratories, and is very similar to the sperm preparation step as described in Section 3.2.4.1. In the present study, the double wash was done according to the WHO manual[3], by diluting the semen sample with an equal volume of HAMS-BSA in a 1:1 ratio. The mixture was then centrifuged for 5 minutes at 300×g, after which the supernatant was removed and discarded. The pellet was resuspended in 2ml of HAMS-BSA and the mixture centrifuged for another 5 minutes, at 300×g. Finally, the pellet (see Figure 3-13) was resuspended in 1ml of HAMS-BSA, after which concentration, motility and viability was assessed. The sample was then used for sex-chromosome ratio determination with FISH.

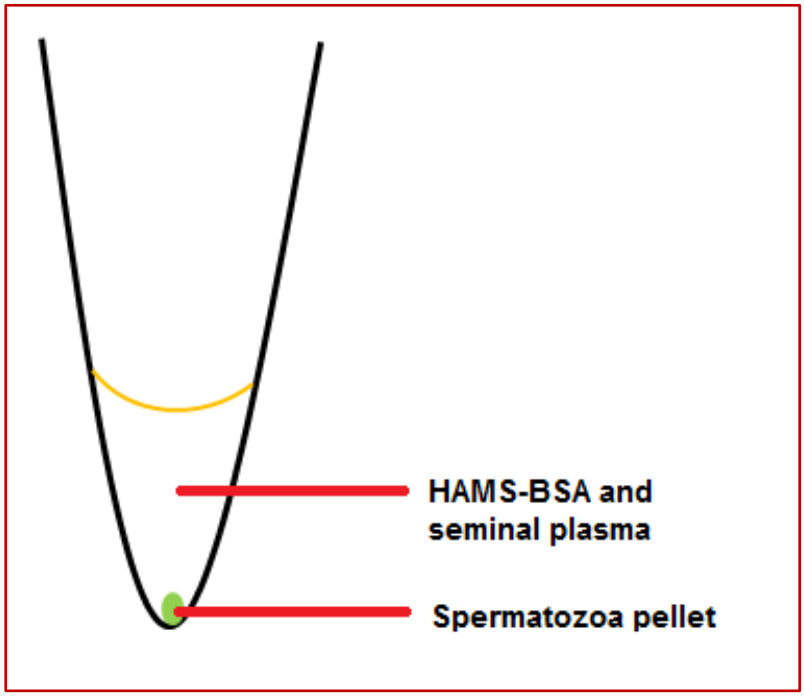

Figure 3-13: Illustration of the simple double wash after centrifugation

3.2.7 STATISTICAL ANALYSIS

FISH data is presented in terms of X:Y ratio, as well as through the percentage difference and absolute percentage increase or decrease in sperm population.

Sperm motility and viability data were analysed using the analysis of variance (ANOVA) method (Statistica, version 5) with Least Significant Difference (LSD) post hoc tests. Data is presented as mean ± standard error of the mean (SEM). The significance level was set as $p<0.05$.

The p-values indicated on graphs represent the mean of the p-values for all the data represented by the graph. Individual p-values are noted in text and also available in Appendices 1 to 7.

CHAPTER 4: RESULTS

INTRODUCTION

The results of all of the experiments as described in Chapter 3 will subsequently be discussed in this chapter. The data for every Research Aim (as set out in Chapter 1) will be reported individually by means of tables and graphs (means ± SEM).

The sex-chromosome ratios of all the neat semen samples were skewed slightly in favour of the X-chromosome bearing spermatozoa prior to any processing. As these values can therefore not be interpreted directly, the changes in sex-chromosome ratio will be portrayed as percentages of absolute change that each population of spermatozoa underwent when compared to the original value. These results will be reported graphically as a percentage absolute increase or percentage absolute decrease from the original value.

With regard to motility and viability data, motility parameters as defined by the WHO are reported: Percentage total motile cells (Type a+b+c), Percentage progressively motile cells (Type a+b) and Percentage static cells (Type d)[3]. Velocity parameters included are the VCL, VSL, LIN and STR. The results of all other motility parameters, including individual p-values, are attached in Appendices 1 to 7.

The percentage of viable spermatozoa will be reported for Aims 1 and 3. The p-values indicated on the graphs are average p-values for all of the data; individual p-values are available in Appendices 1 to 7.

4.1 AIM 1A: SEPARATION OF X- AND Y-CHROMOSOME BEARING SPERMATOZOA ACCORDING TO THEIR RESPECTIVE ABILITIES TO REMAIN VIABLE UPON EXPOSURE TO HOSTILE ENVIRONMENTS.

The results of the sex-chromosome ratios, as determined by the FISH procedure, are summarized in Table 4-1A-C.

Table 4-1A: Sex-chromosome ratios for Aim 1: Viability separation: (pH)

pH incubation (n=10)		
pH value	X-chromosome percentage	Y-chromosome percentage
NEAT	55	45
5.5	62	38
6.5	51	49
7.5	55	45
8.5	53	47
9.5	51	49

Table 4-1B: Sex-chromosome ratios for Aim 1: Viability separation: (Temperature)

Temperature incubation (n=4)		
Temperature	X-chromosome percentage	Y-chromosome percentage
NEAT	52	48
37°C	52	48
41°C	59	41
45°C	54	46

Table 4-1C: Sex-chromosome ratios for Aim 1: Viability separation: (H_2O_2)

Hydrogen peroxide incubation (n=4)		
H_2O_2 concentration	X-chromosome percentage	Y-chromosome percentage
NEAT	54	46
50µM	54	46
750µm	57	43
1000µM	56	44

4.1.1 pH INCUBATION

The effect of pH on the sex-chromosome ratio of the samples, expressed as the absolute changes in the incidence of sex-chromosomes when compared to the original values, is illustrated in Figure 4-1. After the 15-minute incubation period, there was no change

between the X:Y sex-chromosome ratio measured in the neat sample and the neutral pH (7.5) experimental ratio (55%:45%). When compared to the sex-chromosome ratio of the neat sample, a very acidic pH (5.5) led to considerable enrichment of the X-chromosome bearing spermatozoa (55% vs. 62%). However, Y-spermatozoa became enriched in all of the remaining samples in relation to the neat semen sample, at pH level 6.5 (45% vs. 49%) as well as pH levels 8.5 (45% vs. 47%) and 9.5 (45% vs. 49%).

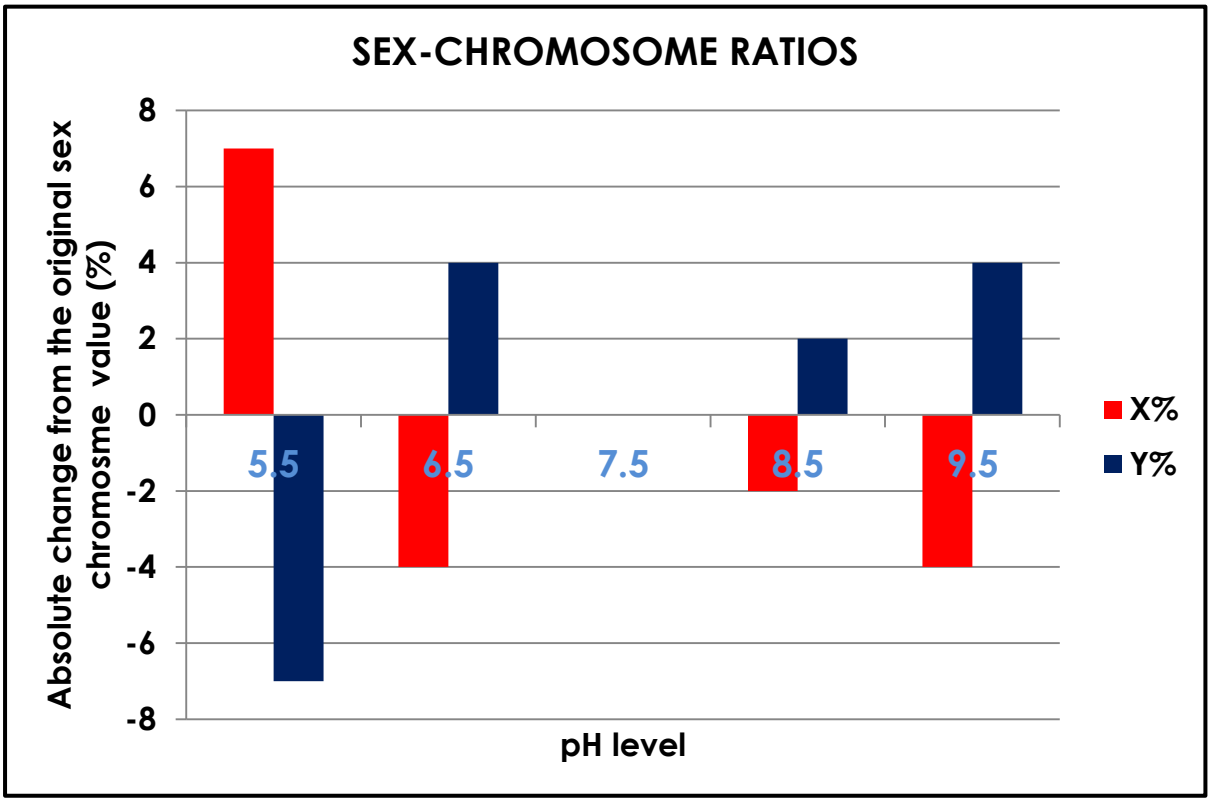

Figure 4-1: Effect of pH on the sex-chromosome ratios of the samples

Figures 4-2(a) and 4-2(b) illustrate the effect of the pH incubation on the percentage of total motile cells and progressively motile cells in the samples, respectively. The same trend can be seen in both instances – total motility and progressive motility

peak at a pH value of 7.5, and decreases when the pH becomes either acidic or alkaline.

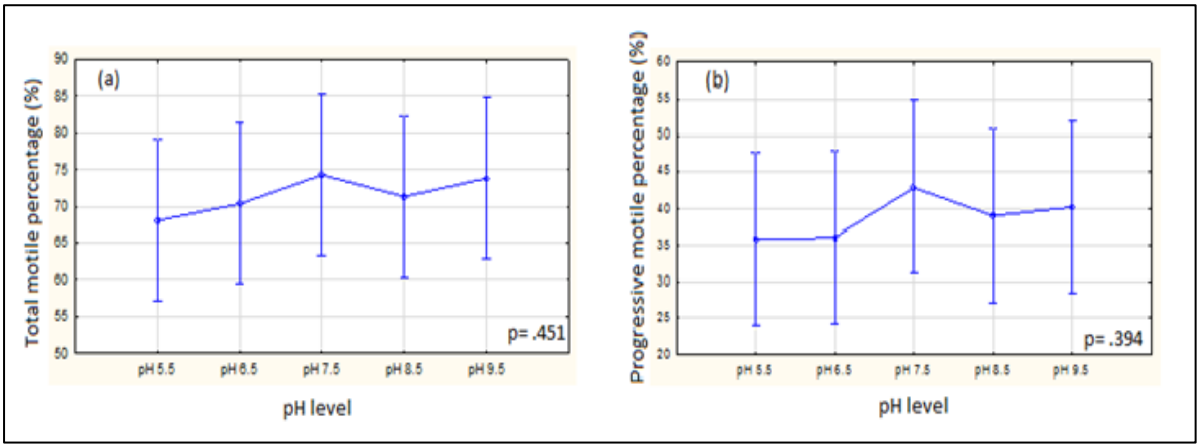

Figure 4-2: Effect of pH on motility parameters. (a) Total motility and (b) Progressive motility

The effect of the pH incubation on the velocity parameters of the spermatozoa is illustrated in Figure 4-3. The same trend is seen in virtually all instances – velocities peak at a pH value of 8.5, and decrease when the pH becomes either acidic or alkaline.

The VCL peaks at 7.5 and remains relatively constant over all the pH levels. The VSL data indicates a peak at pH level 8.5, when compared to all other parameters. There is a statistically significant difference in the VSL between pH level 8.5 and pH level 5.5 (22.58 ± 3.066um/s vs. 16.25 ± 3.066um/s, p=0.039) as well as between pH level 8.5 and pH level 6.5 (22.58 ± 3.066um/s vs. 16.17 ± 3.066um/s, p=0.037).

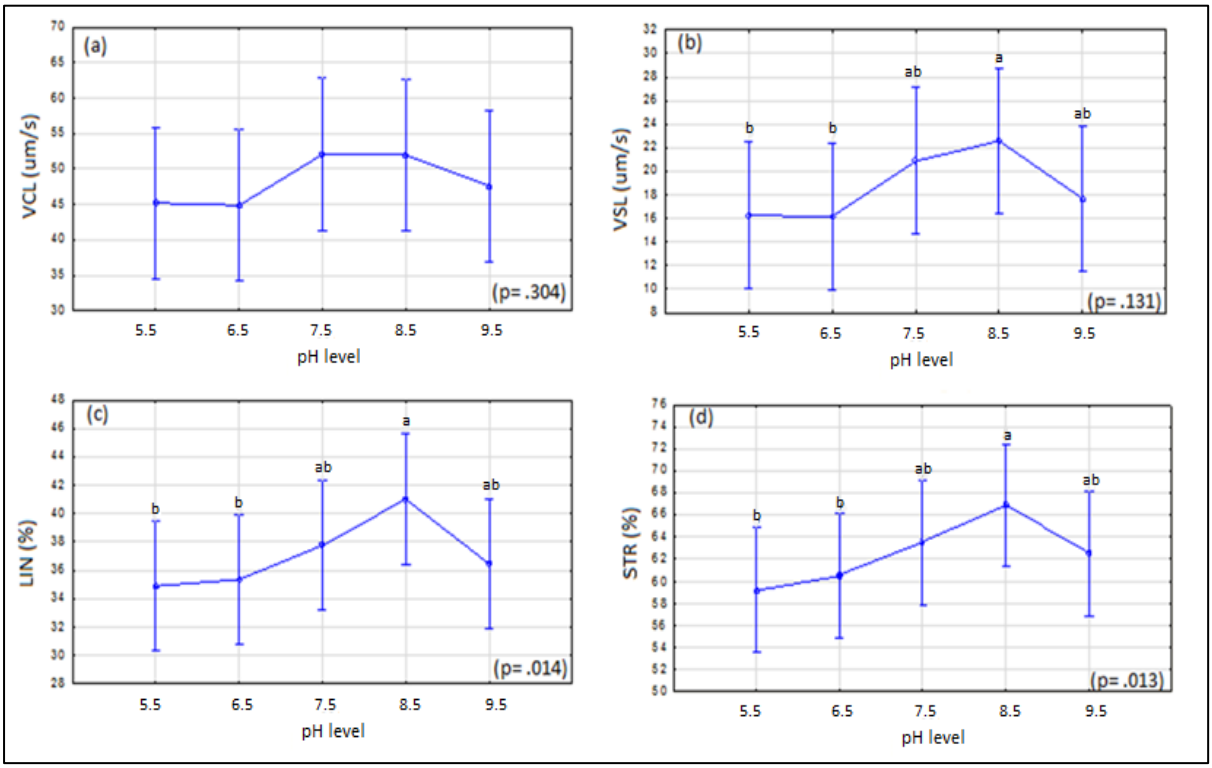

Figure 4-3: Effect of pH on velocity parameters. (a) Curvilinear velocity (VCL) (µm/s), (b) Straight line velocity (VSL) (µm/s), (c) Linearity (LIN) (%) and (d) Straightness (STR) (%)

Significance denoted as: a differs significantly from b; ab does not differ significantly from a or b

When considering the linearity of the movement of the spermatozoa, there is another peak at pH level 8.5. According to the results, there are statistically significant differences between the LIN of the spermatozoa at pH level 8.5 and pH level 5.5 (41.02 ± 2.264% vs. 34.90 ± 2.264%. p=0.002) as well as between pH level 8.5 and 6.5 (41.02 ± 2.264% vs. 35.36 ± 2.264%. p=0.004), and also between pH levels 8.5 and 9.5 (41.02 ± 2.264% vs. 36.47 ± 2.264%. p=0.017). Furthermore, there is a statistically insignificant difference in the LIN between pH levels 8.5 and 7.5 (41.02 ± 2.264% vs. 37.77 ± 2.264%. p=0.083).

The STR data follows a trend that is very similar to the LIN results. The data indicates the greatest STR values at pH level 8.5, with statistically significant differences between the STR at pH levels 8.5 and 5.5 (66.89 ± 2.759% vs. 59.25 ± 2.759%, p=0.001) as well as between pH levels 8.5 and 6.5 (66.89 ± 2.759% vs. 60.54 ± 2.759%, p=0.006). Decreases in the STR was also noted between pH values 8.5 and 7.5 (66.89 ± 2.759% vs. 63.53 ± 2.759%, p=0.132) and between pH levels 8.5 and 9.5 (66.89 ± 2.759% vs. 62.52 ± 2.759%, p=0.052).

The percentage of viable cells (Figure 4-4) is highest at a pH level of 7.5 and declines as the pH increases and decreases. There is a significant difference in the viable cell fraction between pH levels 7.5 and 5.5 (70.75 ± 3.229% vs. 58.70 ± 3.229%, p=0.003) as well as between pH levels 6.5 and 5.5 (67.48 ± 3.229% vs. 58.70 ± 3.229%, p=0.028).

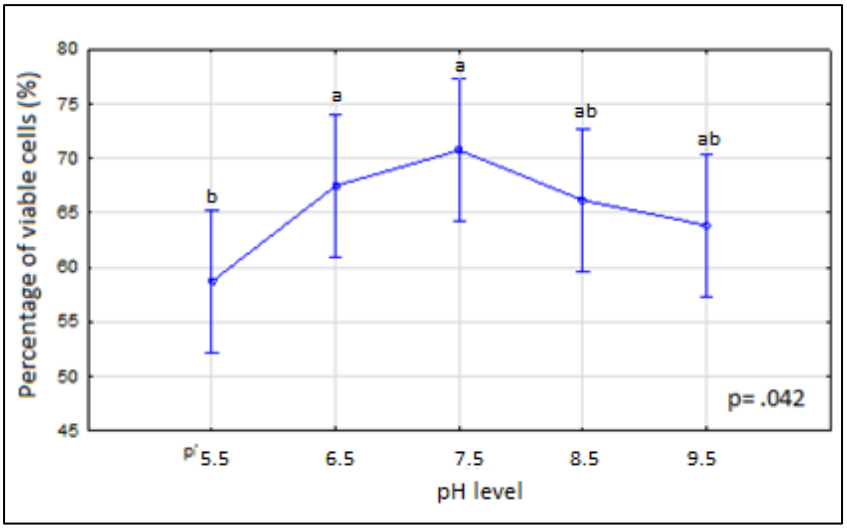

Figure 4-4: Effect of pH on the viability of the spermatozoa

Significance denoted as: a differs significantly from b; ab does not differ significantly from a or b

4.1.2 TEMPERATURE INCUBATION

The effect of increased temperature on the sex-chromosome ratio of the spermatozoa is summarized in Table 4-1 and illustrated in Figure 4-5. At the standard temperature of 37°C and after 25 minutes' incubation, as indicated for normal laboratory processing of human spermatozoa, there was no change in the sex-chromosome ratio of the sample (X 52:52, Y 48:48). At 41°C there was an absolute increase of 7% in the incidence of X-chromosome bearing spermatozoa compared to the sample prior to processing (52% vs. 59%) indicating a 13.46% increase. At 45°C the incidence of X-chromosome bearing spermatozoa increased with 2% when compared to the neat semen sample (52% vs. 54%), which is a 3.84% increase.

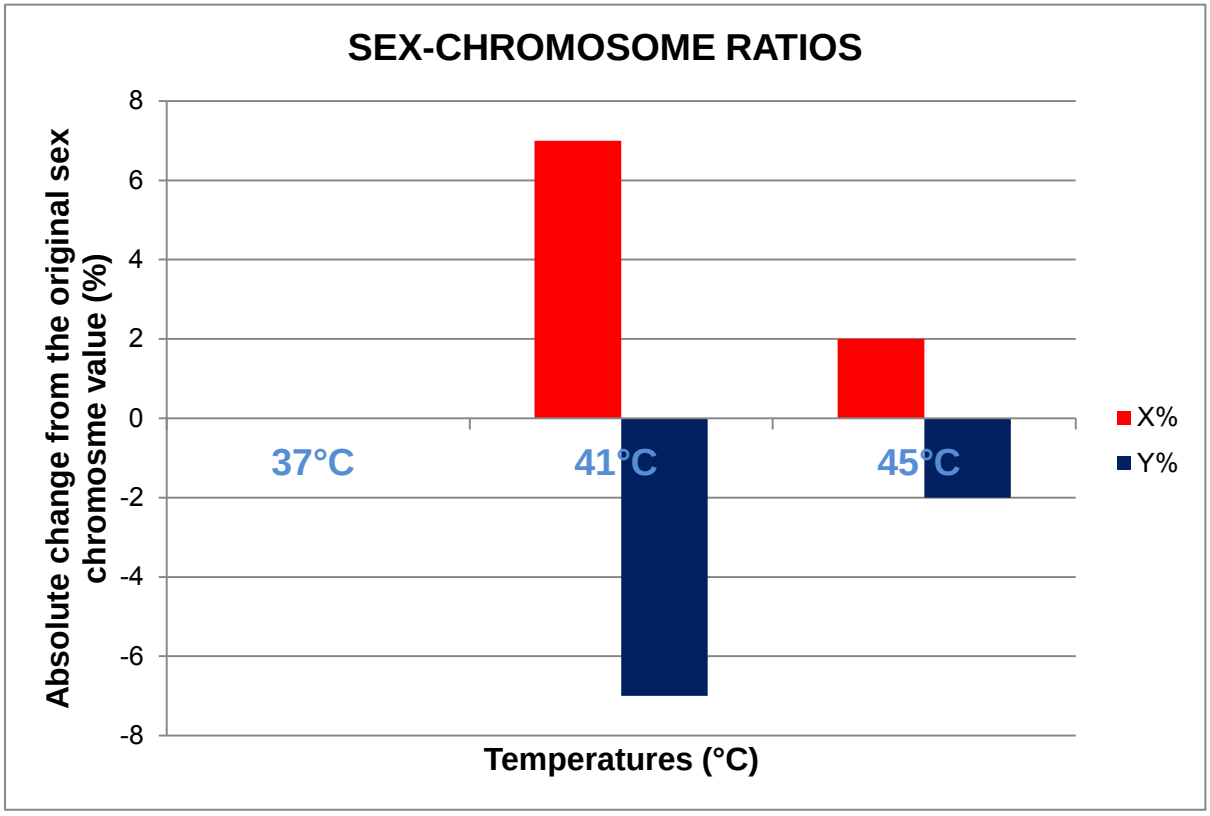

Figure 4-5: Effect of temperature on the sex-chromosome ratio

Figures 4-6(a) and 4-6(b) represent the effect of temperature on the motility of the spermatozoa. The data indicates a statistically significant decrease in the percentage of total motile cells between 37°C and 45°C (74.55 ± 3.883% vs. 55.53 ± 3.883%, p=0.013). The difference in total motility between temperatures 41°C and 45°C (72.28 ± 3.883% vs. 55.53 ± 3.883%, p=0.022) was also statistically significant. Motility data between temperatures 37°C and 41°C did not differ significantly.

Progressive motility (Figure 4-6(b)) followed the same trend and declined significantly between 37°C and 45°C (35.25 ± 2.719% vs. 11.73 ±2.719%, p=0.001) as well as between 41°C and 45°C (39.73 ± 2.719% vs. 11.73 ±2.719%, p=0.0003).

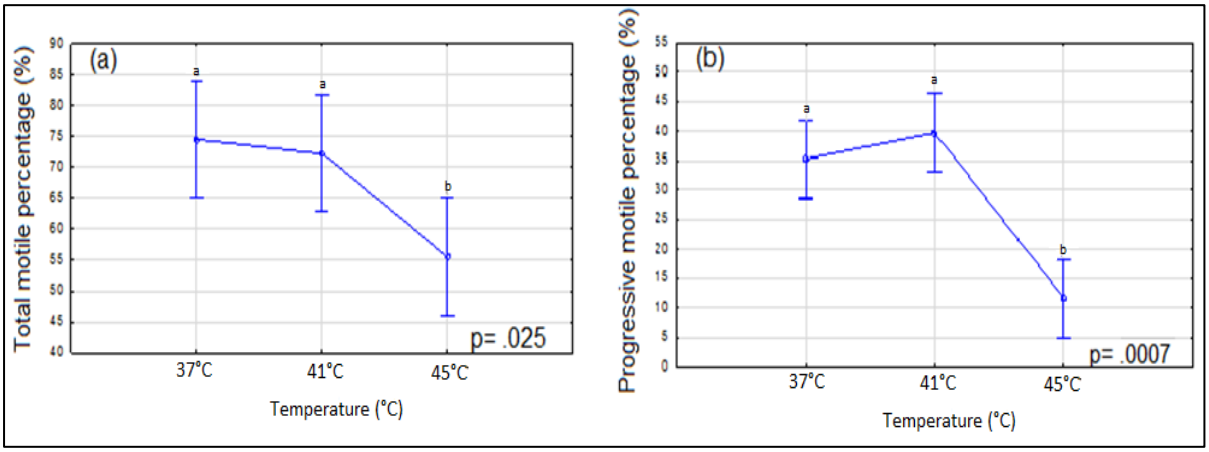

Figure 4-6: Effect of temperature motility parameters. (a) Total motility and (b) Progressive motility

Significance denoted as: a differs significantly from b

The data of the velocity parameters after incubation at different temperatures (see Figure 4-7) all followed the same trend. Values increased slightly as the temperature

increased from 37°C to 41°C and then declined significantly as the temperature increased to 45°C.

The curvilinear velocity increased significantly between 37°C and 41°C (43.43 ± 2.697um/s vs. 53.60 ± 2.697um/s, p=0.037). There was a statistically significant decrease in VCL between 37°C and 45°C (43.43 ± 2.697um/s vs. 26.10 ± 2.697um/s, p=0.004) as well as between 41°C and 45°C (53.60 ± 2.697um/s vs. 26.10 ± 2.697um/s, p=0.0003).

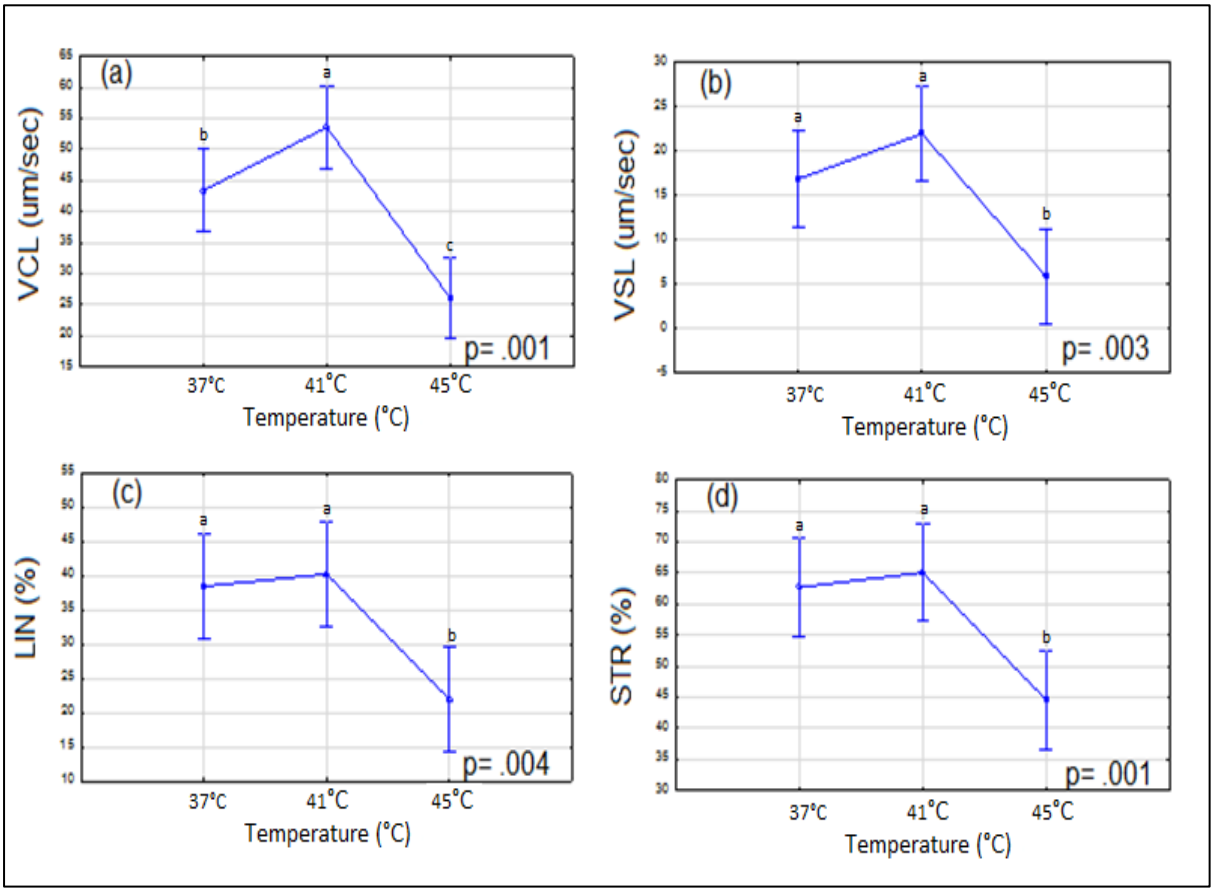

Figure 4-7: Effect of temperature on velocity parameters. (a) Curvilinear velocity (VCL), (b) Straight line velocity (VSL), (c) Linearity (LIN) and (d) Straightness (STR)

Significance denoted as: a differs significantly from b and c; b differs significantly from a and c

The data for VSL indicates a significant decrease between temperatures 37°C and 45°C (16.78 ± 2.180um/s vs. 5.85 ± 2.180um/s, p=0.009). A statistically significant decrease in straight line velocity was also noted between temperatures 41°C and 45°C (21.85 ± 2.180um/s vs. 5.85 ± 2.180um/s, p=0.001).

Results for LIN show a significant decrease in linear movement from temperature 41°C to 45°C (40.23 ± 3.111% vs. 21.93 ± 3.111%, p=0.004) and between 37°C and 45°C (38.45 ± 3.111% vs. 21.93 ± 3.111%, p=0.002). There was virtually no change in LIN between temperatures 37°C and 41°C (38.45 ± 3.111% vs. 40.23 ± 3.111%, p=0.644).

STR results indicate a statistically significant decrease between temperatures 41°C and 45°C (65.10 ± 3.219% vs. 44.58 ± 3.219%, p=0.0006) and temperatures 37°C to 45°C (62.73 ± 3.219% vs. 44.58 ± 3.219%, p=0.001). There was no significant change in STR between temperatures 37°C and 41°C (62.73 ± 3.219% vs. 65.10 ± 3.219%, p=0.477).

The effect of temperature on the percentage of viable spermatozoa is shown in Figure 4-8. Viability declined considerably as the temperature increased. The cells were most viable at 37°C, with the viability decreasing significantly between 37°C and 41°C (65.95 ± 4.257% vs. 47.35 ± 4.257%, p=0.013), as well as between 37°C

and 45°C (65.95 ± 4.257% vs. 38.87 ± 4.257%, p=0.002). Cell viability did not change significantly between temperatures 41°C and 45°C (47.35 ± 4.257% vs. 38.87 ± 4.257%, p=0.66).

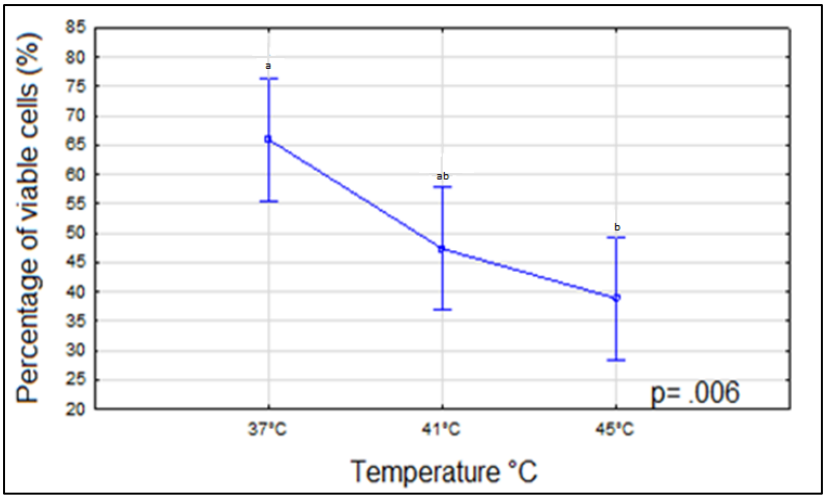

Figure 4-8: Effect of temperature on the viability of the spermatozoa

Significance denoted as: a differs significantly from b; ab does not differ significantly from a or b

4.1.3 H_2O_2 INCUBATION

Upon exposure to H_2O_2 at 50µM, there was no change in the sex-chromosome ratios when compared to the unprocessed semen samples, as is seen in Figure 4-9. At a concentration of 750µM, there was an absolute increase of 3% in the incidence of X-chromosome bearing spermatozoa (54% vs. 57%).Exposure of the spermatozoa to 1000µM H_2O_2 still enriched the X-chromosome bearing spermatozoa, to a lesser extent, by an absolute 2% when compared to the neat semen (54% vs. 56%).

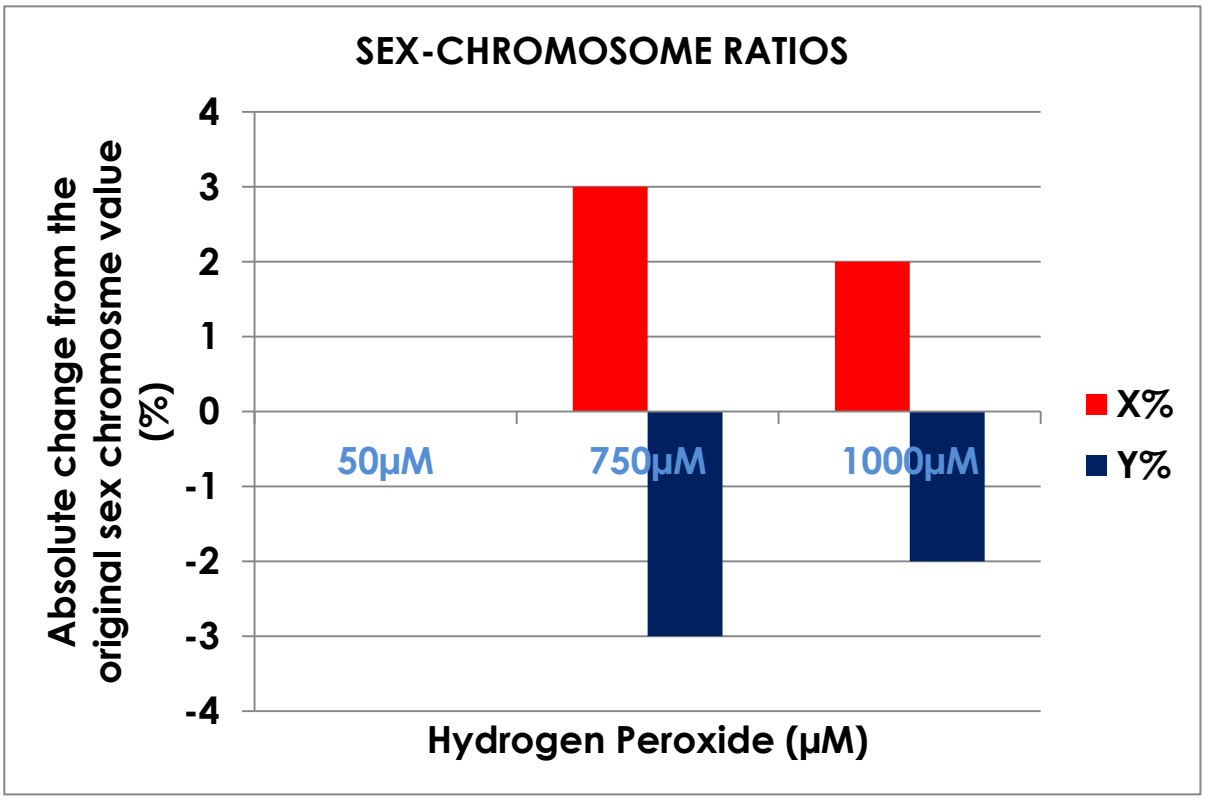

Figure 4-9: Effect of hydrogen peroxide on the sex-chromosome ratios of the samples

The effect of H_2O_2 on motility parameters of the spermatozoa can be seen in Figure 4-10. Both total motility (Figure 4-10(a)) and progressive motility (Figure 4-10(b)) declines significantly between exposure to 50µM and 750µM (total motility, 79.15 ± 9.047% vs. 55.65 ± 9.047%, p=0.016; progressive motility, 53.63 ± 7.697% vs. 22.23 ± 7.697%, p=0.021) and also between 50µM and 1000µM (total motility, 79.15 ± 9.047% vs. 59.63 ± 9.047%, p=0.033; progressive motility, 53.63 ± 7.697% vs16.63 ± 7.697%, p=0.011). There is no significant change in either motility parameter between exposure to 750µM and 1000µM.

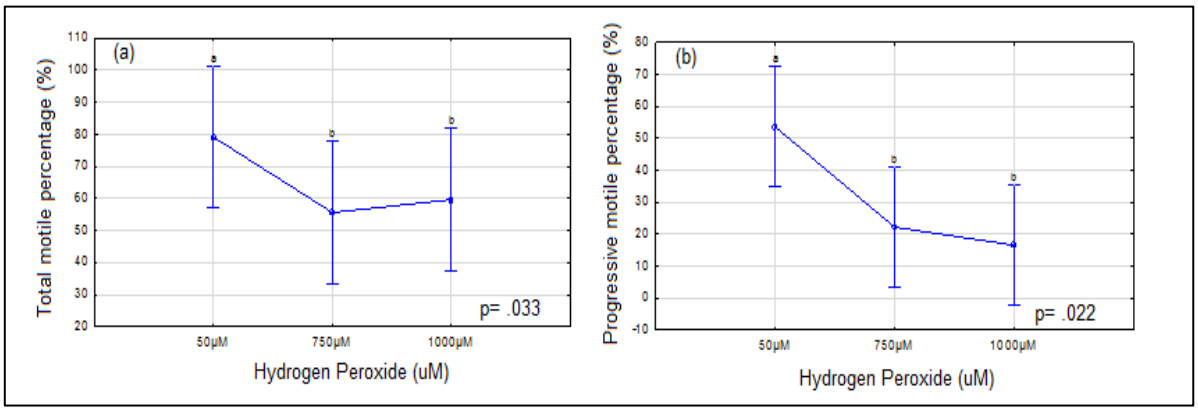

Figure 4-10: Effect of hydrogen peroxide on motility parameters. (a) Total motility and (b) Progressive motility

Significance denoted as: a differs significantly from b

Exposure of spermatozoa to H_2O_2 also compromised the velocity parameters of the cells (Figure 4-11). The same trend could be seen throughout the data: velocity decreased steadily and significantly from exposure to a low H_2O_2 concentration of 50µM to the higher concentrations of 750µM and 1000µM.

The VCL data (Figure 4-11(a)) indicates significant decreases between exposure to 50µM and 750µM H_2O_2 (52.75 ± 4.427µm/s vs. 35.23µM/s, p=0.031) as well as between 50µM and 1000µM (52.75 ± 4.427µm/s vs. 25.05µM/s, p=0.004).

VSL (Figure 4-11(b)) was affected similarly, with a significant decrease upon incubation in 50µM and 750µM H_2O_2 (18.85 ± 2.353µm/s vs. 8.30 ± 2.353µm/s, p=0.019) and also between 50µM and 1000µM H_2O_2 incubation (18.85 ± 2.353µm/s vs. 4.58 ± 2.353µm/s, p=0.005).

The results for LIN and STR indicate a significant decrease between 50µM and 1000µM H_2O_2 incubation (LIN, 35.68 ± 4.017% vs. 16.40 ± 4.017%, p=0.013; STR, 58.60 ± 4.304 vs. 31.35 ± 4.304, p=0.004), while STR also decreased significantly between 50µM and 750µM H_2O_2 exposure (58.60 ± 4.304% vs. 38.83 ± 4.304%, p=0.018).

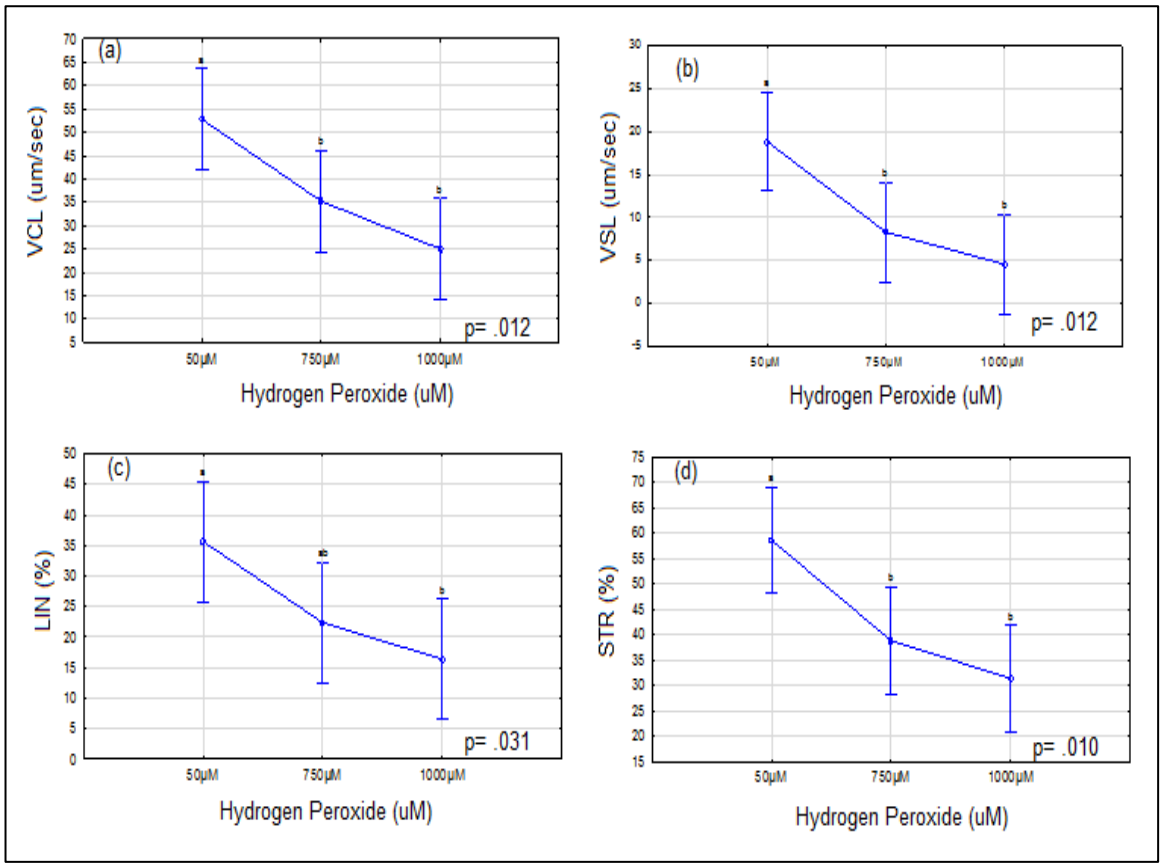

Figure 4-11: Effect of hydrogen peroxide (H_2O_2) on velocity parameters. (a) Curvilinear velocity (VCL), (b) Straight line velocity (VSL), (c) Linearity (LIN) and (d) Straightness (STR)

Significance denoted as: a differs significantly from b; ab does not differ significantly from a or b

The effect of H_2O_2 incubation on the percentage of viable cells is depicted in Figure 4-12. The viability of the cells did not change significantly in any of the incubation groups.

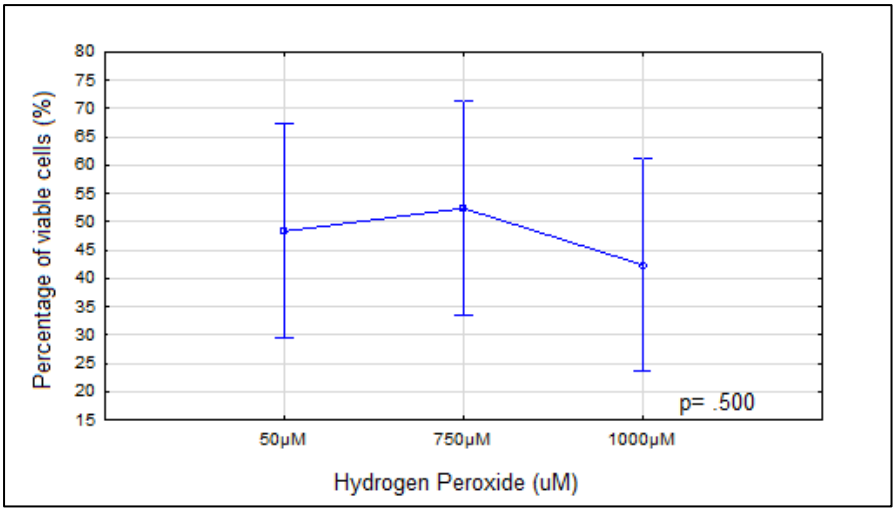

Figure 4-12: Effect of Hydrogen Peroxide on viability of the spermatozoa

4.1.4 Aim 1b: Comparison of the effectiveness of MACS and modified Swim-up techniques in separating live and dead spermatozoa

Data from the preceding chapter with regard to the MACS and swim-up separations will be reported subsequently, and is depicted in Figures 4-13 to 4-15. Graphs were selectively included based on relevance and will include the concentration of viable cells that were selected by each separation method respectively, as well as for the total motility and viability of the spermatozoa in the sample.

4.1.4.1 Concentration

The data representing the concentrations of viable cells that were isolated by each particular method (see Figure 4-13) indicates that during the pH incubation, the MACS yielded a higher concentration than the swim-up method for all pH levels (Figure 4-13(a)). Results from both the temperature (Figure 4-13(b)) and H_2O_2

(Figure 4-13(c)) incubations indicate that concentrations of viable cells that were selected was much more similar, except in the case of 50µM H_2O_2, where the MACS technique was able to select a much higher concentration of spermatozoa when compared to the swim-up method.

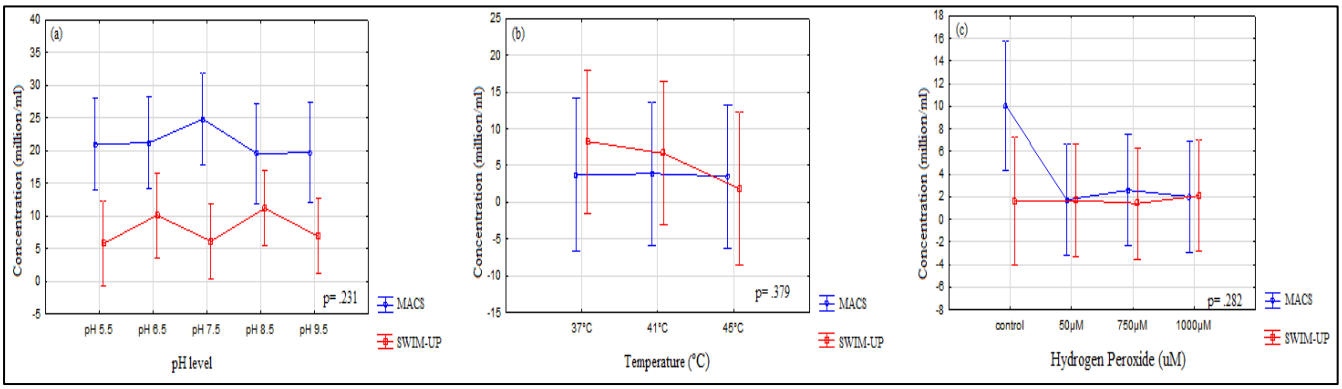

Figure 4-13: Concentrations of viable fractions isolated by MACS and modified Swim-up respectively. (a) pH incubation, (b) Temperature incubation, (c) H_2O_2 incubation

4.1.4.2 Motility

The results as illustrated in Figure 4-14 represent data regarding total motility of the spermatozoa after selection of viable cells. Overall, the spermatozoa selected by the modified swim-up displayed increased total motility percentages, with the exception of incubation in the 6.5 pH medium. Motility parameters of MACS-separated spermatozoa remained relatively constant over all the different pH levels that they were incubated in (Figure 4-14(a)). The motility data of the temperature (Figure 4-14(b)) and H_2O_2 (Figure 4-14(c)) incubations follow similar trends.

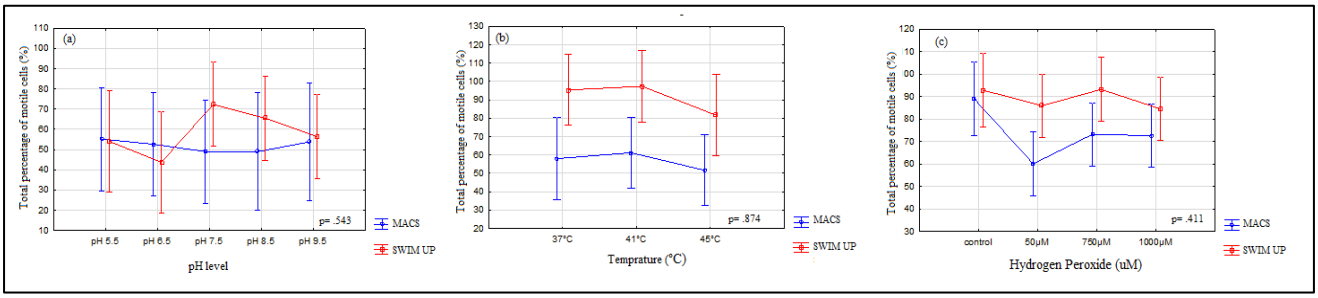

Figure 4-14: Percentage of total motility of the viable fractions of spermatozoa isolated by MACS and modified swim-up, respectively. (a) pH incubation, (b) Temperature incubation, (c) H_2O_2 incubation

4.1.4.3 Viability

While the viability of the spermatozoa incubated at the different pH values remains relatively constant when separated by MACS, the fraction separated by the modified swim-up at pH level 5.5 was considerably less viable (Figure 4-15(a)). In the temperature (Figure 4-15(b)) experiments the spermatozoa isolated by modified swim-up displayed increased percentages of viability.

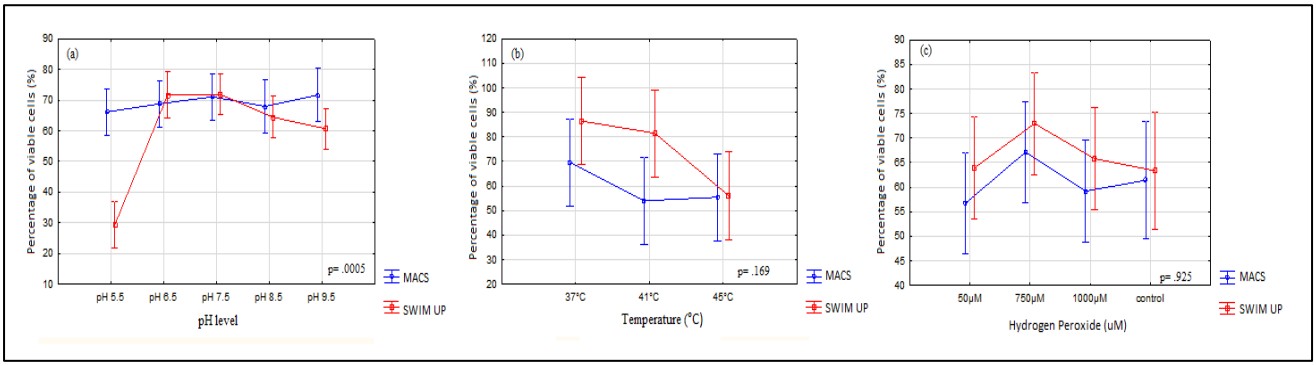

Figure 4-15: Percentage of viable cells in the fraction isolated by MACS and modified Swim-up, respectively. (a) pH incubation, (b) Temperature incubation, (c) H_2O_2 incubation

4.2 RESEARCH AIM 2: SEPARATION OF X- AND Y-CHROMOSOME BEARING SPERMATOZOA BASED ON THEIR PARTICULAR MOTILITY CAPACITIES.

The chromosome ratios of the samples – as determined by FISH - before and after the experiments are summarized in Table 4-2.

Table 4-2: Sex-chromosome ratios for Aim 2: Motility separation

Direct Swim-up		
Isolated fraction	X-chromosome percentage	Y-chromosome percentage
NEAT	56	44
A (top fraction)	52	48
B (middle fraction)	57	43
Capillary Tube		
Isolated fraction	X-chromosome percentage	Y-chromosome percentage
NEAT	56	44
A (6-9cm)	54	46
B (3-6cm)	53	47
C (0-3cm)	54	46

4.2.1 DIRECT SWIM-UP

During the direct swim-up technique, there was considerable enrichment of the Y-chromosome bearing spermatozoa in the top fraction (Fraction A) when compared to the original semen value (44% vs. 48%), equalling an absolute increase of 4% in Y-chromosome bearing spermatozoa (Figure 4-16). The bottom fraction (Fraction B) was slightly enriched with X-chromosome bearing spermatozoa when compared to the unprocessed sample (56% vs. 57%).

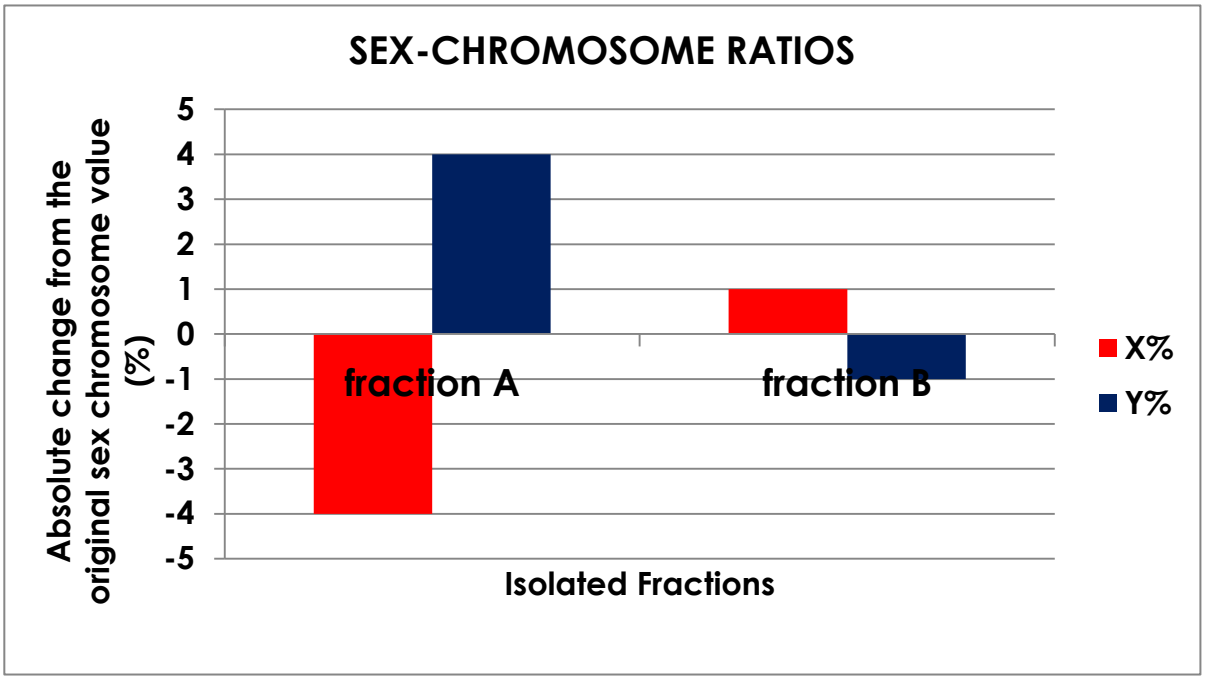

Figure 4-16: Sex-chromosome ratios after incubation

Motility parameters (see Figure 4-17) were different for the individual fractions when compared to the control sample (before processing). Both the total percentage of motile spermatozoa and the percentage of progressively motile spermatozoa increased significantly in both fractions when compared to the control.

The percentage of total motility (Figure 4-17(a)) increased significantly between the neat sample and Fraction A (67.75 ± 4.774% vs. 97.70 ± 4.420%, p=0.0007) as well as between the neat sample and Fraction B (67.75 ± 4.774% vs. 95.77 ± 4.420%, p=0.001). There was no significant difference between the total motility of fraction A and fraction B (97.70% ± 4.420 vs. 95.77 ± 4.420%, p=0.763).

Progressive motility (Figure 4-17(b)) increased significantly between the control and Fraction A (39.72 ± 6.270% vs. 83.53 ± 6.270%, p=0.0001) and also between the control and Fraction B (39.72 ± 6.270% vs. 80.17 ± 6.270%, p=0.0003). The difference between Fractions A and B were not significant (83.53 ± 6.270% vs. 80.17 ± 6.270%, p=0.655).

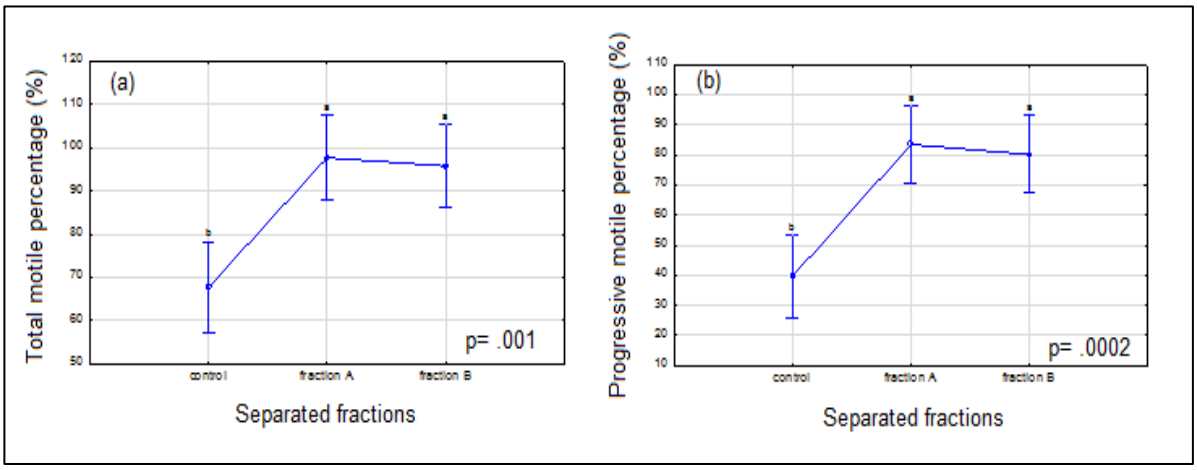

Figure 4-17: Motility parameters after incubation. (a) Total motility and (b) Progressive motility

Significance denoted as: a differs significantly from b

Velocity parameters (Figure 4-18) indicate a significant increase in the VCL and VSL between the control and Fraction A (VCL, 46.42 ± 6.273µm/s vs. 78.84 ± 5.834µm/s, p=0.002; VSL, 16.00 ± 2.792µm/s vs. 26.86 ± 2.707µm/s, p=0.0003) and also

between the control and Fraction B (VCL, 46.42 ± 6.273µm/s vs. 68.69 ± 5.834µm/s, p=0.015; VSL, 16.00 ± 2.792µm/s vs. 24.93 ± 2.707µm/s, p=0.001). The differences in velocity parameters were not significant between Fractions A and B (VCL, 78.84 ± 5.834µm/s vs. 68.69 ± 5.834µm/s, p=0.197; VSL, 26.86 ± 2.707µm/s vs. 24.93 ± 2.707µm/s, p=0.351).

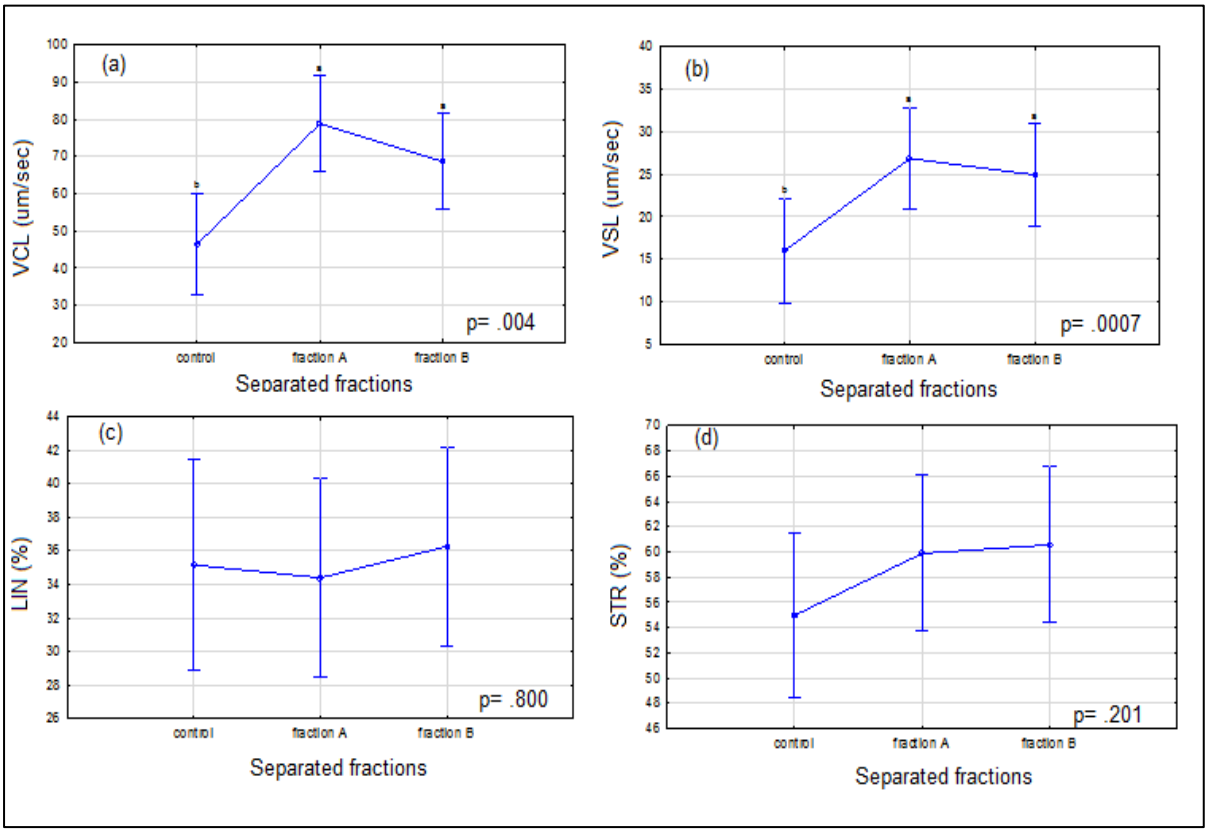

Figure 4-18: Velocity parameters after incubation. (a) Curvilinear velocity (VCL), (b) Straight line velocity (VSL), (c) Linearity (LIN) and (d) Straightness (STR)

Significance denoted as: a differs significantly from b

LIN (Figure 4-18(c)) remained relatively unchanged between the control and Fraction A (35.17 ± 2.841% vs. 34.40 ± 2.687%, p=0.799), and between the control and Fraction B (35.17 ± 2.841% vs. 36.27 ± 2.687%, p=0.714). An increase in STR (Figure 4-18(d)) is seen between the control and Fraction A (54.99 ± 2.978% vs.

59.87 ± 2.814%, p=0.143) and between the control and Fraction B (54.99 ± 2.978% vs. 60.57 ± 2.814%, p=0.099). The differences between Fraction A and Fraction B were not significant for either LIN (34.40 ± 2.687% vs. 36.27 ± 2.687%, p=0.516) or STR (59.87 ± 2.814% vs. 60.57 ± 2.814%, p=0.821).

4.2.2 Capillary Tube

During the capillary tube method, enrichment of Y-chromosome bearing spermatozoa can be observed in all fractions (see Figure 4-19). When compared to the control, the incidence of Y-chromosome bearing spermatozoa increased most in Fraction B (44% vs. 47%), while the percentage increase of Y-chromosome bearing spermatozoa was the same (2%) in both Fraction A (44% vs. 46%) and Fraction C (44% vs. 46%).

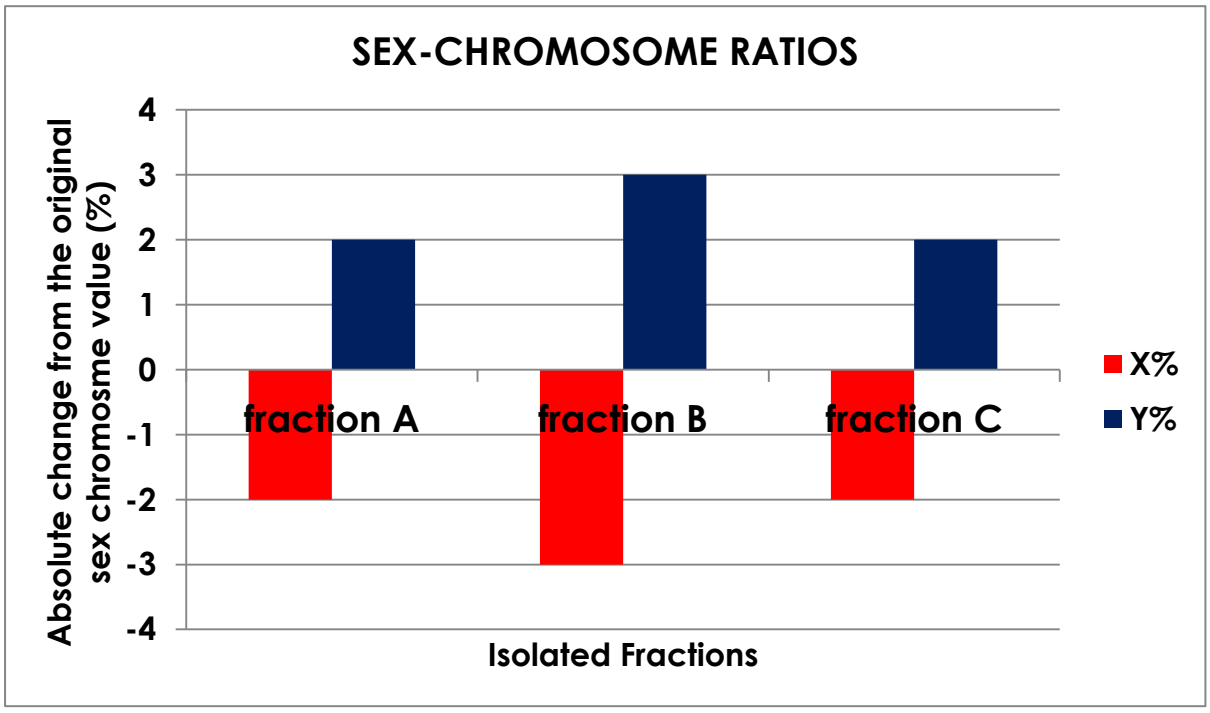

Figure 4-19: Sex-chromosome ratios after incubation

Motility results (Figure 4-20) of both total motility (Figure 4-20(a)) and progressive motility (Figure 4-20(b)) indicate the same trend, with both parameters remaining relatively unchanged between the controls and Fractions A (total motility, 68.42 ± 8.559% vs. 68.24 ± 8.074%, p=0.985; progressive motility, 41.49 ± 9.908% vs. 40.21 ± 9.437%, p=0.898). There was a slight increase between the control and Fraction B in the total motile percentage (68.42 ± 8.559% vs. 73.09 ± 8.074%, p=0.624) as well as in the progressively motile percentage (41.49 ± 9.908% vs. 44.40 ± 9.437%, p=0.771).

The data indicates decreasing trends in both parameters when comparing the controls and Fraction C (total motility, 68.42 ± 8.559% vs. 61.33 ± 8.074%, p=0.459; progressive motility, 41.49 ± 9.908% vs. 31.84 ± 9.437%, p=0.339).

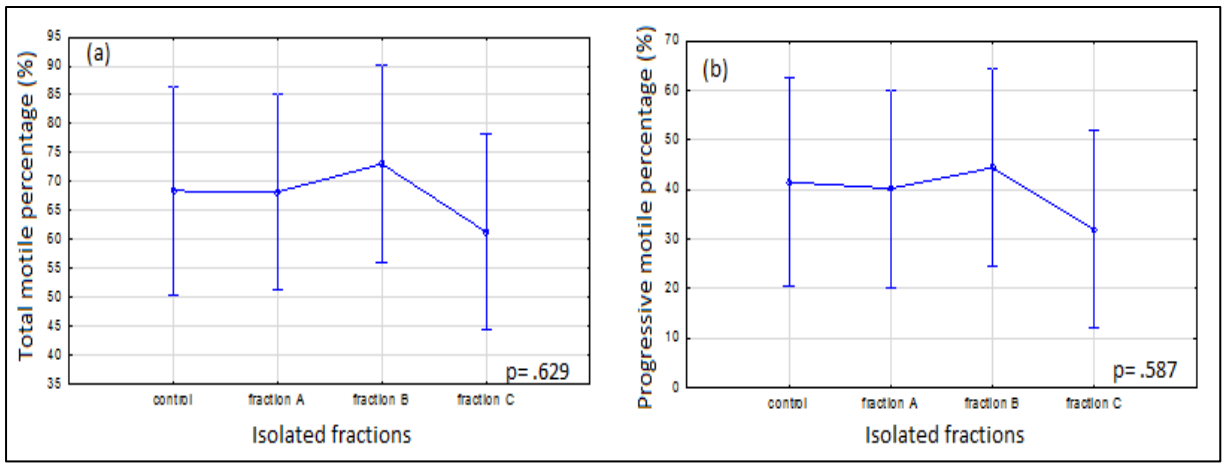

Figure 4-20: Motility parameters after incubation. (a) Total motility and (b) Progressive motility

Velocity parameters of the capillary tube fractions are illustrated in Figure 4-21. The VCL (Figure 4-21(a)) remains relatively constant for the spermatozoa in all fractions

when compared to the control and to each other. The VSL (Figure 4-21(b)) parameters for the control and Fractions B and C remain relatively unchanged with the exception of Fraction A, where the data indicated a more pronounced but still insignificant decrease in the VSL when compared to the control (17.14 ± 2.694µm/s vs. 13.37 ± 2.565µm/s, p=0.177).

Both LIN (Figure 4-21(c)) and STR (Figure 4-21(d)) followed the same trend, with a significant decrease in the parameters between the control and Fraction A (LIN, 35.54 ± 3.762% vs. 25.99 ± 3.538%, p=0.037; STR, 55.70 ± 5.455% vs. 45.84 ± 5.105%, p=0.143). Both LIN and STR are further significantly increased between Fraction A and Fraction C (LIN, 25.99 ± 3.538% vs. 37.33 ± 3.538%, p=0.012; STR, 45.84 ± 5.105% vs. 59.84 ± 5.105%, p=0.036).

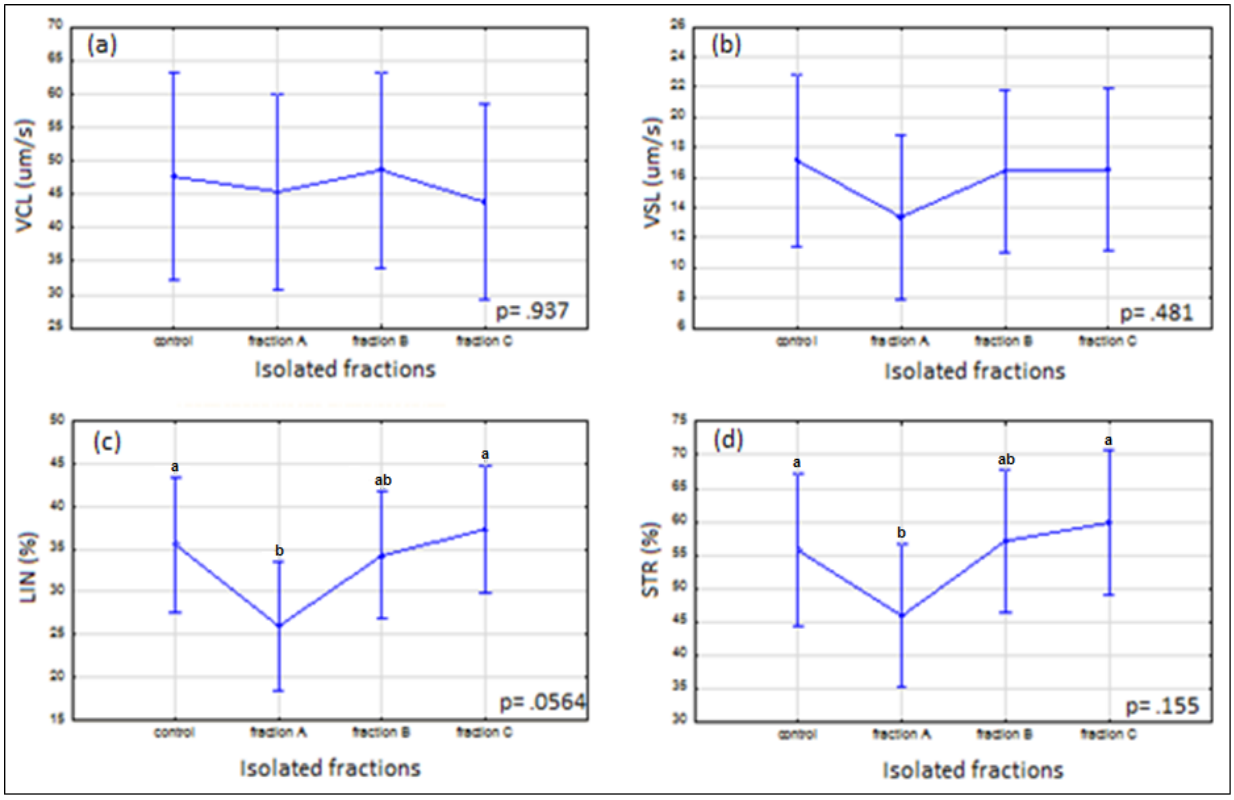

Figure 4-21: Velocity parameters after incubation. (a) Curvilinear velocity (VCL), (b) Straight line velocity (VSL), (c) Linearity (LIN) and (d) Straightness (STR)

Significance denoted as: a differs significantly from b, ab does not differ significantly from a or b

4.3 RESEARCH AIM 3: SEPARATION OF X- AND Y-CHROMOSOME BEARING SPERMATOZOA BASED ON DIFFERENCES IN SIZE/WEIGHT.

The chromosome ratios of the samples before and after centrifugation processing are summarized in Table 4-3.

Table 4-3: Sex-chromosome ratios for Aim 3: Size/Weight Separation

Double Density Gradient Centrifugation		
Isolated fraction	X-chromosome percentage	Y-chromosome percentage
NEAT	56	44
A (top layer)	57	43
B (middle layer)	59	41
C (pellet)	52	48
Simple Double Wash		
Isolated Fraction	X-chromosome percentage	Y-chromosome percentage
NEAT	55	45
Pellet	54	46

4.3.1 Double Density Gradient Centrifugation

Results from the DDG method (see Figure 4-22) showed that, compared to the control, there was a slight increase in the X-chromosome bearing spermatozoa in Fraction A (56% vs. 57%), and a more pronounced enrichment of X-chromosome bearing spermatozoa in Fraction B (56% vs. 59%). There was also enrichment of Y-chromosome bearing spermatozoa in Fraction C (the pellet) when compared to the control sample (44% vs. 48%).

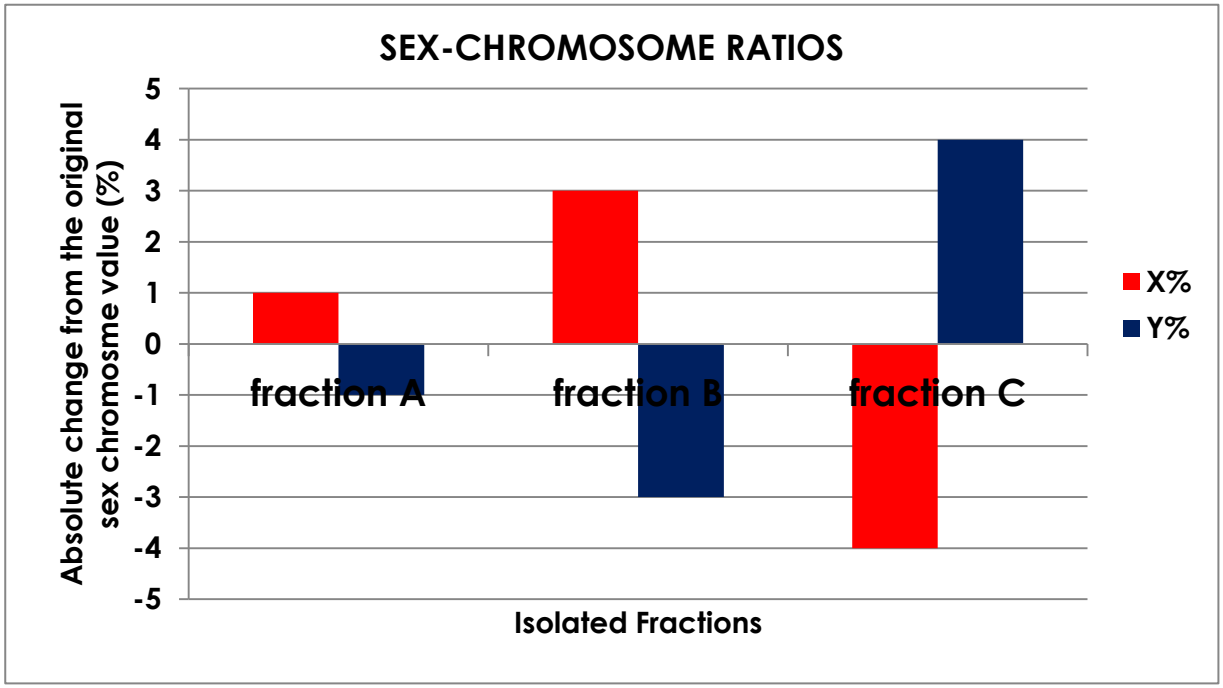

Figure 4-22: Sex-chromosome ratios after DDG centrifugation

The results of the motility parameters (Figure 4-23) indicated that the spermatozoa isolated in Fraction A displayed significantly decreased total motility when compared to the control (59.92 ± 5.442% vs. 88.60 ± 5.442%, p=0.0006), Fraction B (59.92 ± 5.442% vs. 81.52 ± 5.442%, p=0.004) as well as Fraction C (59.92 ± 5.442% vs. 89.92 ± 5.442%, p=0.0004). Data for progressively motile spermatozoa followed the

same trend, with Fraction A displaying significantly decreased progressive motility when compared to the control (27.66 ± 8.789% vs. 62.14 ± 8.789%, p=0.0005), Fraction B (27.66 ± 8.789% vs. 53.48 ± 8.789%, p=0.004) and Fraction C (27.66 ± 8.789% vs. 68.56 ± 8.789%, p=0.0001).

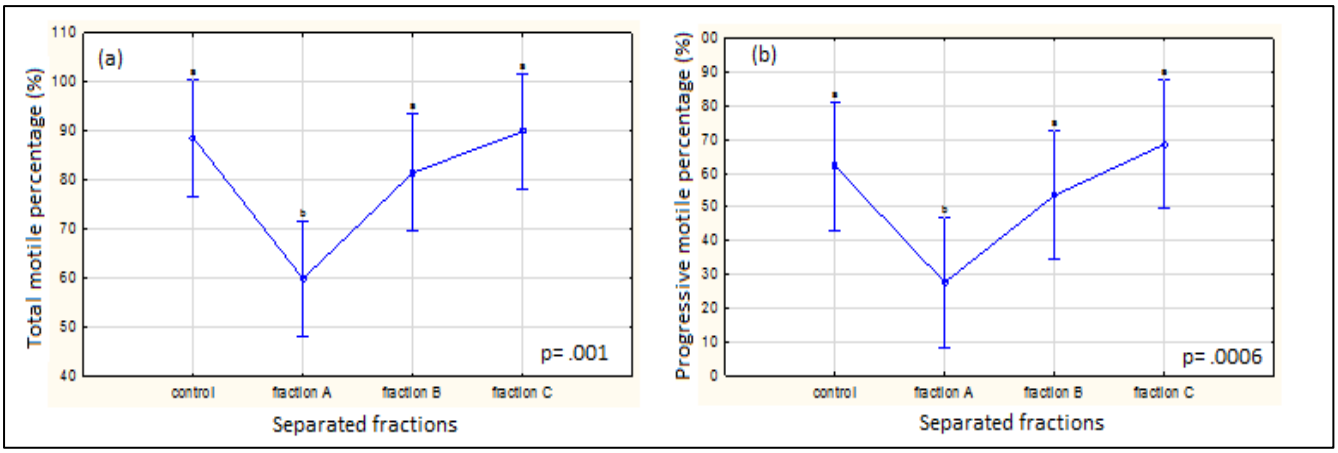

Figure 4-23: Motility parameters after DDG centrifugation. (a) Total motility and (b) Progressive motility

Significance denoted as: a differs significantly from b

The results of the velocity parameters of the spermatozoa isolated in all the fractions follow the same trend as the total and progressive motility results, and are depicted in Figure 4-24. All velocity parameters peak for the spermatozoa isolated in Fraction C. In all instances, Fraction A displays a statistically significantly decreased kinematic results when compared to Fraction C (VCL, 39.26 ± 6.258µm/s vs. 57.28 ± 6.258µm/s, p=0.004; VSL, 8.18 ± 2.946µm/s vs. 21.1 ± 2.946µm/s, p=0.0003; LIN, 20.96 ± 2.886% vs. 35.92 ± 2.886%, p=0.0002; and STR, 40.22 ± 3.687% vs. 56.74 ± 3.687%, p=0.002).

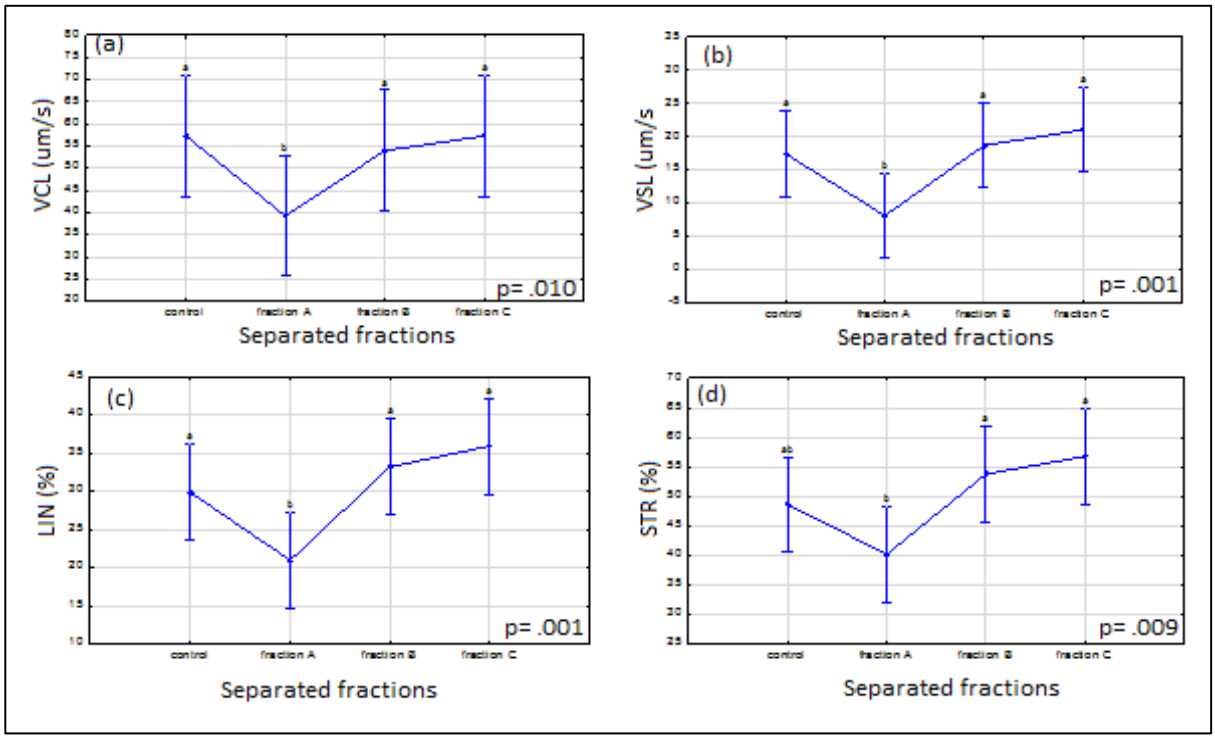

Figure 4-24: Velocity parameters after DDG centrifugation. (a) Curvilinear velocity (VCL), (b) Straight line velocity (VSL), (c) Linearity (LIN) and (d) Straightness (STR)

Significance denoted as: a differs significantly from b; ab does not differ significantly from a or b

Viability of the spermatozoa in the separated fractions is illustrated in Figure 4-25. The viability of spermatozoa is significantly decreased when the control is compared to Fraction A (89.53 ± 2.550 vs. 79.11 ± 2.550, p=0.007), and significantly increased when Fraction A is compared to Fraction B (79.11 ± 2.550 vs. 86.32 ± 2.550, p=0.044) and Fraction C (79.11 ± 2.550 vs. 88.24 ± 2.550, p=0.015).

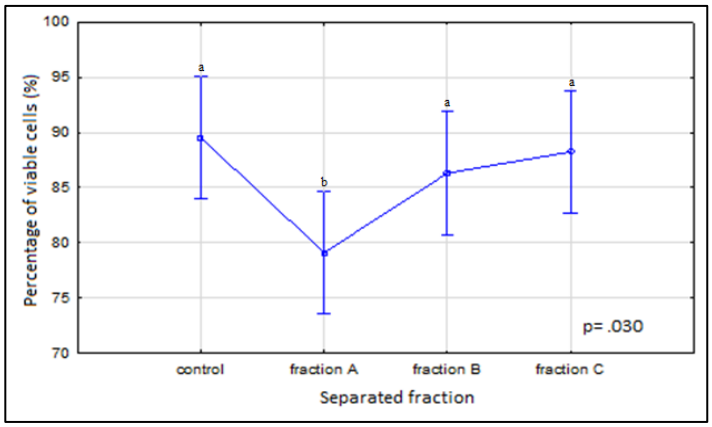

Figure 4-25: Viability of spermatozoa in fractions separated by DDG centrifugation

Significance denoted as: a differs significantly from b

4.3.2 DOUBLE WASH

The double wash preparation method had virtually no effect on the sex-chromosome ratio of the spermatozoa in the samples, as is summarized in Table 4-3. An absolute difference of 1% was observed between the neat sample and the pellet after centrifugation (55:45 vs. 54:46) (see Figure 4-26).

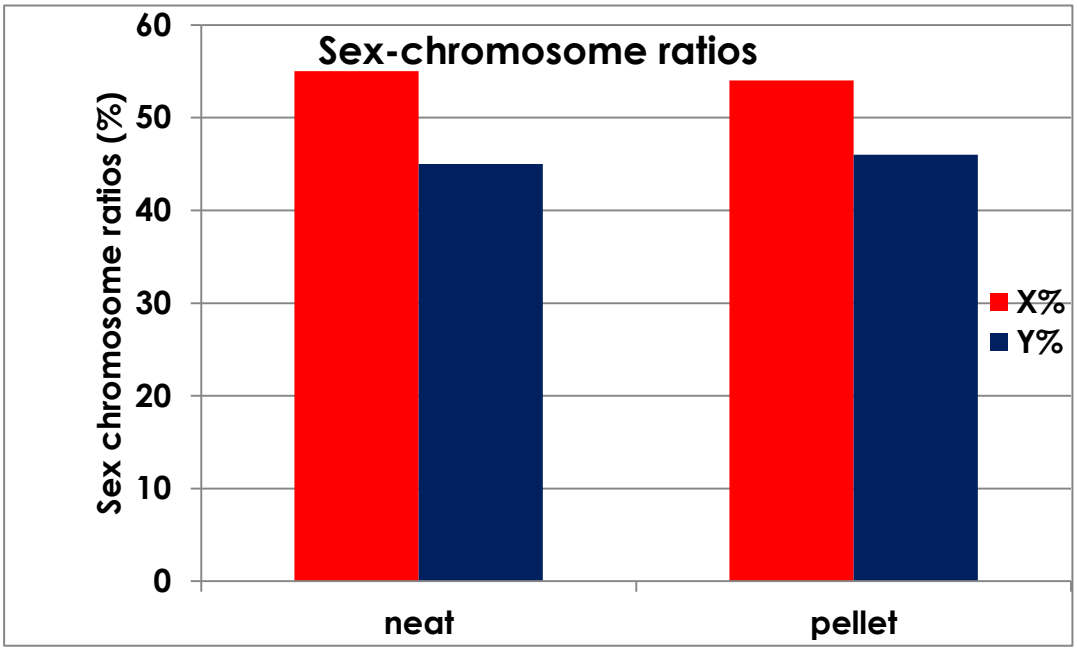

Figure 4-26: Sex-chromosome ratios before and after double-wash centrifugation

The results of the motility parameters before and after centrifugation indicate no significant change in the total motility (73.11 ± 4.517% vs. 68.19 ± 4.517%, p=0.282) but a significant decrease in progressive motility (41.14 ± 3.026% vs. 34.57 ± 3.026%, p=0.064), as indicated in Figure 4-27.

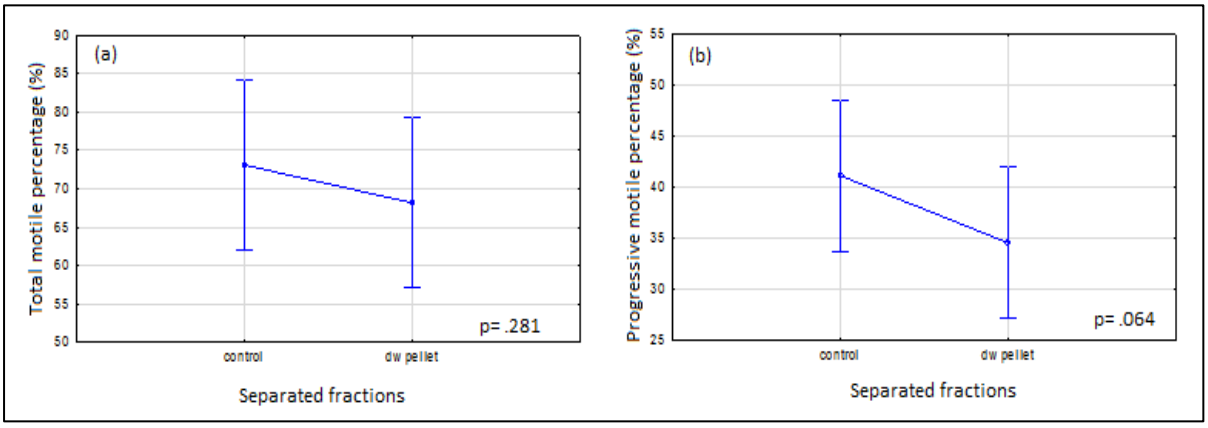

Figure 4-27: Motility parameters before and after double-wash centrifugation. (a) Total motility and (b) Progressive motility

All of the measured velocity parameters (Figure 4-28) displayed significant increases between the control sample and the resuspended pellet after centrifugation (VCL, 46.44 ± 2.138µm/s vs. 50.70 ± 2.138µm/s, p=0.206; VSL, 16.70 ± 1.540µm/s vs. 24.36 ± 1.540µm/s, p=0.010; LIN, 35.57 ± 2.181% vs. 47.96 ± 2.181%, p=0.007; STR 54.64 ± 2.739% vs. 74.31 ± 2.739%, p=0.002).

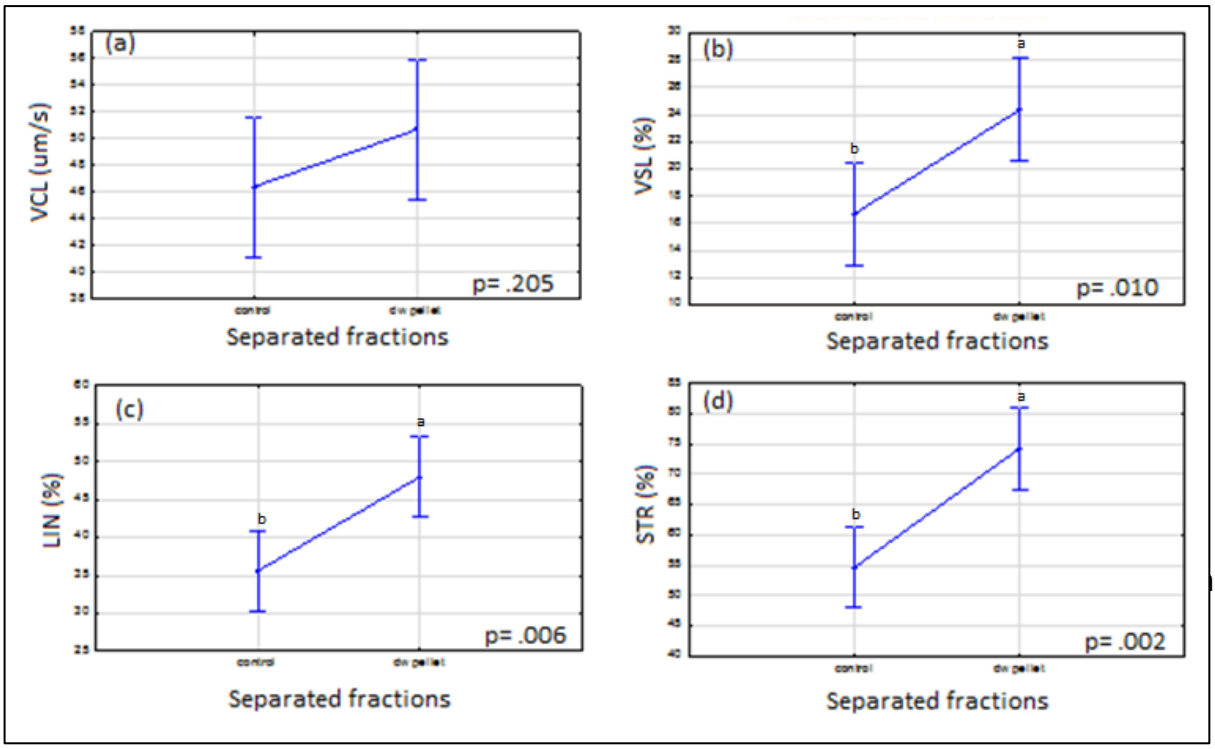

Figure 4-28: Velocity parameters before and after double-wash centrifugation. (a) Curvilinear velocity (VCL), (b) Straight line velocity (VSL), (c) Linearity (LIN) and (d) Straightness (STR)

Significance denoted as: a differs significantly from b

The viability of the spermatozoa in the pellet decreased slightly after centrifugation (80.41 ± 3.154% vs. 69.12 ± 3.154%, p=0.045) as illustrated by Figure 4-29.

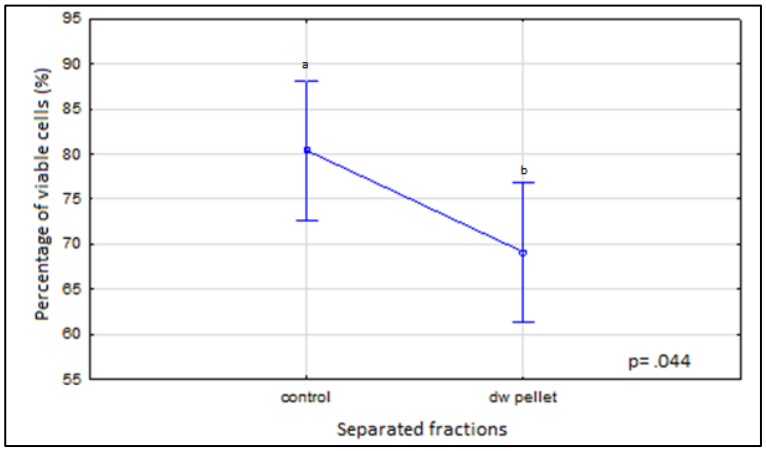

Figure 4-29: Viability of spermatozoa before and after double-wash centrifugation.

Significance denoted as: a differs significantly from b

CHAPTER 5: DISCUSSION

INTRODUCTION

The following chapter will interpret and discuss the results of the present study, and compare them to relevant literature articles. Results will be discussed in the same fashion as they were reported in Chapter 4, according to each Research Aim respectively.

5.1 RESEARCH AIM 1A: SEPARATION OF X- AND Y-CHROMOSOME BEARING SPERMATOZOA ACCORDING TO THEIR RESPECTIVE ABILITIES TO REMAIN VIABLE UPON EXPOSURE TO HOSTILE ENVIRONMENTS.

5.1.1 pH INCUBATION

The standard pH for laboratory processing of human semen as prescribed by the WHO is 7.5[3], at which there was no change observed in the sex-chromosome ratio of the samples. Table 5-1 summarizes the percentage increase of the respective sex-chromosome bearing spermatozoa.

The enrichment of X-chromosome bearing spermatozoa when incubated in 5.5 pH media suggests that these cells are better able to survive exposure to such an acidic environment. It is speculated that the bigger size of the X-chromosome bearing spermatozoa[33] (when compared to the Y-chromosome bearing spermatozoa) may allow for an increased cytoplasmic volume, which in turn could lead to higher levels of intracellular proteins and phosphates that are able to act as an intracellular buffering system, ultimately enabling the X-spermatozoa to survive in the acidic pH.

Table 5-1: Sex chromosome enrichment after pH incubation

pH incubation				
pH value	X:Y ratio		Absolute increase	% enrichment
	Pre-treatment	Post-treatment		
5.5	55:45	62:38	7 % X-chromosome increase	12.7 % X-chromosome enrichment
6.5	55:45	51:49	4% Y-chromosome increase	7% Y-chromosome enrichment
7.5	*55:45*	*55:45*	*No change*	*No change*
8.5	55:45	53:47	2% Y-chromosome increase	3.6% Y-chromosome enrichment
9.5	55:45	51:49	4% Y-chromosome increase	7% Y-chromosome enrichment

Enrichment of Y-chromosome bearing spermatozoa at pH levels 8.5 and 9.5 indicates the existence of optimal pH ranges for isolation of Y-spermatozoa. These results correlate with the findings of Hassan (2008)[55], who also demonstrated significant X-chromosome enrichment at pH values of 5.5 (75.12%) and enrichment of Y-chromosome spermatozoa at pH level 9.5 (60.28%).

When comparing motility data between the X- and Y-chromosome bearing spermatozoa enriched samples, the samples rich in Y-chromosome bearing

spermatozoa (at pH levels 8.5 and 9.5) presented a trend that suggested increased percentages of total and progressively motile spermatozoa. Results of the velocity parameters indicate that a pH of 8.5 yields the fastest swimming post-processed spermatozoa with regards to VSL, as well as LIN and STR, which is also similar to the findings of Hassan (2008)[55].

Viability results indicate that spermatozoa of both populations are most viable at a pH level of 7.5. The statistically significant decrease in viability from pH level 7.5 to pH level 5.5 represents the Y-chromosome bearing spermatozoa that did not remain viable during the incubation. This suggests that upon deposition in the vaginal regions – which often reaches pH levels lower than 4, Y-chromosome bearing spermatozoa might not be able to remain viable for a sufficient amount of time to be able to reach the cervical os. Due to the estrogen surge just prior to ovulation, there is a sudden decrease in the pH of the female reproductive tract[56] which could favour the survival of X-chromosome bearing spermatozoa. This is in accordance with Shettle's method of sex preselection, which states that in order to conceive a girl, intercourse should take place 1-2 days prior to ovulation[2].

Linear progression was significantly higher in the 8.5-pH Fraction, in which Y-chromosome bearing spermatozoa was enriched. After ovulation, as the pH in the female reproductive tract rises[57], Y-chromosome bearing spermatozoa should be

able to survive better. This increased viability, together with their increased motility abilities, should enable the Y-chromosome bearing spermatozoa to be the first to reach and fertilize the oocyte, as proposed by the Shettle's method[2], which suggests that intercourse on the day of or immediately after ovulation will increase the chances of conceiving a boy.

5.1.2 TEMPERATURE INCUBATION

Enrichment of X-chromosome bearing spermatozoa was seen upon exposure to increased temperatures. The control temperature of 37°C, which is similar to human basal body temperature and also prescribed by the WHO for laboratory processing of gametes, had no influence on the ratio of X- and Y-chromosome bearing spermatozoa (see Table 5-2). However, when the temperature was elevated to 41°C, there was a considerable increase in the incidence of X-chromosome bearing spermatozoa in the sample. This increase, when also taking into account the significant decrease in the percentage of viable cells, can be attributed to the Y-chromosome bearing spermatozoa that are not able to withstand this increase in temperature. The Whelan method[2] of preconceptual sex selection is based on the inability of the Y-chromosome bearing spermatozoa to survive exposure to the 1-2°C increase in body temperature of the female after ovulation [58], therefore recommending intercourse after ovulation when aiming to conceive a girl. According

to this method, the decreased pre-ovulatory body temperature of the female is optimal for conceiving a boy (contradicting the Shettle's method).

At 45°C there is still an increase in the X-chromosome bearing spermatozoa, although not as pronounced as during the 41°C incubation, and also an even greater decrease in the viable cell percentage. This could be attributed to the X-spermatozoa also starting to lose their viability at this very high temperature. Significant decreases in both the percentage of total and progressively motile cells at 45°C support this theory.

Table 5-2: Sex chromosome enrichment after temperature incubation

Temperature incubation				
Incubation temperature	X:Y ratio		Absolute increase	% enrichment
	Pre-treatment	Post-treatment		
37°C	*52:48*	*52:48*	*No change*	*No change*
41°C	52:48	59:41	7% X-chromosome increase	13.5% X-chromosome enrichment
45°C	52:48	54:46	2% X-chromosome increase	3.8% X-chromosome enrichment

The effect of temperature on motility parameters is summarized in Figure 5-1. At 45°C, all motility parameters decrease significantly. On average, it seems that the 4°C increase in temperature from the control (37°C to 41°C) has some beneficial effects on the motility parameters of the spermatozoa, specifically with regard to velocity parameters. This could be attributed to spermatozoa starting to become hyperactivated at 41°C.

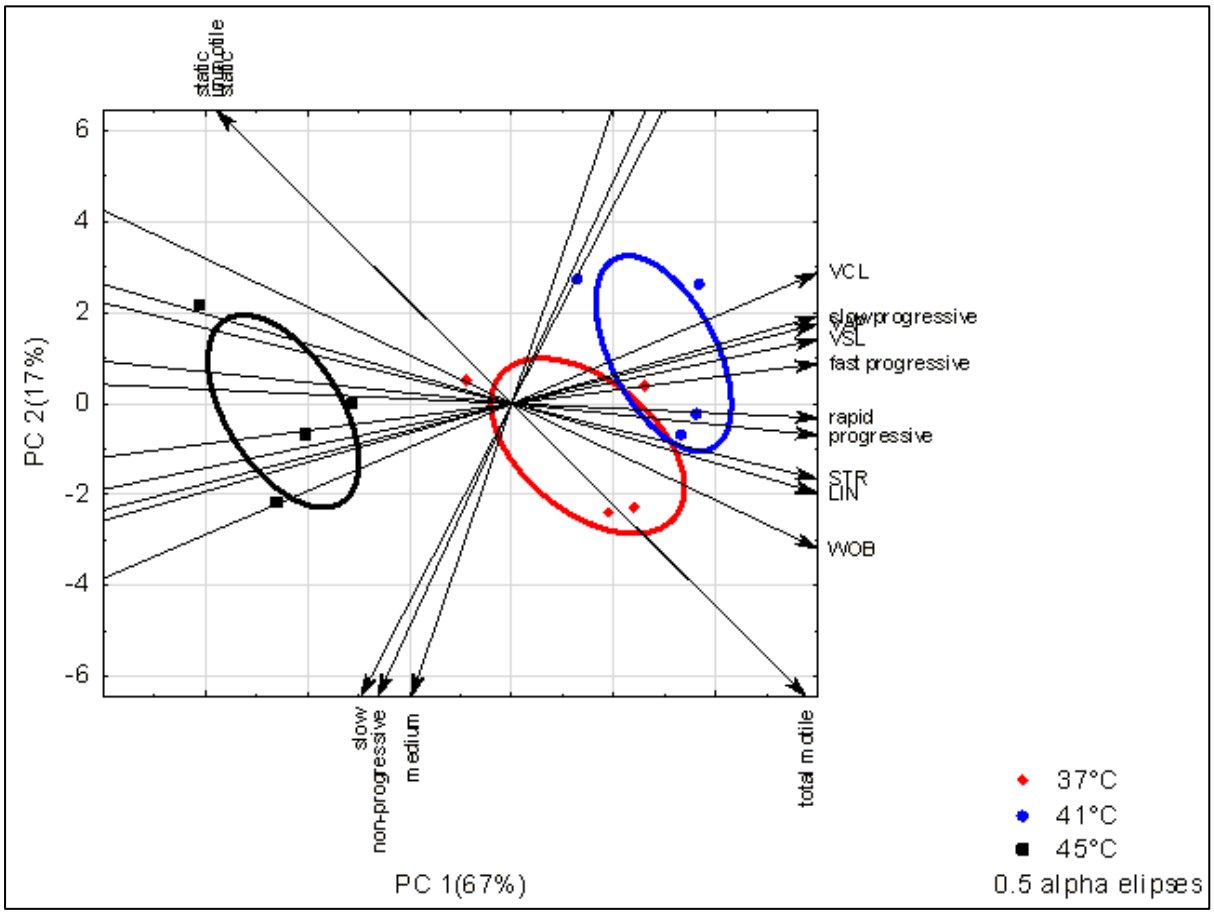

Figure 5-1: Biplot summarizing the effect of temperature on the kinematic parameters of spermatozoa

As far as we know, this study was the first to employ incubation of spermatozoa in elevated temperatures for the purposes of sperm selection, therefore there are no other studies with which the results can be compared.

5.1.3 H_2O_2 INCUBATION

The effect of H_2O_2 on the sex-chromosome ratio follows the same trend as in the temperature incubation, with X-chromosome bearing spermatozoa enriched in both the moderate (750µM) and high (1000µM) H_2O_2 fractions. Duru et al. reported that sublethal doses of H_2O_2 (10-100uM) were not associated with membrane translocation of phosphatidylserine[59] (an indicator of decreased cell viability). In the present study, there was no change in the sex-chromosome ratio or viability of the spermatozoa after incubation with 50µM of H_2O_2. This correlates with Duru et al.'s findings that spermatozoa are capable of surviving exposure to low doses of H_2O_2. In another study on the effect of H_2O_2 on spermatozoa, it was found that low-dose supplementation of H_2O_2 facilitates both hyperactivation and the initiation of the acrosome reaction[60], suggesting a possible in vitro role for low-dose H_2O_2 incubation in IVF settings.

The ability of the X-chromosome bearing spermatozoa to survive exposure to 750µM and even 1000µM of H_2O_2 (Table 5-3), suggests that once again the bigger cells may have a more sophisticated intracellular method of protection against hostile

environments. It is hypothesized that an increased intracellular store of antioxidants may exist, or that the membranes of the X-chromosome bearing spermatozoa might be more resistant to the external environment, which could be attributed to the different surface charges and/or surface protein properties[34] of the X- and Y-chromosome bearing spermatozoa.

Table 5-3: Sex chromosome enrichment after H_2O_2 incubation

H_2O_2 incubation				
H_2O_2 concentration	X:Y ratio		Absolute increase	% enrichment
	Pre-treatment	Post-treatment		
50µM	*54:46*	*54:46*	*No change*	*No change*
750µM	54:46	57:43	3% X-chromosome increase	5.5% X-chromosome enrichment
1000µM	54:46	56:44	2% X-chromosome increase	3.7% X-chromosome enrichment

As far as we know, this was the first study to employ H_2O_2 for the purpose of separating X- and Y-chromosome bearing spermatozoa, and there are no other studies in the literature with which to correlate the results.

Kinematic results – total motility, progressive motility and velocity related - indicate a significant decrease when exposed to 750µM and 1000µM, as is expected with the decrease in Y-chromosome bearing spermatozoa in the samples. The overall changes in motility resulting from the H_2O_2 incubation are summarized in Figure 5-2. As the figure indicates, 50µM H_2O_2 incubation has beneficial effects on the motility parameters of the spermatozoa, especially in terms of the velocity-related parameters. The overlapping ellipses at 750µM and 1000µM H_2O_2 concentrations indicated relatively equal deleterious effects of these elevated concentrations on the motility of the spermatozoa.

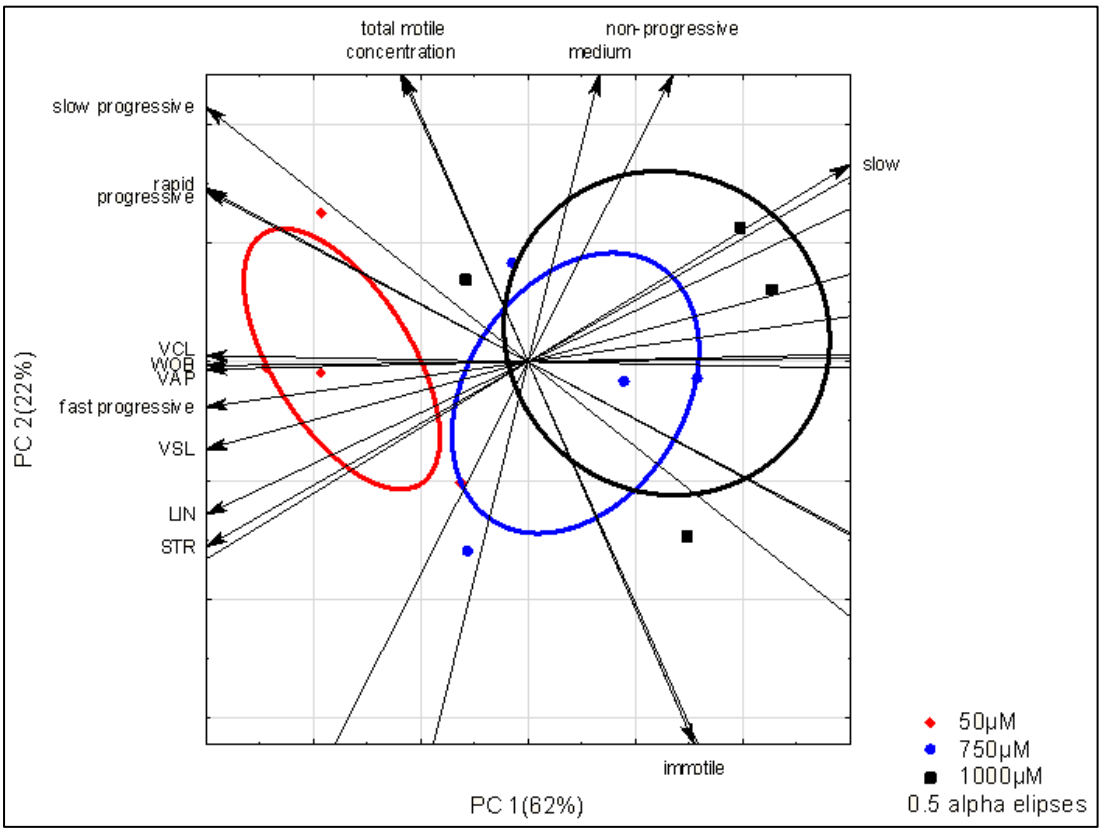

Figure 5-2: Biplot summarizing the effect of hydrogen peroxide on kinematic parameters of spermatozoa

5.1.4 *RESEARCH AIM 1B: COMPARISON OF THE EFFECTIVENESS OF MACS AND MODIFIED SWIM-UP TECHNIQUES IN SEPARATING LIVE AND DEAD SPERMATOZOA*

The results indicate that the MACS technique was consistently able to separate a higher concentration of viable cells when compared to the modified swim-up method, after different pH conditions ranging from 5.5 to 9.5. However, the motility parameters of the spermatozoa separated by each method indicated that spermatozoa separated by the modified swim-up displayed better total and progressive motility, while the viability of spermatozoa separated by modified swim-up was also better than in the case of the MACS separation. This could be due to the MACS reagents' osmolarity and low temperature (as they are refrigerated) suppressing motility and having some detrimental effect on the viability of the cells, as these are reagents are not specially developed for use on spermatozoa. The presence of more non-viable spermatozoa in the MACS fraction can also be attributed to non-specific binding of the magnetic antibodies.

Despite the increased incubation time during the modified swim-up, the method does still, by definition, select for motile cells, and therefore excludes spermatozoa that are immotile yet viable. However, since motility is important for fertilization in all instances with the exception of ICSI, the exclusion of immotile cells in addition to the non-viable spermatozoa may contribute to higher fertilization rates. Due to the

additional stress that processing steps of the MACS technique can place on the spermatozoa, it is therefore recommended that the modified swim-up method be used as an effective alternative for the selection of live spermatozoa.

5.1.5 SUMMARY OF RESULTS

The results of '*Research Aim 1: Separation of X- and Y-chromosome bearing spermatozoa based on viability'* indicate optimal enrichment of X-spermatozoa by incubation in pH-media of 5.5, incubation at 41°C as well as incubation in 750µM of H_2O_2, all of which was followed by successful isolation of viable X-chromosome bearing spermatozoa through MACS and/or modified swim-up. Incubation in alkaline media (pH levels 8.5 and 9.5) was also effective in enriching Y-chromosome bearing spermatozoa.

5.2 RESEARCH AIM 2: SEPARATION OF X- AND Y-CHROMOSOME BEARING SPERMATOZOA BASED ON THEIR PARTICULAR MOTILITY CAPACITIES.

5.2.1 DIRECT SWIM-UP

Results from the direct swim-up separation technique indicated enrichment of Y-spermatozoa in the top fraction (Fraction A) as well as slight enrichment of X-chromosome bearing spermatozoa in the bottom fraction (Fraction B) (see Table 5-4). These results, when compared with the motility data, in which a trend of increased progressive motility was observed, reconfirms the existence of different motility capacities between X- and Y-chromosome bearing spermatozoa. This also suggests that the direct swim-up method can be effective in the application of preconceptual gender selection.

Table 5-4 Sex chromosome enrichment after direct swim-up

Direct swim-up				
Isolated fraction	X:Y ratio		Absolute increase	% enrichment
	Pre-treatment	Post-treatment		
A (top)	56:44	52:48	4% Y-chromosome increase	8.3% Y-chromosome enrichment
B (bottom)	56:44	57:43	1% X-chromosome increase	1.8% X-chromosome enrichment

Studies in which the gender of offspring were recorded after women were inseminated with spermatozoa separated by swim-up, found a 86.7% success rate[61] in conceiving female offspring from the bottom fraction of the swim-up. Male offspring birth success was found to be 89.2% in the same study, and 81%[62] and 88%[41] in two separate studies when using top fractions. In these studies, insemination of the spermatozoa occurred in a timed fashion with regards to ovulation dates. Male sex preselection had the highest success rates in spite of ovulation inducing drugs, which have been reported to favour survival of X-chromosome bearing spermatozoa[63].

These studies, together with the findings of the present studies, suggest that sex preselection can be successfully achieved by sperm separation through the direct swim-up method.

5.2.2 Capillary Tube

As far as we know this was the first study of its kind to employ a capillary tube swim out technique to separate X- and Y-chromosome bearing spermatozoa based on motility. The results indicated enrichment of Y-chromosome bearing spermatozoa in the 3-9cm fractions (see Table 5-5). These fractions contained the cells that were able to swim out of the seminal plasma and into the incubation media. However, the motility data that was analysed after the incubation did not indicate significant

changes in total or progressive motility for any of the fractions. It is hypothesized that the spermatozoa that were able to reach these distances might have exhausted their energy supply by the time of analysis, which would account for the lack of increased progressively motile spermatozoa.

Table 5-5: Sex chromosome enrichment after capillary tube swim out

Capillary tube				
Isolated fraction	X:Y ratio		Absolute increase	% enrichment
	Pre-treatment	Post-treatment		
A (6-9cm)	56:44	54:46	2% Y-chromosome increase	4.5% Y-chromosome enrichment
B (3-6cm)	56:44	53:47	3% Y-chromosome increase	6.8% X-chromosome enrichment
C (0-3cm)	56:44	54:46	2% Y-chromosome increase	4.5% Y-chromosome enrichment

Linear progression and straightness was significantly decreased in Fraction A (6-9cm from semen), which could again be attributed to spermatozoa having drained their energy store, and no longer being able to display much progressive movement.

5.2.3 SUMMARY OF RESULTS

In summary, the direct swim-up protocol – as defined by the WHO – does alter the sex-chromosome ration of a sample, and also reaffirms the theory that Y-chromosome bearing spermatozoa swim faster and more progressively than X-chromosome bearing spermatozoa. The results are supported by a variety of studies in which the outcome was determined in terms of actual births and the ultimate success of sex preselection. The results of the capillary tube – which was also successful in the enrichment of Y-chromosome bearing spermatozoa, indicate that Y-spermatozoa might be able to swim faster and more progressively, but that they probably deplete their energy stores faster, which results in their motility declining by the time of analysis.

5.3 RESEARCH AIM 3: SEPARATION OF X- AND Y-CHROMOSOME BEARING SPERMATOZOA BASED ON DIFFERENCES IN SIZE/WEIGHT.

5.3.1 DOUBLE DENSITY GRADIENT CENTRIFUGATION

The results of the present study for the double density centrifugation method as defined by the WHO indicate successful enrichment of both X- and Y-chromosome bearing spermatozoa in the respective fractions. The increasingly dense layers provide a barrier for spermatozoa to penetrate through, ultimately reaching the bottom of the tube to form a pellet. The pellet – which is generally used after employing this method – was found to be enriched with Y-chromosome bearing spermatozoa (see Table 5-6).

Table 5-6: Sex chromosome enrichment after pH incubation

Double Density Gradient Centrifugation				
Isolated fraction	X:Y ratio		Absolute increase	% enrichment
	Pre-treatment	Post-treatment		
A (top)	56:44	57:43	1% X-chromosome increase	1.7% Y-chromosome enrichment
B (middle)	56:44	59:41	3% X-chromosome increase	5.4% X-chromosome enrichment
C (pellet)	56:44	52:48	4% Y-chromosome increase	9.1% Y-chromosome enrichment

This means that during the centrifugation step, the small size and increased kinematic abilities of the Y-chromosome bearing spermatozoa enabled them to penetrate the barriers to allow faster sedimentation beneath the 80% layer, compared to the sluggish bigger X-chromosome bearing spermatozoa, which only made it into the 40% layer.

Motility and viability results supported these findings, as there was significantly decreased total and progressive motility as well as percentage of viable cells in the top fraction (Fraction A) when compared to all other fractions. This indicates that most of the spermatozoa had migrated out of the seminal plasma and into either the 40% or 80% PureSperm® layer. The same trend was seen in the velocity parameters, as Fraction A displayed significantly decreased VCL and VSL measurements, as well as significantly lower linear progression and straightness.

Various studies have successfully employed double density gradient centrifugation during sex preselection. Enrichment of X-chromosome bearing spermatozoa through percoll gradient centrifugation yielded 94%[47] purity in one study, while the incidence of Y-chromosome bearing spermatozoa was enriched to 73.1%[64] in another.

5.3.2 SIMPLE DOUBLE WASH

The double wash is a relatively simple centrifugation step. In the absence of the discontinuous layers, both populations of spermatozoa sediment equally fast, and there was virtually no change observed in the sex-chromosome ratio (see Table 5-7).

Table 5-7: Sex chromosome enrichment after pH incubation

Double Wash				
Isolated fraction	X:Y ratio		Absolute increase	% enrichment
	Pre-treatment	Post-treatment		
Pellet	55:45	54:46	1% Y-chromosome increase	1.8% Y-chromosome enrichment

Centrifugation is generally considered to be detrimental to spermatozoa, disrupting membranes[65] and causing excessive production of reactive oxygen species (ROS). This fact is supported by the significant decrease in the number of viable cells after centrifugation.

However, a simultaneous increase in the VSL, LIN and STR of the spermatozoa after the wash indicates that centrifugation may have a beneficial effect on the velocity parameters of the spermatozoa, as was also observed by Makler and Jakobi

(1981), whose studies showed that shaking or centrifugation of spermatozoa led to an increase in velocity parameters[66]. The removal of the seminal plasma contributes to energy conservation through the suppression of motility due to removal of decapacitation factors contained in the seminal plasma.

Another theory for the increase in velocity parameters during a procedure that has always been considered detrimental to sperm function and quality is that the short periods of centrifugation led to the production of low, sublethal levels of ROS[67], which has also been shown to be beneficial to hyperactivation and capacitation of the spermatozoa[68]. Hyperactivation can therefore be postulated to account for the increase in velocity parameters.

5.3.3 Summary of Results

In summary, the DDG centrifugation led to enrichment of X- and Y-chromosome bearing spermatozoa in different fractions, respectively. For the purposes of gender selection, this method warrants further investigation. Top and middle layers are often discarded, as the spermatozoa in these layers are considered immature. However, if the quality of the spermatozoa in these layers can be established, the DDG could be a very useful tool for the separation of either sperm population.

The simple double wash is also defined in the WHO manual[3] as a standard method of sperm preparation, and is being utilized in the clinical setting. This method is preferable to the gradient centrifugation when the goal is not to sort spermatozoa, but rather to remove the seminal plasma.

CHAPTER 6: CONCLUSION

The enrichment of X-chromosome bearing spermatozoa through incubation in acidic media or at increased temperatures could be of particular interest in the field of ART. The potential to enrich the X-chromosome bearing spermatozoa even more by combining these (and other) methods warrants further investigation.

The effects of preparation techniques that are prescribed by the WHO – such as direct swim-up and double density gradient centrifugation – on the incidence of Y-chromosome bearing spermatozoa must be considered when preparing spermatozoa for ART. As these standard laboratory protocols do affect the sex-chromosome ratio of a sample, they should be employed with caution, to prevent the inadvertent practice of gender selection.

In conclusion, this study reconfirmed the existence of the differences in viability, motility and size between X- and Y-chromosome bearing spermatozoa. The results indicate that these differences have real potential in the development of preconceptual gender selection methods.

Although this study did yield significant results, the clinical relevance of these findings per se remains arguable. The xxx of this study was predominantly limited due to the cost implications of FISH and MACS. For future reference and studies it is advised to (i) increase sample size; (ii) perform individual FISH analyses per sample to enable establishment of statistical significance; (iii) refinement and expanding of current methods; and (iv) combining of various permutations of selection methods.

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APPENDIX A

pH INCUBATION DATA: MOTILITY

1. Static Cells

1.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=.94442, p=.44959
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	static Mean	static Std.Err.	static -95.00%	static +95.00%	N
1	pH 5.5	31.8800	5.41132	20.9053	42.8546	10
2	pH 6.5	29.6000	5.41132	18.6253	40.5746	10
3	pH 7.5	25.7000	5.41132	14.7253	36.6746	10
4	pH 8.5	28.6000	5.41132	17.6253	39.5746	10
5	pH 9.5	26.1800	5.41132	15.2053	37.1546	10

1.2 Post Hoc Tests (1)

LSD test; variable static (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 31.880	{2} 29.600	{3} 25.700	{4} 28.600	{5} 26.180
1	pH 5.5		0.54124	0.10321	0.38078	0.13181
2	pH 6.5	0.54124		0.29842	0.78829	0.36101
3	pH 7.5	0.10321	0.29842		0.43785	0.89740
4	pH 8.5	0.38078	0.78829	0.43785		0.51683
5	pH 9.5	0.13181	0.36101	0.89740	0.51683	

1.3 Post Hoc Tests (2)

LSD test; variable static (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	2.2800	3.69643	0.54124	-5.2167	9.7767
{1}-{3}	pH 5.5	pH 7.5	6.1800	3.69643	0.10321	-1.3167	13.6767
{1}-{4}	pH 5.5	pH 8.5	3.2800	3.69643	0.38078	-4.2167	10.7767
{1}-{5}	pH 5.5	pH 9.5	5.7000	3.69643	0.13181	-1.7967	13.1967
{2}-{3}	pH 6.5	pH 7.5	3.9000	3.69643	0.29842	-3.5967	11.3967
{2}-{4}	pH 6.5	pH 8.5	1.0000	3.69643	0.78829	-6.4967	8.4967
{2}-{5}	pH 6.5	pH 9.5	3.4200	3.69643	0.36101	-4.0767	10.9167
{3}-{4}	pH 7.5	pH 8.5	-2.9000	3.69643	0.43785	-10.396	4.5967
{3}-{5}	pH 7.5	pH 9.5	-0.4800	3.69643	0.89740	-7.9767	7.0167
{4}-{5}	pH 8.5	pH 9.5	2.4200	3.69643	0.51683	-5.0767	9.9167

2. Non-Progressive Cells

2.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=.32452, p=.85966
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	pH 5.5	32.2600(	2.64569(	26.8942(	37.6257'	10
2	pH 6.5	34.2800(	2.64569(	28.9142(	39.6457'	10
3	pH 7.5	31.3100(	2.64569(	25.9442(	36.6757'	10
4	pH 8.5	32.3500(	2.64569(	26.9842(	37.7157'	10
5	pH 9.5	33.6000(	2.64569(	28.2342(	38.9657'	10

2.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 32.260	{2} 34.280	{3} 31.310	{4} 32.350	{5} 33.600
1	pH 5.5		0.49361:	0.74686:	0.97558'	0.64913:
2	pH 6.5	0.49361:		0.31599:	0.51294-	0.81721:
3	pH 7.5	0.74686:	0.31599:		0.72385:	0.43813
4	pH 8.5	0.97558'	0.51294-	0.72385:		0.67121:
5	pH 9.5	0.64913:	0.81721:	0.43813	0.67121:	

2.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	-2.0200(	2.92067'	0.49361:	-7.9434'	3.90341:
{1}-{3}	pH 5.5	pH 7.5	0.9500(	2.92067'	0.74686:	-4.9734'	6.87341:
{1}-{4}	pH 5.5	pH 8.5	-0.0900(	2.92067'	0.97558'	-6.0134'	5.83341:
{1}-{5}	pH 5.5	pH 9.5	-1.3400(	2.92067'	0.64913:	-7.2634'	4.58341:
{2}-{3}	pH 6.5	pH 7.5	2.9700(	2.92067'	0.31599:	-2.9534'	8.89341:
{2}-{4}	pH 6.5	pH 8.5	1.9300(	2.92067'	0.51294-	-3.9934'	7.85341:
{2}-{5}	pH 6.5	pH 9.5	0.6800(	2.92067'	0.81721:	-5.2434'	6.60341:
{3}-{4}	pH 7.5	pH 8.5	-1.0400(	2.92067'	0.72385:	-6.9634'	4.88341:
{3}-{5}	pH 7.5	pH 9.5	-2.2900(	2.92067'	0.43813	-8.2134'	3.63341:
{4}-{5}	pH 8.5	pH 9.5	-1.2500(	2.92067'	0.67121:	-7.1734'	4.67341:

3. Progressive Cells

3.1.1Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=1.0519, p=.39431
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	progressive Mean	progressive Std.Err.	progressive -95.00%	progressive +95.00%	N
1	pH 5.5	35.8700	5.83710	24.0318	47.7082	10
2	pH 6.5	36.1000	5.83710	24.2618	47.9382	10
3	pH 7.5	42.9700	5.83710	31.1318	54.8082	10
4	pH 8.5	39.0100	5.83710	27.1718	50.8482	10
5	pH 9.5	40.2300	5.83710	28.3918	52.0682	10

3.2 Post Hoc Tests (1)

LSD test; variable progressive (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 35.870	{2} 36.100	{3} 42.970	{4} 39.010	{5} 40.230
1	pH 5.5		0.955563	0.091806	0.448638	0.294551
2	pH 6.5	0.955563		0.102394	0.482314	0.320380
3	pH 7.5	0.091806	0.102394		0.340436	0.508099
4	pH 8.5	0.448638	0.482314	0.340436		0.767690
5	pH 9.5	0.294551	0.320380	0.508099	0.767690	

3.3 Post Hoc Tests (2)

LSD test; variable progressive (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	-0.2300	4.098925	0.955563	-8.543	8.0830
{1}-{3}	pH 5.5	pH 7.5	-7.1000	4.098925	0.091806	-15.413	1.2130
{1}-{4}	pH 5.5	pH 8.5	-3.1400	4.098925	0.448638	-11.453	5.1730
{1}-{5}	pH 5.5	pH 9.5	-4.3600	4.098925	0.294551	-12.673	3.9530
{2}-{3}	pH 6.5	pH 7.5	-6.8700	4.098925	0.102394	-15.183	1.4430
{2}-{4}	pH 6.5	pH 8.5	-2.9100	4.098925	0.482314	-11.223	5.4030
{2}-{5}	pH 6.5	pH 9.5	-4.1300	4.098925	0.320380	-12.443	4.1830
{3}-{4}	pH 7.5	pH 8.5	3.9600	4.098925	0.340436	-4.353	12.2730
{3}-{5}	pH 7.5	pH 9.5	2.7400	4.098925	0.508099	-5.573	11.0530
{4}-{5}	pH 8.5	pH 9.5	-1.2200	4.098925	0.767690	-9.533	7.0930

4. Total Motile

4.1.1Means Table

Cell No.	pH level	total motile Mean	total motile Std.Err.	total motile -95.00%	total motile +95.00%	N
	pH level; LS Means (Spreadsheet2) Current effect: F(4, 36)=.94067, p=.45163 Type III decomposition Include condition: stage="pH"					
1	pH 5.5	68.1300	5.41130	57.1553	79.1046	10
2	pH 6.5	70.3800	5.41130	59.4053	81.3546	10
3	pH 7.5	74.2800	5.41130	63.3053	85.2546	10
4	pH 8.5	71.3600	5.41130	60.3853	82.3346	10
5	pH 9.5	73.8300	5.41130	62.8553	84.8046	10

4.2 Post Hoc Tests (1)

Cell No.	pH level	{1} 68.130	{2} 70.380	{3} 74.280	{4} 71.360	{5} 73.830
	LSD test; variable total motile (Spreadsheet2) Probabilities for Post Hoc Tests Effect: pH level Include condition: stage="pH"					
1	pH 5.5		0.54682	0.10507	0.38834	0.13207
2	pH 6.5	0.54682		0.29875	0.79257	0.35720
3	pH 7.5	0.10507	0.29875		0.43505	0.90385
4	pH 8.5	0.38834	0.79257	0.43505		0.50855
5	pH 9.5	0.13207	0.35720	0.90385	0.50855	

4.3 Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable total motile (Spreadsheet2) Simultaneous confidence intervals Effect: pH level Include condition: stage="pH"						
{1}-{2}	pH 5.5	pH 6.5	-2.2500	3.69903	0.54682	-9.752	5.2519
{1}-{3}	pH 5.5	pH 7.5	-6.1500	3.69903	0.10507	-13.652	1.3519
{1}-{4}	pH 5.5	pH 8.5	-3.2300	3.69903	0.38834	-10.732	4.2719
{1}-{5}	pH 5.5	pH 9.5	-5.7000	3.69903	0.13207	-13.202	1.8019
{2}-{3}	pH 6.5	pH 7.5	-3.9000	3.69903	0.29875	-11.402	3.6019
{2}-{4}	pH 6.5	pH 8.5	-0.9800	3.69903	0.79257	-8.482	6.5219
{2}-{5}	pH 6.5	pH 9.5	-3.4500	3.69903	0.35720	-10.952	4.0519
{3}-{4}	pH 7.5	pH 8.5	2.9200	3.69903	0.43505	-4.582	10.4219
{3}-{5}	pH 7.5	pH 9.5	0.4500	3.69903	0.90385	-7.052	7.9519
{4}-{5}	pH 8.5	pH 9.5	-2.4700	3.69903	0.50855	-9.972	5.0319

5. Fast Progressive

5.1.1Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=1.8979, p=.13203
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	fast progressive Mean	fast progressive Std.Err.	fast progressive -95.00%	fast progressive +95.00%	N
1	pH 5.5	8.8900(	3.34862(	2.09867	15.68133	10
2	pH 6.5	9.4100(	3.34862(	2.61867	16.20133	10
3	pH 7.5	15.1500(	3.34862(	8.35867	21.94133	10
4	pH 8.5	16.9900(	3.34862(	10.19867	23.78133	10
5	pH 9.5	11.2000(	3.34862(	4.40867	17.99133	10

5.2 Post Hoc Tests (1)

LSD test; variable fast progressive (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 8.8900	{2} 9.4100	{3} 15.150	{4} 16.990	{5} 11.200
1	pH 5.5		0.88831	0.09724	0.03406	0.53376
2	pH 6.5	0.88831		0.12720	0.04650	0.62929
3	pH 7.5	0.09724	0.12720		0.61978	0.28979
4	pH 8.5	0.03406	0.04650	0.61978		0.12403
5	pH 9.5	0.53376	0.62929	0.28979	0.12403	

5.3 Post Hoc Tests (2)

LSD test; variable fast progressive (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	-0.5200(	3.67647	0.88831	-7.976	6.9362
{1}-{3}	pH 5.5	pH 7.5	-6.2600(	3.67647	0.09724	-13.716	1.1962
{1}-{4}	pH 5.5	pH 8.5	-8.1000(	3.67647	0.03406	-15.556	-0.6437(
{1}-{5}	pH 5.5	pH 9.5	-2.3100(	3.67647	0.53376	-9.766	5.1462
{2}-{3}	pH 6.5	pH 7.5	-5.7400(	3.67647	0.12720	-13.196	1.7162
{2}-{4}	pH 6.5	pH 8.5	-7.5800(	3.67647	0.04650	-15.036	-0.1237(
{2}-{5}	pH 6.5	pH 9.5	-1.7900(	3.67647	0.62929	-9.246	5.6662
{3}-{4}	pH 7.5	pH 8.5	-1.8400(	3.67647	0.61978	-9.296	5.6162
{3}-{5}	pH 7.5	pH 9.5	3.9500(	3.67647	0.28979	-3.506	11.4062
{4}-{5}	pH 8.5	pH 9.5	5.7900(	3.67647	0.12403	-1.666	13.2462

6. Slow Progressive

6.1.1Means Table

Cell No.	pH level	slow progressive Mean	slow progressive Std.Err.	slow progressive -95.00%	slow progressive +95.00%	N
	pH level; LS Means (Spreadsheet2) Current effect: F(4, 36)=1.4945, p=.22435 Type III decomposition Include condition: stage="pH"					
1	pH 5.5	26.9700(	3.59662	19.6757	34.2642	10
2	pH 6.5	26.6700(	3.59662	19.3757	33.9642	10
3	pH 7.5	27.8200(	3.59662	20.5257	35.1142	10
4	pH 8.5	22.0500(	3.59662	14.7557	29.3442	10
5	pH 9.5	29.0300(	3.59662	21.7357	36.3242	10

6.1.2Post Hoc Tests (1)

Cell No.	pH level	{1} 26.970	{2} 26.670	{3} 27.820	{4} 22.050	{5} 29.030
	LSD test; variable slow progressive (Spreadsheet2) Probabilities for Post Hoc Tests Effect: pH level Include condition: stage="pH"					
1	pH 5.5		0.92273	0.78355	0.11791	0.50668
2	pH 6.5	0.92273		0.71028	0.14124	0.44726
3	pH 7.5	0.78355	0.71028		0.06840	0.69593
4	pH 8.5	0.11791	0.14124	0.06840		0.02911
5	pH 9.5	0.50668	0.44726	0.69593	0.02911	

6.1.3Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable slow progressive (Spreadsheet2) Simultaneous confidence intervals Effect: pH level Include condition: stage="pH"						
{1}-{2}	pH 5.5	pH 6.5	0.3000(	3.07133	0.92273	-5.929(	6.5289
{1}-{3}	pH 5.5	pH 7.5	-0.8500(	3.07133	0.78355	-7.079(	5.3789
{1}-{4}	pH 5.5	pH 8.5	4.9200(	3.07133	0.11791	-1.309(	11.1489
{1}-{5}	pH 5.5	pH 9.5	-2.0600(	3.07133	0.50668	-8.289(	4.1689
{2}-{3}	pH 6.5	pH 7.5	-1.1500(	3.07133	0.71028	-7.379(	5.0789
{2}-{4}	pH 6.5	pH 8.5	4.6200(	3.07133	0.14124	-1.609(	10.8489
{2}-{5}	pH 6.5	pH 9.5	-2.3600(	3.07133	0.44726	-8.589(	3.8689
{3}-{4}	pH 7.5	pH 8.5	5.7700(	3.07133	0.06840	-0.459(	11.9989
{3}-{5}	pH 7.5	pH 9.5	-1.2100(	3.07133	0.69593	-7.439(	5.0189
{4}-{5}	pH 8.5	pH 9.5	-6.9800(	3.07133	0.02911	-13.209(	-0.7510

7. Non-Progressive

7.1.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=.32452, p=.85966
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	pH 5.5	32.2600	2.64569	26.8942	37.6257	10
2	pH 6.5	34.2800	2.64569	28.9142	39.6457	10
3	pH 7.5	31.3100	2.64569	25.9442	36.6757	10
4	pH 8.5	32.3500	2.64569	26.9842	37.7157	10
5	pH 9.5	33.6000	2.64569	28.2342	38.9657	10

7.1.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 32.260	{2} 34.280	{3} 31.310	{4} 32.350	{5} 33.600
1	pH 5.5		0.49361	0.74686	0.97558	0.64913
2	pH 6.5	0.49361		0.31599	0.51294	0.81721
3	pH 7.5	0.74686	0.31599		0.72385	0.43813
4	pH 8.5	0.97558	0.51294	0.72385		0.67121
5	pH 9.5	0.64913	0.81721	0.43813	0.67121	

7.1.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	-2.0200	2.92067	0.49361	-7.9434	3.90341
{1}-{3}	pH 5.5	pH 7.5	0.9500	2.92067	0.74686	-4.9734	6.87341
{1}-{4}	pH 5.5	pH 8.5	-0.0900	2.92067	0.97558	-6.0134	5.83341
{1}-{5}	pH 5.5	pH 9.5	-1.3400	2.92067	0.64913	-7.2634	4.58341
{2}-{3}	pH 6.5	pH 7.5	2.9700	2.92067	0.31599	-2.9534	8.89341
{2}-{4}	pH 6.5	pH 8.5	1.9300	2.92067	0.51294	-3.9934	7.85341
{2}-{5}	pH 6.5	pH 9.5	0.6800	2.92067	0.81721	-5.2434	6.60341
{3}-{4}	pH 7.5	pH 8.5	-1.0400	2.92067	0.72385	-6.9634	4.88341
{3}-{5}	pH 7.5	pH 9.5	-2.2900	2.92067	0.43813	-8.2134	3.63341
{4}-{5}	pH 8.5	pH 9.5	-1.2500	2.92067	0.67121	-7.1734	4.67341

8. VCL

8.1.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=1.2580, p=.30437
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	VCL Mean	VCL Std.Err.	VCL -95.00%	VCL +95.00%	N
1	pH 5.5	45.2200	5.27763	34.5164	55.9235	10
2	pH 6.5	44.8900	5.27763	34.1864	55.5935	10
3	pH 7.5	52.0900	5.27763	41.3864	62.7935	10
4	pH 8.5	51.9900	5.27763	41.2864	62.6935	10
5	pH 9.5	47.6500	5.27763	36.9464	58.3535	10

8.1.2 Post Hoc Tests (1)

LSD test; variable VCL (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 45.220	{2} 44.890	{3} 52.090	{4} 51.990	{5} 47.650
1	pH 5.5		0.941101	0.130143	0.135650	0.587156
2	pH 6.5	0.941101		0.113241	0.118161	0.537677
3	pH 7.5	0.130143	0.113241		0.982137	0.323477
4	pH 8.5	0.135650	0.118161	0.982137		0.334349
5	pH 9.5	0.587156	0.537677	0.323477	0.334349	

8.1.3 Post Hoc Tests (2)

LSD test; variable VCL (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	0.3300	4.43528	0.941101	-8.6652	9.3251
{1}-{3}	pH 5.5	pH 7.5	-6.8700	4.43528	0.130143	-15.8652	2.1251
{1}-{4}	pH 5.5	pH 8.5	-6.7700	4.43528	0.135650	-15.7652	2.2251
{1}-{5}	pH 5.5	pH 9.5	-2.4300	4.43528	0.587156	-11.4252	6.5651
{2}-{3}	pH 6.5	pH 7.5	-7.2000	4.43528	0.113241	-16.1952	1.7951
{2}-{4}	pH 6.5	pH 8.5	-7.1000	4.43528	0.118161	-16.0952	1.8951
{2}-{5}	pH 6.5	pH 9.5	-2.7600	4.43528	0.537677	-11.7552	6.2351
{3}-{4}	pH 7.5	pH 8.5	0.1000	4.43528	0.982137	-8.8952	9.0951
{3}-{5}	pH 7.5	pH 9.5	4.4400	4.43528	0.323477	-4.5552	13.4351
{4}-{5}	pH 8.5	pH 9.5	4.3400	4.43528	0.334349	-4.6552	13.3351

9. VSL

9.1.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=1.9024, p=.13125
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	VSL Mean	VSL Std.Err.	VSL -95.00%	VSL +95.00%	N
1	pH 5.5	16.2500	3.06649	10.0308	22.4691	10
2	pH 6.5	16.1700	3.06649	9.9508	22.3891	10
3	pH 7.5	20.8700	3.06649	14.6508	27.0891	10
4	pH 8.5	22.5800	3.06649	16.3608	28.7991	10
5	pH 9.5	17.6800	3.06649	11.4608	23.8991	10

9.1.2 Post Hoc Tests (1)

LSD test; variable VSL (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 16.250	{2} 16.170	{3} 20.870	{4} 22.580	{5} 17.680
1	pH 5.5		0.97854	0.12660	0.03897	0.63128
2	pH 6.5	0.97854		0.12037	0.03670	0.61238
3	pH 7.5	0.12660	0.12037		0.56631	0.28741
4	pH 8.5	0.03897	0.03670	0.56631		0.10588
5	pH 9.5	0.63128	0.61238	0.28741	0.10588	

9.1.3 Post Hoc Tests (2)

LSD test; variable VSL (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	0.0800	2.95427	0.97854	-5.911	6.0715
{1}-{3}	pH 5.5	pH 7.5	-4.6200	2.95427	0.12660	-10.611	1.3715
{1}-{4}	pH 5.5	pH 8.5	-6.3300	2.95427	0.03897	-12.321	-0.3384
{1}-{5}	pH 5.5	pH 9.5	-1.4300	2.95427	0.63128	-7.421	4.5615
{2}-{3}	pH 6.5	pH 7.5	-4.7000	2.95427	0.12037	-10.691	1.2915
{2}-{4}	pH 6.5	pH 8.5	-6.4100	2.95427	0.03670	-12.401	-0.4184
{2}-{5}	pH 6.5	pH 9.5	-1.5100	2.95427	0.61238	-7.501	4.4815
{3}-{4}	pH 7.5	pH 8.5	-1.7100	2.95427	0.56631	-7.701	4.2815
{3}-{5}	pH 7.5	pH 9.5	3.1900	2.95427	0.28741	-2.801	9.1815
{4}-{5}	pH 8.5	pH 9.5	4.9000	2.95427	0.10588	-1.091	10.8915

10. VAP

10.1.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=1.4444, p=.23944
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	VAP Mean	VAP Std.Err.	VAP -95.00%	VAP +95.00%	N
1	pH 5.5	26.8200	3.54945	19.6213	34.0186	10
2	pH 6.5	26.2200	3.54945	19.0213	33.4186	10
3	pH 7.5	31.2300	3.54945	24.0313	38.4286	10
4	pH 8.5	32.1000	3.54945	24.9013	39.2986	10
5	pH 9.5	27.8200	3.54945	20.6213	35.0186	10

10.1.2 Post Hoc Tests (1)

LSD test; variable VAP (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 26.820	{2} 26.220	{3} 31.230	{4} 32.100	{5} 27.820
1	pH 5.5		0.84912	0.16760	0.10040	0.75130
2	pH 6.5	0.84912		0.11834	0.06852	0.61249
3	pH 7.5	0.16760	0.11834		0.78272	0.28338
4	pH 8.5	0.10040	0.06852	0.78272		0.18015
5	pH 9.5	0.75130	0.61249	0.28338	0.18015	

10.1.3 Post Hoc Tests (2)

LSD test; variable VAP (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	0.60000	3.13130	0.84912	-5.7506	6.95057
{1}-{3}	pH 5.5	pH 7.5	-4.4100	3.13130	0.16760	-10.760	1.94057
{1}-{4}	pH 5.5	pH 8.5	-5.2800	3.13130	0.10040	-11.630	1.07057
{1}-{5}	pH 5.5	pH 9.5	-1.0000	3.13130	0.75130	-7.350	5.35057
{2}-{3}	pH 6.5	pH 7.5	-5.0100	3.13130	0.11834	-11.360	1.34057
{2}-{4}	pH 6.5	pH 8.5	-5.8800	3.13130	0.06852	-12.230	0.47057
{2}-{5}	pH 6.5	pH 9.5	-1.6000	3.13130	0.61249	-7.950	4.75057
{3}-{4}	pH 7.5	pH 8.5	-0.8700	3.13130	0.78272	-7.220	5.48057
{3}-{5}	pH 7.5	pH 9.5	3.4100	3.13130	0.28338	-2.940	9.76057
{4}-{5}	pH 8.5	pH 9.5	4.2800	3.13130	0.18015	-2.070	10.63057

11. LIN

11.1.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=3.6155, p=.01411
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	LIN Mean	LIN Std.Err.	LIN -95.00%	LIN +95.00%	N
1	pH 5.5	34.9000	2.26408	30.3082	39.4917	10
2	pH 6.5	35.3600	2.26408	30.7682	39.9517	10
3	pH 7.5	37.7700	2.26408	33.1782	42.3617	10
4	pH 8.5	41.0200	2.26408	36.4282	45.6117	10
5	pH 9.5	36.4700	2.26408	31.8782	41.0617	10

11.1.2 Post Hoc Tests (1)

LSD test; variable LIN (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 34.900	{2} 35.360	{3} 37.770	{4} 41.020	{5} 36.470
1	pH 5.5		0.80241	0.12453	0.00188	0.39529
2	pH 6.5	0.80241		0.19495	0.00373	0.54682
3	pH 7.5	0.12453	0.19495		0.08335	0.48081
4	pH 8.5	0.00188	0.00373	0.08335		0.01738
5	pH 9.5	0.39529	0.54682	0.48081	0.01738	

11.1.3 Post Hoc Tests (2)

LSD test; variable LIN (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	-0.4600	1.82485	0.80241	-4.1609	3.2409
{1}-{3}	pH 5.5	pH 7.5	-2.8700	1.82485	0.12453	-6.5709	0.8309
{1}-{4}	pH 5.5	pH 8.5	-6.1200	1.82485	0.00188	-9.8209	-2.4190
{1}-{5}	pH 5.5	pH 9.5	-1.5700	1.82485	0.39529	-5.2709	2.1309
{2}-{3}	pH 6.5	pH 7.5	-2.4100	1.82485	0.19495	-6.1109	1.2909
{2}-{4}	pH 6.5	pH 8.5	-5.6600	1.82485	0.00373	-9.3609	-1.9590
{2}-{5}	pH 6.5	pH 9.5	-1.1100	1.82485	0.54682	-4.8109	2.5909
{3}-{4}	pH 7.5	pH 8.5	-3.2500	1.82485	0.08335	-6.9509	0.4509
{3}-{5}	pH 7.5	pH 9.5	1.3000	1.82485	0.48081	-2.4009	5.0009
{4}-{5}	pH 8.5	pH 9.5	4.5500	1.82485	0.01738	0.8490	8.2509

12. STR

12.1.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=3.6450, p=.01360
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	STR Mean	STR Std.Err.	STR -95.00%	STR +95.00%	N
1	pH 5.5	59.2500	2.75944	53.6535	64.8464	10
2	pH 6.5	60.5400	2.75944	54.9435	66.1364	10
3	pH 7.5	63.5300	2.75944	57.9335	69.1264	10
4	pH 8.5	66.8900	2.75944	61.2935	72.4864	10
5	pH 9.5	62.5200	2.75944	56.9235	68.1164	10

12.1.2 Post Hoc Tests (1)

LSD test; variable STR (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 59.250	{2} 60.540	{3} 63.530	{4} 66.890	{5} 62.520
1	pH 5.5		0.55818	0.05765	0.001257	0.14279
2	pH 6.5	0.55818		0.17919	0.006172	0.37034
3	pH 7.5	0.05765	0.17919		0.13243	0.64632
4	pH 8.5	0.001257	0.006172	0.13243		0.05283
5	pH 9.5	0.14279	0.37034	0.64632	0.05283	

12.1.3 Post Hoc Tests (2)

LSD test; variable STR (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	-1.2900	2.18257	0.55818	-5.7165	3.13647
{1}-{3}	pH 5.5	pH 7.5	-4.2800	2.18257	0.05765	-8.7065	0.14647
{1}-{4}	pH 5.5	pH 8.5	-7.6400	2.18257	0.00125	-12.0665	-3.21353
{1}-{5}	pH 5.5	pH 9.5	-3.2700	2.18257	0.14279	-7.6965	1.15647
{2}-{3}	pH 6.5	pH 7.5	-2.9900	2.18257	0.17919	-7.4165	1.43647
{2}-{4}	pH 6.5	pH 8.5	-6.3500	2.18257	0.00617	-10.7765	-1.92353
{2}-{5}	pH 6.5	pH 9.5	-1.9800	2.18257	0.37034	-6.4065	2.44647
{3}-{4}	pH 7.5	pH 8.5	-3.3600	2.18257	0.13243	-7.7865	1.06647
{3}-{5}	pH 7.5	pH 9.5	1.0100	2.18257	0.64632	-3.4165	5.43647
{4}-{5}	pH 8.5	pH 9.5	4.3700	2.18257	0.05283	-0.0565	8.79647

13. WOB

13.1.1 Means Table

pH level; LS Means (Spreadsheet2)
Current effect: F(4, 36)=1.8981, p=.13199
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	WOB Mean	WOB Std.Err.	WOB -95.00%	WOB +95.00%	N
1	pH 5.5	58.3800	1.40354	55.5334	61.2265	10
2	pH 6.5	58.1900	1.40354	55.3434	61.0365	10
3	pH 7.5	58.8400	1.40354	55.9934	61.6865	10
4	pH 8.5	60.8600	1.40354	58.0134	63.7065	10
5	pH 9.5	58.0300	1.40354	55.1834	60.8765	10

13.1.2 Post Hoc Tests (1)

LSD test; variable WOB (Spreadsheet2)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 58.380	{2} 58.190	{3} 58.840	{4} 60.860	{5} 58.030
1	pH 5.5		0.87395	0.70117	0.04418	0.77020
2	pH 6.5	0.87395		0.58803	0.03099	0.89372
3	pH 7.5	0.70117	0.58803		0.09802	0.50015
4	pH 8.5	0.04418	0.03099	0.09802		0.02274
5	pH 9.5	0.77020	0.89372	0.50015	0.02274	

13.1.3 Post Hoc Tests (2)

LSD test; variable WOB (Spreadsheet2)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	0.19000	1.18920	0.87395	-2.22182	2.60182
{1}-{3}	pH 5.5	pH 7.5	-0.46000	1.18920	0.70117	-2.87182	1.95182
{1}-{4}	pH 5.5	pH 8.5	-2.48000	1.18920	0.04418	-4.89182	-0.06817
{1}-{5}	pH 5.5	pH 9.5	0.35000	1.18920	0.77020	-2.06182	2.76182
{2}-{3}	pH 6.5	pH 7.5	-0.65000	1.18920	0.58803	-3.06182	1.76182
{2}-{4}	pH 6.5	pH 8.5	-2.67000	1.18920	0.03099	-5.08182	-0.25817
{2}-{5}	pH 6.5	pH 9.5	0.16000	1.18920	0.89372	-2.25182	2.57182
{3}-{4}	pH 7.5	pH 8.5	-2.02000	1.18920	0.09802	-4.43182	0.39182
{3}-{5}	pH 7.5	pH 9.5	0.81000	1.18920	0.50015	-1.60182	3.22182
{4}-{5}	pH 8.5	pH 9.5	2.83000	1.18920	0.02274	0.41818	5.24182

pH Incubation Data: Viability

14. Viability

14.1.1 Means Table

Cell No.	pH level	viable percentage Mean	viable percentage Std.Err.	viable percentage -95.00%	viable percentage +95.00%	N
	pH level; LS Means (Spreadsheet3) Current effect: F(4, 32)=2.7960, p=.04251 Type III decomposition Include condition: stage="pH"					
1	pH 5.5	58.6959	3.22953	52.1176	65.2743	9
2	pH 6.5	67.4762	3.22953	60.8978	74.0545	9
3	pH 7.5	70.7456	3.22953	64.1673	77.3240	9
4	pH 8.5	66.1493	3.22953	59.5709	72.7276	9
5	pH 9.5	63.8433	3.22953	57.2650	70.4217	9

14.1.2 Post Hoc Tests (1)

Cell No.	pH level	{1} 58.696	{2} 67.476	{3} 70.746	{4} 66.149	{5} 63.843
	LSD test; variable viable percentage (Spreadsheet3) Probabilities for Post Hoc Tests Effect: pH level Include condition: stage="pH"					
1	pH 5.5		0.02751	0.00335	0.05867	0.18521
2	pH 6.5	0.02751		0.39616	0.72934	0.34642
3	pH 7.5	0.00335	0.39616		0.23549	0.07880
4	pH 8.5	0.05867	0.72934	0.23549		0.54841
5	pH 9.5	0.18521	0.34642	0.07880	0.54841	

14.1.3 Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable viable percentage (Spreadsheet3) Simultaneous confidence intervals Effect: pH level Include condition: stage="pH"						
{1}-{2}	pH 5.5	pH 6.5	-8.7802	3.80154	0.027513	-16.5237	-1.03675
{1}-{3}	pH 5.5	pH 7.5	-12.0497	3.80154	0.003352	-19.7932	-4.30620
{1}-{4}	pH 5.5	pH 8.5	-7.4533	3.80154	0.058679	-15.1968	0.29016
{1}-{5}	pH 5.5	pH 9.5	-5.1474	3.80154	0.185213	-12.8909	2.59609
{2}-{3}	pH 6.5	pH 7.5	-3.2695	3.80154	0.396168	-11.0129	4.47403
{2}-{4}	pH 6.5	pH 8.5	1.3269	3.80154	0.729343	-6.4166	9.07039
{2}-{5}	pH 6.5	pH 9.5	3.6328	3.80154	0.346427	-4.1106	11.37632
{3}-{4}	pH 7.5	pH 8.5	4.5964	3.80154	0.235491	-3.1471	12.33984
{3}-{5}	pH 7.5	pH 9.5	6.9023	3.80154	0.078804	-0.8412	14.64577
{4}-{5}	pH 8.5	pH 9.5	2.3059	3.80154	0.548410	-5.4376	10.04942

APPENDIX B

TEMPERATURE INCUBATION DATA: MOTILITY

1. Static

1.1. Means Table

temp; LS Means (Spreadsheet649 in MOTILITY ST
Current effect: F(2, 6)=7.2379, p=.02516
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	static Mean	static Std.Err.	static -95.00%	static +95.00%	N
1	37°C	25.4500	3.86508	15.9924	34.9075	4
2	41°C	27.7500	3.86508	18.2924	37.2075	4
3	45°C	44.5000	3.86508	35.0424	53.9575	4

1.2. Post Hoc Tests (1)

LSD test; variable static (Spreadsheet6
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 25.450	{2} 27.750	{3} 44.500
1	37°C		0.68857	0.01306
2	41°C	0.68857		0.02210
3	45°C	0.01306	0.02210	

1.3. Post Hoc Tests (2)

LSD test; variable static (Spreadsheet649 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-2.300	5.46605	0.68857	-15.675	11.0749
{1}-{3}	37°C	45°C	-19.050	5.46605	0.01306	-32.425	-5.6750
{2}-{3}	41°C	45°C	-16.750	5.46605	0.02210	-30.125	-3.3750

2. Non-Progressive

2.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=7.7027, p=.02202
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	37°C	39.3000	2.04022	34.3077	44.2922	4
2	41°C	32.5500	2.04022	27.5577	37.5422	4
3	45°C	43.8000	2.04022	38.8077	48.7922	4

2.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Spr
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 39.300	{2} 32.550	{3} 43.800
1	37°C		0.05788	0.16986
2	41°C	0.05788		0.00799
3	45°C	0.16986	0.00799	

2.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet649 in MOTILITY ST
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	6.7500	2.88530	0.05788	-0.3101	13.8100
{1}-{3}	37°C	45°C	-4.5000	2.88530	0.16986	-11.5601	2.5600
{2}-{3}	41°C	45°C	-11.2500	2.88530	0.00799	-18.3101	-4.1899

3. Progressive

3.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STATS DATA.s
Current effect: F(2, 6)=30.596, p=.00071
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	progressive Mean	progressive Std.Err.	progressive -95.00%	progressive +95.00%	N
1	37°C	35.2500	2.71929	28.5961	41.9038	4
2	41°C	39.7250	2.71929	33.0711	46.3788	4
3	45°C	11.7250	2.71929	5.0711	18.3788	4

3.2 Post Hoc Tests (1)

LSD test; variable progressive (Spreads
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 35.250	{2} 39.725	{3} 11.725
1	37°C		0.28874	0.00087
2	41°C	0.28874		0.00034
3	45°C	0.00087	0.00034	

3.3 Post Hoc Tests (2)

LSD test; variable progressive (Spreadsheet649 in MOTILITY STATS
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-4.4750	3.84566	0.28874	-13.885	4.9350
{1}-{3}	37°C	45°C	23.5250	3.84566	0.00087	14.115	32.9350
{2}-{3}	41°C	45°C	28.0000	3.84566	0.00034	18.590	37.4100

4. Total Motile

4.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STATS DATA.s
Current effect: F(2, 6)=7.1572, p=.02577
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	total motile Mean	total motile Std.Err.	total motile -95.00%	total motile +95.00%	N
1	37°C	74.5500	3.88360	65.0471	84.0528	4
2	41°C	72.2750	3.88360	62.7721	81.7778	4
3	45°C	55.5250	3.88360	46.0221	65.0278	4

4.2 Post Hoc Tests (1)

LSD test; variable total motile (Spread
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 74.550	{2} 72.275	{3} 55.525
1	37°C		0.69311	0.01340
2	41°C	0.69311		0.02251
3	45°C	0.01340	0.02251	

4.3 Post Hoc Tests (2)

LSD test; variable total motile (Spreadsheet649 in MOTILITY STATS
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	2.2750	5.49224	0.69311	-11.164	15.7140
{1}-{3}	37°C	45°C	19.0250	5.49224	0.01340	5.586	32.4640
{2}-{3}	41°C	45°C	16.7500	5.49224	0.02251	3.311	30.1890

5. Fast Progressive

5.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=19.948, p=.00223
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	fast progressive Mean	fast progressive Std.Err.	fast progressive -95.00%	fast progressive +95.00%	N
1	37°C	10.0500(	1.81004'	5.6209(	14.4790'	4
2	41°C	14.1750(	1.81004'	9.7459(	18.6040'	4
3	45°C	0.6250(	1.81004'	-3.80401	5.0540'	4

5.2 Post Hoc Tests (1)

LSD test; variable fast progressive (Spr
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 10.050	{2} 14.175	{3} .62500
1	37°C		0.10982(	0.00517'
2	41°C	0.10982(		0.00083(
3	45°C	0.00517'	0.00083(	

5.3 Post Hoc Tests (2)

LSD test; variable fast progressive (Spreadsheet649 in MOTILITY STA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-4.1250(	2.19927'	0.10982(	-9.5064(	1.2564(
{1}-{3}	37°C	45°C	9.4250(	2.19927'	0.00517'	4.0435(	14.8064(
{2}-{3}	41°C	45°C	13.5500(	2.19927'	0.00083(	8.1685(	18.9314(

6. Slow Progressive

6.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=5.5877, p=.04263
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	slow progressive Mean	slow progressive Std.Err.	slow progressive -95.00%	slow progressive +95.00%	N
1	37°C	25.2000	2.38832	19.3559	31.0440	4
2	41°C	25.5000	2.38832	19.6559	31.3440	4
3	45°C	15.5750	2.38832	9.7309	21.4190	4

6.2 Post Hoc Tests (1)

LSD test; variable slow progressive (Sp
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 25.200	{2} 25.500	{3} 15.575
1	37°C		0.93211	0.02919
2	41°C	0.93211		0.02599
3	45°C	0.02919	0.02599	

6.3 Post Hoc Tests (2)

LSD test; variable slow progressive (Spreadsheet649 in MOTILITY STA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-0.30000	3.37760	0.93211	-8.5646	7.9646
{1}-{3}	37°C	45°C	9.62500	3.37760	0.02919	1.3603	17.8896
{2}-{3}	41°C	45°C	9.92500	3.37760	0.02599	1.6603	18.1896

7. Non-Progressive

7.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=7.7027, p=.02202
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	37°C	39.3000	2.04022	34.3077	44.2922	4
2	41°C	32.5500	2.04022	27.5577	37.5422	4
3	45°C	43.8000	2.04022	38.8077	48.7922	4

7.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Spr
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 39.300	{2} 32.550	{3} 43.800
1	37°C		0.05788	0.16986
2	41°C	0.05788		0.00799
3	45°C	0.16986	0.00799	

7.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet649 in MOTILITY ST
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	6.750	2.88530	0.05788	-0.310	13.8100
{1}-{3}	37°C	45°C	-4.500	2.88530	0.16986	-11.560	2.5600
{2}-{3}	41°C	45°C	-11.250	2.88530	0.00799	-18.310	-4.1899

8. VCL

8.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STA
Current effect: F(2, 6)=26.561, p=.00105
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	VCL Mean	VCL Std.Err.	VCL -95.00%	VCL +95.00%	N
1	37°C	43.4250(	2.697877	36.82353	50.02647	4
2	41°C	53.6000(	2.697877	46.99853	60.20147	4
3	45°C	26.1000(	2.697877	19.49853	32.70147	4

8.2 Post Hoc Tests (1)

LSD test; variable VCL (Spreadsheet64
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 43.425	{2} 53.600	{3} 26.100
1	37°C		0.037183	0.00393(
2	41°C	0.037183		0.00036
3	45°C	0.00393(	0.00036	

8.3 Post Hoc Tests (2)

LSD test; variable VCL (Spreadsheet649 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-10.175(	3.815375	0.037183	-19.5109	-0.8391
{1}-{3}	37°C	45°C	17.325(	3.815375	0.00393(	7.989	26.6608
{2}-{3}	41°C	45°C	27.500(	3.815375	0.00036	18.164	36.8358

9. VSL

9.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY ST
Current effect: F(2, 6)=16.075, p=.00389
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	VSL Mean	VSL Std.Err.	VSL -95.00%	VSL +95.00%	N
1	37°C	16.7750	2.18064	11.4391	22.1108	4
2	41°C	21.8500	2.18064	16.5141	27.1858	4
3	45°C	5.8500	2.18064	0.5141	11.1858	4

9.2 Post Hoc Tests (1)

LSD test; variable VSL (Spreadsheet64
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 16.775	{2} 21.850	{3} 5.8500
1	37°C		0.12895	0.00909
2	41°C	0.12895		0.00144
3	45°C	0.00909	0.00144	

9.3 Post Hoc Tests (2)

LSD test; variable VSL (Spreadsheet649 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-5.0750	2.88403	0.12895	-12.132	1.9819
{1}-{3}	37°C	45°C	10.9250	2.88403	0.00909	3.868	17.9819
{2}-{3}	41°C	45°C	16.0000	2.88403	0.00144	8.943	23.0569

10. VAP

10.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY ST
Current effect: F(2, 6)=19.771, p=.00229
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	VAP Mean	VAP Std.Err.	VAP -95.00%	VAP +95.00%	N
1	37°C	26.5750	2.30676	20.9305	32.2194	4
2	41°C	33.0750	2.30676	27.4305	38.7194	4
3	45°C	12.9750	2.30676	7.3305	18.6194	4

10.2 Post Hoc Tests (1)

LSD test; variable VAP (Spreadsheet64
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 26.575	{2} 33.075	{3} 12.975
1	37°C		0.09339	0.00588
2	41°C	0.09339		0.00083
3	45°C	0.00588	0.00083	

10.3 Post Hoc Tests (2)

LSD test; variable VAP (Spreadsheet649 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-6.5000	3.26226	0.09339	-14.482	1.4824
{1}-{3}	37°C	45°C	13.6000	3.26226	0.00588	5.617	21.5824
{2}-{3}	41°C	45°C	20.1000	3.26226	0.00083	12.117	28.0824

11. LIN

11.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY ST
Current effect: F(2, 6)=15.331, p=.00438
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	LIN Mean	LIN Std.Err.	LIN -95.00%	LIN +95.00%	N
1	37°C	38.4500	3.11138	30.8367	46.0632	4
2	41°C	40.2250	3.11138	32.6117	47.8382	4
3	45°C	21.9250	3.11138	14.3117	29.5382	4

11.2 Post Hoc Tests (1)

LSD test; variable LIN (Spreadsheet64
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 38.450	{2} 40.225	{3} 21.925
1	37°C		0.64357	0.00396
2	41°C	0.64357		0.00240
3	45°C	0.00396	0.00240	

11.3 Post Hoc Tests (2)

LSD test; variable LIN (Spreadsheet649 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-1.7750	3.64514	0.64357	-10.694	7.1443
{1}-{3}	37°C	45°C	16.5250	3.64514	0.00396	7.6057	25.4443
{2}-{3}	41°C	45°C	18.3000	3.64514	0.00240	9.3807	27.2193

12. STR

12.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STA
Current effect: F(2, 6)=25.695, p=.00114
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	STR Mean	STR Std.Err.	STR -95.00%	STR +95.00%	N
1	37°C	62.7250(	3.21979(	54.8464⁴	70.6035(	4
2	41°C	65.1000(	3.21979(	57.2214⁴	72.9785(	4
3	45°C	44.5750(	3.21979(	36.6964⁴	52.4535(	4

12.2 Post Hoc Tests (1)

LSD test; variable STR (Spreadsheet6⁴
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 62.725	{2} 65.100	{3} 44.575
1	37°C		0.47704	0.00115
2	41°C	0.47704		0.00060
3	45°C	0.00115	0.00060	

12.3 Post Hoc Tests (2)

LSD test; variable STR (Spreadsheet649 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-2.3750(	3.13238(	0.47704	-10.0397	5.2896(
{1}-{3}	37°C	45°C	18.1500(	3.13238(	0.00115	10.485	25.8146(
{2}-{3}	41°C	45°C	20.5250(	3.13238(	0.00060	12.860	28.1896(

13. WOB

13.1 Means Table

temp; LS Means (Spreadsheet649 in MOTILITY STA
Current effect: F(2, 6)=7.7330, p=.02184
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	WOB Mean	WOB Std.Err.	WOB -95.00%	WOB +95.00%	N
1	37°C	60.9750(	2.58092(	54.6597(	67.2903(	4
2	41°C	61.4250(	2.58092(	55.1097(	67.7403(	4
3	45°C	48.7750(	2.58092(	42.4597(	55.0903(	4

13.2 Post Hoc Tests (1)

LSD test; variable WOB (Spreadsheet6
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 60.975	{2} 61.425	{3} 48.775
1	37°C		0.90590	0.01556
2	41°C	0.90590		0.01337
3	45°C	0.01556	0.01337	

13.3 Post Hoc Tests (2)

LSD test; variable WOB (Spreadsheet649 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	-0.4500(	3.64998	0.90590	-9.3811	8.4811
{1}-{3}	37°C	45°C	12.2000(	3.64998	0.01556	3.2688	21.1311
{2}-{3}	41°C	45°C	12.6500(	3.64998	0.01337	3.7188	21.5811

14. Viability

14.1 Means Table

temp; LS Means (Spreadsheet20 in VIABILITY & HA STATS DATA.stw)
Current effect: F(2, 6)=13.306, p=.00623
Type III decomposition
Include condition: stage="inc"

Cell No.	temp	viable percentage Mean	viable percentage Std.Err.	viable percentage -95.00%	viable percentage +95.00%	N
1	37°C	65.9541	4.25722	55.5371	76.3712	4
2	41°C	47.3482	4.25722	36.9311	57.7652	4
3	45°C	38.8714	4.25722	28.4544	49.2885	4

14.2 Post Hoc Tests (1)

LSD test; variable viable percentage (S
Probabilities for Post Hoc Tests
Effect: temp
Include condition: stage="inc"

Cell No.	temp	{1} 65.954	{2} 47.348	{3} 38.871
1	37°C		0.01339	0.00235
2	41°C	0.01339		0.16558
3	45°C	0.00235	0.16558	

14.3 Post Hoc Tests (2)

LSD test; variable viable percentage (Spreadsheet20 in VIABILITY &
Simultaneous confidence intervals
Effect: temp
Include condition: stage="inc"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	37°C	41°C	18.6059	5.37095	0.01339	5.4637	31.7482
{1}-{3}	37°C	45°C	27.0827	5.37095	0.00235	13.9404	40.2249
{2}-{3}	41°C	45°C	8.4767	5.37095	0.16558	-4.6655	21.6190

APPENDIX C

HYDROGEN PEROXIDE INCUBATION DATA: MOTILITY

1. Static

1.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DA
Current effect: F(2, 6)=6.2738, p=.03385
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	static Mean	static Std.Err.	static -95.00%	static +95.00%	N
1	50µM	20.8500	9.05042	-1.2955	42.9955	4
2	750µM	44.3750	9.05042	22.2294	66.5205	4
3	1000µM	40.3750	9.05042	18.2294	62.5205	4

1.2 Post Hoc Tests (1)

LSD test; variable static (Spreadsheet3 in
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 20.850	{2} 44.375	{3} 40.375
1	50µM		0.01620	0.03341
2	750µM	0.01620		0.59394
3	1000µM	0.03341	0.59394	

1.3 Post Hoc Tests (2)

LSD test; variable static (Spreadsheet3 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	-23.525	7.10695	0.01620	-40.915	-6.1349
{1}-{3}	50µM	1000µM	-19.525	7.10695	0.03341	-36.915	-2.1349
{2}-{3}	750µM	1000µM	4.000	7.10695	0.59394	-13.390	21.3900

2. Non-Progressive

2.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=3.7269, p=.08870
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	50µM	25.5250	6.06363	10.6878	40.3621	4
2	750µM	33.4250	6.06363	18.5878	48.2621	4
3	1000µM	43.0000	6.06363	28.1628	57.8371	4

2.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Sprea
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 25.525	{2} 33.425	{3} 43.000
1	50µM		0.26391	0.03436
2	750µM	0.26391		0.18588
3	1000µM	0.03436	0.18588	

2.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet3 in MOTILITY STATS
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	-7.900	6.41052	0.26391	-23.586	7.7860
{1}-{3}	50µM	1000µM	-17.475	6.41052	0.03436	-33.161	-1.7890
{2}-{3}	750µM	1000µM	-9.575	6.41052	0.18588	-25.261	6.1110

3. Progressive

3.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=7.6947, p=.02207
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	progressive Mean	progressive Std.Err.	progressive -95.00%	progressive +95.00%	N
1	50µM	53.6250	7.69791	34.7888	72.4611	4
2	750µM	22.2250	7.69791	3.3888	41.0611	4
3	1000µM	16.6250	7.69791	-2.2111	35.4611	4

3.2 Post Hoc Tests (1)

LSD test; variable progressive (Spreadshe
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 53.625	{2} 22.225	{3} 16.625
1	50µM		0.02143	0.01084
2	750µM	0.02143		0.60167
3	1000µM	0.01084	0.60167	

3.3 Post Hoc Tests (2)

LSD test; variable progressive (Spreadsheet3 in MOTILITY STATS DAT
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	31.4000	10.1673	0.02143	6.5213	56.2786
{1}-{3}	50µM	1000µM	37.0000	10.1673	0.01084	12.1213	61.8786
{2}-{3}	750µM	1000µM	5.6000	10.1673	0.60167	-19.278	30.4786

4. Total Motile

4.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=6.3176, p=.03338
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	total motile Mean	total motile Std.Err.	total motile -95.00%	total motile +95.00%	N
1	50µM	79.1500	9.04704	57.0126	101.287	4
2	750µM	55.6500	9.04704	33.5126	77.787	4
3	1000µM	59.6250	9.04704	37.4876	81.762	4

4.2 Post Hoc Tests (1)

LSD test; variable total motile (Spreadsh
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 79.150	{2} 55.650	{3} 59.625
1	50µM		0.01599	0.03290
2	750µM	0.01599		0.59468
3	1000µM	0.03290	0.59468	

4.3 Post Hoc Tests (2)

LSD test; variable total motile (Spreadsheet3 in MOTILITY STATS DAT
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	23.5000	7.07718	0.01599	6.182	40.8172
{1}-{3}	50µM	1000µM	19.5250	7.07718	0.03290	2.207	36.8422
{2}-{3}	750µM	1000µM	-3.9750	7.07718	0.59468	-21.292	13.3422

5. Fast Progressive

5.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=8.4675, p=.01790
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	fast progressive Mean	fast progressive Std.Err.	fast progressive -95.00%	fast progressive +95.00%	N
1	50µM	13.0250	2.49335	6.9239	19.1260	4
2	750µM	0.6500	2.49335	-5.4510	6.7510	4
3	1000µM	0.2750	2.49335	-5.8260	6.3760	4

5.2 Post Hoc Tests (1)

LSD test; variable fast progressive (Sprea
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 13.025	{2} .65000	{3} .27500
1	50µM		0.012679	0.011152
2	750µM	0.012679		0.918772
3	1000µM	0.011152	0.918772	

5.3 Post Hoc Tests (2)

LSD test; variable fast progressive (Spreadsheet3 in MOTILITY STATS D
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	12.3750	3.52613	0.01267	3.7468	21.0031
{1}-{3}	50µM	1000µM	12.7500	3.52613	0.01115	4.1218	21.3781
{2}-{3}	750µM	1000µM	0.3750	3.52613	0.91877	-8.2531	9.0031

6. Slow Progressive

6.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=3.1084, p=.11846
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	slow progressive Mean	slow progressive Std.Err.	slow progressive -95.00%	slow progressive +95.00%	N
1	50µM	40.6000	7.66051	21.8553	59.3446	4
2	750µM	21.6000	7.66051	2.8553	40.3446	4
3	1000µM	16.3750	7.66051	-2.3696	35.1196	4

6.2 Post Hoc Tests (1)

LSD test; variable slow progressive (Spre
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 40.600	{2} 21.600	{3} 16.375
1	50µM		0.11253	0.05560
2	750µM	0.11253		0.62763
3	1000µM	0.05560	0.62763	

6.3 Post Hoc Tests (2)

LSD test; variable slow progressive (Spreadsheet3 in MOTILITY STATS
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	19.0000	10.2260	0.11253	-6.022	44.0222
{1}-{3}	50µM	1000µM	24.2250	10.2260	0.05560	-0.797	49.2472
{2}-{3}	750µM	1000µM	5.2250	10.2260	0.62763	-19.797	30.2472

7. Non-Progressive

7.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DATA.stw)
Current effect: F(2, 6)=3.7269, p=.08870
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	50µM	25.5250	6.06363	10.6878	40.3621	4
2	750µM	33.4250	6.06363	18.5878	48.2621	4
3	1000µM	43.0000	6.06363	28.1628	57.8371	4

7.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Sprea
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 25.525	{2} 33.425	{3} 43.000
1	50µM		0.26391	0.03436
2	750µM	0.26391		0.18588
3	1000µM	0.03436	0.18588	

7.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet3 in MOTILITY STATS
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	-7.900	6.41052	0.26391	-23.586	7.7860
{1}-{3}	50µM	1000µM	-17.475	6.41052	0.03436	-33.161	-1.7890
{2}-{3}	750µM	1000µM	-9.575	6.41052	0.18588	-25.261	6.1110

8. VCL

8.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DA
Current effect: F(2, 6)=10.017, p=.01224
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	VCL Mean	VCL Std.Err.	VCL -95.00%	VCL +95.00%	N
1	50µM	52.7500	4.42708	41.9173	63.5826	4
2	750µM	35.2250	4.42708	24.3923	46.0576	4
3	1000µM	25.0500	4.42708	14.2173	35.8826	4

8.2 Post Hoc Tests (1)

LSD test; variable VCL (Spreadsheet3 in
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 52.750	{2} 35.225	{3} 25.050
1	50µM		0.03119	0.00445
2	750µM	0.03119		0.15525
3	1000µM	0.00445	0.15525	

8.3 Post Hoc Tests (2)

LSD test; variable VCL (Spreadsheet3 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	17.5250	6.26084	0.03119	2.2052	32.8447
{1}-{3}	50µM	1000µM	27.7000	6.26084	0.00445	12.3802	43.0197
{2}-{3}	750µM	1000µM	10.1750	6.26084	0.15525	-5.1447	25.4947

9. VSL

9.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DA
Current effect: F(2, 6)=9.8994, p=.01258
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	VSL Mean	VSL Std.Err.	VSL -95.00%	VSL +95.00%	N
1	50µM	18.8500	2.35335	13.0915	24.6084	4
2	750µM	8.3000	2.35335	2.5415	14.0584	4
3	1000µM	4.5750	2.35335	-1.1834	10.3334	4

9.2 Post Hoc Tests (1)

LSD test; variable VSL (Spreadsheet3 in
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 18.850	{2} 8.3000	{3} 4.5750
1	50µM		0.01932	0.00515
2	750µM	0.01932		0.30583
3	1000µM	0.00515	0.30583	

9.3 Post Hoc Tests (2)

LSD test; variable VSL (Spreadsheet3 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	10.5500	3.32814	0.01932	2.4063	18.6936
{1}-{3}	50µM	1000µM	14.2750	3.32814	0.00515	6.1313	22.4186
{2}-{3}	750µM	1000µM	3.7250	3.32814	0.30583	-4.4186	11.8686

10. VAP

10.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DA
Current effect: F(2, 6)=6.2491, p=.03412
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	VAP Mean	VAP Std.Err.	VAP -95.00%	VAP +95.00%	N
1	50µM	31.7250	3.79333	22.4430	41.0069	4
2	750µM	20.4000	3.79333	11.1180	29.6819	4
3	1000µM	13.8500	3.79333	4.5680	23.1319	4

10.2 Post Hoc Tests (1)

LSD test; variable VAP (Spreadsheet3 in
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 31.725	{2} 20.400	{3} 13.850
1	50µM		0.06879	0.01292
2	750µM	0.06879		0.24770
3	1000µM	0.01292	0.24770	

10.3 Post Hoc Tests (2)

LSD test; variable VAP (Spreadsheet3 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	11.3250	5.11594	0.06879	-1.1932	23.8432
{1}-{3}	50µM	1000µM	17.8750	5.11594	0.01292	5.3567	30.3932
{2}-{3}	750µM	1000µM	6.5500	5.11594	0.24770	-5.9682	19.0682

11. LIN

11.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DA
Current effect: F(2, 6)=6.4618, p=.03187
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	LIN Mean	LIN Std.Err.	LIN -95.00%	LIN +95.00%	N
1	50µM	35.6750	4.01717	25.8453	45.5046	4
2	750µM	22.3500	4.01717	12.5203	32.1796	4
3	1000µM	16.4000	4.01717	6.5703	26.2296	4

11.2 Post Hoc Tests (1)

LSD test; variable LIN (Spreadsheet3 in M
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 35.675	{2} 22.350	{3} 16.400
1	50µM		0.05139	0.01266
2	750µM	0.05139		0.32015
3	1000µM	0.01266	0.32015	

11.3 Post Hoc Tests (2)

LSD test; variable LIN (Spreadsheet3 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	13.3250	5.49096	0.05139	-0.1109	26.7609
{1}-{3}	50µM	1000µM	19.2750	5.49096	0.01266	5.8390	32.7109
{2}-{3}	750µM	1000µM	5.9500	5.49096	0.32015	-7.4859	19.3859

12. STR

12.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DA
Current effect: F(2, 6)=10.698, p=.01051
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	STR Mean	STR Std.Err.	STR -95.00%	STR +95.00%	N
1	50µM	58.6000	4.30483	48.0664	69.1335	4
2	750µM	38.8250	4.30483	28.2914	49.3585	4
3	1000µM	31.3500	4.30483	20.8164	41.8835	4

12.2 Post Hoc Tests (1)

LSD test; variable STR (Spreadsheet3 in
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 58.600	{2} 38.825	{3} 31.350
1	50µM		0.01750	0.00421
2	750µM	0.01750		0.26548
3	1000µM	0.00421	0.26548	

12.3 Post Hoc Tests (2)

LSD test; variable STR (Spreadsheet3 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	19.7750	6.08795	0.01750	4.87831	34.6716
{1}-{3}	50µM	1000µM	27.2500	6.08795	0.00421	12.3533	42.1466
{2}-{3}	750µM	1000µM	7.4750	6.08795	0.26548	-7.4216	22.3716

13. WOB

13.1 Means Table

HP; LS Means (Spreadsheet3 in MOTILITY STATS DA
Current effect: F(2, 6)=1.4800, p=.30028
Type III decomposition
Include condition: stage="incubation"

Cell No.	HP	WOB Mean	WOB Std.Err.	WOB -95.00%	WOB +95.00%	N
1	50µM	60.1750(	4.79069(	48.4525(	71.8974(	4
2	750µM	56.5500(	4.79069(	44.8275(	68.2724(	4
3	1000µM	51.8500(	4.79069(	40.1275(	63.5724(	4

13.2 Post Hoc Tests (1)

LSD test; variable WOB (Spreadsheet3 in
Probabilities for Post Hoc Tests
Effect: HP
Include condition: stage="incubation"

Cell No.	HP	{1} 60.175	{2} 56.550	{3} 51.850
1	50µM		0.48325(	0.13703(
2	750µM	0.48325(		0.37013(
3	1000µM	0.13703(	0.37013(	

13.3 Post Hoc Tests (2)

LSD test; variable WOB (Spreadsheet3 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: HP
Include condition: stage="incubation"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	50µM	750µM	3.62500(	4.85219(	0.48325(	-8.2478(	15.4978(
{1}-{3}	50µM	1000µM	8.32500(	4.85219(	0.13703(	-3.5478(	20.1978(
{2}-{3}	750µM	1000µM	4.70000(	4.85219(	0.37013(	-7.1728(	16.5728(

HYDROGEN PEROXIDE INCUBATION DATA: VIABILITY

14. Viability

14.1 Means Table

pH level; LS Means (Spreadsheet3)
Current effect: F(4, 32)=2.7960, p=.04251
Type III decomposition
Include condition: stage="pH"

Cell No.	pH level	viable percentage Mean	viable percentage Std.Err.	viable percentage -95.00%	viable percentage +95.00%	N
1	pH 5.5	58.6959	3.22953	52.1176	65.2743	9
2	pH 6.5	67.4762	3.22953	60.8978	74.0545	9
3	pH 7.5	70.7456	3.22953	64.1673	77.3240	9
4	pH 8.5	66.1493	3.22953	59.5709	72.7276	9
5	pH 9.5	63.8433	3.22953	57.2650	70.4217	9

14.2 Post Hoc Tests (1)

LSD test; variable viable percentage (Spreadsheet3)
Probabilities for Post Hoc Tests
Effect: pH level
Include condition: stage="pH"

Cell No.	pH level	{1} 58.696	{2} 67.476	{3} 70.746	{4} 66.149	{5} 63.843
1	pH 5.5		0.02751	0.00335	0.05867	0.18521
2	pH 6.5	0.02751		0.39616	0.72934	0.34642
3	pH 7.5	0.00335	0.39616		0.23549	0.07880
4	pH 8.5	0.05867	0.72934	0.23549		0.54841
5	pH 9.5	0.18521	0.34642	0.07880	0.54841	

14.3 Post Hoc Tests (2)

LSD test; variable viable percentage (Spreadsheet3)
Simultaneous confidence intervals
Effect: pH level
Include condition: stage="pH"

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	pH 5.5	pH 6.5	-8.7802	3.80154	0.027513	-16.5237	-1.03675
{1}-{3}	pH 5.5	pH 7.5	-12.0497	3.80154	0.003352	-19.7932	-4.30620
{1}-{4}	pH 5.5	pH 8.5	-7.4533	3.80154	0.058679	-15.1968	0.29016
{1}-{5}	pH 5.5	pH 9.5	-5.1474	3.80154	0.185213	-12.8909	2.59609
{2}-{3}	pH 6.5	pH 7.5	-3.2695	3.80154	0.396168	-11.0129	4.47403
{2}-{4}	pH 6.5	pH 8.5	1.3269	3.80154	0.729343	-6.4166	9.07039
{2}-{5}	pH 6.5	pH 9.5	3.6328	3.80154	0.346427	-4.1106	11.37632
{3}-{4}	pH 7.5	pH 8.5	4.5964	3.80154	0.235491	-3.1471	12.33984
{3}-{5}	pH 7.5	pH 9.5	6.9023	3.80154	0.078804	-0.8412	14.64577
{4}-{5}	pH 8.5	pH 9.5	2.3059	3.80154	0.548410	-5.4376	10.04942

APPENDIX D

DIRECT SWIM-UP DATA: MOTILITY

15. Static

15.1 Means Table

Cell No.	stage	static Mean	static Std.Err.	static -95.00%	static +95.00%	N
	stage; LS Means (Spreadsheet159) Current effect: F(2, 11)=12.931, p=.00129 Type III decomposition					
1	contro	32.2333	4.77708	21.7190	42.7476	6
2	fraction A	2.2857	4.42271	-7.4486	12.0200	7
3	fraction B	4.2142	4.42271	-5.5200	13.9486	7

15.2 Post Hoc Tests (1)

Cell No.	stage	{1} 32.233	{2} 2.2857	{3} 4.2143
	LSD test; variable static (Spreadsheet159 Probabilities for Post Hoc Tests Effect: stage			
1	contro		0.00076	0.00124
2	fraction A	0.00076		0.76358
3	fraction B	0.00124	0.76358	

15.3 Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable static (Spreadsheet159) Simultaneous confidence intervals Effect: stage						
{1}-{2}	contro	fraction A	29.9476	6.51006	0.00076	15.619	44.2761
{1}-{3}	contro	fraction B	28.0190	6.51006	0.00124	13.690	42.3476
{2}-{3}	fraction A	fraction B	-1.9285	6.25466	0.76358	-15.695	11.8378

2. Non-Progressive

2.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=42.840, p=.00001
Type III decomposition

Cell No.	stage	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	contro	29.2693	2.95724	22.7605	35.7782	6
2	fraction A	14.1714	2.89938	7.7899	20.5529	7
3	fraction B	15.6000	2.89938	9.2184	21.9815	7

2.2. Post Hoc Tests (1)

LSD test; variable non-progressive (Spread
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 29.269	{2} 14.171	{3} 15.600
1	contro		0.00000	0.00000
2	fraction A	0.00000		0.41108
3	fraction B	0.00000	0.41108	

2.3. Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	15.0979	1.77039	0.00000	11.2013	18.9945
{1}-{3}	contro	fraction B	13.6693	1.77039	0.00000	9.7727	17.5659
{2}-{3}	fraction A	fraction B	-1.4285	1.67195	0.41108	-5.1085	2.2513

3. Progressive

3.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=19.742, p=.00023
Type III decomposition

Cell No.	stage	progressive Mean	progressive Std.Err.	progressive -95.00%	progressive +95.00%	N
1	contro	39.7214	6.27043	25.9202	53.5225	6
2	fraction A	83.5285	5.83804	70.6791	96.3780	7
3	fraction B	80.1714	5.83804	67.3219	93.0208	7

3.2. Post Hoc Tests (1)

LSD test; variable progressive (Spreadshee
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 39.721	{2} 83.529	{3} 80.171
1	contro		0.00013	0.00025
2	fraction A	0.00013		0.65451
3	fraction B	0.00025	0.65451	

3.3. Post Hoc Tests (2)

LSD test; variable progressive (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	-43.807	7.64926	0.00013	-60.643	-26.971
{1}-{3}	contro	fraction B	-40.450	7.64926	0.00025	-57.285	-23.614
{2}-{3}	fraction A	fraction B	3.357	7.29902	0.65451	-12.707	19.422

4. Total Motile

4.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=12.945, p=.00129
Type III decomposition

Cell No.	stage	total motile Mean	total motile Std.Err.	total motile -95.00%	total motile +95.00%	N
1	contro	67.7500	4.77492	57.2404	78.259	6
2	fraction A	97.7000	4.42072	87.9700	107.429	7
3	fraction B	95.7714	4.42072	86.0414	105.501	7

4.2. Post Hoc Tests (1)

LSD test; variable total motile (Spreadshee
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 67.750	{2} 97.700	{3} 95.771
1	contro		0.00076	0.00124
2	fraction A	0.00076		0.76348
3	fraction B	0.00124	0.76348	

Post Hoc Tests (2)

LSD test; variable total motile (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	-29.950	6.50712	0.00076	-44.2721	-15.627
{1}-{3}	control	fraction B	-28.0214	6.50712	0.00124	-42.3435	-13.699
{2}-{3}	fraction A	fraction B	1.928	6.25184	0.76348	-11.831	15.688

5. Fast Progressive

5.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=8.4721, p=.00593
Type III decomposition

Cell No.	stage	fast progressive Mean	fast progressive Std.Err.	fast progressive -95.00%	fast progressive +95.00%	N
1	control	5.9262	4.25629	-3.4417	15.2942	6
2	fraction A	19.4285	4.10456	10.3944	28.4626	7
3	fraction B	17.3714	4.10456	8.3373	26.4055	7

5.2. Post Hoc Tests (1)

LSD test; variable fast progressive (Spreads
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 5.9263	{2} 19.429	{3} 17.371
1	contro		0.00256	0.00721
2	fraction A	0.00256		0.54485
3	fraction B	0.00721	0.54485	

5.3. Post Hoc Tests (2)

LSD test; variable fast progressive (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	-13.502	3.47987	0.00256	-21.161	-5.8431
{1}-{3}	control	fraction B	-11.445	3.47987	0.00721	-19.104	-3.7860
{2}-{3}	fraction A	fraction B	2.0571	3.29256	0.54485	-5.189	9.3040

6. Slow Progressive

6.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=8.1070, p=.00686
Type III decomposition

Cell No.	stage	slow progressive Mean	slow progressive Std.Err.	slow progressive -95.00%	slow progressive +95.00%	N
1	control	33.2242	6.36900	19.2061	47.2423	6
2	fraction A	64.1142	5.89717	51.1346	77.0938	7
3	fraction B	62.8285	5.89717	49.8489	75.8081	7

6.2. Post Hoc Tests (1)

LSD test; variable slow progressive (Spread
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 33.224	{2} 64.114	{3} 62.829
1	control		0.00413	0.00538
2	fraction A	0.00413		0.87859
3	fraction B	0.00538	0.87859	

6.3. Post Hoc Tests (2)

LSD test; variable slow progressive (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	-30.890	8.56848	0.00413	-49.749	-12.030
{1}-{3}	contro	fraction B	-29.604	8.56848	0.00538	-48.463	-10.745
{2}-{3}	fraction A	fraction B	1.285	8.22383	0.87859	-16.814	19.386

7. Non-Progressive

7.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=42.840, p=.00001
Type III decomposition

Cell No.	stage	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	contro	29.2693	2.95724	22.7605	35.7782	6
2	fraction A	14.1714	2.89938	7.7899	20.5529	7
3	fraction E	15.6000	2.89938	9.2184	21.9815	7

7.2. Post Hoc Tests (1)

LSD test; variable non-progressive (Spread
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 29.269	{2} 14.171	{3} 15.600
1	control		0.000004	0.000009
2	fraction A	0.000004		0.411087
3	fraction E	0.000009	0.411087	

7.3. Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	15.0979	1.77039	0.00000	11.2013	18.9945
{1}-{3}	contro	fraction E	13.6693	1.77039	0.00000	9.7727	17.5659
{2}-{3}	fraction A	fraction E	-1.4285	1.67195	0.41108	-5.1085	2.2513

8. VCL

8.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=9.0154, p=.00481
Type III decomposition

Cell No.	stage	VCL Mean	VCL Std.Err.	VCL -95.00%	VCL +95.00%	N
1	contro	46.4196	6.27301	32.6128	60.2264	6
2	fraction A	78.8428	5.83475	66.0006	91.6850	7
3	fraction B	68.6857	5.83475	55.8435	81.5279	7

8.2. Post Hoc Tests (1)

LSD test; variable VCL (Spreadsheet159)
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 46.420	{2} 78.843	{3} 68.686
1	contro		0.00152	0.01510
2	fraction A	0.00152		0.19691
3	fraction B	0.01510	0.19691	

8.3. Post Hoc Tests (2)

LSD test; variable VCL (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	-32.4232	7.74507	0.00152	-49.4700	-15.3764
{1}-{3}	control	fraction B	-22.2661	7.74507	0.01510	-39.3129	-5.2193
{2}-{3}	fraction A	fraction B	10.1571	7.39458	0.19691	-6.1182	26.4325

9. VSL

9.1. Means Table

Cell No.	stage	VSL Mean	VSL Std.Err.	VSL -95.00%	VSL +95.00%	N
	stage; LS Means (Spreadsheet159) Current effect: F(2, 11)=14.820, p=.00076 Type III decomposition					
1	contro	16.0030	2.79231	9.8572	22.1489	6
2	fraction A	26.8571	2.70770	20.8975	32.8167	7
3	fraction E	24.9285	2.70770	18.9689	30.8882	7

9.2. Post Hoc Tests (1)

Cell No.	stage	{1} 16.003	{2} 26.857	{3} 24.929
	LSD test; variable VSL (Spreadsheet15 Probabilities for Post Hoc Tests Effect: stage			
1	control		0.000304	0.001343
2	fraction A	0.000304		0.351228
3	fraction E	0.001343	0.351228	

9.3. Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable VSL (Spreadsheet159) Simultaneous confidence intervals Effect: stage						
{1}-{2}	contro	fraction A	-10.854	2.09522	0.00030	-15.465	-6.2425
{1}-{3}	contro	fraction E	-8.925	2.09522	0.00134	-13.537	-4.3139
{2}-{3}	fraction A	fraction E	1.928	1.98107	0.35122	-2.4317	6.2888

10. VAP

10.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=8.2006, p=.00661
Type III decomposition

Cell No.	stage	VAP Mean	VAP Std.Err.	VAP -95.00%	VAP +95.00%	N
1	control	29.23756	3.838019	20.79014	37.68499	6
2	fraction A	44.54286	3.636048	36.53997	52.54574	7
3	fraction B	40.84286	3.636048	32.83997	48.84574	7

10.2. Post Hoc Tests (1)

LSD test; variable VAP (Spreadsheet159
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 29.238	{2} 44.543	{3} 40.843
1	contro		0.00235	0.01253
2	fraction A	0.00235		0.33834
3	fraction B	0.01253	0.33834	

10.3. Post Hoc Tests (2)

LSD test; variable VAP (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	-15.3053	3.895188	0.00235	-23.8785	-6.73204
{1}-{3}	control	fraction B	-11.6053	3.895188	0.01253	-20.1785	-3.03204
{2}-{3}	fraction A	fraction B	3.7000	3.696342	0.33834	-4.4356	11.83559

11. LIN

11.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=.22757, p=.80013
Type III decomposition

Cell No.	stage	LIN Mean	LIN Std.Err.	LIN -95.00%	LIN +95.00%	N
1	contro	35.1654	2.84128	28.9118	41.4190	6
2	fraction A	34.4000	2.68716	28.4855	40.3144	7
3	fraction B	36.2714	2.68716	30.3570	42.1858	7

11.2. Post Hoc Tests (1)

LSD test; variable LIN (Spreadsheet159
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 35.165	{2} 34.400	{3} 36.271
1	control		0.799149	0.713576
2	fraction A	0.799149		0.515817
3	fraction B	0.713576	0.515817	

11.3. Post Hoc Tests (2)

LSD test; variable LIN (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	0.7654	2.93617	0.79914	-5.6970	7.22790
{1}-{3}	contro	fraction B	-1.1060	2.93617	0.71357	-7.5684	5.35647
{2}-{3}	fraction A	fraction B	-1.8714	2.78731	0.51581	-8.0062	4.26340

12. STR

12.1. Means Table

stage; LS Means (Spreadsheet159)
Current effect: F(2, 11)=1.8611, p=.20129
Type III decomposition

Cell No.	stage	STR Mean	STR Std.Err.	STR -95.00%	STR +95.00%	N
1	contro	54.9878	2.97887	48.4314	61.5443	6
2	fraction A	59.8857	2.81462	53.6907	66.0806	7
3	fraction B	60.5714	2.81462	54.3764	66.7663	7

12.2. Post Hoc Tests (1)

LSD test; variable STR (Spreadsheet159
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 54.988	{2} 59.886	{3} 60.571
1	contro		0.14347	0.09999
2	fraction A	0.14347		0.82058
3	fraction B	0.09999	0.82058	

12.3. Post Hoc Tests (2)

LSD test; variable STR (Spreadsheet159)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	-4.8978	3.10903	0.14347	-11.740	1.94507
{1}-{3}	contro	fraction B	-5.5835	3.10903	0.09999	-12.426	1.25936
{2}-{3}	fraction A	fraction B	-0.6857	2.95203	0.82058	-7.183	5.81167

13. WOB

13.1. Means Table

Cell No.	stage	WOB Mean	WOB Std.Err.	WOB -95.00%	WOB +95.00%	N
	stage; LS Means (Spreadsheet159) Current effect: F(2, 11)=3.9042, p=.05233 Type III decomposition					
1	contro	63.6478	2.30461	58.5754	68.7202	6
2	fraction A	56.9285	2.17686	52.1373	61.7198	7
3	fraction B	59.4857	2.17686	54.6944	64.2769	7

13.2. Post Hoc Tests (1)

Cell No.	stage	{1} 63.648	{2} 56.929	{3} 59.486
	LSD test; variable WOB (Spreadsheet159 Probabilities for Post Hoc Tests Effect: stage			
1	contro		0.01775	0.11250
2	fraction A	0.01775		0.28821
3	fraction B	0.11250	0.28821	

13.3. Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable WOB (Spreadsheet159) Simultaneous confidence intervals Effect: stage						
{1}-{2}	contro	fraction A	6.7192	2.41307	0.01775	1.4081	12.0304
{1}-{3}	contro	fraction B	4.1621	2.41307	0.11250	-1.1490	9.4732
{2}-{3}	fraction A	fraction B	-2.5571	2.29138	0.28821	-7.6004	2.4861

APPENDIX E

CAPILLARY TUBE DATA: MOTILITY

1. Static

1.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=.58334, p=.63403
Type III decomposition

Cell No.	stage	static Mean	static Std.Err.	static -95.00%	static +95.00%	N
1	contro	31.5627	8.56995	13.4816	49.6437	6
2	fraction A	31.7571	8.08414	14.7010	48.8131	7
3	fraction B	26.9428	8.08414	9.8868	43.9989	7
4	fraction C	38.6428	8.08414	21.5868	55.6989	7

1.2 Post Hoc Tests (1)

LSD test; variable static (Spreadsheet416)
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 31.563	{2} 31.757	{3} 26.943	{4} 38.643
1	contro		0.98367	0.62812	0.46000
2	fraction A	0.98367		0.59651	0.45090
3	fraction B	0.62812	0.59651		0.20722
4	fraction C	0.46000	0.45090	0.20722	

1.3 Post Hoc Tests (2)

LSD test; variable static (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	-0.194	9.36551	0.98367	-19.953	19.5650
{1}-{3}	contro	fraction B	4.619	9.36551	0.62812	-15.139	24.3793
{1}-{4}	contro	fraction C	-7.080	9.36551	0.46000	-26.839	12.6793
{2}-{3}	fraction A	fraction B	4.814	8.92312	0.59651	-14.011	23.6404
{2}-{4}	fraction A	fraction C	-6.885	8.92312	0.45090	-25.711	11.9404
{3}-{4}	fraction B	fraction C	-11.700	8.92312	0.20722	-30.526	7.1261

2. Non-Progressive

2.1 Means Table

Cell No.	stage	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
	stage; LS Means (Spreadsheet416) Current effect: F(3, 17)=.06823, p=.97607 Type III decomposition					
1	contro	27.2031	4.01717	18.7276	35.6786	6
2	fraction A	28.0285	3.72346	20.1727	35.8843	7
3	fraction B	28.6857	3.72346	20.8298	36.5415	7
4	fraction C	29.4857	3.72346	21.6298	37.3415	7

2.2 Post Hoc Tests (1)

Cell No.	stage	{1} 27.203	{2} 28.029	{3} 28.686	{4} 29.486
	LSD test; variable non-progressive (Spreadsheet41 Probabilities for Post Hoc Tests Effect: stage				
1	contro		0.87747	0.78202	0.67060
2	fraction A	0.87747		0.89807	0.77659
3	fraction B	0.78202	0.89807		0.87609
4	fraction C	0.67060	0.77659	0.87609	

2.3 Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable non-progressive (Spreadsheet416) Simultaneous confidence intervals Effect: stage						
{1}-{2}	contro	fraction A	-0.8254	5.27414	0.87747	-11.952	10.3020
{1}-{3}	contro	fraction B	-1.4825	5.27414	0.78202	-12.610	9.6448
{1}-{4}	contro	fraction C	-2.2825	5.27414	0.67060	-13.410	8.8448
{2}-{3}	fraction A	fraction B	-0.6571	5.05401	0.89807	-11.320	10.0059
{2}-{4}	fraction A	fraction C	-1.4571	5.05401	0.77659	-12.120	9.2059
{3}-{4}	fraction B	fraction C	-0.8000	5.05401	0.87609	-11.463	9.8630

3. Progressive

3.1 Means Table

Cell No.	stage	progressive Mean	progressive Std.Err.	progressive -95.00%	progressive +95.00%	N
	stage; LS Means (Spreadsheet416) Current effect: F(3, 17)=.66000, p=.58786 Type III decomposition					
1	contro	41.4910	9.90877	20.5853	62.3966	6
2	fraction A	40.2142	9.43798	20.3018	60.1267	7
3	fraction B	44.4000	9.43798	24.4875	64.3124	7
4	fraction C	31.8428	9.43798	11.9304	51.7552	7

3.2 Post Hoc Tests (1)

Cell No.	stage	{1} 41.491	{2} 40.214	{3} 44.400	{4} 31.843
	LSD test; variable progressive (Spreadsheet416) Probabilities for Post Hoc Tests Effect: stage				
1	contro		0.89805	0.77057	0.33948
2	fraction A	0.89805		0.65975	0.38268
3	fraction B	0.77057	0.65975		0.19653
4	fraction C	0.33948	0.38268	0.19653	

3.3 Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable progressive (Spreadsheet416) Simultaneous confidence intervals Effect: stage						
{1}-{2}	contro	fraction A	1.2767	9.81690	0.89805	-19.435	21.9885
{1}-{3}	contro	fraction B	-2.9090	9.81690	0.77057	-23.620	17.8028
{1}-{4}	contro	fraction C	9.6481	9.81690	0.33948	-11.063	30.3600
{2}-{3}	fraction A	fraction B	-4.1857	9.34148	0.65975	-23.894	15.5231
{2}-{4}	fraction A	fraction C	8.3714	9.34148	0.38268	-11.337	28.0802
{3}-{4}	fraction B	fraction C	12.5571	9.34148	0.19653	-7.1517	32.2659

4. Total Motile

4.1 Means Table

Cell No.	stage	total motile Mean	total motile Std.Err.	total motile -95.00%	total motile +95.00%	N
	stage; LS Means (Spreadsheet416) Current effect: F(3, 17)=.59067, p=.62949 Type III decomposition					
1	contro	68.4211	8.55975	50.3616	86.4806	6
2	fraction A	68.2428	8.07485	51.2064	85.2793	7
3	fraction B	73.0857	8.07485	56.0492	90.1221	7
4	fraction C	61.3285	8.07485	44.2921	78.3650	7

4.2 Post Hoc Tests (1)

Cell No.	stage	{1} 68.421	{2} 68.243	{3} 73.086	{4} 61.329
	LSD test; variable total motile (Spreadsheet416) Probabilities for Post Hoc Tests Effect: stage				
1	contro		0.98500	0.62427	0.45853
2	fraction A	0.98500		0.59377	0.44834
3	fraction B	0.62427	0.59377		0.20443
4	fraction C	0.45853	0.44834	0.20443	

4.3 Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable total motile (Spreadsheet416) Simultaneous confidence intervals Effect: stage						
{1}-{2}	contro	fraction A	0.1782	9.35064	0.98500	-19.549	19.9064
{1}-{3}	contro	fraction B	-4.6645	9.35064	0.62427	-24.392	15.0635
{1}-{4}	contro	fraction C	7.0925	9.35064	0.45853	-12.635	26.8207
{2}-{3}	fraction A	fraction B	-4.8428	8.90890	0.59377	-23.639	13.9532
{2}-{4}	fraction A	fraction C	6.9142	8.90890	0.44834	-11.881	25.7104
{3}-{4}	fraction B	fraction C	11.7571	8.90890	0.20443	-7.039	30.5532

5. Fast Progressive

5.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=.21689, p=.88332
Type III decomposition

Cell No.	stage	fast progressive Mean	fast progressive Std.Err.	fast progressive -95.00%	fast progressive +95.00%	N
1	contro	7.32505	2.00615	3.09244	11.5576	6
2	fraction A	6.74285	1.88050	2.77533	10.7103	7
3	fraction B	6.78571	1.88050	2.81818	10.7532	7
4	fraction C	8.31428	1.88050	4.34675	12.2818	7

5.2 Post Hoc Tests (1)

LSD test; variable fast progressive (Spreadsheet41
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 7.3251	{2} 6.7429	{3} 6.7857	{4} 8.3143
1	contro		0.80549	0.81953	0.67618
2	fraction A	0.80549		0.98482	0.48869
3	fraction B	0.81953	0.98482		0.50046
4	fraction C	0.67618	0.48869	0.50046	

5.3 Post Hoc Tests (2)

LSD test; variable fast progressive (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	0.5822	2.32772	0.80549	-4.3288	5.49326
{1}-{3}	control	fraction B	0.5393	2.32772	0.81953	-4.3717	5.45040
{1}-{4}	control	fraction C	-0.9892	2.32772	0.67618	-5.9002	3.92183
{2}-{3}	fraction A	fraction B	-0.0428	2.22035	0.98482	-4.7273	4.64167
{2}-{4}	fraction A	fraction C	-1.5714	2.22035	0.48869	-6.2559	3.11310
{3}-{4}	fraction B	fraction C	-1.5285	2.22035	0.50046	-6.2131	3.15595

6. Slow Progressive

6.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=1.0104, p=.41240
Type III decomposition

Cell No.	stage	slow progressive Mean	slow progressive Std.Err.	slow progressive -95.00%	slow progressive +95.00%	N
1	contro	34.1542	8.83090	15.5226	52.7858	6
2	fraction A	33.4857	8.39497	15.7738	51.1975	7
3	fraction B	37.6142	8.39497	19.9024	55.3261	7
4	fraction C	23.5142	8.39497	5.8024	41.2261	7

6.2 Post Hoc Tests (1)

LSD test; variable slow progressive (Spreadsheet41
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 34.154	{2} 33.486	{3} 37.614	{4} 23.514
1	contro		0.94121	0.70328	0.24994
2	fraction A	0.94121		0.63342	0.25700
3	fraction B	0.70328	0.63342		0.11554
4	fraction C	0.24994	0.25700	0.11554	

6.3 Post Hoc Tests (2)

LSD test; variable slow progressive (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	0.6685	8.93219	0.94121	-18.176	19.5137
{1}-{3}	contro	fraction B	-3.4600	8.93219	0.70328	-22.305	15.3852
{1}-{4}	contro	fraction C	10.6399	8.93219	0.24994	-8.205	29.4852
{2}-{3}	fraction A	fraction B	-4.1285	8.50146	0.63342	-22.065	13.8079
{2}-{4}	fraction A	fraction C	9.9714	8.50146	0.25700	-7.965	27.9079
{3}-{4}	fraction B	fraction C	14.1000	8.50146	0.11554	-3.836	32.0365

7. Non-Progressive

7.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=.06823, p=.97607
Type III decomposition

Cell No.	stage	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	contro	27.2031	4.01717	18.7276	35.6786	6
2	fraction A	28.0285	3.72346	20.1727	35.8843	7
3	fraction B	28.6857	3.72346	20.8298	36.5415	7
4	fraction C	29.4857	3.72346	21.6298	37.3415	7

7.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Spreadsheet41
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 27.203	{2} 28.029	{3} 28.686	{4} 29.486
1	contro		0.87747	0.78202	0.67060
2	fraction A	0.87747		0.89807	0.77659
3	fraction B	0.78202	0.89807		0.87609
4	fraction C	0.67060	0.77659	0.87609	

7.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	-0.8254	5.27414	0.87747	-11.952	10.3020
{1}-{3}	contro	fraction B	-1.4825	5.27414	0.78202	-12.610	9.6448
{1}-{4}	contro	fraction C	-2.2825	5.27414	0.67060	-13.410	8.8448
{2}-{3}	fraction A	fraction B	-0.6571	5.05401	0.89807	-11.320	10.0059
{2}-{4}	fraction A	fraction C	-1.4571	5.05401	0.77659	-12.120	9.2059
{3}-{4}	fraction B	fraction C	-0.8000	5.05401	0.87609	-11.463	9.8630

8. VCL

8.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=.13566, p=.93740
Type III decomposition

Cell No.	stage	VCL Mean	VCL Std.Err.	VCL -95.00%	VCL +95.00%	N
1	contro	47.6414	7.37155	32.0888	63.1941	6
2	fraction A	45.3857	6.90487	30.8177	59.9537	7
3	fraction B	48.6000	6.90487	34.0319	63.1680	7
4	fraction C	43.8285	6.90487	29.2605	58.3965	7

8.2 Post Hoc Tests (1)

LSD test; variable VCL (Spreadsheet416)
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 47.641	{2} 45.386	{3} 48.600	{4} 43.829
1	control		0.796519	0.912682	0.663532
2	fraction A	0.796519		0.700495	0.851926
3	fraction B	0.912682	0.700495		0.569033
4	fraction C	0.663532	0.851926	0.569033	

8.3 Post Hoc Tests (2)

LSD test; variable VCL (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	2.25577	8.611979	0.796519	-15.9139	20.4254
{1}-{3}	control	fraction B	-0.95851	8.611979	0.912682	-19.1282	17.2111
{1}-{4}	control	fraction C	3.81292	8.611979	0.663532	-14.3568	21.9826
{2}-{3}	fraction A	fraction B	-3.21429	8.216064	0.700495	-20.5487	14.1200
{2}-{4}	fraction A	fraction C	1.55714	8.216064	0.851926	-15.7772	18.8915
{3}-{4}	fraction B	fraction C	4.77143	8.216064	0.569033	-12.5630	22.1058

9. VSL

9.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=.85930, p=.48105
Type III decomposition

Cell No.	stage	VSL Mean	VSL Std.Err.	VSL -95.00%	VSL +95.00%	N
1	contro	17.1402	2.69437	11.4556	22.8249	6
2	fraction A	13.3714	2.56591	7.9578	18.7850	7
3	fraction B	16.4428	2.56591	11.0292	21.8564	7
4	fraction C	16.5428	2.56591	11.1292	21.9564	7

9.2 Post Hoc Tests (1)

LSD test; variable VSL (Spreadsheet416)
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 17.140	{2} 13.371	{3} 16.443	{4} 16.543
1	contro		0.17679	0.79739	0.82589
2	fraction A	0.17679		0.24400	0.22960
3	fraction B	0.79739	0.24400		0.96911
4	fraction C	0.82589	0.22960	0.96911	

9.3 Post Hoc Tests (2)

LSD test; variable VSL (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	3.7688	2.67440	0.17679	-1.8736	9.41135
{1}-{3}	control	fraction B	0.6974	2.67440	0.79739	-4.9450	6.33992
{1}-{4}	control	fraction C	0.5974	2.67440	0.82589	-5.0450	6.23992
{2}-{3}	fraction A	fraction B	-3.0714	2.54493	0.24400	-8.4407	2.29792
{2}-{4}	fraction A	fraction C	-3.1714	2.54493	0.22960	-8.5407	2.19792
{3}-{4}	fraction B	fraction C	-0.1000	2.54493	0.96911	-5.4693	5.26935

10. VAP

10.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=.39957, p=.75507
Type III decomposition

Cell No.	stage	VAP Mean	VAP Std.Err.	VAP -95.00%	VAP +95.00%	N
1	contro	30.3714	4.58186	20.7045	40.0383	6
2	fraction A	25.2142	4.31206	16.1166	34.3119	7
3	fraction B	29.1714	4.31206	20.0737	38.2690	7
4	fraction C	27.3000	4.31206	18.2023	36.3976	7

10.2 Post Hoc Tests (1)

LSD test; variable VAP (Spreadsheet416)
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 30.371	{2} 25.214	{3} 29.171	{4} 27.300
1	control		0.327922	0.81749	0.556482
2	fraction A	0.327922		0.428625	0.674445
3	fraction B	0.81749	0.428625		0.706104
4	fraction C	0.556482	0.674445	0.706104	

10.3 Post Hoc Tests (2)

LSD test; variable VAP (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	5.15713	5.11984	0.327922	-5.6448	15.9590
{1}-{3}	control	fraction B	1.19999	5.11984	0.81749	-9.6019	12.0019
{1}-{4}	control	fraction C	3.07142	5.11984	0.556482	-7.7305	13.8733
{2}-{3}	fraction A	fraction B	-3.95714	4.87988	0.428625	-14.2528	6.3385
{2}-{4}	fraction A	fraction C	-2.08571	4.87988	0.674445	-12.3814	8.2099
{3}-{4}	fraction B	fraction C	1.87143	4.87988	0.706104	-8.4242	12.1670

11. LIN

11.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=3.0603, p=.05644
Type III decomposition

Cell No.	stage	LIN Mean	LIN Std.Err.	LIN -95.00%	LIN +95.00%	N
1	contro	35.5404	3.76234	27.6026	43.4783	6
2	fraction A	25.9857	3.53818	18.5208	33.4506	7
3	fraction B	34.2571	3.53818	26.7922	41.7220	7
4	fraction C	37.3285	3.53818	29.8636	44.7934	7

11.2 Post Hoc Tests (1)

LSD test; variable LIN (Spreadsheet416)
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 35.540	{2} 25.986	{3} 34.257	{4} 37.329
1	contro		0.03747	0.76547	0.67806
2	fraction A	0.03747		0.05616	0.01203
3	fraction B	0.76547	0.05616		0.45705
4	fraction C	0.67806	0.01203	0.45705	

11.3 Post Hoc Tests (2)

LSD test; variable LIN (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	9.5548	4.23369	0.03747	0.6224	18.4871
{1}-{3}	control	fraction B	1.2833	4.23369	0.76547	-7.6490	10.2156
{1}-{4}	control	fraction C	-1.7881	4.23369	0.67806	-10.7204	7.1442
{2}-{3}	fraction A	fraction B	-8.2714	4.03580	0.05616	-16.7862	0.2433
{2}-{4}	fraction A	fraction C	-11.3429	4.03580	0.01203	-19.8577	-2.8280
{3}-{4}	fraction B	fraction C	-3.0714	4.03580	0.45705	-11.5862	5.4433

12. STR

12.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=1.9773, p=.15566
Type III decomposition

Cell No.	stage	STR Mean	STR Std.Err.	STR -95.00%	STR +95.00%	N
1	contro	55.7007	5.45528	44.1910	67.2103	6
2	fraction A	45.8428	5.10551	35.0711	56.6145	7
3	fraction B	57.1142	5.10551	46.3425	67.8859	7
4	fraction C	59.8428	5.10551	49.0711	70.6145	7

12.2 Post Hoc Tests (1)

LSD test; variable STR (Spreadsheet416)
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 55.701	{2} 45.843	{3} 57.114	{4} 59.843
1	contro		0.14339	0.82850	0.52777
2	fraction A	0.14339		0.08356	0.03555
3	fraction B	0.82850	0.08356		0.66192
4	fraction C	0.52777	0.03555	0.66192	

12.3 Post Hoc Tests (2)

LSD test; variable STR (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	9.857	6.42569	0.14339	-3.699	23.4148
{1}-{3}	contro	fraction B	-1.413	6.42569	0.82850	-14.970	12.1434
{1}-{4}	contro	fraction C	-4.142	6.42569	0.52777	-17.699	9.4148
{2}-{3}	fraction A	fraction B	-11.271	6.13153	0.08356	-24.207	1.6649
{2}-{4}	fraction A	fraction C	-14.000	6.13153	0.03555	-26.936	-1.0635
{3}-{4}	fraction B	fraction C	-2.728	6.13153	0.66192	-15.665	10.2078

13. WOB

13.1 Means Table

stage; LS Means (Spreadsheet416)
Current effect: F(3, 17)=2.6408, p=.08268
Type III decomposition

Cell No.	stage	WOB Mean	WOB Std.Err.	WOB -95.00%	WOB +95.00%	N
1	contro	63.7433	4.98903	53.2174	74.2693	6
2	fraction A	47.8857	4.63051	38.1161	57.6552	7
3	fraction B	59.6571	4.63051	49.8876	69.4266	7
4	fraction C	62.0714	4.63051	52.3018	71.8409	7

13.2 Post Hoc Tests (1)

LSD test; variable WOB (Spreadsheet416)
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 63.743	{2} 47.886	{3} 59.657	{4} 62.071
1	contro		0.02433	0.53274	0.79757
2	fraction A	0.02433		0.07228	0.03374
3	fraction B	0.53274	0.07228		0.69916
4	fraction C	0.79757	0.03374	0.69916	

13.3 Post Hoc Tests (2)

LSD test; variable WOB (Spreadsheet416)
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	15.8577	6.41700	0.02433	2.319	29.3963
{1}-{3}	control	fraction B	4.0862	6.41700	0.53274	-9.4525	17.6249
{1}-{4}	control	fraction C	1.6719	6.41700	0.79757	-11.8668	15.2106
{2}-{3}	fraction A	fraction B	-11.7714	6.14241	0.07228	-24.7308	1.1879
{2}-{4}	fraction A	fraction C	-14.1857	6.14241	0.03374	-27.1451	-1.2263
{3}-{4}	fraction B	fraction C	-2.4143	6.14241	0.69916	-15.3736	10.5450

APPENDIX F

DOUBLE DENSITY GRADIENT CENTRIFUGATION DATA: MOTILITY

1. Static

1.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS
Current effect: F(3, 12)=10.073, p=.00134
Type III decomposition

Cell No.	stage	static Mean	static Std.Err.	static -95.00%	static +95.00%	N
1	control	11.4000(	5.433912	-0.4394{	23.2394{	5
2	fraction A	40.0600(	5.433912	28.2205{	51.8994{	5
3	fraction B	18.4400(	5.433912	6.6005{	30.2794{	5
4	fraction C	10.0800(	5.433912	-1.7594{	21.9194{	5

1.2 Post Hoc Tests (1)

LSD test; variable static (Spreadsheet923 in MOTILIT
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 11.400	{2} 40.060	{3} 18.440	{4} 10.080
1	control		0.000573	0.27693{	0.83447{
2	fraction A	0.000573		0.00439{	0.00039{
3	fraction B	0.27693{	0.00439{		0.20113{
4	fraction C	0.83447{	0.00039{	0.20113{	

1.3 Post Hoc Tests (2)

LSD test; variable static (Spreadsheet923 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	-28.660(	6.18087	0.00057	-42.127(	-15.193(
{1}-{3}	contro	fraction B	-7.040(	6.18087	0.27693	-20.507(	6.427(
{1}-{4}	contro	fraction C	1.320(	6.18087	0.83447{	-12.147(	14.787(
{2}-{3}	fraction A	fraction B	21.620(	6.18087	0.00439	8.153(	35.087(
{2}-{4}	fraction A	fraction C	29.980(	6.18087	0.00039	16.513(	43.447(
{3}-{4}	fraction B	fraction C	8.360(	6.18087	0.20113{	-5.107(	21.827(

2. Non-Progressive

2.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS DATA.stw)
Current effect: F(3, 12)=8.5008, p=.00268
Type III decomposition

Cell No.	stage	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	contro	26.4600	4.11635	17.4912	35.4287	5
2	fraction A	32.2600	4.11635	23.2912	41.2287	5
3	fraction B	28.0400	4.11635	19.0712	37.0087	5
4	fraction C	21.3600	4.11635	12.3912	30.3287	5

2.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Spreadsheet923 i
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 26.460	{2} 32.260	{3} 28.040	{4} 21.360
1	contro		0.02098	0.48336	0.03774
2	fraction A	0.02098		0.07733	0.00031
3	fraction B	0.48336	0.07733		0.00993
4	fraction C	0.03774	0.00031	0.00993	

2.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet923 in MOTILITY STATS DA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	-5.8000	2.18446	0.02098	-10.559	-1.0404
{1}-{3}	contro	fraction B	-1.5800	2.18446	0.48336	-6.339	3.1795
{1}-{4}	contro	fraction C	5.1000	2.18446	0.03774	0.340	9.8595
{2}-{3}	fraction A	fraction B	4.2200	2.18446	0.07733	-0.539	8.9795
{2}-{4}	fraction A	fraction C	10.9000	2.18446	0.00031	6.140	15.6595
{3}-{4}	fraction B	fraction C	6.6800	2.18446	0.00993	1.920	11.4395

3. Progressive

3.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS DATA.s
Current effect: F(3, 12)=12.068, p=.00062
Type III decomposition

Cell No.	stage	progressive Mean	progressive Std.Err.	progressive -95.00%	progressive +95.00%	N
1	contro	62.1400	8.78921	42.9899	81.2900	5
2	fraction A	27.6600	8.78921	8.5099	46.8100	5
3	fraction B	53.4800	8.78921	34.3299	72.6300	5
4	fraction C	68.5600	8.78921	49.4099	87.7100	5

3.2 Post Hoc Tests (1)

LSD test; variable progressive (Spreadsheet923 in MC
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 62.140	{2} 27.660	{3} 53.480	{4} 68.560
1	contro		0.00050	0.25924	0.39722
2	fraction A	0.00050		0.00414	0.00011
3	fraction B	0.25924	0.00414		0.06153
4	fraction C	0.39722	0.00011	0.06153	

3.3 Post Hoc Tests (2)

LSD test; variable progressive (Spreadsheet923 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	34.480	7.31276	0.00050	18.546	50.413
{1}-{3}	contro	fraction B	8.660	7.31276	0.25924	-7.273	24.593
{1}-{4}	contro	fraction C	-6.420	7.31276	0.39722	-22.353	9.513
{2}-{3}	fraction A	fraction B	-25.820	7.31276	0.00414	-41.753	-9.886
{2}-{4}	fraction A	fraction C	-40.900	7.31276	0.00011	-56.833	-24.966
{3}-{4}	fraction B	fraction C	-15.080	7.31276	0.06153	-31.013	0.853

4. Total Motile

4.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS DATA.
Current effect: F(3, 12)=10.044, p=.00136
Type III decomposition

Cell No.	stage	total motile Mean	total motile Std.Err.	total motile -95.00%	total motile +95.00%	N
1	contro	88.6000	5.44247	76.7418	100.458	5
2	fraction A	59.9200	5.44247	48.0618	71.778	5
3	fraction B	81.5200	5.44247	69.6618	93.378	5
4	fraction C	89.9200	5.44247	78.0618	101.778	5

4.2 Post Hoc Tests (1)

LSD test; variable total motile (Spreadsheet923 in MC
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 88.600	{2} 59.920	{3} 81.520	{4} 89.920
1	contro		0.00057	0.27526	0.83480
2	fraction A	0.00057		0.00448	0.00040
3	fraction B	0.27526	0.00448		0.19997
4	fraction C	0.83480	0.00040	0.19997	

4.3 Post Hoc Tests (2)

LSD test; variable total motile (Spreadsheet923 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	28.680	6.19337	0.00057	15.185	42.174
{1}-{3}	control	fraction B	7.080	6.19337	0.27526	-6.414	20.574
{1}-{4}	control	fraction C	-1.320	6.19337	0.83480	-14.814	12.174
{2}-{3}	fraction A	fraction B	-21.600	6.19337	0.00448	-35.094	-8.105
{2}-{4}	fraction A	fraction C	-30.000	6.19337	0.00040	-43.494	-16.505
{3}-{4}	fraction B	fraction C	-8.400	6.19337	0.19997	-21.894	5.094

5. Fast Progressive

5.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS DATA.stw)
Current effect: F(3, 12)=4.5854, p=.02322
Type III decomposition

Cell No.	stage	fast progressive Mean	fast progressive Std.Err.	fast progressive -95.00%	fast progressive +95.00%	N
1	control	6.9200(	3.24483(	-0.1498(	13.9898(	5
2	fraction A	1.8200(	3.24483(	-5.2498(	8.8898(	5
3	fraction B	12.9200(	3.24483(	5.8501(	19.9898(	5
4	fraction C	14.6600(	3.24483(	7.5901(	21.7298(	5

5.2 Post Hoc Tests (1)

LSD test; variable fast progressive (Spreadsheet923 in
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 6.9200	{2} 1.8200	{3} 12.920	{4} 14.660
1	contro		0.21266(	0.14744(	0.06893(
2	fraction A	0.21266(		0.01422(	0.00617(
3	fraction B	0.14744(	0.01422(		0.66137(
4	fraction C	0.06893(	0.00617(	0.66137(	

5.3 Post Hoc Tests (2)

LSD test; variable fast progressive (Spreadsheet923 in MOTILITY STATS DA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	5.100(	3.87454(	0.21266(	-3.341(	13.5419(
{1}-{3}	contro	fraction B	-6.000(	3.87454(	0.14744(	-14.441(	2.4419(
{1}-{4}	contro	fraction C	-7.740(	3.87454(	0.06893(	-16.181(	0.7019(
{2}-{3}	fraction A	fraction B	-11.100(	3.87454(	0.01422(	-19.541(	-2.6580(
{2}-{4}	fraction A	fraction C	-12.840(	3.87454(	0.00617(	-21.281(	-4.3980(
{3}-{4}	fraction B	fraction C	-1.740(	3.87454(	0.66137(	-10.181(	6.7019(

6. Slow Progressive

6.1 Means Table

Cell No.	stage	slow progressive Mean	slow progressive Std.Err.	slow progressive -95.00%	slow progressive +95.00%	N
	stage; LS Means (Spreadsheet923 in MOTILITY STATS DATA.stw) Current effect: F(3, 12)=8.3827, p=.00283 Type III decomposition					
1	contro	55.2400	7.00591	39.9754	70.5045	5
2	fraction A	25.8600	7.00591	10.5954	41.1245	5
3	fraction B	40.5600	7.00591	25.2954	55.8245	5
4	fraction C	53.8800	7.00591	38.6154	69.1445	5

6.2 Post Hoc Tests (1)

Cell No.	stage	{1} 55.240	{2} 25.860	{3} 40.560	{4} 53.880
	LSD test; variable slow progressive (Spreadsheet923 i Probabilities for Post Hoc Tests Effect: stage				
1	contro		0.00089	0.04897	0.84259
2	fraction A	0.00089		0.04871	0.00127
3	fraction B	0.04897	0.04871		0.07018
4	fraction C	0.84259	0.00127	0.07018	

6.3 Post Hoc Tests (2)

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
	LSD test; variable slow progressive (Spreadsheet923 in MOTILITY STATS D Simultaneous confidence intervals Effect: stage						
{1}-{2}	contro	fraction A	29.380	6.70204	0.00089	14.777	43.982
{1}-{3}	contro	fraction B	14.680	6.70204	0.04897	0.077	29.282
{1}-{4}	contro	fraction C	1.360	6.70204	0.84259	-13.242	15.962
{2}-{3}	fraction A	fraction B	-14.700	6.70204	0.04871	-29.302	-0.097
{2}-{4}	fraction A	fraction C	-28.020	6.70204	0.00127	-42.622	-13.417
{3}-{4}	fraction B	fraction C	-13.320	6.70204	0.07018	-27.922	1.282

7. Non-Progressive

7.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS DATA.stw)
Current effect: F(3, 12)=8.5008, p=.00268
Type III decomposition

Cell No.	stage	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	contro	26.4600	4.11635	17.4912	35.4287	5
2	fraction A	32.2600	4.11635	23.2912	41.2287	5
3	fraction B	28.0400	4.11635	19.0712	37.0087	5
4	fraction C	21.3600	4.11635	12.3912	30.3287	5

7.2 Post Hoc Tests (1)

LSD test; variable non-progressive (Spreadsheet923 i
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 26.460	{2} 32.260	{3} 28.040	{4} 21.360
1	contro		0.02098	0.48336	0.03774
2	fraction A	0.02098		0.07733	0.00031
3	fraction B	0.48336	0.07733		0.00993
4	fraction C	0.03774	0.00031	0.00993	

7.3 Post Hoc Tests (2)

LSD test; variable non-progressive (Spreadsheet923 in MOTILITY STATS DA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	-5.8000	2.18446	0.02098	-10.559	-1.0404
{1}-{3}	contro	fraction B	-1.5800	2.18446	0.48336	-6.339	3.1795
{1}-{4}	contro	fraction C	5.1000	2.18446	0.03774	0.340	9.8595
{2}-{3}	fraction A	fraction B	4.2200	2.18446	0.07733	-0.539	8.9795
{2}-{4}	fraction A	fraction C	10.9000	2.18446	0.00031	6.140	15.6595
{3}-{4}	fraction B	fraction C	6.6800	2.18446	0.00993	1.920	11.4395

8. VCL

8.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS
Current effect: F(3, 12)=5.7909, p=.01098
Type III decomposition

Cell No.	stage	VCL Mean	VCL Std.Err.	VCL -95.00%	VCL +95.00%	N
1	contro	57.3400	6.25835	43.7042	70.9757	5
2	fraction A	39.2600	6.25835	25.6242	52.8957	5
3	fraction B	53.9600	6.25835	40.3242	67.5957	5
4	fraction C	57.2800	6.25835	43.6442	70.9157	5

8.2 Post Hoc Tests (1)

LSD test; variable VCL (Spreadsheet923 in MOTILITY
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 57.340	{2} 39.260	{3} 53.960	{4} 57.280
1	contro		0.00383	0.51691	0.99073
2	fraction A	0.00383		0.01322	0.00392
3	fraction B	0.51691	0.01322		0.52424
4	fraction C	0.99073	0.00392	0.52424	

8.3 Post Hoc Tests (2)

LSD test; variable VCL (Spreadsheet923 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	18.080	5.06152	0.00383	7.051	29.1081
{1}-{3}	control	fraction B	3.380	5.06152	0.51691	-7.6481	14.4081
{1}-{4}	control	fraction C	0.060	5.06152	0.99073	-10.9681	11.0881
{2}-{3}	fraction A	fraction B	-14.700	5.06152	0.01322	-25.7281	-3.6718
{2}-{4}	fraction A	fraction C	-18.020	5.06152	0.00392	-29.0481	-6.9918
{3}-{4}	fraction B	fraction C	-3.320	5.06152	0.52424	-14.3481	7.7081

9. VSL

9.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS
Current effect: F(3, 12)=9.8300, p=.00149
Type III decomposition

Cell No.	stage	VSL Mean	VSL Std.Err.	VSL -95.00%	VSL +95.00%	N
1	contro	17.4600	2.94641	11.0403	23.8796	5
2	fraction A	8.1800	2.94641	1.7603	14.5996	5
3	fraction B	18.7200	2.94641	12.3003	25.1396	5
4	fraction C	21.1000	2.94641	14.6803	27.5196	5

9.2 Post Hoc Tests (1)

LSD test; variable VSL (Spreadsheet923 in MOTILITY
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 17.460	{2} 8.1800	{3} 18.720	{4} 21.100
1	contro		0.003426	0.630638	0.179539
2	fraction A	0.003426		0.001402	0.000280
3	fraction B	0.630638	0.001402		0.369723
4	fraction C	0.179539	0.000280	0.369723	

9.3 Post Hoc Tests (2)

LSD test; variable VSL (Spreadsheet923 in MOTILITY STATS DATA.
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	9.2800	2.55372	0.00342	3.7159	14.8440
{1}-{3}	contro	fraction B	-1.2600	2.55372	0.63063	-6.824	4.3040
{1}-{4}	contro	fraction C	-3.6400	2.55372	0.17953	-9.204	1.9240
{2}-{3}	fraction A	fraction B	-10.5400	2.55372	0.00140	-16.104	-4.9759
{2}-{4}	fraction A	fraction C	-12.9200	2.55372	0.00028	-18.484	-7.3559
{3}-{4}	fraction B	fraction C	-2.3800	2.55372	0.36972	-7.944	3.1840

10. VAP

10.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS
Current effect: F(3, 12)=14.126, p=.00030
Type III decomposition

Cell No.	stage	VAP Mean	VAP Std.Err.	VAP -95.00%	VAP +95.00%	N
1	contro	35.4400	4.11438	26.4755	44.4044	5
2	fraction A	20.3800	4.11438	11.4155	29.3444	5
3	fraction B	33.1200	4.11438	24.1555	42.0844	5
4	fraction C	36.2800	4.11438	27.3155	45.2444	5

10.2 Post Hoc Tests (1)

LSD test; variable VAP (Spreadsheet923 in MOTILIT
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 35.440	{2} 20.380	{3} 33.120	{4} 36.280
1	contro		0.00015	0.42130	0.76821
2	fraction A	0.00015		0.00064	0.00009
3	fraction B	0.42130	0.00064		0.27888
4	fraction C	0.76821	0.00009	0.27888	

10.3 Post Hoc Tests (2)

LSD test; variable VAP (Spreadsheet923 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	15.060	2.78627	0.00015	8.989	21.1307
{1}-{3}	contro	fraction B	2.320	2.78627	0.42130	-3.750	8.3907
{1}-{4}	contro	fraction C	-0.840	2.78627	0.76821	-6.910	5.2307
{2}-{3}	fraction A	fraction B	-12.740	2.78627	0.00064	-18.810	-6.6692
{2}-{4}	fraction A	fraction C	-15.900	2.78627	0.00009	-21.970	-9.8292
{3}-{4}	fraction B	fraction C	-3.160	2.78627	0.27888	-9.230	2.9107

11. LIN

11.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS
Current effect: F(3, 12)=10.298, p=.00123
Type III decomposition

Cell No.	stage	LIN Mean	LIN Std.Err.	LIN -95.00%	LIN +95.00%	N
1	control	29.8600(	2.886997	23.56977	36.15023	5
2	fraction A	20.9600(	2.886997	14.66977	27.25023	5
3	fraction B	33.1800(	2.886997	26.88977	39.47023	5
4	fraction C	35.9200(	2.886997	29.62977	42.21023	5

11.2 Post Hoc Tests (1)

LSD test; variable LIN (Spreadsheet923 in MOTILITY
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 29.860	{2} 20.960	{3} 33.180	{4} 35.920
1	contro		0.00910:	0.26925	0.05609
2	fraction A	0.00910:		0.00110	0.00021
3	fraction B	0.26925	0.00110		0.35794
4	fraction C	0.05609	0.00021	0.35794	

11.3 Post Hoc Tests (2)

LSD test; variable LIN (Spreadsheet923 in MOTILITY STATS DATA.s
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	8.900(	2.86617	0.00910:	2.655:	15.1448
{1}-{3}	contro	fraction B	-3.320(	2.86617	0.26925	-9.564	2.9248
{1}-{4}	contro	fraction C	-6.060(	2.86617	0.05609	-12.304	0.1848
{2}-{3}	fraction A	fraction B	-12.220(	2.86617	0.00110	-18.464	-5.9751
{2}-{4}	fraction A	fraction C	-14.960(	2.86617	0.00021	-21.204	-8.7151
{3}-{4}	fraction B	fraction C	-2.740(	2.86617	0.35794	-8.984	3.5048

12. STR

12.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS
Current effect: F(3, 12)=6.1214, p=.00908
Type III decomposition

Cell No.	stage	STR Mean	STR Std.Err.	STR -95.00%	STR +95.00%	N
1	contro	48.6200	3.68769	40.5852	56.6548	5
2	fraction A	40.2200	3.68769	32.1852	48.2548	5
3	fraction B	53.8000	3.68769	45.7652	61.8348	5
4	fraction C	56.7400	3.68769	48.7052	64.7748	5

12.2 Post Hoc Tests (1)

LSD test; variable STR (Spreadsheet923 in MOTILIT
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 48.620	{2} 40.220	{3} 53.800	{4} 56.740
1	contro		0.06520	0.23462	0.07341
2	fraction A	0.06520		0.00657	0.00179
3	fraction B	0.23462	0.00657		0.49111
4	fraction C	0.07341	0.00179	0.49111	

12.3 Post Hoc Tests (2)

LSD test; variable STR (Spreadsheet923 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	contro	fraction A	8.400	4.13930	0.06520	-0.618	17.4187
{1}-{3}	contro	fraction B	-5.180	4.13930	0.23462	-14.198	3.8387
{1}-{4}	contro	fraction C	-8.120	4.13930	0.07341	-17.138	0.8987
{2}-{3}	fraction A	fraction B	-13.580	4.13930	0.00657	-22.598	-4.5612
{2}-{4}	fraction A	fraction C	-16.520	4.13930	0.00179	-25.538	-7.5012
{3}-{4}	fraction B	fraction C	-2.940	4.13930	0.49111	-11.958	6.0787

13. WOB

13.1 Means Table

stage; LS Means (Spreadsheet923 in MOTILITY STATS
Current effect: F(3, 12)=23.491, p=.00003
Type III decomposition

Cell No.	stage	WOB Mean	WOB Std.Err.	WOB -95.00%	WOB +95.00%	N
1	contro	61.2600	1.73613	57.4773	65.0427	5
2	fraction A	51.9400	1.73613	48.1573	55.7227	5
3	fraction B	61.0800	1.73613	57.2973	64.8627	5
4	fraction C	63.1000	1.73613	59.3173	66.8827	5

13.2 Post Hoc Tests (1)

LSD test; variable WOB (Spreadsheet923 in MOTILIT
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 61.260	{2} 51.940	{3} 61.080	{4} 63.100
1	contro		0.00003	0.90423	0.23298
2	fraction A	0.00003		0.00004	0.00000
3	fraction B	0.90423	0.00004		0.19306
4	fraction C	0.23298	0.00000	0.19306	

13.3 Post Hoc Tests (2)

LSD test; variable WOB (Spreadsheet923 in MOTILITY STATS DATA
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	9.320	1.46486	0.00003	6.128	12.5116
{1}-{3}	control	fraction B	0.180	1.46486	0.90423	-3.0117	3.3716
{1}-{4}	control	fraction C	-1.840	1.46486	0.23298	-5.0317	1.3516
{2}-{3}	fraction A	fraction B	-9.140	1.46486	0.00004	-12.3317	-5.9483
{2}-{4}	fraction A	fraction C	-11.160	1.46486	0.00000	-14.3517	-7.9683
{3}-{4}	fraction B	fraction C	-2.020	1.46486	0.19306	-5.2117	1.1716

DOUBLE DENSITY GRADIENT CENTRIFUGATION DATA: VIABILITY

14. Viability

14.1 Means Table

stage; LS Means (Spreadsheet88 in VIABILITY & HA STATS DATA.stw)
Current effect: F(3, 12)=4.2025, p=.03005
Type III decomposition

Cell No.	stage	viable percentage Mean	viable percentage Std.Err.	viable percentage -95.00%	viable percentage +95.00%	N
1	control	89.52877	2.550844	83.97096	95.08658	5
2	fraction A	79.10811	2.550844	73.55030	84.66592	5
3	fraction B	86.32018	2.550844	80.76237	91.87799	5
4	fraction C	88.24316	2.550844	82.68534	93.80097	5

14.2 Post Hoc Tests (1)

LSD test; variable viable percentage (Spreadsheet88
Probabilities for Post Hoc Tests
Effect: stage

Cell No.	stage	{1} 89.529	{2} 79.108	{3} 86.320	{4} 88.243
1	control		0.006995	0.337147	0.695760
2	fraction A	0.006995		0.044214	0.014719
3	fraction B	0.337147	0.044214		0.560186
4	fraction C	0.695760	0.014719	0.560186	

14.3 Post Hoc Tests (2)

LSD test; variable viable percentage (Spreadsheet88 in VIABILITY & HA ST
Simultaneous confidence intervals
Effect: stage

Comparisons Cell {#1}-{#2}	1st Mean	2nd Mean	Mean Differ.	Standard Error	p	-95.00% Cnf.Lmt	+95.00% Cnf.Lmt
{1}-{2}	control	fraction A	10.42066	3.209264	0.006995	3.4283	17.41305
{1}-{3}	control	fraction B	3.20858	3.209264	0.337147	-3.7838	10.20097
{1}-{4}	control	fraction C	1.28561	3.209264	0.695760	-5.7068	8.27800
{2}-{3}	fraction A	fraction B	-7.21207	3.209264	0.044214	-14.2045	-0.21969
{2}-{4}	fraction A	fraction C	-9.13505	3.209264	0.014719	-16.1274	-2.14266
{3}-{4}	fraction B	fraction C	-1.92297	3.209264	0.560186	-8.9154	5.06941

APPENDIX G

DOUBLE WASH CENTRIFUGATION DATA: MOTILITY

1. Static

1.1 Means Table

Cell No.	stage	static Mean	static Std.Err.	static -95.00%	static +95.00%	N
	stage; LS Means (Spreadsheet2) Current effect: F(1, 6)=1.4167, p=.27889 Type III decomposition					
1	contro	26.8857	4.51185	15.8455	37.9258	7
2	dw pelle	31.8142	4.51185	20.7741	42.8544	7

1.2 Descriptive Statistics (1)

Effect	Num. DF	Den. DF	F	p
	Fixed Effect Test for static (Spreadshee Restricted Maximum Likelihood (REML Type III decomposition grouping vars: sample, stage Fixed: stage Random: sample			
stage	1	6	1.41673	0.27889

1.3 Descriptive Statistics (2)

Effect	Level of Factor	N	static Mean	static Std.Dev.	static Std.Err	static -95.00%	static +95.00%
	Descriptive Statistics (Spreadsheet2)						
Total		14	29.3500	11.7506	3.14047	22.565	36.134
stage	contro	7	26.8857	12.3854	4.68124	15.431	38.340
stage	dw pelle	7	31.8142	11.4716	4.33586	21.205	42.423

2. Non-Progressive

2.1 Means Table

stage; LS Means (Spreadsheet2)
Current effect: F(1, 6)=.34950, p=.57597
Type III decomposition

Cell No.	stage	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	contro	31.9714	2.37246	26.1662	37.7766	7
2	dw pelle	33.6142	2.37246	27.8090	39.4195	7

2.2 Descriptive Statistics (1)

Fixed Effect Test for non-progressive (Spre
Restricted Maximum Likelihood (REML)
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	0.34950	0.57597

2.3 Descriptive Statistics (2)

Descriptive Statistics (Spreadsheet2)

Effect	Level of Factor	N	non-progressive Mean	non-progressive Std.Dev.	non-progressive Std.Err	non-progressive -95.00%	non-progressive +95.00%
Total		14	32.7928	6.09065	1.62779	29.276	36.309
stage	contro	7	31.9714	4.35496	1.64602	27.943	35.999
stage	dw pelle	7	33.6142	7.73529	2.92366	26.460	40.768

3. Progressive

3.1 Means Table

stage; LS Means (Spreadsheet2)
Current effect: F(1, 6)=5.0997, p=.06470
Type III decomposition

Cell No.	stage	progressive Mean	progressive Std.Err.	progressive -95.00%	progressive +95.00%	N
1	contro	41.1428	3.02570	33.7392	48.5464	7
2	dw pelle	34.5714	3.02570	27.1677	41.9750	7

3.2 Descriptive Statistics (1)

Fixed Effect Test for progressive (Spreadsh
Restricted Maximum Likelihood (REML)
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	5.09965	0.06470

3.3 Descriptive Statistics (2)

Descriptive Statistics (Spreadsheet2)

Effect	Level of Factor	N	progressive Mean	progressive Std.Dev.	progressive Std.Err	progressive -95.00%	progressive +95.00%
Total		14	37.8571	8.4131	2.2485	32.999	42.714
stage	control	7	41.1428	9.8188	3.7111	32.061	50.223
stage	dw pelle	7	34.5714	5.6355	2.1300	29.359	39.783

4. Total Motile

4.1 Means Table

Cell No.	stage	total motile Mean	total motile Std.Err.	total motile -95.00%	total motile +95.00%	N
	stage; LS Means (Spreadsheet2) Current effect: F(1, 6)=1.3982, p=.28176 Type III decomposition					
1	contro	73.1142	4.51702	62.0615	84.1670	7
2	dw pelle	68.1857	4.51702	57.1329	79.2384	7

4.2 Descriptive Statistics (1)

Effect	Num. DF	Den. DF	F	p
	Fixed Effect Test for total motile (Spreads Restricted Maximum Likelihood (REML) Type III decomposition grouping vars: sample, stage Fixed: stage Random: sample			
stage	1	6	1.39817	0.28175

4.3 Descriptive Statistics (2)

Effect	Level of Factor	N	total motile Mean	total motile Std.Dev.	total motile Std.Err	total motile -95.00%	total motile +95.00%
	Descriptive Statistics (Spreadsheet2)						
Total		14	70.6500	11.7634	3.14390	63.858	77.442
stage	contro	7	73.1142	12.3926	4.68398	61.653	84.575
stage	dw pelle	7	68.1857	11.4922	4.34365	57.557	78.814

5. Fast Progressive

5.1 Means Table

Cell No.	stage	fast progressive Mean	fast progressive Std.Err.	fast progressive -95.00%	fast progressive +95.00%	N
	stage; LS Means (Spreadsheet2) Current effect: F(1, 6)=31.859, p=.00133 Type III decomposition					
1	contro	7.7714	1.33610	4.5021	11.0407	7
2	dw pelle	17.0000	1.33610	13.7306	20.2693	7

5.2 Descriptive Statistics (1)

Effect	Num. DF	Den. DF	F	p
	Fixed Effect Test for fast progressive (Spre Restricted Maximum Likelihood (REML) Type III decomposition grouping vars: sample, stage Fixed: stage Random: sample			
stage	1	6	31.8594	0.00132

5.3 Descriptive Statistics (2)

Effect	Level of Factor	N	fast progressive Mean	fast progressive Std.Dev.	fast progressive Std.Err	fast progressive -95.00%	fast progressive +95.00%
	Descriptive Statistics (Spreadsheet2)						
Total		14	12.3857	5.8706	1.56899	8.996	15.775
stage	contro	7	7.7714	4.0202	1.51951	4.053	11.489
stage	dw pelle	7	17.0000	2.9715	1.12313	14.251	19.748

6. Slow Progressive

6.1 Means Table

stage; LS Means (Spreadsheet2)
Current effect: F(1, 6)=39.518, p=.00075
Type III decomposition

Cell No.	stage	slow progressive Mean	slow progressive Std.Err.	slow progressive -95.00%	slow progressive +95.00%	N
1	contro	33.3857	2.36743	27.5928	39.1786	7
2	dw pelle	17.5857	2.36743	11.7928	23.3786	7

6.2 Descriptive Statistics (1)

Fixed Effect Test for slow progressive (Spre
Restricted Maximum Likelihood (REML)
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	39.51787	0.00075

6.3 Descriptive Statistics (2)

Descriptive Statistics (Spreadsheet2)

Effect	Level of Factor	N	slow progressive Mean	slow progressive Std.Dev.	slow progressive Std.Err	slow progressive -95.00%	slow progres +95.00%
Total		14	25.4857	10.1698	2.7180	19.614	31.
stage	contro	7	33.3857	7.5074	2.8375	26.443	40.
stage	dw pelle	7	17.5857	4.7015	1.7770	13.237	21.

7. Non-Progressive

7.1 Means Table

stage; LS Means (Spreadsheet2)
Current effect: F(1, 6)=.34950, p=.57597
Type III decomposition

Cell No.	stage	non-progressive Mean	non-progressive Std.Err.	non-progressive -95.00%	non-progressive +95.00%	N
1	contro	31.9714	2.37246	26.1662	37.7766	7
2	dw pelle	33.6142	2.37246	27.8090	39.4195	7

7.2 Descriptive Statistics (1)

Fixed Effect Test for non-progressive (Spre
Restricted Maximum Likelihood (REML)
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	0.34950	0.57597

8. VCL

8.1 Means Table

stage; LS Means (Spreadsheet2)
Current effect: F(1, 6)=2.0117, p=.20588
Type III decomposition

Cell No.	stage	VCL Mean	VCL Std.Err.	VCL -95.00%	VCL +95.00%	N
1	control	46.4428(	2.13841(	41.2103-	51.6753(	7
2	dw pelle	50.7000(	2.13841(	45.4674(	55.9325(	7

8.2 Descriptive Statistics (1)

Fixed Effect Test for VCL (Spreadshee
Restricted Maximum Likelihood (REMI
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	2.01168(	0.20588(

8.3 Descriptive Statistics (2)

Descriptive Statistics (Spreadsheet2)

Effect	Level of Factor	N	VCL Mean	VCL Std.Dev.	VCL Std.Err	VCL -95.00%	VCL +95.00%
Total		14	48.5714(	5.86743(	1.56813(	45.183(	51.959(
stage	contro	7	46.4428(	6.79383(	2.56782(	40.159(	52.726(
stage	dw pelle	7	50.7000(	4.22650(	1.59746(	46.791(	54.608(

9. VSL

9.1 Means Table

Cell No.	stage	VSL Mean	VSL Std.Err.	VSL -95.00%	VSL +95.00%	N
	stage; LS Means (Spreadsheet2) Current effect: F(1, 6)=13.492, p=.01042 Type III decomposition					
1	control	16.7000(	1.53970'	12.9324!	20.4675'	7
2	dw pelle	24.3571-	1.53970'	20.5896:	28.1246(	7

9.2 Descriptive Statistics (1)

Effect	Num. DF	Den. DF	F	p
	Fixed Effect Test for VSL (Spreadshee Restricted Maximum Likelihood (REMI Type III decomposition grouping vars: sample, stage Fixed: stage Random: sample			
stage	1	6	13.4920!	0.01041!

9.3 Descriptive Statistics (2)

Effect	Level of Factor	N	VSL Mean	VSL Std.Dev.	VSL Std.Err	VSL -95.00%	VSL +95.00%
	Descriptive Statistics (Spreadsheet2)						
Total		14	20.5285'	5.5770'	1.49053!	17.308!	23.748'
stage	contro	7	16.7000(	4.6263'	1.74860!	12.421:	20.978'
stage	dw pelle	7	24.3571-	3.4331(	1.29759'	21.182'	27.532:

10. VAP

10.1 Means Table

stage; LS Means (Spreadsheet2)
Current effect: F(1, 6)=1.6938, p=.24084
Type III decomposition

Cell No.	stage	VAP Mean	VAP Std.Err.	VAP -95.00%	VAP +95.00%	N
1	contro	30.2428	1.68609	26.1171	34.3685	7
2	dw pelle	32.7142	1.68609	28.5885	36.8400	7

10.2 Descriptive Statistics (1)

Fixed Effect Test for VAP (Spreadshee
Restricted Maximum Likelihood (REM
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	1.69380	0.24083

10.3 Descriptive Statistics (2)

Descriptive Statistics (Spreadsheet2)

Effect	Level of Factor	N	VAP Mean	VAP Std.Dev.	VAP Std.Err	VAP -95.00%	VAP +95.00%
Total		14	31.4785	4.47371	1.19565	28.895	34.0616
stage	contro	7	30.2428	5.24876	1.98384	25.388	35.0971
stage	dw pelle	7	32.7142	3.50020	1.32295	29.477	35.9514

11. LIN

11.1 Means Table

stage; LS Means (Spreadsheet2)
Current effect: F(1, 6)=16.124, p=.00699
Type III decomposition

Cell No.	stage	LIN Mean	LIN Std.Err.	LIN -95.00%	LIN +95.00%	N
1	control	35.5714	2.18106	30.2345	40.9083	7
2	dw pelle	47.9571	2.18106	42.6202	53.2940	7

11.2 Descriptive Statistics (1)

Fixed Effect Test for LIN (Spreadsheet
Restricted Maximum Likelihood (REM
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	16.1240	0.00699

11.3 Descriptive Statistics (2)

Descriptive Statistics (Spreadsheet2)

Effect	Level of Factor	N	LIN Mean	LIN Std.Dev.	LIN Std.Err	LIN -95.00%	LIN +95.00%
Total		14	41.7642	8.4876	2.2684	36.864	46.664
stage	control	7	35.5714	7.0165	2.6520	29.082	42.060
stage	dw pelle	7	47.9571	4.1672	1.5750	44.103	51.811

12. STR

12.1 Means Table

Cell No.	stage; LS Means (Spreadsheet2) Current effect: F(1, 6)=25.794, p=.00227 Type III decomposition stage	STR Mean	STR Std.Err.	STR -95.00%	STR +95.00%	N
1	control	54.6428	2.73879	47.9412	61.3444	7
2	dw pelle	74.3142	2.73879	67.6126	81.0158	7

12.2 Descriptive Statistics (1)

Effect	Fixed Effect Test for STR (Spreadshee Restricted Maximum Likelihood (REMI Type III decomposition grouping vars: sample, stage Fixed: stage Random: sample Num. DF	Den. DF	F	p
stage	1	6	25.7941	0.00226

12.3 Descriptive Statistics (2)

Effect	Descriptive Statistics (Spreadsheet2) Level of Factor	N	STR Mean	STR Std.Dev.	STR Std.Err	STR -95.00%	STR +95.00%
Total		14	64.4785	12.3552	3.3020	57.345	71.612
stage	control	7	54.6428	9.4005	3.5530	45.949	63.336
stage	dw pelle	7	74.3142	4.0798	1.5420	70.541	78.087

13. WOB

13.1 Means Table

stage; LS Means (Spreadsheet2)
Current effect: F(1, 6)=.12032, p=.74053
Type III decomposition

Cell No.	stage	WOB Mean	WOB Std.Err.	WOB -95.00%	WOB +95.00%	N
1	contro	65.0571	1.48945	61.4125	68.7017	7
2	dw pelle	64.4714	1.48945	60.8268	68.1159	7

13.2 Descriptive Statistics (1)

Fixed Effect Test for WOB (Spreadsheet2
Restricted Maximum Likelihood (REML)
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	0.12032	0.74052

13.3 Descriptive Statistics (2)

Descriptive Statistics (Spreadsheet2)

Effect	Level of Factor	N	WOB Mean	WOB Std.Dev.	WOB Std.Err	WOB -95.00%	WOB +95.00%
Total		14	64.7642	3.79830	1.01513	62.5712	66.957
stage	contro	7	65.0571	4.51030	1.70473	60.8858	69.228
stage	dw pelle	7	64.4714	3.27348	1.23726	61.4439	67.498

DOUBLE WASH CENTRIFUGATION DATA: VIABILITY

14. Viability

14.1 Means Table

stage; LS Means (Spreadsheet101 in VIABILITY & HA STATS DATA.stw)
Current effect: F(1, 6)=6.4010, p=.04468
Type III decomposition

Cell No.	stage	viable percentage Mean	viable percentage Std.Err.	viable percentage -95.00%	viable percentage +95.00%	N
1	contro	80.4073	3.15371	72.6904	88.1241	7
2	dw pelle	69.1233	3.15371	61.4064	76.8402	7

14.2 Descriptive Statistics (1)

Fixed Effect Test for viable percentage (S
Restricted Maximum Likelihood (REML)
Type III decomposition
grouping vars: sample, stage
Fixed: stage
Random: sample

Effect	Num. DF	Den. DF	F	p
stage	1	6	6.40103	0.04467

14.3 Descriptive Statistics (2)

Descriptive Statistics (Spreadsheet101 in VIABILITY & HA STATS DATA.stw)

Effect	Level of Factor	N	viable percentage Mean	viable percentage Std.Dev.	viable percentage Std.Err	viable percentage -95.00%	viable +9
Total		14	74.7653	9.92707	2.65312	69.034	
stage	control	7	80.4073	8.49593	3.21116	72.550	
stage	dw pelle	7	69.1233	8.18914	3.09520	61.550	

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