
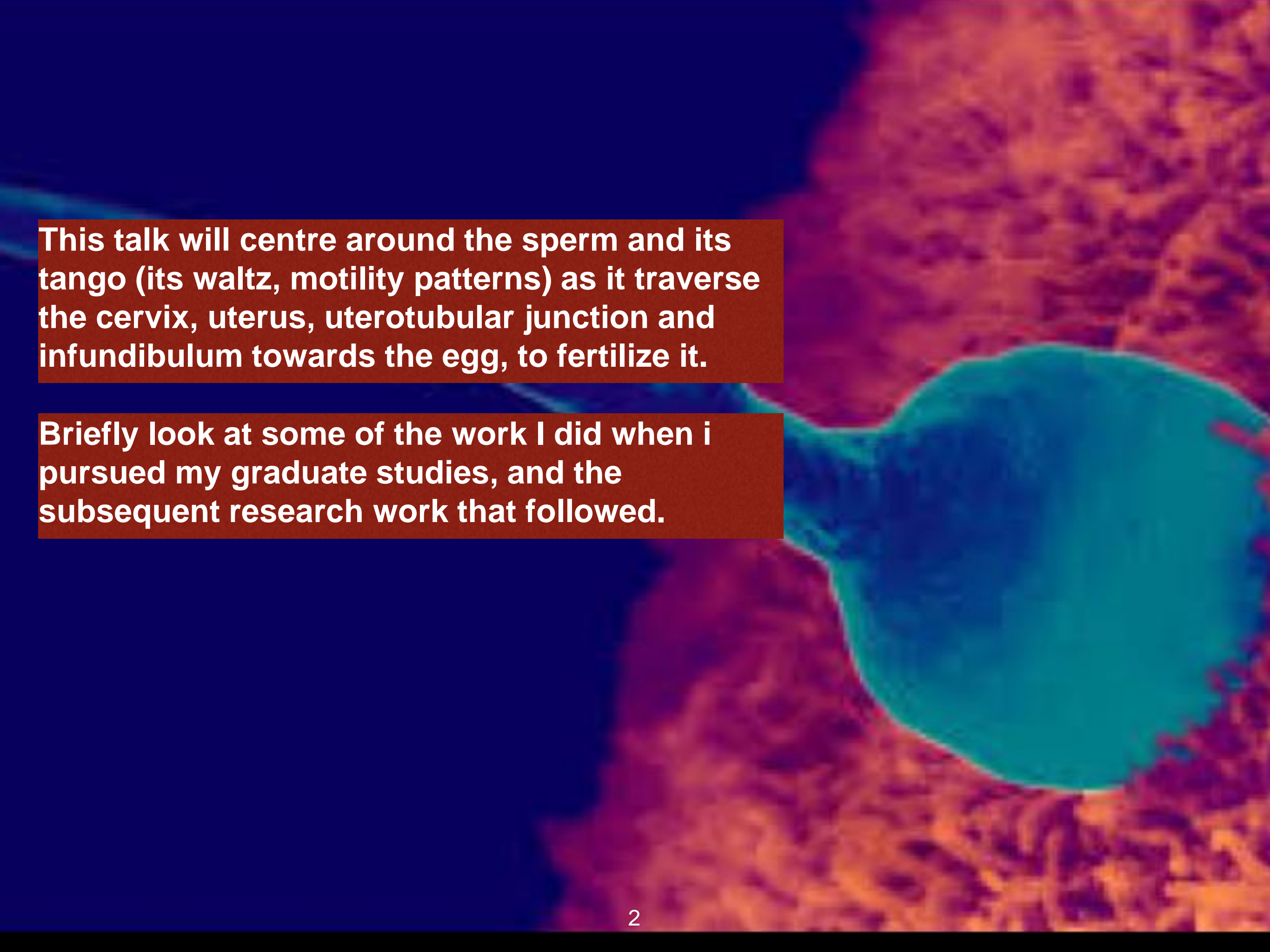


Tango of the Sperm - An Odyssey into Being

A microscopic image showing a sperm cell with a long, wavy tail (flagellum) swimming towards a large, textured egg cell. Several other sperm cells are visible in the background, some appearing to be in the process of fertilization.

Mohd Azam Khan Goriman Khan
DVM (UPM), Ph.D (Glasgow), FMCVS

A microscopic image showing a sperm cell on the left and an egg cell on the right. The sperm cell is smaller and has a distinct head and tail. The egg cell is much larger and more rounded. The background is a textured, reddish-brown color.

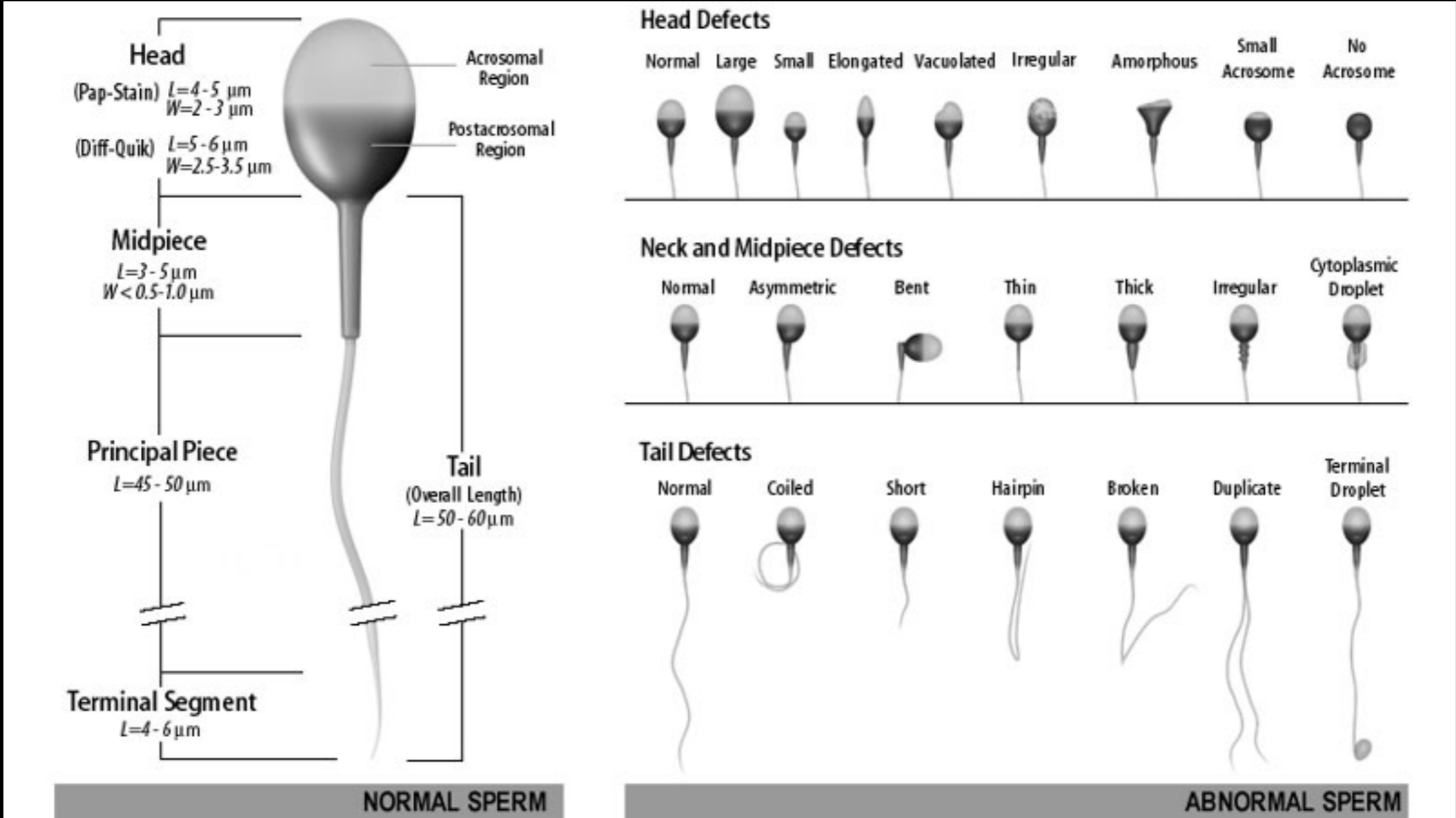
This talk will centre around the sperm and its tango (its waltz, motility patterns) as it traverse the cervix, uterus, uterotubular junction and infundibulum towards the egg, to fertilize it.

Briefly look at some of the work I did when i pursued my graduate studies, and the subsequent research work that followed.

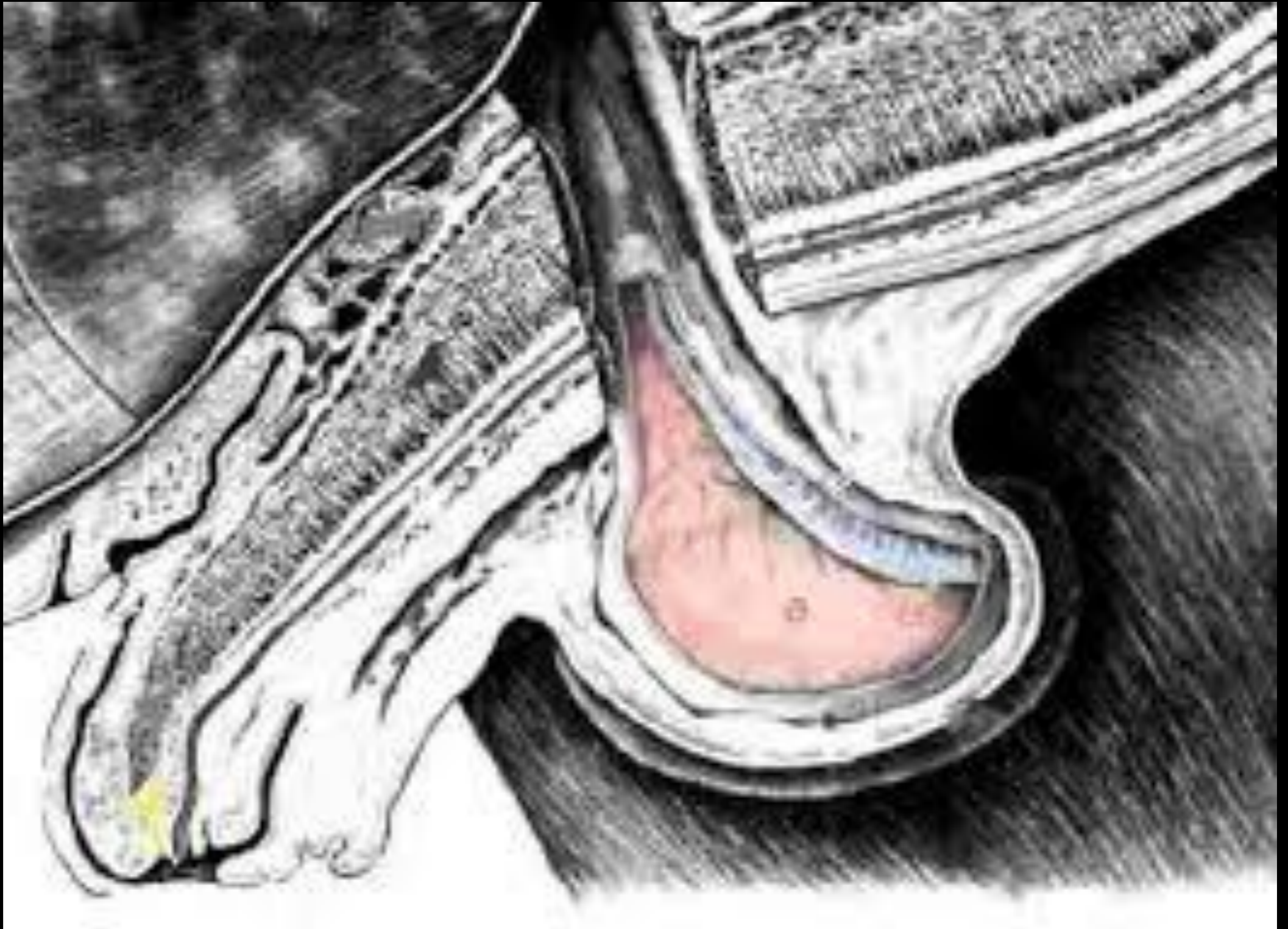
We created man from the purest kind of clay; then made him a drop in a secure receptacle; then formed the drop into a clot and formed the clot into a lump and formed the lump into bones and clothed the bones in flesh; and then brought him into being as another creature. Blessed be God, the Best of Creators! (Qur'an, 23: 12-14)



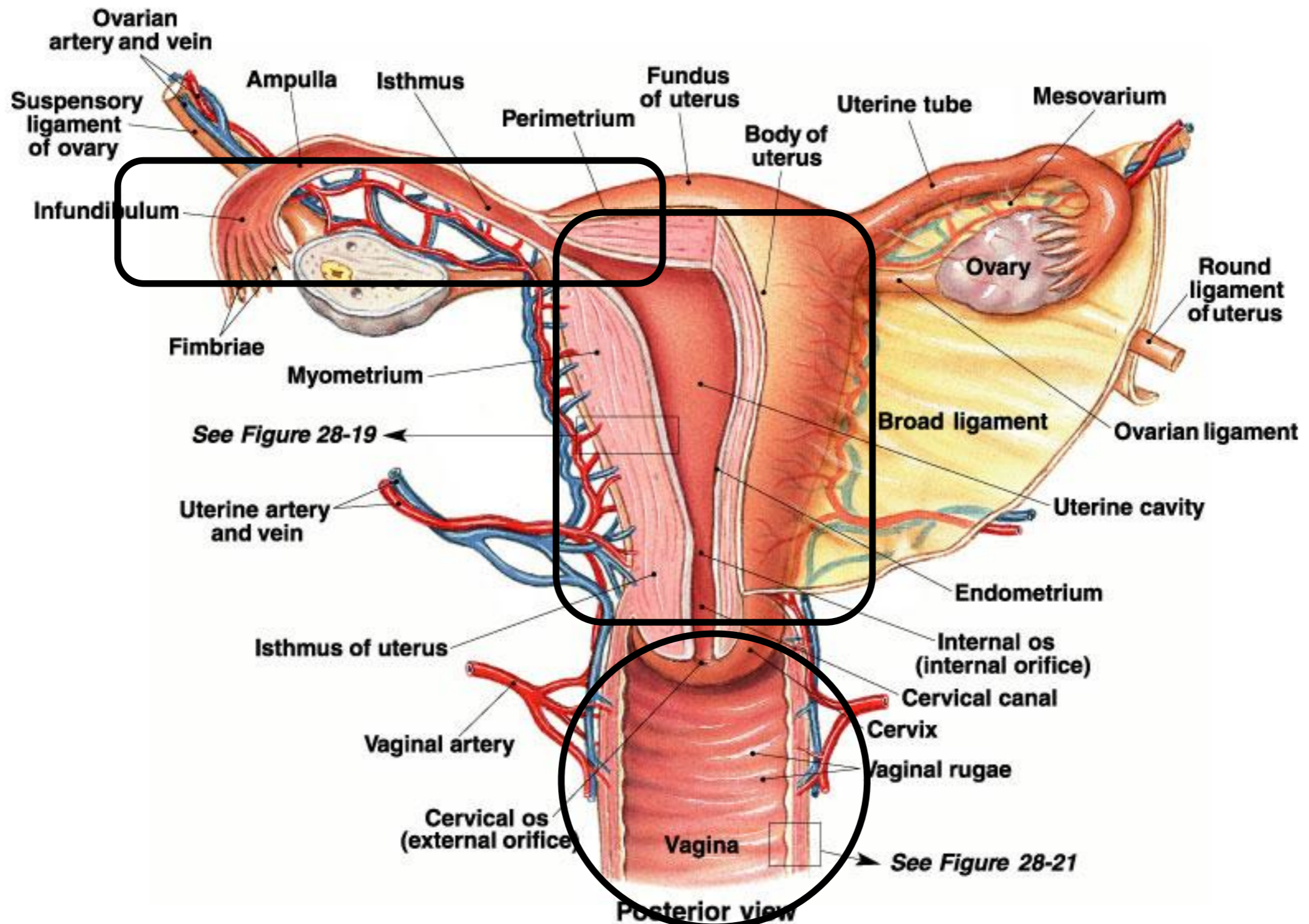
Normal and Abnormal Sperm



Male reproductive system



Female reproductive system



Sperm once deposited

Once deposited within the vaginal vestibule, at the cervical os, or in the uterus, spermatozoa will then make the journey towards the eggs.

How do they find their way towards the egg? How does the spermatozoa zoom onto the egg?



28 March issue of the journal, *Science* (2003), German and U.S. researchers report an odorant receptor (hOR17-4) found on the surface of sperm cells. Receptor triggers a series of physiological events that may result in the directed movement of human sperm towards the egg.

In this chemosensory response, the egg produces a sperm-attracting substance called "bourgeonal."

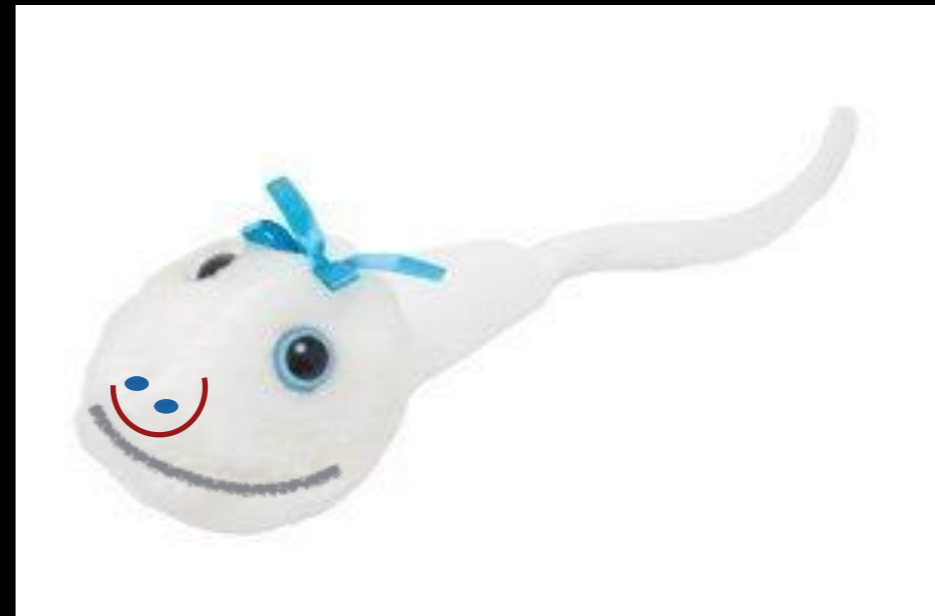


WRONG WAY

Odorant receptor

Sperm cells possess the same sort of odor receptors that allow the nose to smell, suggesting that swimming sperm navigate toward a fertile egg by detecting its scent, scientists have found.

Sperm get their kicks from lily of the valley - New Scientist, 2003

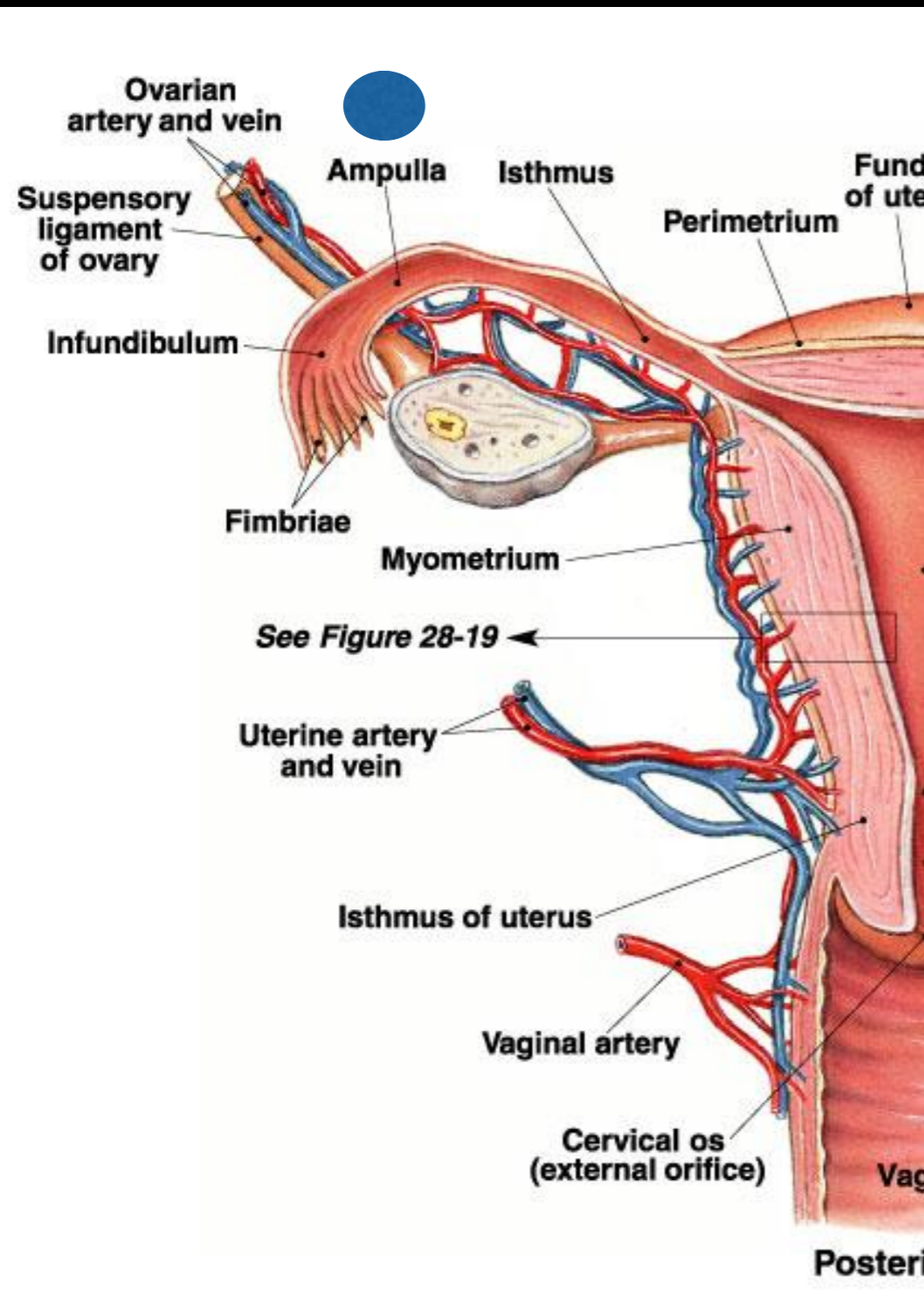


Thermotaxis



There is good evidence from animal studies, of a guidance system that helps sperm reach the oocyte. Long-range thermotaxis guides capacitated sperm released from intimate contact with the endosalpinx towards the fertilisation zone.

--Much like guided missiles that sense the heat of a plane's engine, sperm are guided to the fertilization site by temperature, reports a Weizmann Institute study published in the January 31, 2003 issue of Nature Medicine.



Thermotaxis

Animal studies show that sperm tends to swim towards warmer temperatures and the ampulla is two degrees warmer than the isthmus (Bahat et al, 2003).

How many sperm in an ejaculate ?



Only a few thousands reach the uterotubal junction and gain entry to the fallopian tubes and only one is needed to fertilise the egg

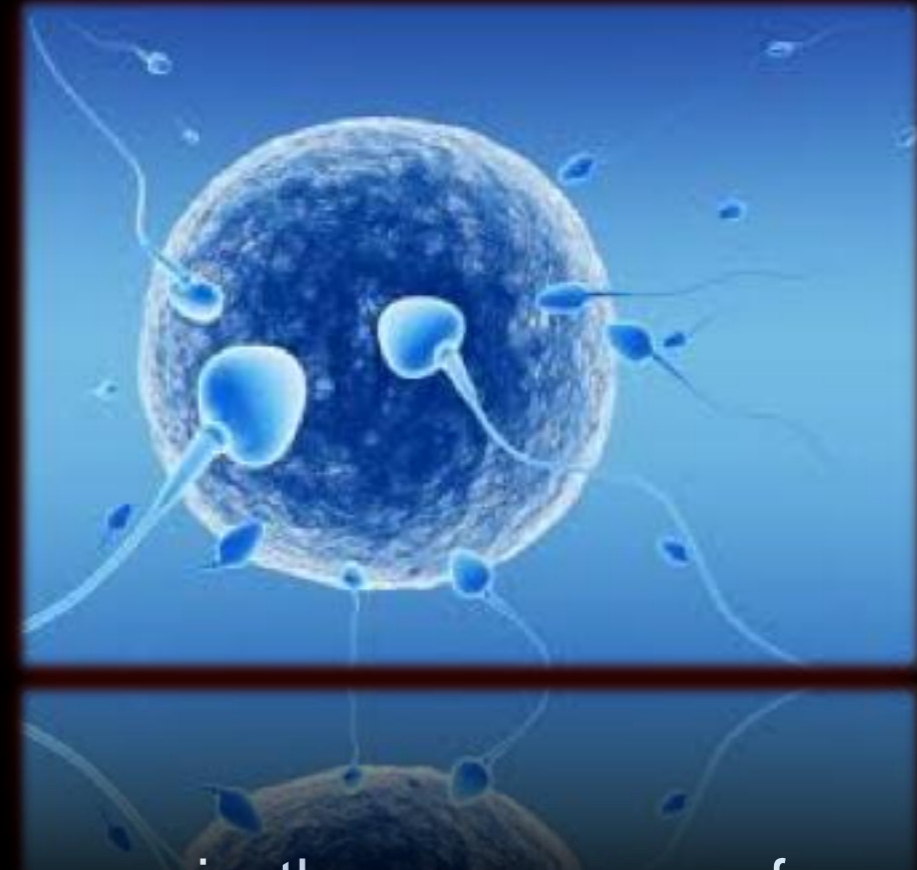
competetion
or
complementing

Some research evidence/theories:

1 Intense selection that occurs in the female tract, with only a relatively few spermatozoa reaching its goal. Especially between the ejaculates of different males.

2 SPERM PLEOMORPHISM - to cover every likely eventuality of the selective process (Holt and Van Look, 2004). Then again in species such as the bull, pleiomorphism is uncommon.

3 The acidic atmosphere of the vagina, the mucus of the cervix, the narrowness of the uterotubal junction, the white blood cells of the immune system, which see the sperm as a pathogenic 'foreign invader' to destroy, and what scientists think is a molecular screening process like at a "checkpoint" that allows only some sperm through.



4 Cervical Crypts

Cervical crypts are thought to entrap and store sperm (Fawcett and Raviola, 1994; Harper, 1994) and scanning electron microscopy of the human cervix indicates that the mucosal grooves may form a preferential pathway for sperm.

5 Cervical Defences

Vaginal insemination stimulates the migration of leukocytes, particularly neutrophils and macrophages into the cervix as well as into the vagina. (Tyler, 1977; Pandya and Cohen, 1985; Barrett and Pockley, 1998). There is evidence from animal work (Taylor, 1982) that the immune response may be a selective one.



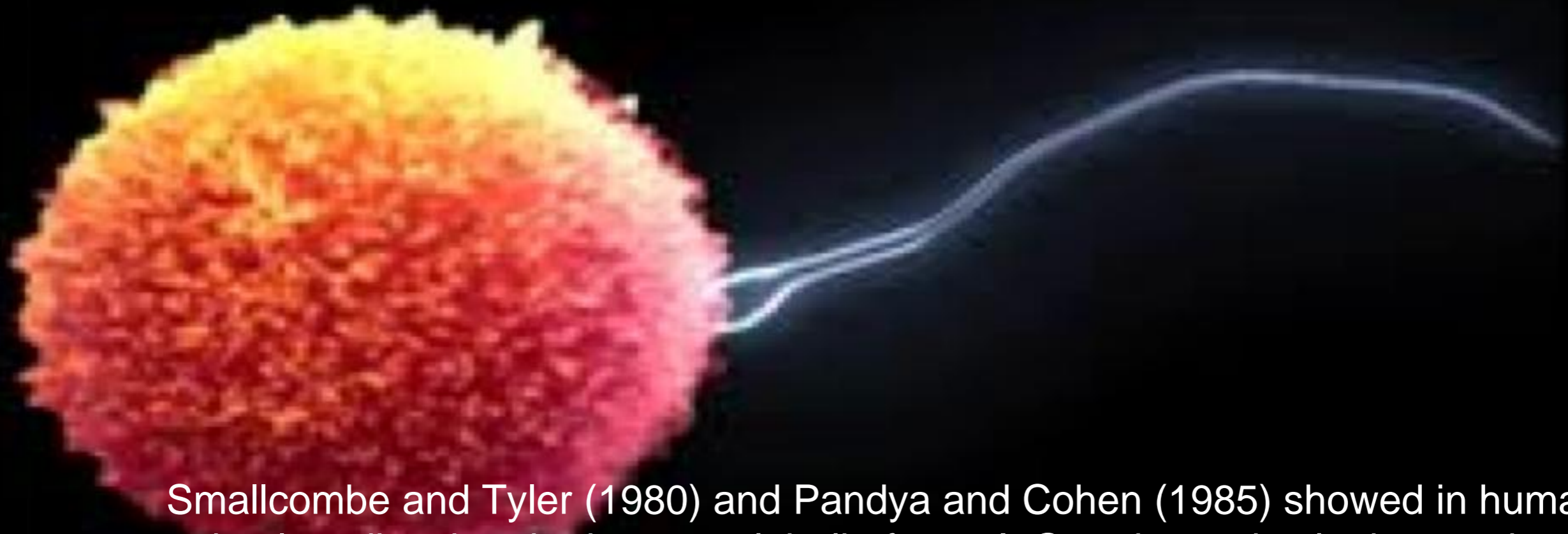
5 Cervical Defences

Animal experiments have shown that highly fertile sperm that make it through the cervix and uterus to the oviduct are uncoated with IgG (Cohen and Tyler, 1980) and that these sperms are not attacked by leukocytes (Taylor, 1982).

It has also been demonstrated that neutrophils will bind to human sperm and ingest them only if serum that contains both serological complement and anti-sperm antibodies is present (D'Cruz et al, 1992). Complement proteins are also present in cervical mucus (Matthur et al, 1988) along with regulators of complement activity (Jensen et al, 1995).

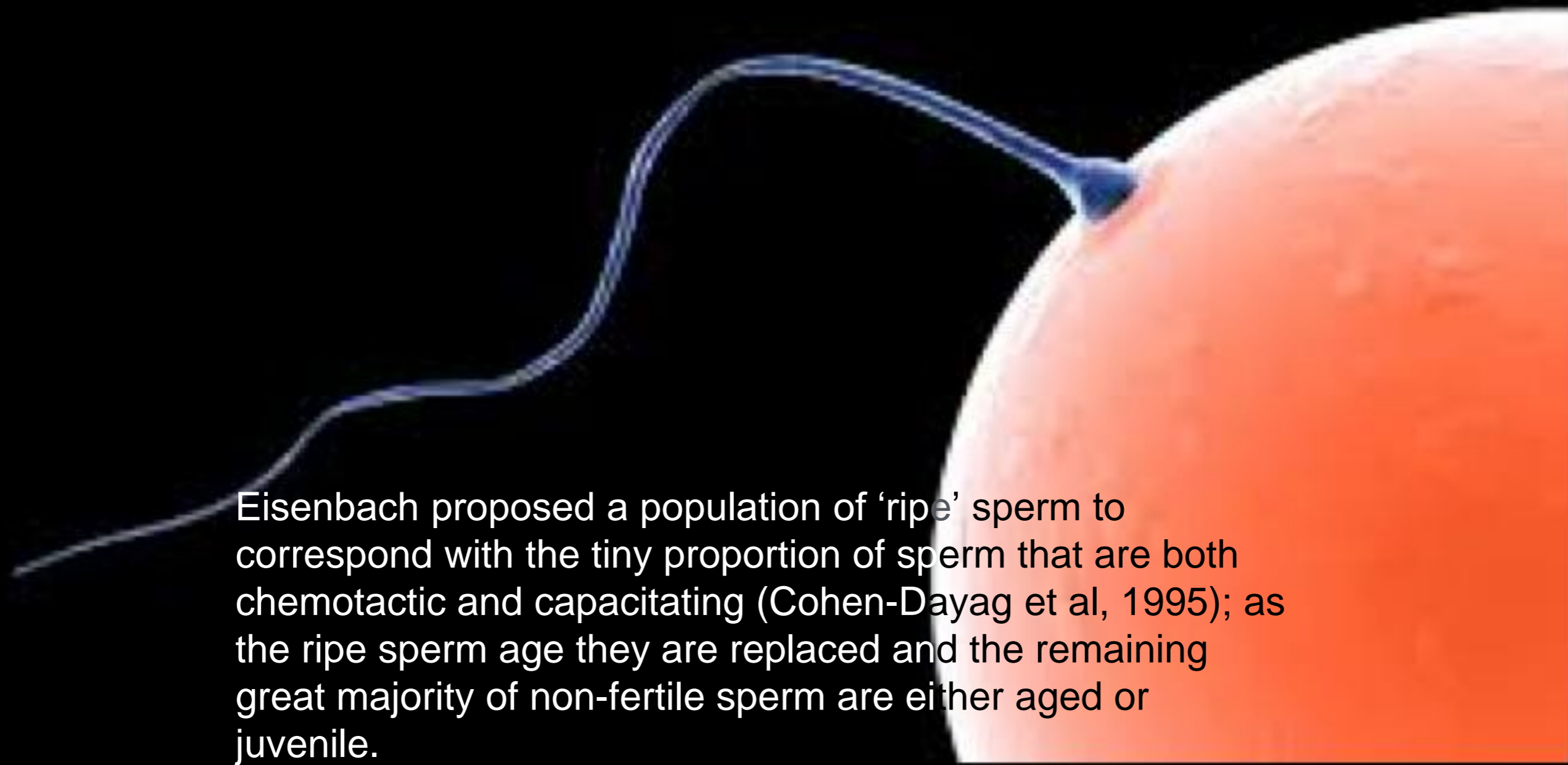


6 COHEN'S TWO-POPULATION THEORY



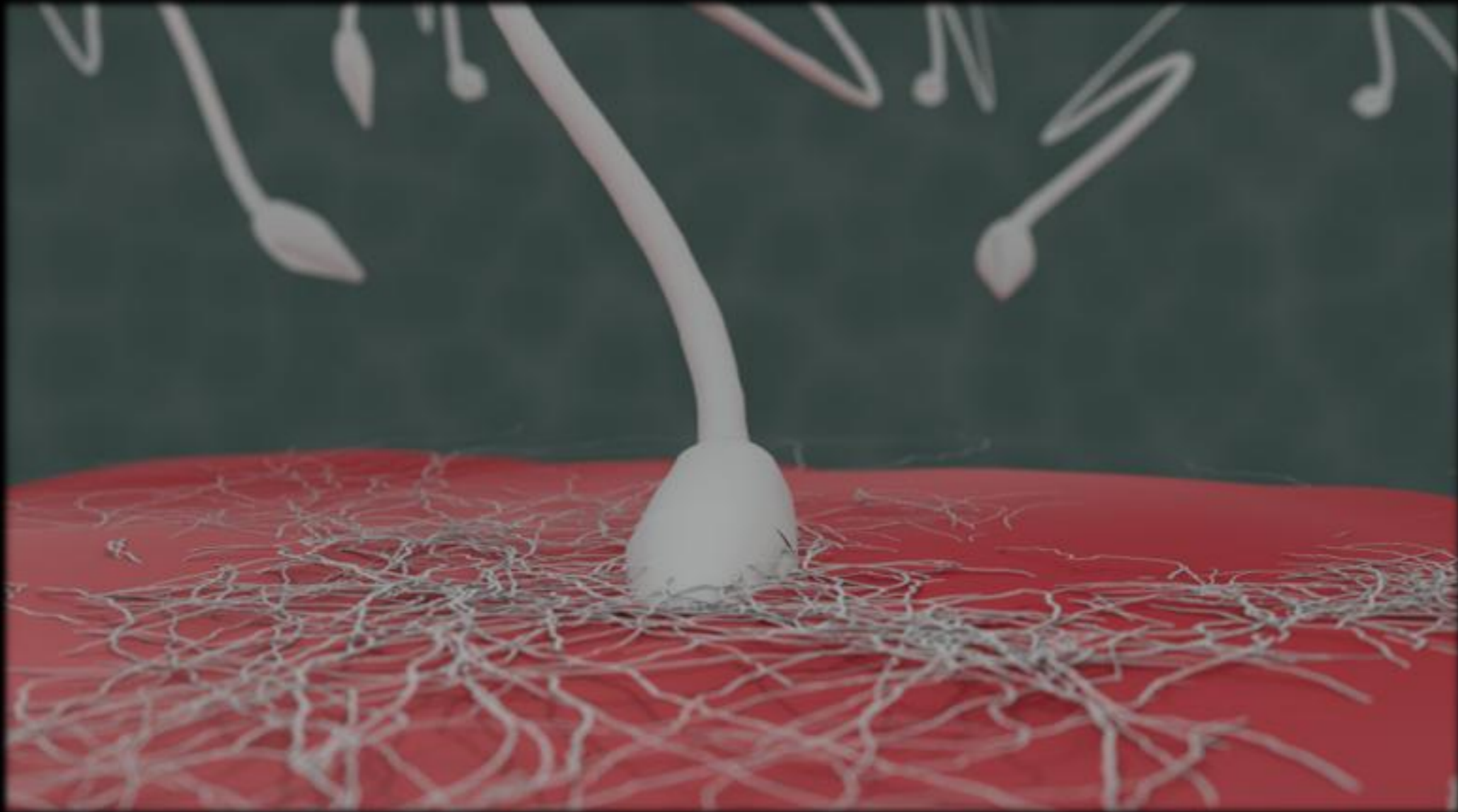
Smallcombe and Tyler (1980) and Pandya and Cohen (1985) showed in human and animal studies that the immunoglobulin factor IgG and massive leukocytosis was elicited by spermatozoa, not by mere copulation. Cohen postulated that spermatozoa emerge from the seminal coagulate up to 15 minutes after pooling to coincide with the female immune response. Spermatozoa comprise of two distinct groups: one that results from errors in the meiotic process and is coated with IgG, and a tiny minority that remain uncoated. The coated majority exit the coagulate first and are immediately targeted and phagocytosed by the circulating leukocytes; the second uncoated minority travel unchallenged (Cohen, 1990; 1998). Advances in DNA assessment have not yet provided direct support for Cohen's theory though it is still described as an attractive and logical hypothesis (Holt and Van Look, 2004).

7 EISENBACH'S THEORY OF RIPE SPERM



Eisenbach proposed a population of 'ripe' sperm to correspond with the tiny proportion of sperm that are both chemotactic and capacitating (Cohen-Dayag et al, 1995); as the ripe sperm age they are replaced and the remaining great majority of non-fertile sperm are either aged or juvenile.

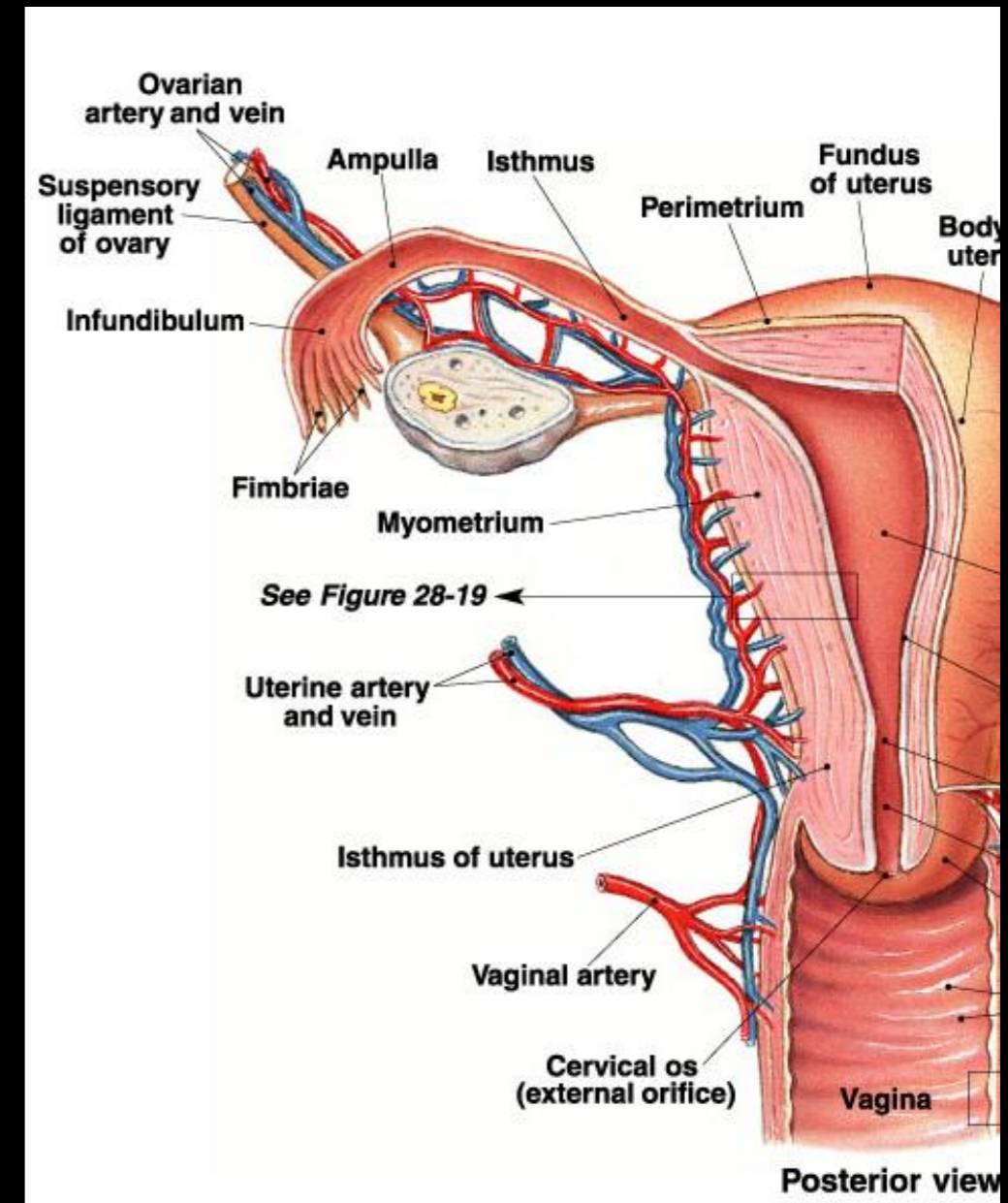
8 THE 'KAMIKAZE' THEORY OF SPERM



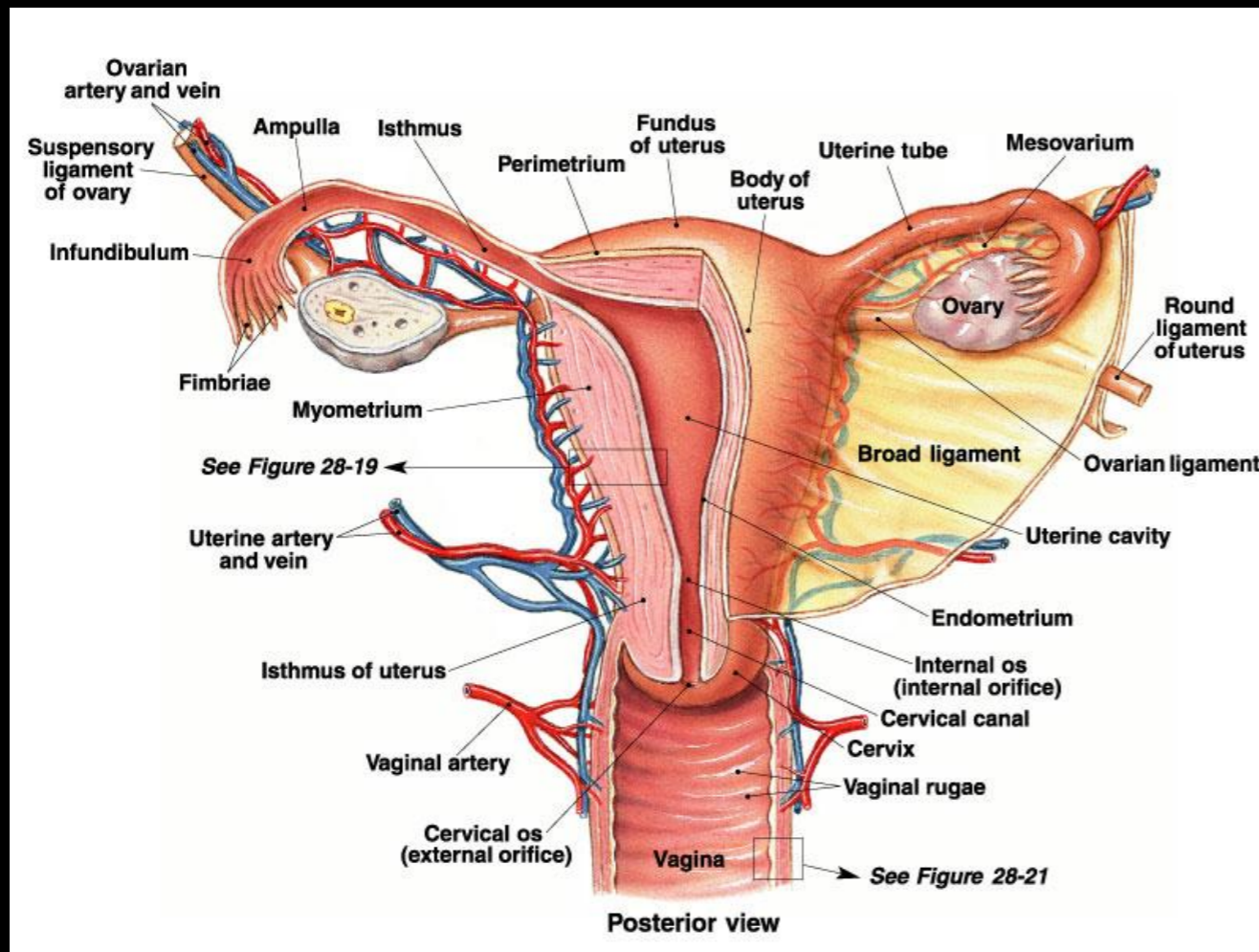
The most notorious theory of sperm heterogeneity is the 'kamikaze' sperm hypothesis of Baker and Bellis (1998 and 1989) that proposed a functionally adaptive division of labour between sperm: 'egg-getters', 'blockers' and 'killers'. The Baker and Bellis 'kamikaze' theory was hypothesised within the context of sperm competition theory. Sperm competition occurs when spermatozoa from more than one male have the opportunity to fertilise a single female during the same fertile period (Parker, 1970; 1998).

TRANSPORT THROUGH THE UTERUS

Uterine cavity is a few cms in length and could be traversed in less than 10 mins by sperm swimming at about 5 mm/min, which is the swimming rate of sperm in aqueous medium (Mortimer and Swan, 1995). Transport of sperm is aided by contractions of the myometrium. (Lyons et al 1991; Fukuda and Fukuda, 1994 Kunz et al, 1996; de Ziegler et al, 2001). There is evidence that there is preferential transport to the isthmus ipsilateral to the dominant follicle than to the contralateral isthmus. Rapid transport of sperm through the uterus by myometrial contractions may enhance sperm survival by propelling them past immunological defences of the female



TRANSPORT THROUGH THE UTEROTUBULAR JUNCTION



The uterotubal junction (UTJ) presents anatomical, physiological and mucous barriers to sperm passage in humans. The entrance traverses a thick muscular layer of uterine wall (Hafez and Black, 1969) and the narrow lumen may be mucus-filled (Jansen, 1980), which impedes the progress of sperm.

It has been observed that capacitated sperm are unable to swim through the narrow junction (Nakanishi et al, 2004) and in rodents that sperm with linear, progressive motility are more successful at passing through (Gaddum-Rosse, 1981; Shalgi et al 1992).



There is likely to be some form of molecular selection at work also. Male mice that are null mutants for genes encoding different sperm surface proteins, including calmeglin and Angiotensin Converting Enzyme, are infertile because their sperm fail to get through the UTJ, despite having normal sperm morphology/motility. Thus it's likely that certain epitopes are available and exposed on the surface of sperm to interact with the UTJ and somehow regulate sperm passage (Krege et al, 1995; Ikawa et al, 1997; Cho et al, 1998; Hagaman et al, 1998; Yamagata et al, 2002).

THE OVIDUCTS- A SAFE HAVEN

The Oviducts provides a haven for sperm. Unlike the vagina, cervix and uterus, the tube does not respond to insemination with an influx of leukocytes (Rodriguez- Martinez et al. 1990).



IN PREPARATION FOR FERTILIZATION

Sperm undergo two changes in preparation for fertilisation: **capacitation** and **hyperactivation** that together may serve to firstly detach spermatozoa from their isthmic 'reservoir' and then speed sperm movement to the ampulla as the time of ovulation approaches.

CAPACITATION

Capacitation involves changes in the plasma membrane, including loss or modification of proteins on the surface of the plasma could reduce affinity for endosalpingeal epithelium and the shedding of proteins and cholesterol, that prepare sperm to undergo the acrosome reaction and fertilise oocytes (De Jonge, 2005)

HYPERACTIVATION

Hyperactivation is a change in flagellar beating that typically involves an increase in the flagellar bend amplitude. This can provide the force necessary for overcoming the attraction between sperm and epithelium (Ho and Suarez, 2001).

Animal experiments have shown that only hyperactivated spermatozoa detach from endosalpingeal epithelium (DeMott and Suarez, 1992) and that capacitated sperm bind less well (Lefebvre and Suarez, 1996) indicating that capacitation-induced changes in the sperm head surface are responsible for reducing binding affinity, though the pull produced by hyperactivation can almost certainly hasten detachment of bound sperm from the epithelium.

FERTILIZATION

At this stage in the fertilization process there are probably no more than tens of sperm that have reached and begun to penetrate through the cumulus.

The cumulus is an extracellular matrix of cells held together primarily by hyaluronate (hyaluronic acid) (De Jonge, 2005).

A scanning electron micrograph showing the head of a spermatozoon. The head is covered in a dense, granular texture, likely representing the acrosomal membrane and the plasma membrane. The background is dark, making the light-colored head stand out.

FERTILIZATION

The membranes in the head of the spermatozoon are reorganised; multiple fusions occur between the outer region of the acrosomal membrane and the plasma membrane that overlies the acrosome

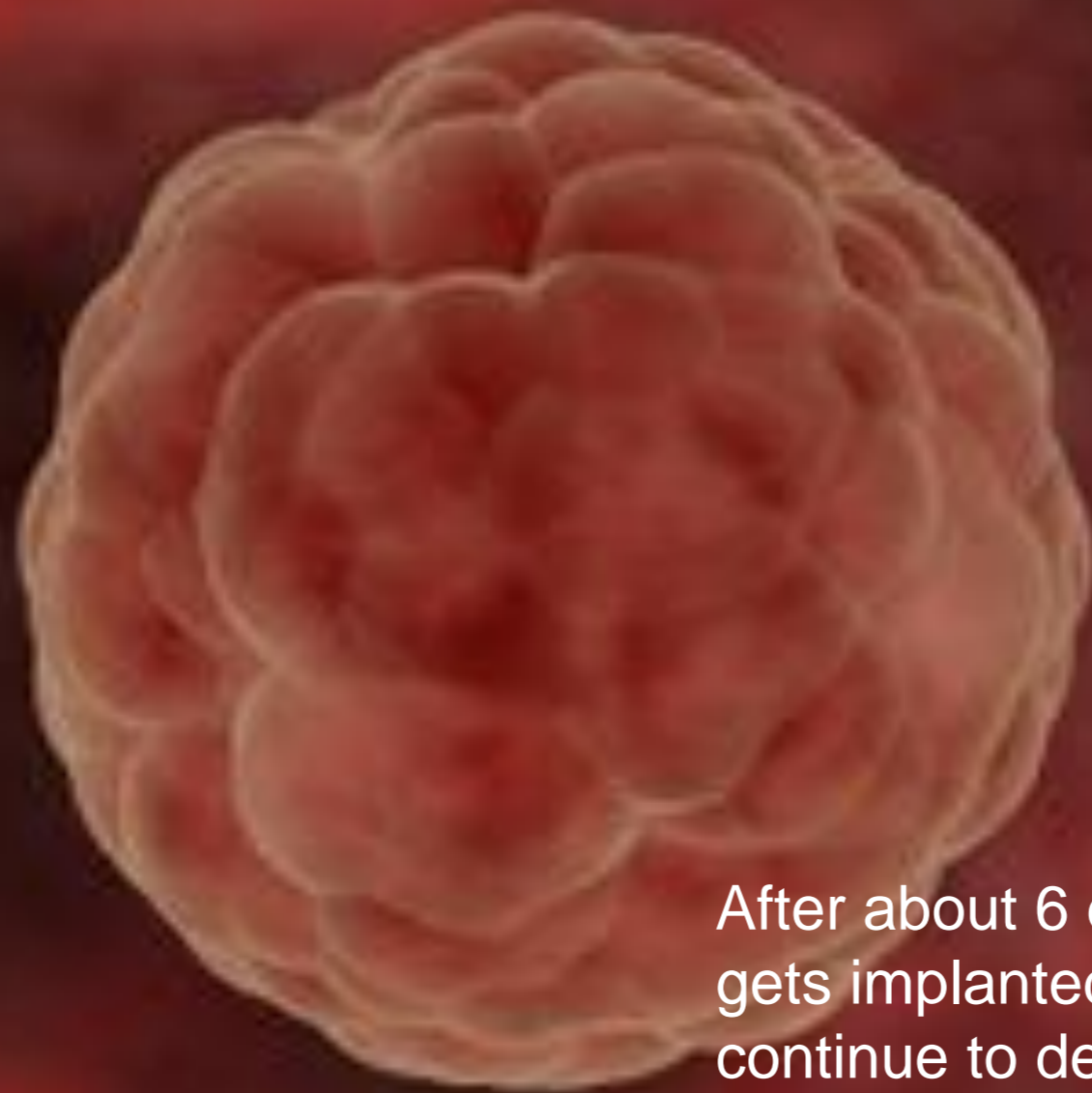
Hyaluronidase activity by the sperm plasma membrane protein PH-20 acting on its hyaluronate substrate facilitates sperm penetration of the cumulus matrix (Lin et al, 1994).

The very few spermatozoa that have reached and now bind to the zona pellucida receive a signal to acrosome react i.e. release by Ca^{2+} stimulated exocytosis of the contents of their large secretory granule, the acrosome

When the cell membranes of the sperm and oocyte merge, the sperm enters the ooplasm of the oocyte, triggering oocyte activation, a series of changes in metabolic activity including a rise in metabolic rate. The cell membrane of the oocyte undergoes immediate electrical changes that block the entry of other sperm and enzymes produced by the fertilized ovum alter receptor sites so that sperm already bound are detached and others are prevented from binding

After fertilisation the cell now termed a zygote, splits into two, then the two cells double to four, four to eight, eight to sixteen and so on. Because the cell cluster looks superficially like a berry it is called the morula (Latin for "mulberry")

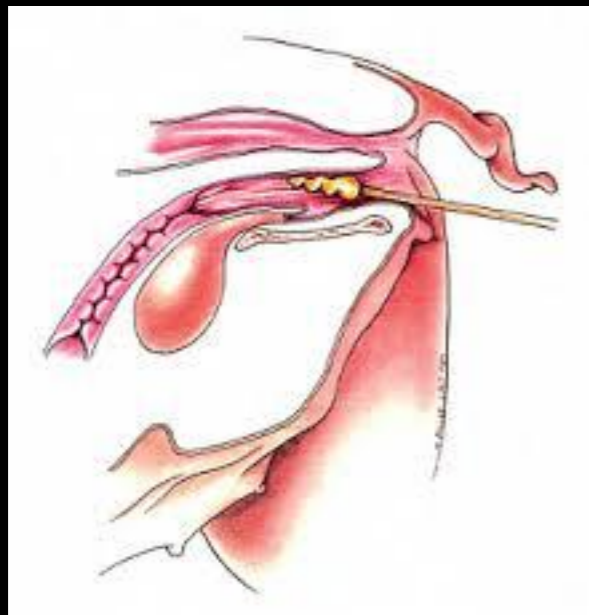
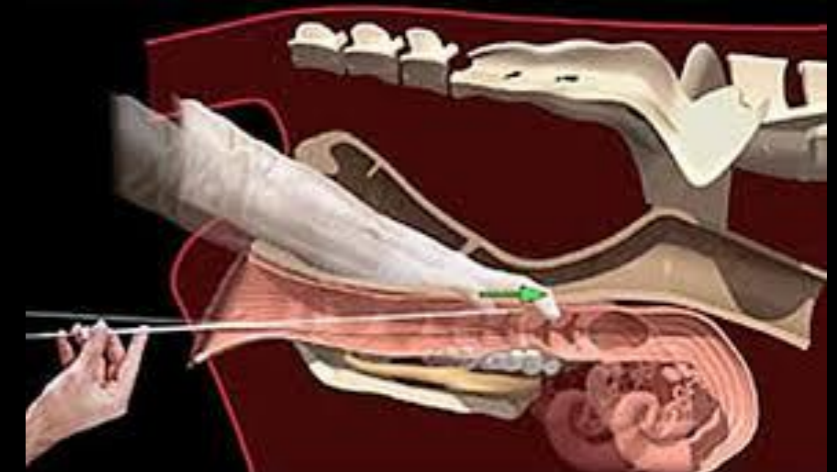




After about 6 days, the (blastocysts) embryo gets implanted in the wall of the womb and continue to develop until it forms a complete being.

Artificial insemination

Artificial insemination (AI) is one of the most effective tools available in the food animal producing sector to improve productivity and profitability of their operation.



Semen Collection



Semen Processing

- Removal gel fraction
- Centrifuge to separate sperm cells from seminal plasma
- Extending in a medium suitable for cooling and low temperature storage
- if keeping longer than 96 hours - extending in freezing medium



Changes in sperm function during slow cooling and low temperature storage



Sperm viability

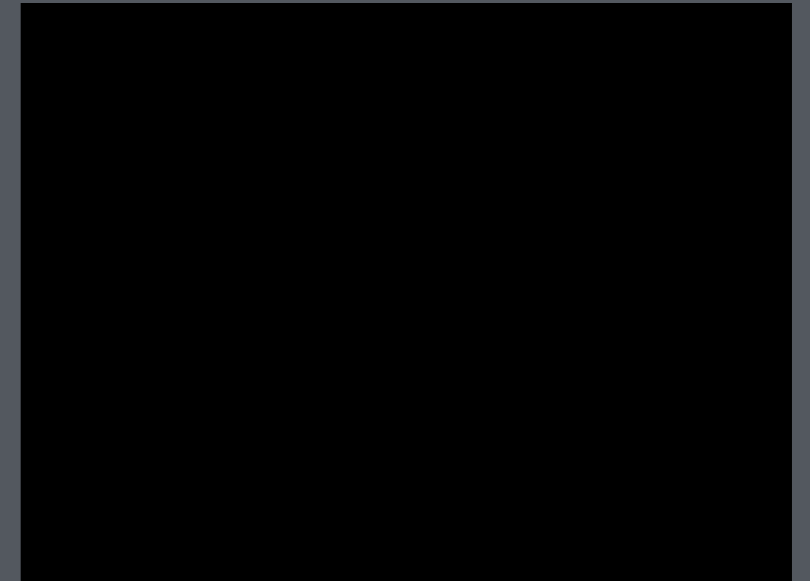
Cooled from 37C to 5C, using a controlled rate freezer. Evaluated their viability, motility patterns and ability to regulate $[Ca^{2+}]_i$

Compared a single (37C to 5C at 0.05C/min) vs a two step cooling protocol, (37C to 22 at 0.5C/min and 22C to 5C at 0.05)

Live dead percentage declined significantly after cooling (80% before) to about 65% immediately after and continued to decline to between 35 and 50%, after 72 hours of low temperature storage (5C)

Extending the period of cooling at the slower rate of 0.05C to cover the entire cooling period from 37C to 5C did not result in marked conservation of viability or velocity. Instead, there were more cells showing non-progressive motility

Sperm Motility Using CASA



Objectively measures curvilinear velocity, straight line velocity and average path velocity

Analysis of the movement parameters, showed a decline in the velocity parameters; ranging between 15% to 25% immediately after cooling. Further decline was noted during subsequent storage (up to 72 hours)

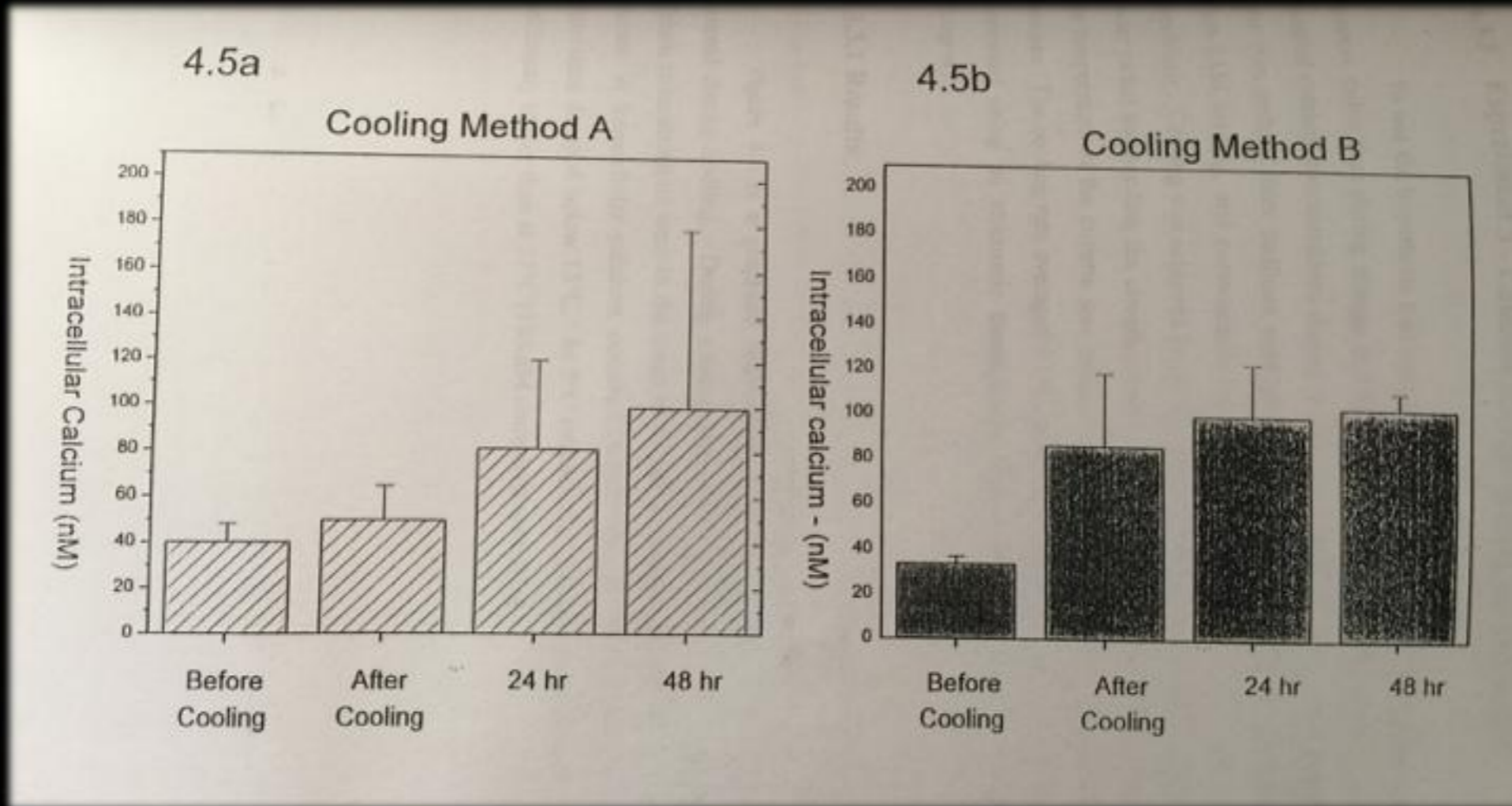
Intracellular Calcium Regulation

Although many cations (Ca^{2+} , Na^+ , K^+ and H^+) have been implicated in the modulation of sperm function, contemporary evidence suggest that Ca^{2+} is the key regulator, and involved in promoting sperm motility, capacitation and hyper activation



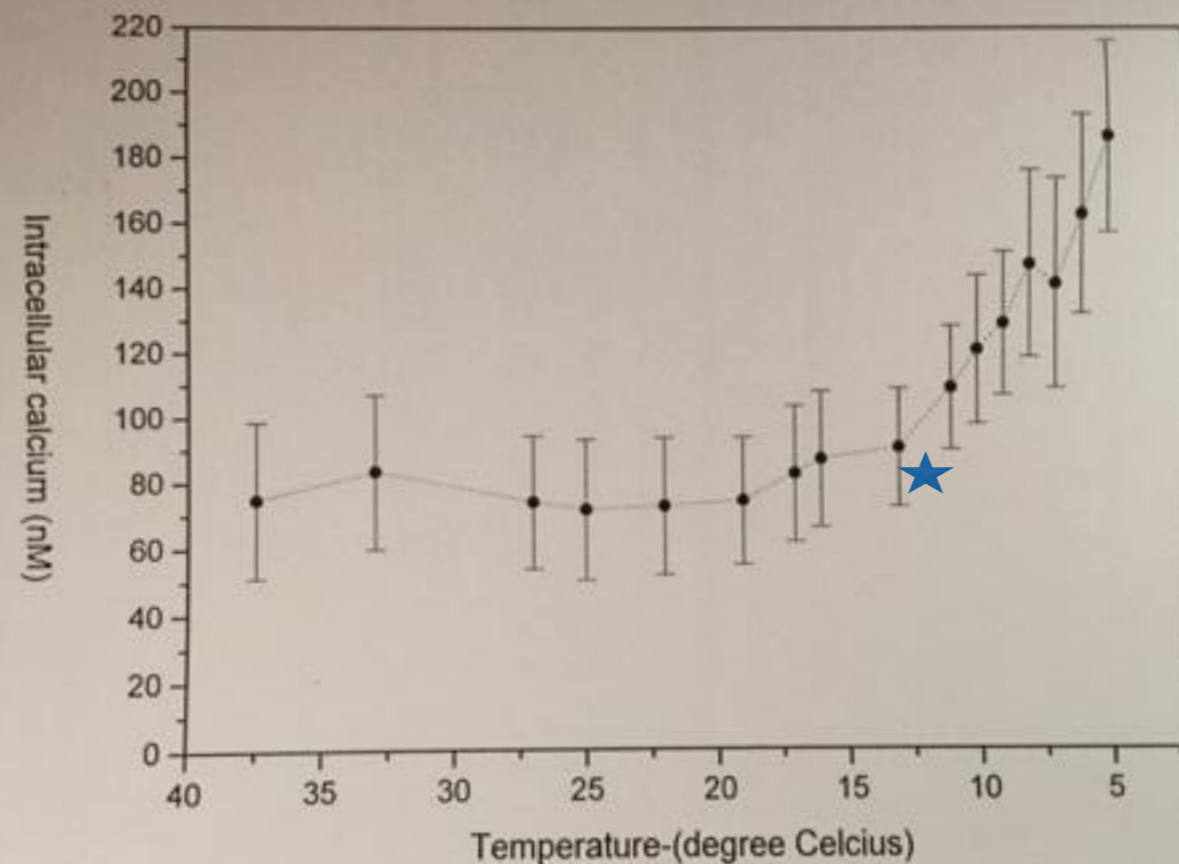
We developed methods to measure measured the ability of sperm cells to regulate $[\text{Ca}^{2+}]_i$ - using Fur-2AM and we measured the fluorescence intensity using a dual wavelength spectrofluorimeter.

Intracellular Calcium Regulation



We noted a general increase of $[Ca^{2+}]_i$ in the population of sperm cells, and $[Ca^{2+}]_i$ continue to increase over 48 hours and plateaus off after that.

When during cooling, will a calcium reflux take place?



[Ca²⁺]_i were in the range of 72nM to 87nM between 37C and 16C; and below 13C a gradual increase in [Ca²⁺]_i occurred which became more rapid when the temperature dropped below 13C. At 5C [Ca²⁺]_i were significantly higher at 187nM.

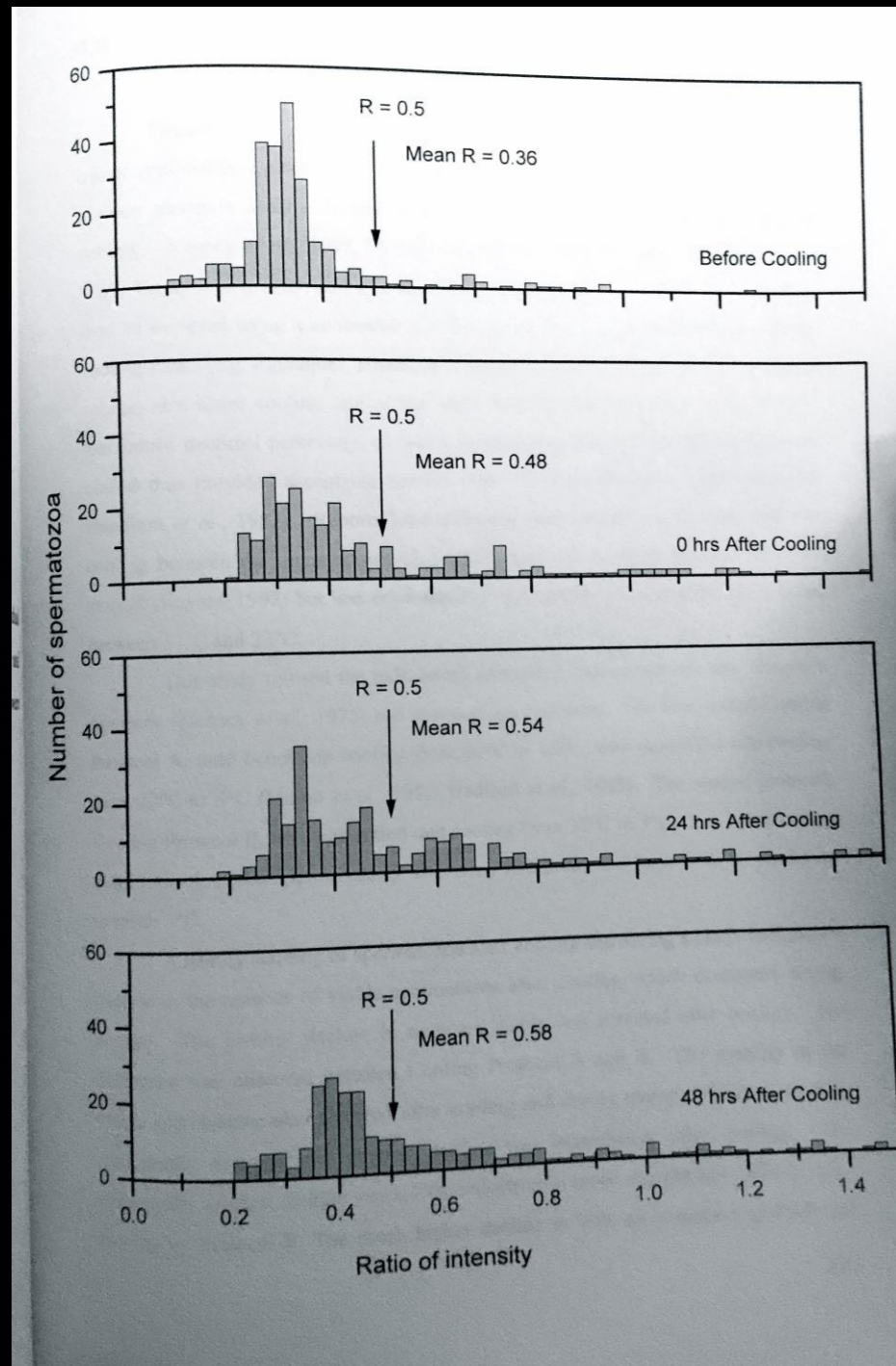
Intracellular calcium (nM; Mean +/- SEM; n=3) of stallion spermatozoa cooled from 37C to 5C

We wanted to know how individual cells regulate $[Ca^{2+}]_i$

We devised a method to immobilize the sperm cells and we determine the $[Ca^{2+}]_i$ in the head of each sperm cell, by measuring the ratio of fluorescence intensity (R) using an inverted fluorescence microscope.

The proportion of cells regulating calcium at proceed levels fell from 68% to 33% and then 18% by 48 hours of storage

The data from the single cell study showed that while the mean R value of the sperm cells sampled increased during cooling and with subsequent storage, there was a discrete sub-population of cells that were still capable of regulating calcium at pre-cooled levels



Ratio of intensity (R; f_{340}/f_{380}) measured from 250 individual sperm cells cooled from 37C to 5C, measured before cooling, at 0, 24 and 48 hours

Significance of the cooling and low temperature storage work

Use of AI in horses is limited (unlike the cattle and swine industry), as it's use is not approved for breeding in the racing industry. So a lot of the breeding work in horses is carried out naturally.

Artificial insemination using cryopreserved semen were not as successful (compared to cattle and swine) - poor fertility rates reported, so breeders use liquid semen chilled to 5C

Whilst it was generally accepted that cooling and low temperature storage will result in loss of viability (function) of some sperms, not much is know about the extent of loss of viability (function).

Filling in the gaps of knowledge particularly in the

- extent of loss of viability
- motility patterns
- ability to regulate calcium in relation to viability and changes in motility patterns.

Dual staining studies to characterise capacitation

We now know that the regulation of calcium is compromised during cooling and low temperature storage. Evidence from the work of others, suggested that intracellular calcium has a role in the functional states of sperm, in particular **capacitation** and **hyperactivation** - both necessary functional states for successful fertilisation.

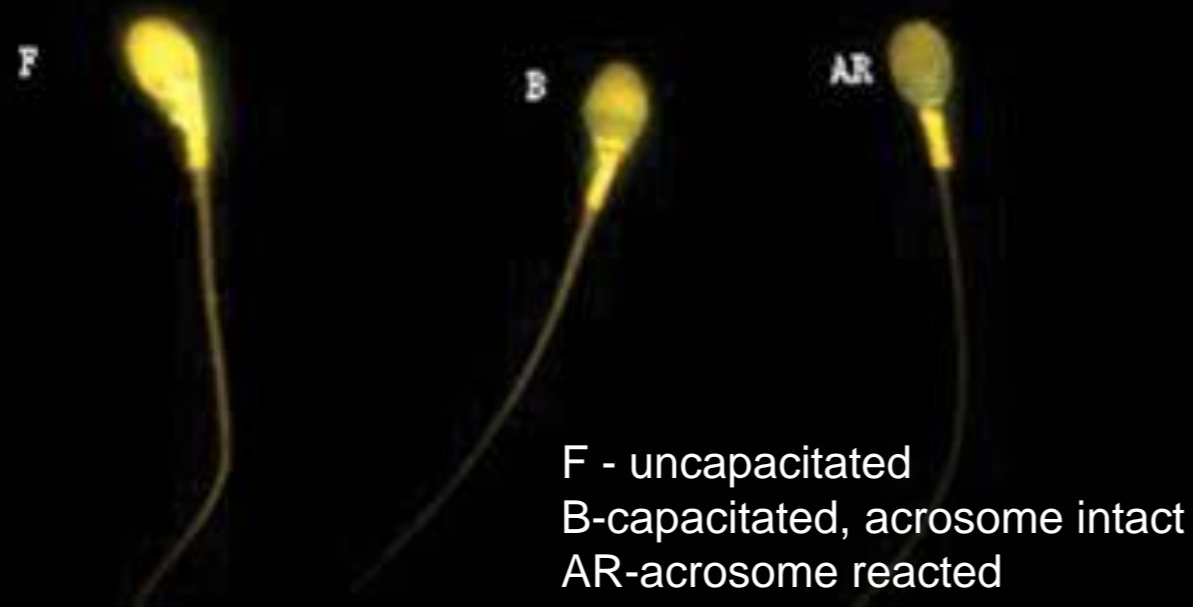
In the equine species, sperm capacitation is relatively poorly studied. It is difficult to repeatedly capacitate stallion semen *in vitro*. The principle problem is the lack of morphological evidence that the spermatozoon is undergoing the process. To circumvent this, studies have evaluated chemical induction of the acrosome reaction, using the rationale that capacitation must be completed to allow acrosome reaction to proceed (Graham *et al.*, 1987; Sampar *et al.*, 1989; Zhang *et al.*, 1991)



Dual staining studies to characterise capacitation

The capacitation process requires prolonged hours of incubation *in vitro*, hence it was necessary to establish a system that would support sperm viability during incubation. Countless hours were spent investigating media that would support this.

Stallion sperm were incubated in test media for up to 6 hours, time for which we expect capacitation to have taken place. We used a dual staining technique with Chlortetracycline and Hoechst 33258 to evaluate sperm functional states.



Semen re-extended in seminal plasma was used as control against known treatments that support capacitation.

We noted, that at the end of the 6 hours period, only uncapacitated sperms were detected in the control sample. A significant increase in the percentage of capacitated-acrosome intact sperms were noted in the other treatments.

To further tests the hypothesis that capacitated sub-population were present within the whole population, we attempted to identify stallion sperm hyperactivation using CASA.

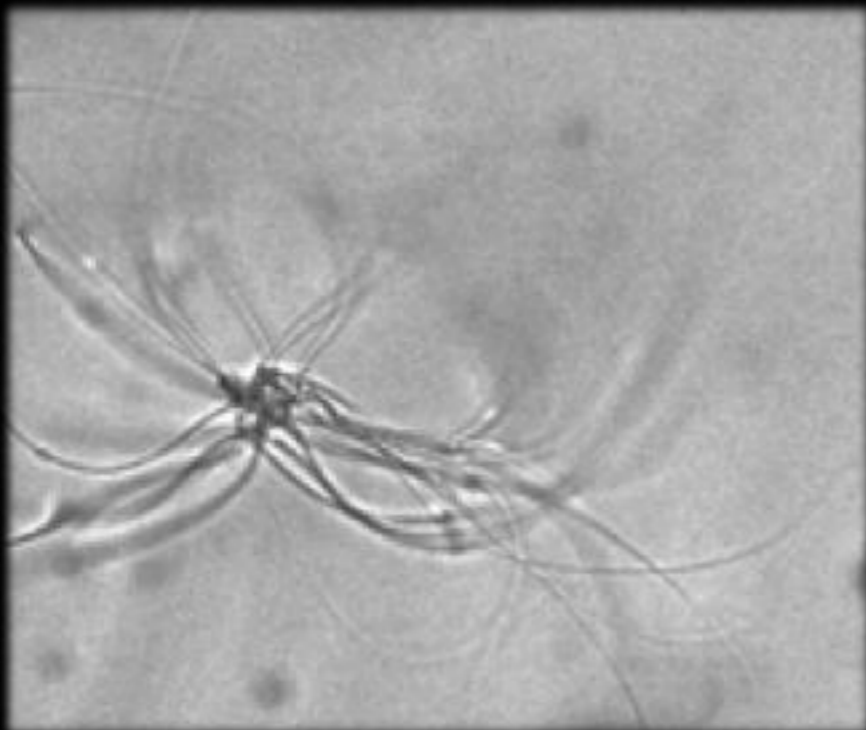
Because studies of hyper activated motility were well establish in human spermatozoa, initial experiments were carried out to quantify human sperm hyper activation under capacitating and non-capacitating conditions.

Characterising hyperactivation

we extended these experience with stallion sperm and concluded that the criteria used to identify human sperm hyper activation, $ALH > 7.5 \mu\text{m}$, LIN of $< 65\%$ and VCL of $> 100 \mu\text{ms}^{-1}$ were insufficiently discriminating for stallion spermatozoa.

So we visually examined 1134 sperm trajectories, and classified them non hyperactivated, transition phase and star-spin pattern as described by Robertson et al., 1988. Based on these observed patterns, a new criteria was derived for stallion sperm.

We went on to examine sperm motility in detail, under the same conditions as the CTC dual staining technique, using the new criteria derived for stallion sperm, and observed 29 transition and 8 star-pin trajectories.



During this work it was observed that stallion sperm exhibited “head to head” agglutination and clumping after 120 minutes. This phenomena is possibly an indication of changes to the sperm plasma membrane. It was not possible to note than, if these changes are part of capacitation.

Progesterone and Calcium Regulation

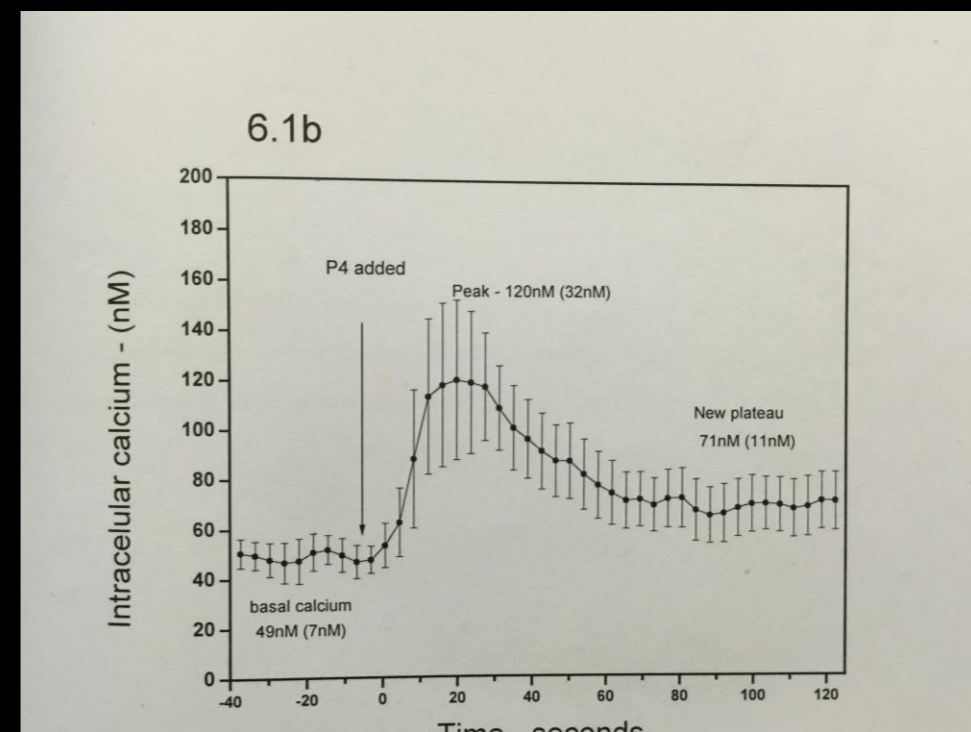
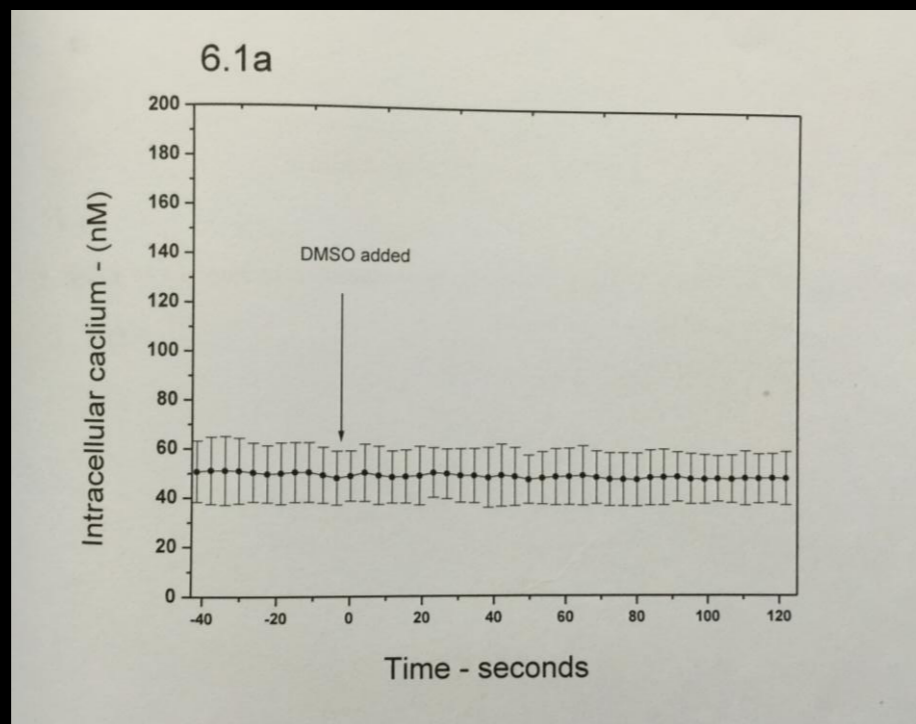
Fluxes of intracellular calcium are a component of the regulatory mechanisms of capacitation, hyperactivation and acrosome reaction. Well established by other work (Fraser, 1987; Adeoya-Osiguwa and Fraser, 1993; Das Gupta et al, 1993), and also in the series of experiments we carried out.

In the early 90's, the steroids progesterone and 17α -hydroxyprogesterone has been shown to stimulate a rapid influx of calcium in human spermatozoa (Thomas and Meizel, 1989; Blackmore and Lattanzio, 1991; Blackmore et al., 1990, Baldi et al., 1991). It has since been proposed that progesterone present in the ovulatory milieu in the fallopian tubes after ovulation play a physiological role by facilitating acrosomal exocytosis when the oocyte reaches the site of fertilisation. In one study (Roldan et al., 1994) with mouse spermatozoa, it was shown that when sperm was exposed first to progesterone and then to zone pellucida, exocytosis was enhanced over that seen when the agonists were not presented together or in the inverse order, suggesting that the steroid exerts a priming effect on the sperms.

Progesterone and Calcium Regulation

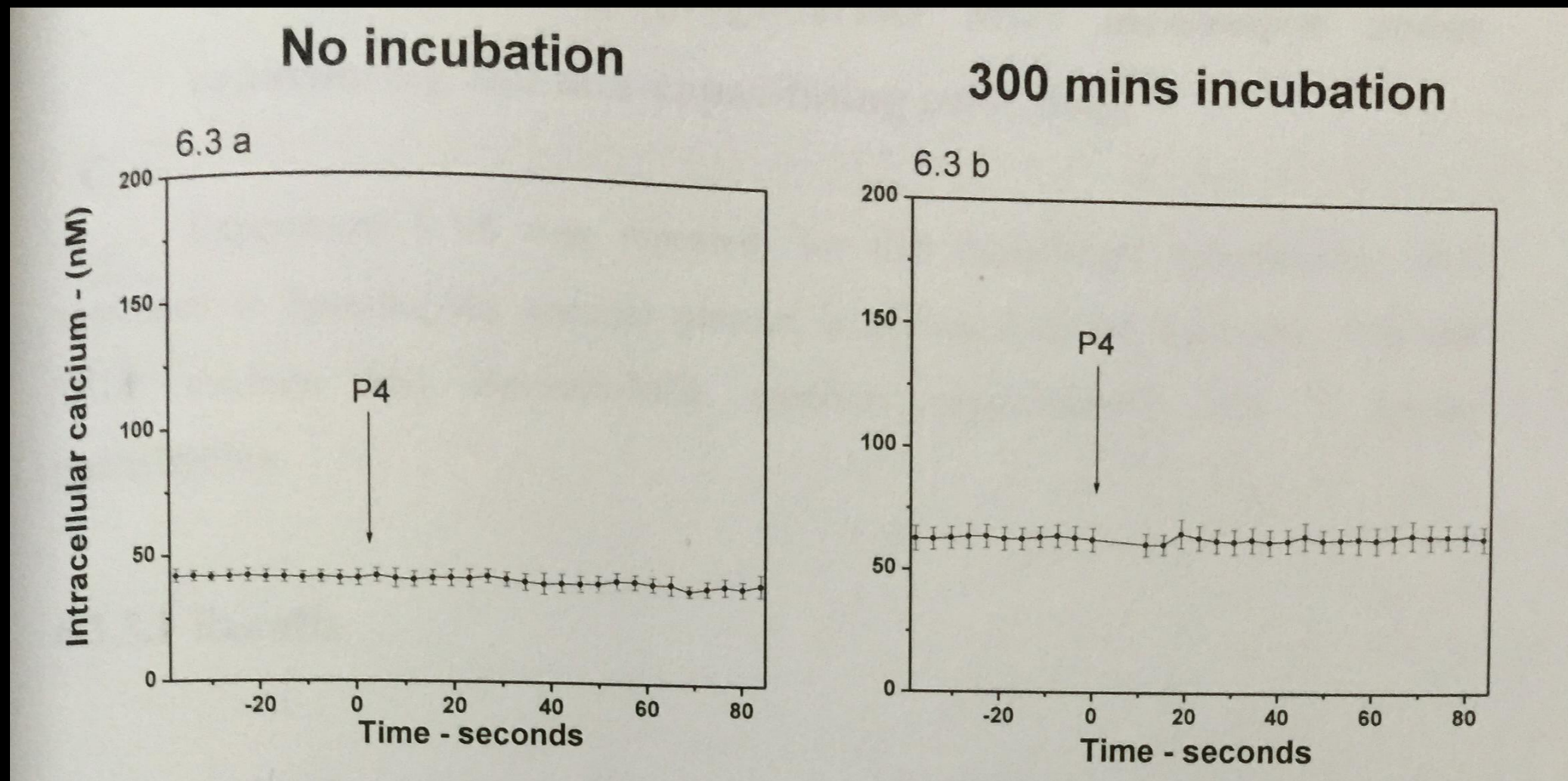
In light of the importance of maintaining the ability to regulate intracellular calcium (as demonstrated in my cooling and low temperature storage work) we embarked on a series of experiments to examine the role of progesterone in calcium regulation under capacitating conditions. The incubation conditions were established in my work using the CTC stains.

Using human sperms and established methods in the labs of other researchers (Blackmore et al., 1990; Baldi et al., 1991; Forresta et al, 1993; Krausz et al., 1995), we noted that a calcium transient was readily produced in human sperms.



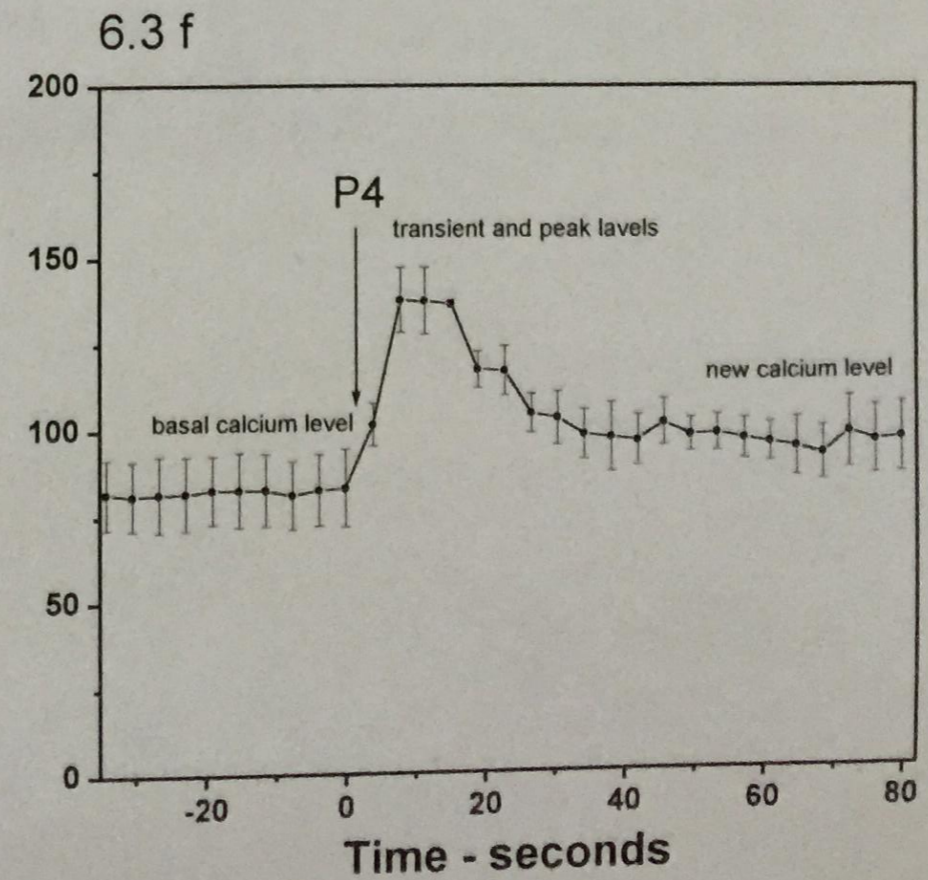
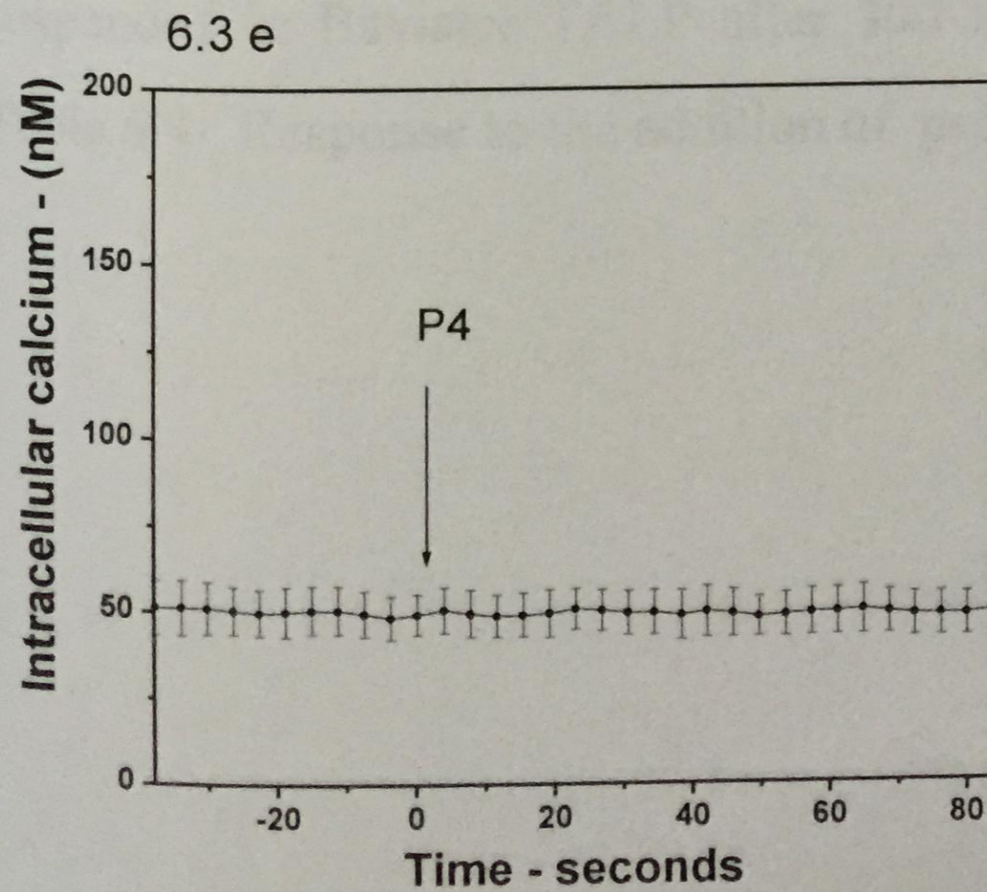
Progesterone and Calcium Regulation

We extended the methods establish in human sperms on stallion sperm



Progesterone and Calcium Regulation

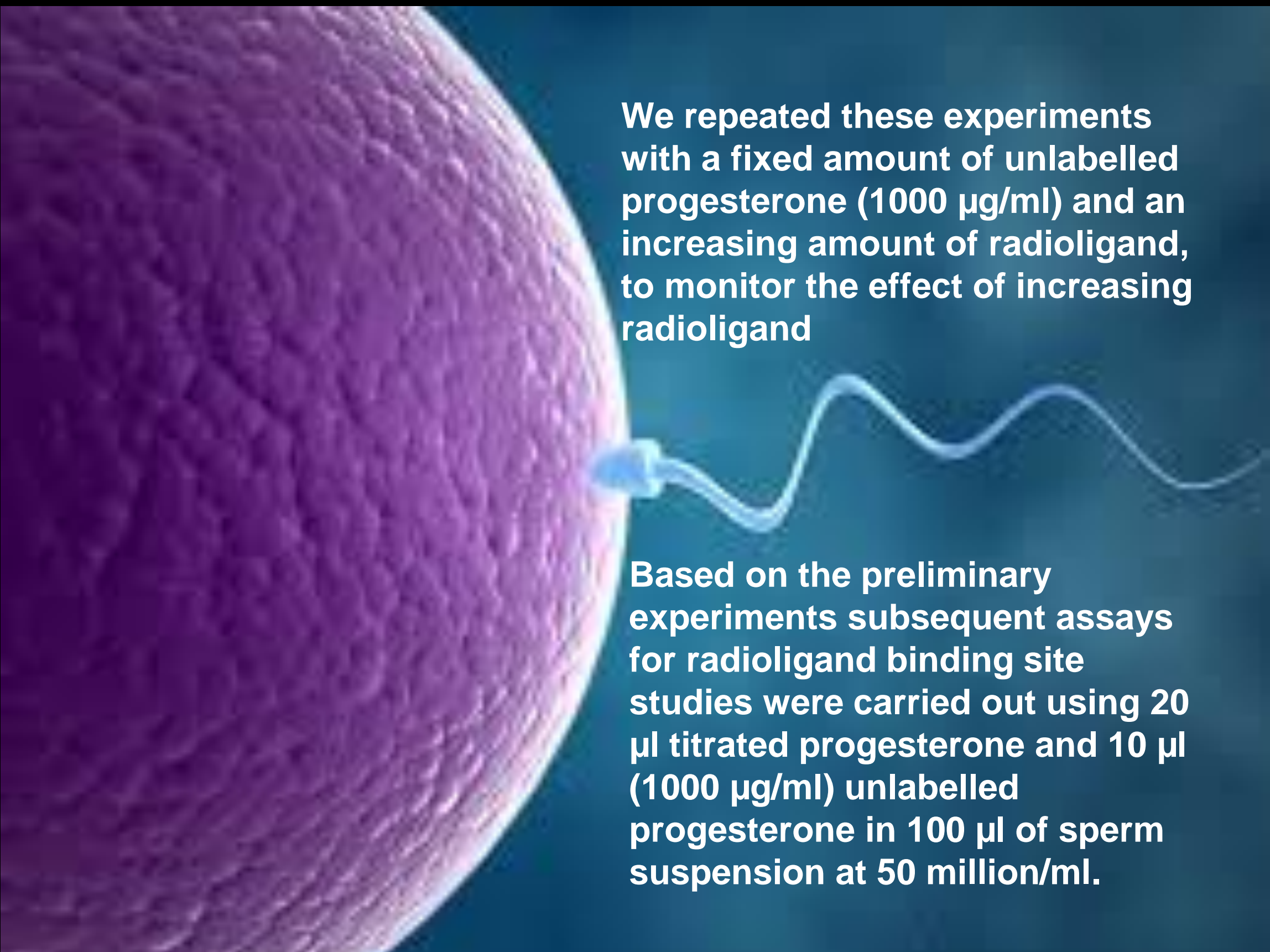
More work was carried out to develop methods for equine spermatozoa.



Progesterone Binding Studies

Having elicited an increase in intracellular calcium after adding progesterone, we set out to identify progesterone binding sites and to characterise receptor site numbers on the membranes of human and stallion spermatozoa, using tritium labelled progesterone

Preliminary work explored mixing aliquots of sperm suspension (100 μ l; 50 million/ml) with a fixed amount (20 μ l) of labelled progesterone and unlabelled progesterone (10 μ l) of different concentration (0, 10, 100 and 1000 μ g/ml)

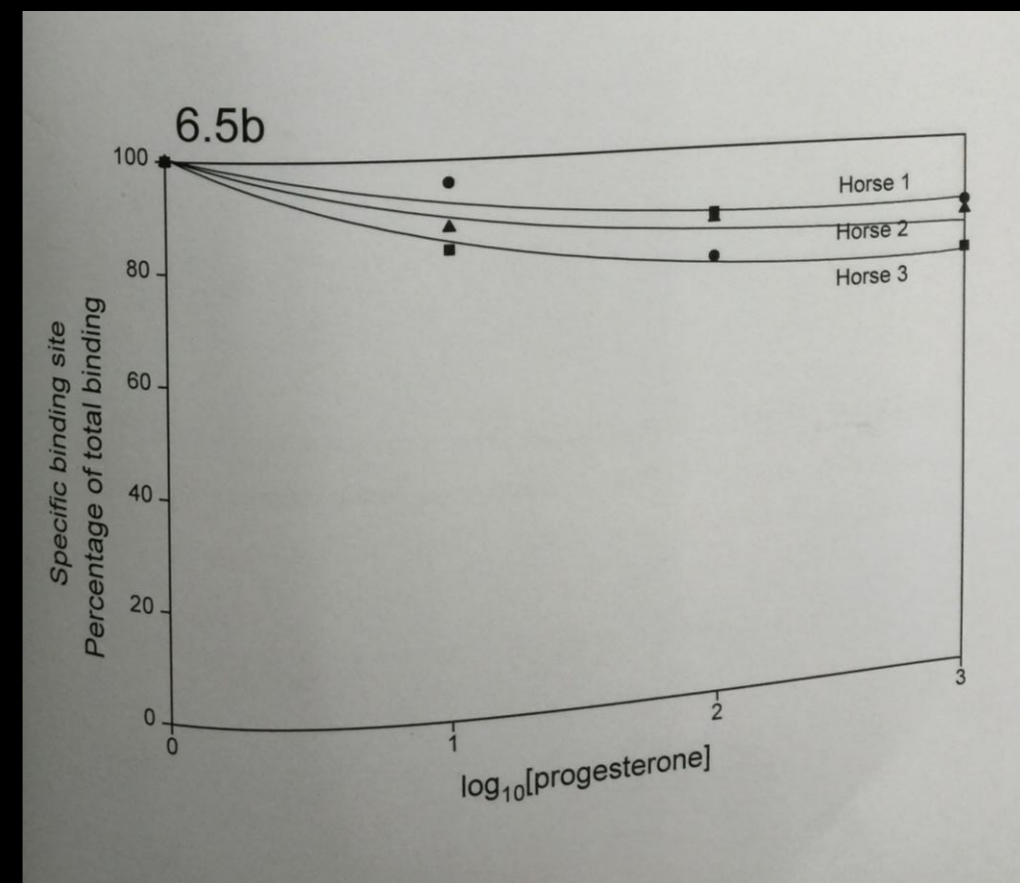
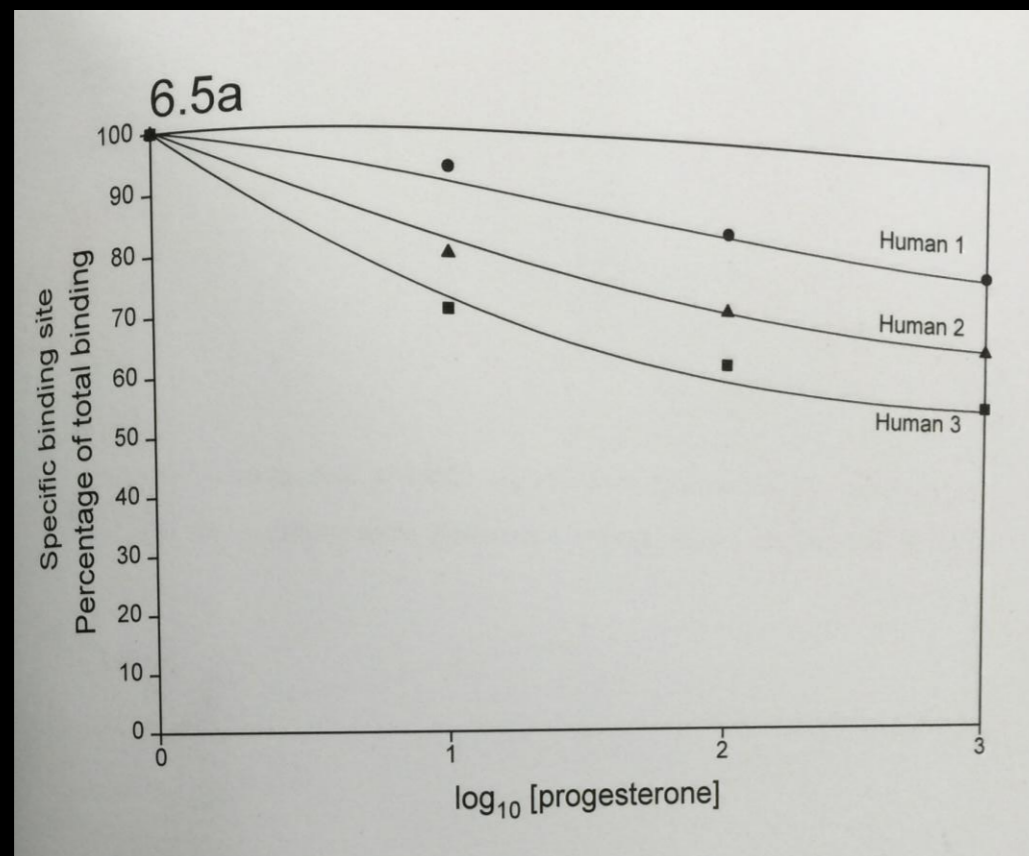


We repeated these experiments with a fixed amount of unlabelled progesterone (1000 $\mu\text{g}/\text{ml}$) and an increasing amount of radioligand, to monitor the effect of increasing radioligand

Based on the preliminary experiments subsequent assays for radioligand binding site studies were carried out using 20 μl titrated progesterone and 10 μl (1000 $\mu\text{g}/\text{ml}$) unlabelled progesterone in 100 μl of sperm suspension at 50 million/ml.

Progesterone Binding Studies

The principle behind this is that a radioligand will bind to receptors in a saturable manner, whereas attachment to a non-receptor tissue component is less saturable. Therefore unlabelled progesterone will selectively inhibit radioligand binding to the receptors.



Our results showed that the numbers of binding sites on the sperm declined as the concentration of unlabelled progesterone was increased, giving evidence of the presence of specific progesterone receptors.

Progesterone Binding Studies

We went on to examine for progesterone binding sites on equine sperm, following incubation in capacitating conditions. Our hypothesis was that higher binding activity would be seen as more receptor sites become available after incubation in a medium supporting capacitation.

This was not the case, instead there was a decline in the binding sites after incubation. It was hypothesised that this may not have been evident because of the decline in percentage of live sperm after incubation for 300 minutes.

Summary

We establish calcium dynamics with cooling and low temperature storage.

It was difficult to maintain stallion sperms *in vitro* cultures for long enough periods of time to establish capacitation. We were able to characterise CTC patterns for capacitated and acrosome reacted sperms.

We attempted to corroborate the CTC evidence of successful capacitation by measuring the capacitation related motility pattern of hyper activation. We were not really successful?

We establish a progesterone-induced transient increase in intracellular calcium in stallion sperms. it was beyond our scope to study if this is a feature of the whole cell population or peculiar to a functionally distinct sub-population.

Life Lessons

Integrity is vital
The need to adapt
Endurance, to see through and respond to change
Creativity in finding solutions





Animal Biotechnology

Assisted Reproductive Technology





Boer Goat Nucleus Farm







SENI SILAT CEKAK



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Lahirnya Cekak kerana kesedaran
Bukan bermusuh sebagai tujuan
Untuk mendaulatkan seni kebangsaan
Mensesuaikan dengan makna kemerdekaan