THE STRUCTURE AND FUNCTION OF THE GAMETES OF THE STRIPED FIELD MOUSE (*RHABDOMYS PUMILIO*)

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ABSTRACT

This study was initiated in an attempt to add the four-striped field mouse (*Rhabdomys pumilio*) to the small group of mammals that are used to study and develop assisted reproductive technologies. To accomplish this, a complete knowledge of both the male and female gametes was necessary, as well as a repeatable and successful IVF protocol.

The gametes of the field mouse, both the falciform sperm and the mature oocytes, were shown to be similar in several respects to those of many other rodent species. The induction of both the capacitated state and the acrosome reaction in the sperm were readily achieved. The success of retrieving mature oocytes was very low, with the rare occurrence of polar bodies within ovulated oocytes. The difficulties with oocytes was either related to the lack of a regular oestrous cycle, or to the hormonal regime used to induce superovulation. Although several protocols were used to accomplish superovulation in this species, it was seldom achieved.

In *in vitro* fertilization trials, sperm were found to attach to both the cumulus oophorus and the zona pellucida of the oocytes. However, fertilization was never accomplished. This failure to achieve fertilization was probably related to the immaturity of the oocytes.

Without further studies on *Rhabdomys pumilio* and further attempts at accomplishing *in vitro* fertilization, it would seem that this species would not easily be introduced as an animal model for assisted reproduction.

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CHAPTER ONE

INTRODUCTION

Our understanding of, and ability to manipulate, the events of reproduction have increased markedly as a result of intensive research. Assisted reproductive technologies are those procedures that have been developed to enhance reproduction in infertile, subfertile or geographically distant individuals (Durrant, 1999). These biotechniques have advanced rapidly and include artificial insemination (AI), *in vitro* maturation (IM), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and gamete and embryo cryopreservation or transfer (Brewis & Wong, 1999).

In recent years, concern for the future well-being of the global environment has grown to an unprecedented level (Volt & Pickard, 1999). Among the major worries is the possibility that many species will be forced into extinction by unnatural causes, rather than through the slower evolutionary processes. Reproductive technology can have only a very limited role in the conservation of biodiversity if the primary aim is simply to preserve species (Volt & Pickard, 1999). However, the aims of conservation should be considered in terms of ensuring the survival and evolutionary development of ecosystems, species and populations of animals, plants and micro-organisms in their native habitats. In this context, therefore, reproductive biology as a discipline has valuable contributions to make towards the broad aims of conservation. It provides insight into the many reproductive specialities and adaptations of different species and is crucial for understanding the novel factors that may deleteriously affect the survival of populations (Volt & Pickard, 1999). In so doing, reproductive technologies also provide information for making strategic management decisions aimed at alleviating these threats to survival and play an important role in sustaining genetic diversity in the management of both captive and natural populations (Wildt *et al.*, 1992).

Fertilization bridges the fields of reproductive and developmental biology and a full understanding of the cellular and molecular events during fertilization is thus essential (Schatten, 1999). In addition, many clinical problems, including sophisticated infertility treatments and contraception, hinge on a more complete knowledge underlying this crucial process. The study of the mechanisms of the reactions that take place during the fertilization of the mammalian oocyte was considerably facilitated by the development of IVF methods (Chang, 1959). This is one of the assisted reproductive technologies, in which mature oocytes are retrieved, co-incubated with capacitated sperm for fertilization in the laboratory and allowed to grow and develop in culture medium (Durrant, 1999; Knobil & Neill, 1999). Usually a few of the highest quality embryos are then chosen for placement into the uterus with the goal that one of these will successfully implant to develop into a new individual.

The development of IVF systems has enabled fertilizing conditions to be manipulated and specific requirements to be investigated (Stock *et al.*, 1989), as well as permitting visual observations of direct interactions between sperm and oocytes (Wakayama *et al.*, 1995). Since the first report of complete IVF in hamsters (Yanagimachi & Chang, 1964), the technique has been applied successfully to an increasing number of mammalian species (Moore & Taggart, 1993). With IVF of mammals, including humans, now routine, it has been possible to solve many of the critically important problems of fertilization at the molecular cell biological level (Schatten, 1999). Studies to characterize the biological aspects of reproduction require a repeatedly successful IVF regime in the study species. To accomplish this, it is essential to evaluate sperm motility, capacitation and the subsequent acrosome reaction, both *in vivo* and *in vitro*.

Early IVF procedures usually incorporated biological materials, i.e., blood serum, cumulus oophorus, or follicular fluid, in the culture medium in order to maintain sperm viability and to support capacitation and acrosome reactions (Bavister, 1989). These ill-defined conditions have been replaced with defined IVF culture medium in which all of the components are known. By using defined culture media, results become much more reproducible, data are more meaningful and expertise is easier to acquire.

IVF is a complex, minimally successful technique even in species with significant market-driven research such as cattle and humans (Durrant, 1999). However, extrapolation of methodologies from those species to exotic bovids and nonhuman primates, respectively, offers some hope for individuals for whom other, less invasive assisted reproduction procedures have failed. The basic technique of IVF has not only led to production of offspring in several species, but has led to alternative procedures with wide-ranging applications (Dukelow, 1993). Thus, IVF has broad application in improving breeding of domestic animals, alleviating infertility in humans and conserving endangered species (Barnett & Bavister, 1992; Wakayama *et al.*, 1995). The development of defined embryo culture systems holds promise for ongoing efforts in reproductive physiology and for utility in the embryo transfer industry (Keskintepe & Brackett, 1996). These techniques, designed to enhance reproduction in humans and domestic animals, are being gradually incorporated into management schemes for exotic species too (Durrant, 1999).

The development of IVF systems has further application. The presence of environmental contaminants and by-products of modern manufacturing processes have been shown to affect reproduction adversely. Several man-made chemicals in the environment have weak oestrogenic or anti-androgenic activity and may exert subtle deleterious effects on endocrine functions in many animal species (Volt & Pickard, 1999). Increasing numbers of studies are reporting adverse effects of environmental contamination by, for example, heavy metals on wildlife. Those wild animals that are struggling to survive this harsh situation represent at least part of the variety of species on earth encompassed by the term 'biodiversity'. Through the development of procedures for IVF in several species, these animals can be utilized to examine the effects of pollutants on the gametes and the fertilization process. From these studies, extrapolation can be made in terms of the effects the same compounds might induce in other species, including man.

Furthermore, in order to analyze fertilization events in detail, reproductive biologists improve existing methods and develop new sophisticated techniques (Oura and Toshimori, 1990). Sperm and oocytes are often ideal systems to study general mechanisms and occasionally provide interesting insights. Examples include the assembly of actin filaments, as found in the acrosome reaction of invertebrate sperm; the activation of a perioxidative system in the fertilized egg that is similar to that seen in the phagocytic white blood cell; and the activation of egg metabolism by increasing intracellular pH (Shapiro & Eddy, 1980).

It is generally accepted that the clinical success of human *in vitro* fertilization, as well as those for valuable domestic animals, is a result of studies using animal models (Nohara *et al.*, 1998). However, since the process of reproduction is a relatively species-specific event, it is also important to evaluate and compare physiological, biochemical and molecular characteristics of fertilization in various species. Therefore, the establishment of a new rodent animal model should help clarify the general field of reproduction.

There are few species of rodent for which *in vitro* fertilization protocols have been established and only one indigenous African rodent, mastomys (*Praomys coucha*) (Nohara *et al.*, 1998). Consequently the common four-striped field mouse, *Rhabdomys pumilio*, was considered for introduction as a model into assisted reproductive technologies and related fields. *Rhabdomys pumilio* is a small (30-40g), diurnal rodent characterized by four black stripes that run longitudinally along the back, from which they obtain their colloquial name (Brooks, 1974; David & Jarvis, 1985; De Graaf, 1981; Skinner & Smithers, 1990). Their distribution is discontinuous through most of Africa, south of the Sahara (Brooks, 1974; De Graaf, 1981; Skinner & Smithers, 1990; Yom-Tov, 1993).

The field mouse occupies a wide variety of habitat types, but is essentially a grassland species, occurring only where there is good grass cover, providing protection from avian predators (De Graaf, 1981; Dewsbury & Dawson, 1979; Skinner & Smithers, 1990). It has been called an opportunistic omnivore, but is mainly granivorous, feeding predominantly on grass seeds, although it also feeds on insects and green vegetable matter (David & Jarvis, 1985; De Graaf, 1981; Perrin, 1986; Skinner & Smithers, 1990). Due to its diet, the four-striped field mouse is often attracted to cultivated lands, where it is known to cause considerable damage (Brooks, 1974; Davis, 1942).

Rhabdomys pumilio ovulates spontaneously throughout the year and is thus relatively easy to breed within the laboratory (*pers. obs.*). In the wild the breeding season is reported to extend from September to April, followed by a four-month period of anoestrus (Brooks, 1974; David & Jarvis, 1985). *Rhabdomys pumilio* starts breeding at an early age (57 days), has short intervals between litters (23 days), large litters ranging from 3 to 9 (mean 6.5) and are weaned at an early age (16 days) (Brooks, 1982; De Graaf, 1981; Skinner & Smithers, 1990).

The overall objective of this project was to establish *Rhabdomys pumilio* as a new animal model in the field of reproductive studies. In this project, investigations into the basic biological characterization of the field mouse oocyte and sperm are reported. Assays were also performed to evaluate sperm capacitation and the acrosome reaction. On the basis of these studies attempts to develop an IVF protocol in this species were carried out.

CHAPTER TWO

GENERAL PROCEDURES

2.1 ANIMAL COLLECTION AND HUSBANDRY

Specimens of the four-striped field mouse (*Rhabdomys pumlio*) were caught near Grahamstown in the Eastern Cape Province of South Africa. Trapping was carried out either at Thomas Baines Nature Reserve or on a farm on the outskirts of Grahamstown itself (33°18'S; 26°32'E).

Trapping was accomplished using aluminium Sherman traps baited with balls of peanut butter and rolled oats. Approximately 100 traps were laid in the grasslands, below the mat of grass. Where travel runways or mouse holes in the ground were observed, the traps were set facing them. Traps were opened in the late afternoon and closed in the early morning, checking them all on both occasions. This was done to avoid dehydration of any animals that might have been caught during the hot hours of the day.

Any field mice caught were removed from the traps and sexed. The male field mouse has a bi-lobed, flattened penis, while the female exhibits a rounded protrusion and vaginal opening. Mice were taken back to the laboratory and placed individually into plastic cages (41x26x15cm) with wire covers. Shredded paper was given as bedding and a toilet roll was placed into the cage to be used as refuge. Cages were cleaned out every two weeks, when new shredded paper was placed into the cages.

The mice were held in a constant environment room under long day conditions (14L:10D - lights on 06:00, lights off 20:00). The room temperature was monitored and maintained at approximately 25°C.

The mice were fed *ad libitum* on a 2:1 mixture of rabbit pellets (Epol, Johannesburg) and striped sunflower seeds (Agricol, Brackenfel) and water was given*ad libitum*. Animals were maintained under these conditions for a minimum of 1 and a maximum of 7 months before they were used in an experiment.

Breeding using the captive mice was carried out by placing an adult male with an adult female between 19:00 and 19:30. If there were no signs of aggression, the mice were left together overnight and the male was removed early the following morning. If the female did not give birth within a month, she was either used again for breeding, or was sacrifices in an experimental procedure.

2.2. LABORATORY CONDITIONS AND STERILE TECHNIQUES

The room used for dissection and other experimental procedures was approximately 3 x 2m, with minimal through-flow of air. The walls, benches and floor were dusted and then washed down with water and the benches, floor and all technical equipment sterilized with 70% alcohol. This procedure was repeated every two weeks.

A water-jacketed incubator (Forma Scientific, Ohio) was used whenever constant, ideal conditions were required for gamete incubation, or for equilibration of culture medium and paraffin oil. The incubator was set at 36°C and 5% CO_2 and a high internal humidity was maintained by placing two glass bowls filled with distilled water into the bottom of the incubator. A desiccator bowl was placed into the incubator and was used to hold tubes or petri dishes containing medium and gametes. This was done to prevent fluctuations in temperature and CO_2 in the air around the medium when the incubator door was opened.

The night prior to any experimental procedure the room and equipment were sterilised using 70% alcohol. The dissecting equipment was dipped into the alcohol and then flamed. The benches, floor, microscope and other apparatus were wiped down with paper towelling soaked with 70% alcohol.

The medium used in all experiments is referred to in the following text as supplemented medium. Unless otherwise specified, it was constituted by adding 0.075g fatty acid free bovine serum albumin (BSA; Boehringer Mannheim, Germany) and 0.015g penicillin (*Novopen*, Novo Nordisk, Johannesburg) to 25ml Dulbecco's Modified Eagles medium 12-707 (DMEM; BioWhittaker, Maryland). Fraser (1985) and Harrison *et al.* (1982) indicate that albumin is required for mouse spermatozoa to achieve efficient fertilization. The supplemented medium was placed into the incubator overnight to equilibrate. Paraffin oil was used in most experiments to overlay medium and culture drops. The oil was also placed into the incubator approximately 12 hours before an experimental procedure to equilibrate. The oil was used primarily in an attempt to minimise changes in osmolarity or temperature within the medium.

CHAPTER THREE

SPERM STRUCTURE AND FUNCTION

3.1 INTRODUCTION

Spermatozoa (sperm) are the haploid male gametes arising from the process of spermatogenesis, occurring in the seminiferous tubules of the testes. Sperm leaving the testes and those in the caput epididymides are functionally immature and incapable of accomplishing fertilization with an oocyte, while those in the cauda epididymides are mature and able to achieve fertilization once capacitation has occurred within the female reproductive tract (Oura & Toshimori, 1990). During epididymal transit, sperm undergo a number of structural, biochemical and functional changes resulting in their ability to fertilize (Biegler *et al.*, 1994). The plasma membrane is the site of the most prominent changes occurring during epididymal maturation and is directly involved in a number of significant prefertilization events within the female, including capacitation, zona binding, the acrosome reaction and sperm-oocyte fusion (Biegler *et al.*, 1994).

The nucleus in mammalian sperm is capped by a sac-like organelle, the acrosome, that stores several hydrolytic enzymes including acrosin, hyaluronidase, neuramindase, esterases and acid-phosphatases (Sidhu & Guraya, 1989). As a prerequisite for fertilization, the sperm must undergo an acrosome reaction after which the acrosomal enzymes are exposed to the oocyte, enabling the sperm to pass through its investments, particularly the zona pellucida (Barros *et al.*, 1984; Biegler *et al.*, 1994; Oura & Toshimori, 1990). The acrosome reaction allows for this release of acrosomal contents, while still preserving surface receptors on the sperm for interaction with the zona pellucida of the oocyte (Sidhu & Guraya, 1989).

During the initial stages of the acrosome reaction the outer acrosomal membrane fuses with the sperm plasma membrane at several sites, the enzymes are released and, at the most anterior region, the inner acrosomal membrane becomes the limiting membrane of the sperm (Barros *et al.*, 1984; Cross *et al.*, 1988; Kholkute *et al.*, 1995). Fusion of the acrosomal and plasma membranes starts at the junction of the anterior and equatorial segments of the sperm head, and progresses forward into the anterior segment (Oura & Toshimori, 1990).

There has been a great deal of controversy over the years as to where the acrosome reaction occurs in relation to the oocyte's cumulus matrix and zona pellucida and whether or not sperm that have already completed the acrosome reaction can still fertilize oocytes (Ivani & Seidel, 1991). In most mammals the acrosome reaction is known to be necessary for sperm penetration through the zona pellucida and for sperm to fuse with the oolemma (Cross *et al.*, 1988; Lee & Storey, 1985; Saling & Storey, 1979). However, in the guinea pig, it has been shown that the acrosome must in fact be intact in order to bind to the zona prior to penetration (Huang *et al.*, 1981).

Various authors have indicated that for sperm to undergo the acrosome reaction, they must be exposed to and interact with chemicals such as bicarbonate, potassium and calcium (Barros *et al.*, 1984; Kholkute *et al.*, 1995; Lee & Storey, 1986; Sidhu & Guraya, 1989). It has also been reported that albumin is necessary for the induction of the acrosome reaction (Fraser, 1985; Oura & Toshimori, 1990; Stewart-Savage, 1993; Yanagamachi, 1982). It has been noted, however, that it is unlikely that albumin actually triggers the acrosome reaction, but rather that it induces surface alterations on the sperm surface in preparation for the reaction (Fraser, 1985). It has been proposed that the oocyte's zona pellucida is responsible for specifically initiating the acrosome reaction in the fertilizing sperm (Fraser, 1985).

The process of capacitation is also an essential prerequisite for the induction of the acrosome reaction. This is a process of biochemical modifications that sperm undergo before they acquire the ability to acrosome react and before they can increase metabolism to induce hyperactive motility (Biegler *et al.*, 1994; Boatman & Robbins, 1991; Cross & Overstreet, 1987; Mate & Rodger, 1991; Oura & Toshimori, 1990; Sidhu & Guraya, 1989). Capacitation normally occurs in the female reproductive tract, but has been achieved in some species in chemically defined media *in vitro* (Biegler *et al.*, 1994). Medium for *in vitro* studies must be pH-stabilized, substrate-rich and contain albumin and sperm motility factors (Boatman & Robbins, 1991). Fraser (1985) reported that albumin is not required to support capacitation *in vitro*, while Stewart-Savage (1993) indicates that hamster sperm capacitation has both an albumin-dependent and albumin-independent phase.

The changes that occur during capacitation are more difficult to assess than the changes associated with the acrosome reaction. Capacitation consists of subtle modifications including removal of peripheral glycoproteins, rearrangement of integral proteins, reduction in cholesterol content and changes in the distribution and composition of membrane phospholipids (Perez *et al.*, 1996). A decreased cholesterol: phospholipid ratio in the sperm plasma membrane and a removal of zinc from the sperm surface facilitates capacitation, eventually leading to the acrosome reaction (Sidhu & Guraya, 1989; Stewart-Savage, 1993).

As mentioned above, removal or alteration of seminal plasma components that normally coat the sperm surface are part of the capacitation process (Brackett & Oliphant, 1975; Sidhu & Guraya, 1989). It has been suggested that epididymal sperm are covered with decapacitation factors that protect zona binding sites and inhibit sperm motility (Biegler *et al.*, 1994; Sidhu & Guraya, 1989). Brackett & Oliphant (1975) and Fraser (1985) used mice to demonstrate the commencement of capacitation only once the removal of these surface seminal plasma components, including decapacitation factors, had occurred. Some seminal plasma components may limit membrane fluidity, thus preventing sperm from undergoing the acrosome reaction (Biegler *et al.*, 1994). Sidhu & Guraya (1989) have shown that, during capacitation, areas depleted of membrane proteins are formed, and it is suggested that these may be the sites for membrane fusion in the acrosome reaction (Sidhu & Guraya, 1989).

Capacitation also involves a modification in the pattern of the flagellar beat and sperm trajectory, resulting in a condition known as hyperactivation. Temporally, the onset of hyperactivation precedes the onset of the acrosome reaction (Boatman & Robbins, 1991). It is suggested that hyperactivated motility might augment the thrust needed to penetrate the oocyte's investments (Sidhu & Guraya, 1989).The change in sperm motility reflects an increased metabolic rate which is most likely related to a dramatic increase in oxygen uptake (Brackett & Oliphant, 1975). Sidhu & Guraya (1989) show that the well-organised strands of small particles enveloping the mid-piece of the sperm tail become disorganised during incubation of sperm in capacitation medium and this structural change correlates with the onset of hyperactivity. The onset of hyperactivation is often used as a signal of the induction of the capacitated state.

Although the structure and function of sperm and associated processes, like the acrosome reaction and capacitation, have been studied in many species and reviewed several times (Eddy, 1988; Fawcett, 1975; Millette, 1999; Sidhu & Guraya, 1989), it was necessary to investigate and describe the structure of the sperm of the field mouse. The mechanisms associated with fertilization are largely dependent on the sperm and thus, in order to eventually attain *in vitro* fertilization, it was necessary to study the processes of capacitation and the acrosome reaction in this species. By doing so, the optimal conditions for incubation of the male gametes could be established and thus the ideal environment for fertilization. It was also essential to determine both the external and internal structure of the intact sperm, in order that comparisons might be made with sperm that had undergone physical alteration in preparation for, during the process of, or after the accomplishment of capacitation, the acrosome reaction and fertilization.

3.2 *SPERM STRUCTURE*

3.2.1 METHODS AND MATERIALS

Sperm were collected from male field mice held under constant conditions as described in Section 2.1. The mice were sacrificed by intra-thoracic injection of 0.35ml Euthenaze (Centaur Labs, Johannesburg) using 0.5ml insulin syringes, and the abdomen and testis region sterilized using 70% alcohol. The cauda epididymides were excised and placed into 5ml of supplemented medium on a sterile plastic petri dish. Caput epididymal sperm are known to be immotile and unable to bind to the zona pellucida of eggs, while cauda epididymal sperm are motile and bind readily (Biegler *et al.*, 1994), thus, in all experiments, cauda epididymal sperm were utilised.

The epididymides were washed free of blood and hair and cleaned of excess fat. They were then each placed into separate wells of a four-well plate (Nunc, USA) containing 1ml of supplemented medium in each well. The tissue was punctured several times with watchmakers forceps and a period of 10 minutes was allowed for sperm to swim out into the medium (Cross *et al.*, 1988; Cross & Overstreet, 1987), after which the tissue was removed.

3.2.1.1 Scanning electron microscopy

For scanning electron microscopy (SEM) preparations, drops of sperm solution, prepared as described above, were placed on 10mm circles of 0.2µm millipore paper. The fluid was then drawn through this paper using filter paper, leaving sperm on the upper surface of the millipore paper. The circle was then cut into smaller pieces (3mm^2), attached to brass SEM stubs using double-sided carbon tape, critically-point dried, coated with gold and viewed using a JEOL JSM 840 microscope.

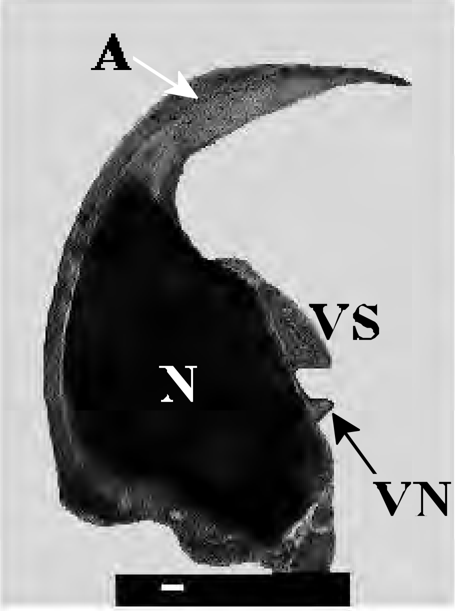
3.2.1.2 Transmission electron microscopy

For transmission electron microscopy (TEM) work, sperm solutions prepared as described above were placed into 1.5ml eppendorf tubes and centrifuged at 1600*g* for 5 minutes. The resultant pellets were removed, resuspended in 2.5% glutaraldehyde in 0.2M cacodylate buffer and repelleted as before. The pellets were then put through the chemical protocol for TEM preparation (Appendix 1a) and embedded in Araldite / TAAB 812 resin mixture (Cross, 1989). 100nm ultra-thin sections were cut on a microtome (LKB 8800A ultratome III), stained (Appendix 1b) and viewed using a JEOL JEM 1210 microscope.

3.2.2 RESULTS

The head of the sperm of the field mouse was falciform, with an extended anterior section curving round to an apex (Fig. 3.2). Posteriorly, the sperm head possessed a ventral spur and smaller ventral node (Figs 3.1, 3.2 & 3.7) which contained neither nucleus nor acrosome. The extended, arched eave was triangular in transverse section with two lateral arms (Figs 3.3 - 3.6). The right arm of the eave was longer than the left (Fig. 3.3). This extension in the arm originated from the ventral spur, continued through the eave and diminished in extent towards the sperm apex (Figs 3.2 & 3.7). The ventral and dorsal surfaces of this eave were concave (Fig. 3.6). The eave contained the anterior extension of the

3.1.



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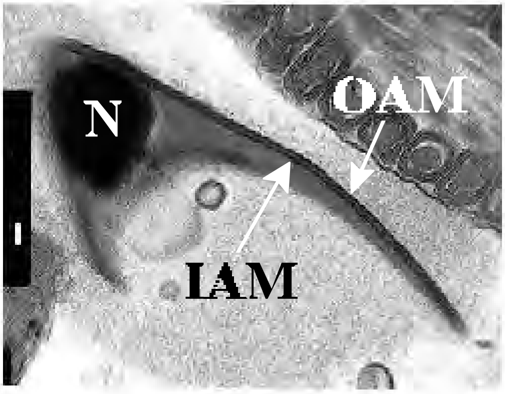

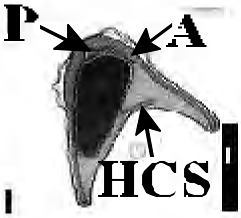

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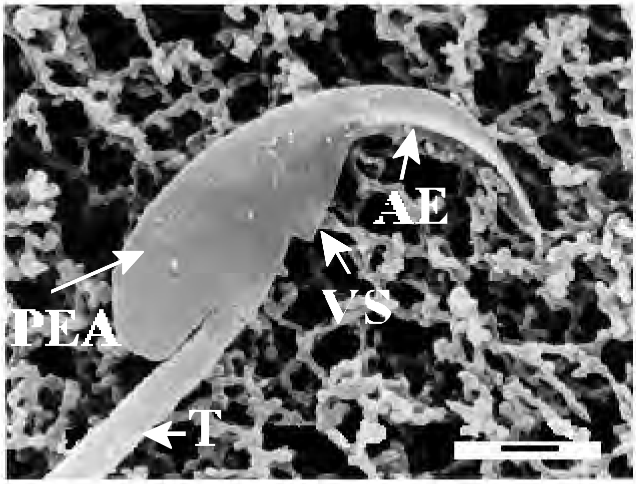

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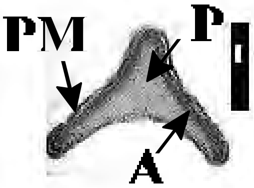


3.6.

Figure 3.1. Longitudinal section through the sperm head of the field mouse.
White scale bar represents 200nm.

Figure 3.2. SEM showing the falciform sperm head, indicating the triangular-shaped anterior acrosomal eave (AE), the posterior extent of the acrosome (PEA) and the posterior nuclear bulge. Black scale bar represents 1µm.

Figures 3.3. - 3.6. Transverse sections through various regions of the anterior portion of the sperm head, indicating changes in nucleus (N) and acrosome (A) along the length of the eave. Labels: HCS, head cap segment; IAM, inner acrosomal membrane; NM, nuclear membrane; OAM, outer acrosomal membrane; P, perforatorium; PM, plasma membrane; T, sperm tail; VN, ventral node; VS, ventral spur.
White scale bars represent 100nm and 50nm in Figures 3.3.- 3.5. and Figure 3.6. respectively.

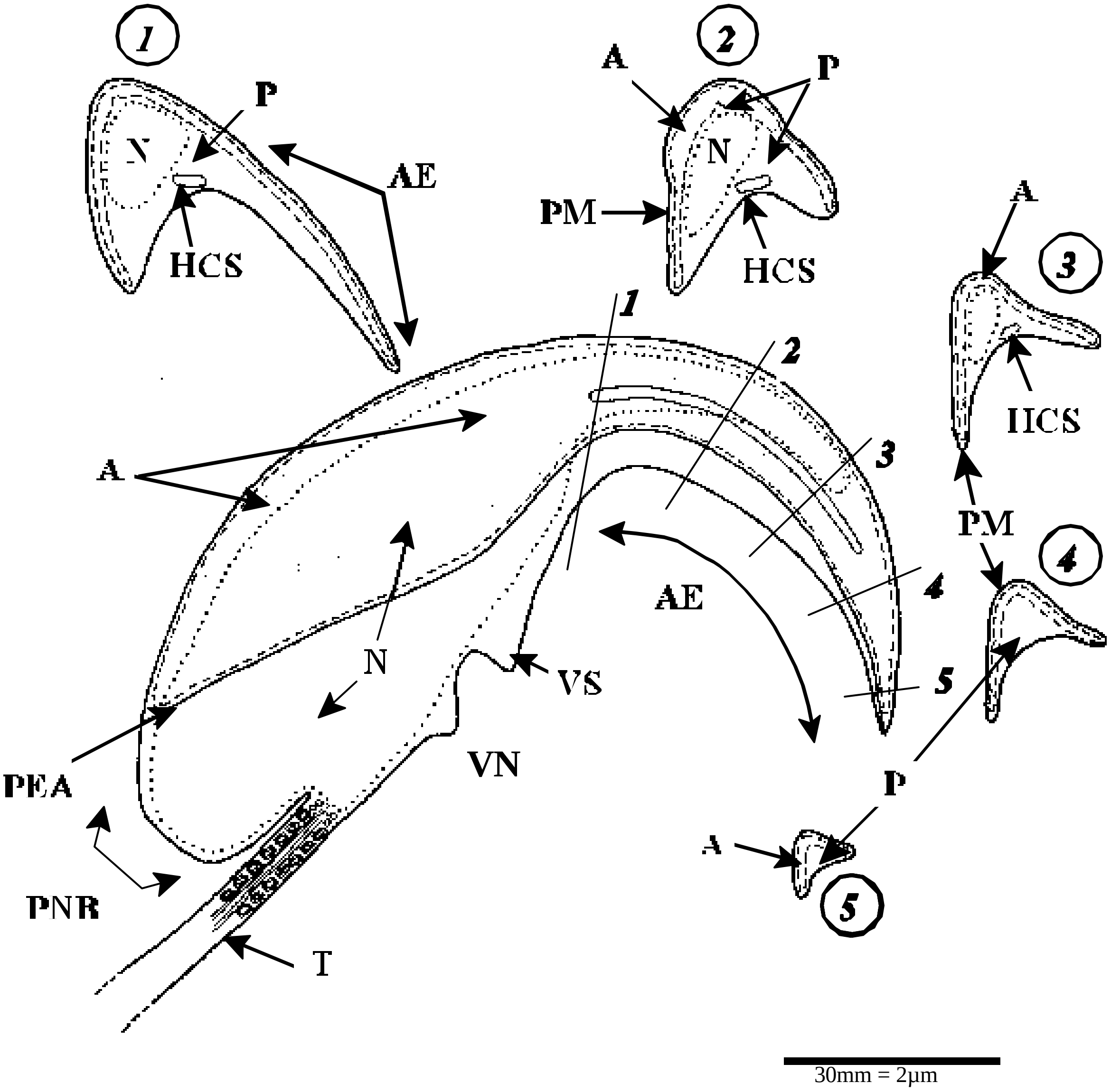


Figure 3.7. Drawing of the head of a field mouse spermatozoon as seen from the side and accompanied by representative transverse sections. The area indicated by the dashed line shows the extent of the acrosome (A), which covers approximately the anterior two-thirds of the nucleus (N) (dotted line). The displaced head cap segment (HCS) lies on the ventral surface of the nucleus, in the acrosomal eave (AE).

Labels: P, perforatorium; PEA posterior extent of the acrosome; PM plasma membrane; PNB posterior nuclear bulge; T sperm tail; VN, ventral node; VS, ventral spur.

Scale: 30mm represents 2µm.

nucleus and the acrosome, including a displaced head cap segment (Fig. 3.7).

The nucleus stretched through much of the sperm head, filling most of the posterior po n (Fig. 3.1). It began to narrow at the start of the eave ending approximately 2.5µm from the sp apex. The gradual narrowing of the nucleus is evident through comparison of Figures 3.3, 3.4 d 3.5 and is shown in the transverse sections of the drawing of the sperm head (Fig. 3.7). Th ucleus lay asymmetrically in the eave, with most in the left lateral arm (Figs 3.3-3.5). There was al an extension of the nucleus beyond the origin of the tail, forming a posterior nuclear bulge (Fig. 3.2).

The acrosome was positioned dorsally over the nucleus, with a thicker dorsal band in the eave and thinner lateral extensions in the posterior region of the head (Figs 3.2 & 3.8). The band was thickest in the middle of the posterior portion of the head and decreased in thickness anteriorly. This decrease in thickness is evident by comparing the dorsal portion of the acrosome in Figures 3.4, 3.5 and 3.6. The thinner lateral extensions lay posteriorly to the start of the eave and formed the posterior extent of the acrosome, clearly visible on SEM photographs (Fig. 3.2) and shown in the reconstructive drawing of the sperm (Fig. 3.7). The transition between the thick and thin portions of the acrosome is distinct, and is clearly visible in Figure 3.8.

A portion of the acrosomal apparatus was displaced and lay ventrally to the nucleus, within the eave (Figs 3.7 & 3.8). This displaced head cap segment (Oko & Clermont, 1988) did not extend all the way through to the sperm apex and was absent from transverse sections through the most anterior region (Fig. 3.6).

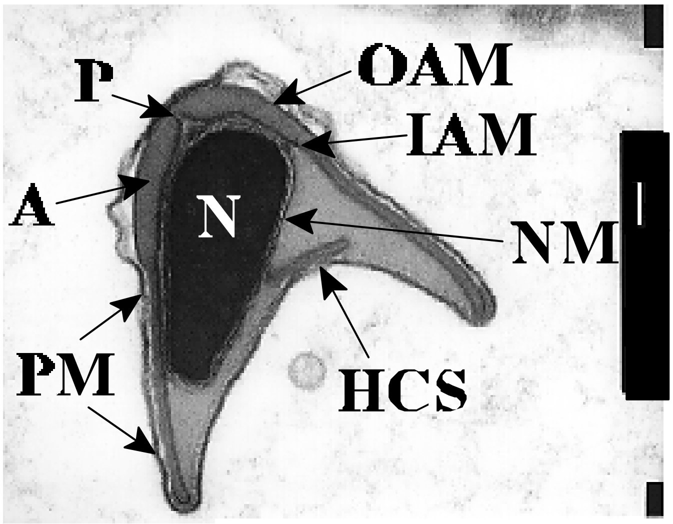


Figure 3.8 Transverse section through the anterior portion of a field mouse sperm head. Note the transition from thick to thin regions of the acrosome.
Labels: A, acrosome; HCS, head cap segment; IAM, inner acrosomal membrane; NM, nuclear membrane; OAM, outer acrosomal membrane; P, perforatorium; PM, plasma membrane.
White scale bar represents 100nm.

Between the nuclear and acrosomal membranes lay the subacrosomal cytoskeleton, or perforatorium. In transverse section the perforatorium extended ventrally down the length of the lateral extensions and, dorsally, lay in a thin band between the acrosome and nucleus (Fig. 3.8). At the posterior extent of the eave, but only within the eave, the perforatorium filled a small dorsal indentation in the inner acrosomal membrane (Fig. 3.8). This indentation decreases in extent towards the anterior of the head and is absent in Figure 3.5. Behind of the posterior extent of the acrosome, the perforatorium lay between the plasma membrane and nucleus and is referred to as the postacrosomal cytoskeleton.

3.3 *SPERM COUNTS AND SPERM MOTILITY*

3.3.1 METHODS AND MATERIALS

Male field mice were sacrificed by intra-thoracic injection of 0.35ml Euthenaze and the abdomen and testis region sterilized using 70% alcohol. The cauda epididymides were excised, cleaned of any blood or hair and dissected free of excess fat. The epididymides were then placed into two separate wells of a four-well plate, each containing 1ml of supplemented medium.

3.3.1.1 Counts

To obtain total cauda epididymal sperm counts (motile and immotile), the epididymides were punctured several times within the medium with watchmakers forceps and the entire contents forced out by pressing the tissue between the forceps.

To count only the motile sperm in the cauda epididymides the epididymides were punctured as before, however, instead of forcible extrusion of the contents, the solution and tissue were left in the incubator for 10 minutes to allow motile sperm to actively swim out into the medium (Lee & Storey, 1985).

Sperm solutions were mixed using an autopipette and 100µl of the sperm solution was then transferred into a single well of a four-well plate. 100µl acetic acid:absolute alcohol fixative (1:3) was then added to the sperm solution and the solution was mixed as before. This was pipetted under the cover slip of an improved Neubauer haemocytometer (Superior; Germany) and examined at 40x magnification under a Nikon light microscope. Sperm counts were carried out and the results recorded. Three replicate counts were performed per experiment, the results averaged and converted to a ‘sperm / ml’ value. Six experiments were carried out for total sperm counts and three for motile sperm counts.

3.3.1.2 Motility assessment

The motility of sperm was assessed in order that optimal samples might be selected for fertilization experiments. For assessment of motility, the cauda epididymides were excised and treated as described in Section 3.3.1. The tissue was then punctured several times within the supplemented medium using watchmakers forceps and left for 10 minutes in the incubator to allow sperm to swim out. The tissue was then discarded.

The 2ml of sperm solution were placed into 10 eppendorf tubes in 200µl volumes and each overlaid with 100µl paraffin oil. The tubes were then placed into a rack and set at a 45° angle in the incubator, thus increasing the surface area for sperm swim-up (Cross & Overstreet, 1987; Mate & Rodger, 1991; White *et al.*, 1990). Over time, the motile sperm swam into the top layers of the solutions, while any immotile or dead sperm sank to the bottom of the tubes. When samples were selected every 50 minutes for motility assessment, they were pipetted off the top layer of the solution as would be done in a fertilization experiment, thus ensuring that mostly motile sperm were selected.

Sperm samples were assessed according to the percentages of forwardly progressive and immotile sperm; the percentage of agglutinated sperm; and the extent of whiplash movement. Sperm were considered to be agglutinated when two or more swimming sperm were attached by their heads. They were considered to be showing whiplash motion when the head moved rapidly 180° from side to side. Whiplash swimming is taken to be one of the signs of sperm capacitation (Section 3.4). The extent of whiplash was scored on a scale of 0 to 4, with 0 representing no evidence of whiplash, with only snake-like swimming observed and 4 representing highly active swimming with extreme whiplash (‘figure-of-eight’) movement (Bavister & Andrews, 1988). Three experimental replicates were carried out.

Sperm progression was rated and classed into five categories according to Mortimer (1994):

0 = *None* - absence of forward progression;

1 = *Poor* - sluggish forward progression;

2 = *Moderate* - definite forward progression;

3 = *Good* - good forward progression; and

4 = *Excellent* - vigorous, rapid forward progression.

A motility index was then calculated as follows (Mortimer, 1994):

Motility Index = (% Progressive sperm x Progression rating x 2.5) + % Non-progressive sperm.

3.3.2 RESULTS

3.3.2.1 Counts

The mean sperm count (motile plus immotile) from the cauda epididymides was $3.75 \times 10^6 \pm 1.15 \times 10^6$ sperm/ml. The mean count for motile sperm from the cauda epididymides was $1.82 \times 10^6 \pm 0.5 \times 10^6$ sperm/ml.

3.3.2.2 Motility assessment

The percentage of motile sperm decreased over time, with the highest percentage of motile sperm (85%) in the initial sample (Table 3.1). The percentage of forwardly progressive sperm followed a similar trend, with 80% of the first sample forwardly progressive. The percentage of immotile sperm increased over time, with an initial value of 15% increasing up to 72% at 350 minutes. The scores given for whiplash swimming were initially high and then, after 100 minutes, decreased by half, subsequently dropping to zero by 350 minutes.

The motility indices indicate that there was a decrease in motility over time (Table 3.1). The initial sample had the highest motility index (883), with the index for the second sample (693) also at a high level. Thereafter, however, there was a rapid decrease in motility down to a low of 76 after 350 minutes.

Table 3.1 Motility assessment scores over time for sperm samples, including motility indices at each time period. Values recorded are means for the three experimental replicates, with standard deviations given in brackets. (No data were obtained for T=150 and T=300 minutes).

	Time (min)					
	0	**50**	**100**	**200**	**250**	**350**
% Motile	85 (6)	73 (12)	60 (10)	50 (10)	43 (6)	28 (21)
% Immotile	15 (6)	27 (12)	40 (10)	50 (10)	57 (6)	72 (20)
% Forwardly progressive	80 (10)	67 (23)	47 (32)	33 (21)	25 (22)	15 (19)
% Agglutination	1.3 (1.5)	1.7 (2.9)	0.7 (1.2)	0 (0)	0.7 (0.6)	0 (0)
Progression rating	4 (0)	3.7 (0.6)	3.3 (0.6)	2 (1)	1 (0)	1.3 (0.6)
Whiplash swimming score	4 (0)	4 (0)	2 (1.5)	2 (0.6)	1 (0.7)	0 (0.6)
Motility index	**883**	**693**	**448**	**215**	**106**	**76**

3.4 *CAPACITATION AND ACROSOME REACTION*

3.4.1 METHODS AND MATERIALS

Cauda epididymides were obtained and treated as described in Section 3.2.1. They were pricked several times within supplemented medium using watchmakers forceps and left for 10 minutes for sperm swim-out. The tissue was then discarded, the solutions mixed and 150µl aliquots of the suspensions placed into eppendorf tubes. The samples were overlaid with paraffin oil and then placed at an angle of 45° into the incubator at 37°C. Fleming & Kuehl (1985) showed that, in the mouse, although sperm capacitation occurred at a temperature of 20°C, there was no accompanying hyperactivation or fertilizing ability. However, when the temperature of incubation was increased to 37°C, as was used in the present experiments, these characteristics developed.

At 45 minute time intervals samples were taken from the top layer of the solutions and the chlortetracycline (CTC) assay carried out (Fraser & Herod, 1990; Lee & Storey, 1985; Mortimer, 1994; Perez *et al.*, 1996). CTC binds membrane calcium, whose distribution changes through capacitation and is readily visualised by fluorescence microscopy (Perez *et al.*, 1996). Preparation took place in a darkened room, where 2.5µl of the sperm solution was placed onto a glass microscope slide and 2.5µl of the CTC solution (Sigma Chemical Co.; Germany) mixed with it. After 10 seconds, 0.25µl of glutaraldehyde fixative was added and mixed in with a plastic pipette tip and cover slips laid over the preparations. In order to preserve the fluorescence on the sperm surface, which deteriorated rapidly if exposed to light, the slides were held in a light-shielded case prior to examination. Two slides were prepared at each time period, one being used for capacitation assessment and the other for photographs.

The prepared slides were viewed under epifluorescence illumination using a U-MU filter on an Olympus BX60 microscope (Perez *et al.*, 1996). According to the pattern of fluorescence exhibited on the sperm head, the sperm were classified into one of three groups (Perez *et al.*, 1996):

1. Uncapacitated, acrosome-intact sperm (UAI) = fluorescence over the entire head;
2. Capacitated, acrosome-intact sperm (CAI) = fluorescence over the anterior head (acrosome) only and not over the posterior section (nucleus); and
3. Capacitated, acrosome-reacted sperm (CAR) = no fluorescence over the head.

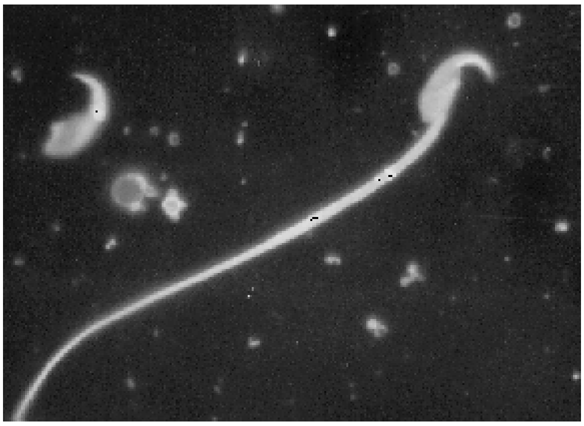
Three experimental replicates were carried out, examining and classifying two hundred sperm per slide at each time period. The results were used to calculate the percentage incidence of sperm within each class.

At each 45 minute time interval samples were also removed and fixed in 2.5% glutaraldehyde in cacodylate buffer. The solutions were then centrifuged at 1600*g* for 5 minutes and the resulting pellets prepared for TEM observation (Appendix 1). 100nm ultra-thin sections were cut on a microtome and these were examined and photographed on a JEOL JEM 1210 transmission electron microscope. Evidence of any changes in sperm structure associated with either capacitation or acrosome reaction were noted.

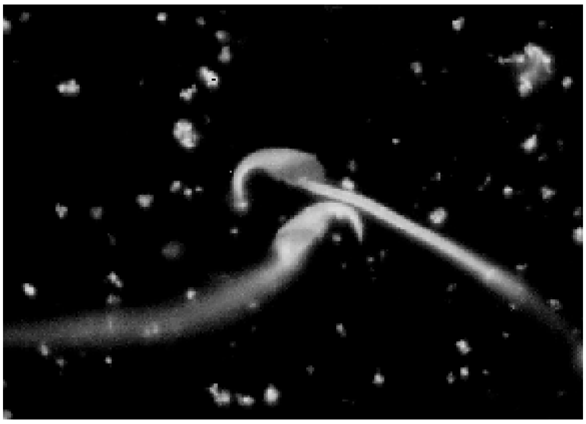
3.4.2 RESULTS

Typical examples of sperm at various stages of the capacitation process, as described by the CTC assay, are shown in Figure 3.9. Sperm exhibiting fluorescence over the entire head (Fig. 3.9.1) were classed as UAI. Figure 3.9.2 shows sperm exhibiting fluorescence over only the anterior portion of the sperm head, indicative of the CAI class, and when the fluorescence was totally absent from the

3.9.1.



3.9.2.



3.9.3.

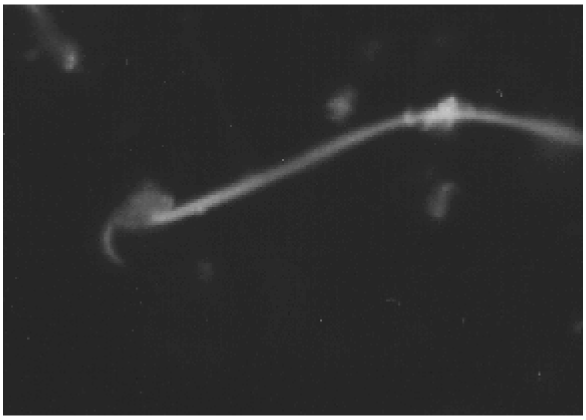


Figure 3.9. Representative images of the three categories of sperm capacitation after staining with CTC.

Figure 3.9.1. shows sperm with fluorescence over its entire surface. This represents an uncapacitated, acrosome-intact (UAI) sperm.

Figure 3.9.2. shows two sperm with fluorescence over the anterior portion of the head only. These sperm represent those that are capacitated and acrosome-intact (CAI).

Figure 3.9.3. shows a sperm with no fluorescence over the surface of the head, representing capacitated, acrosome-reacted (CAR) sperm.
1643 x magnification.

sperm surface (Fig. 3.9.3), the sperm were classified as the CAR group.

There was a significant decrease in the percentage of UAI sperm over time (RM ANOVA; P<0.0001), with the highest value at the start of the experiment (Fig. 3.10). The percentages of sperm in both CAI and CAR classes increased significantly over time (P<0.05 in both cases), with the highest percentage of CAI sperm apparent after 4.5 hours. After 6.0 hours, the percentage of UAI sperm in the sample was low, while the percentages of CAI and CAR sperm were approximately equal (Fig. 3.10).

The investigation using TEM indicated that there was no change in sperm morphology during capacitation. However, there was evidence of alteration in the sperm membranes associated with the induction of the acrosome reaction. From TEM evidence it was not possible to make a distinction between UAI and CAI sperm, where the acrosome was intact in both cases. However, it was possible to identify CAR sperm by the obvious fusion between the outer acrosomal membrane and the plasma membrane.

Throughout the experiment there were some sperm in each of the three classes of the CTC assay (Fig 3.10) and the TEM preparations at each time period have representative sections of the sperm in each of these three classes. An example of this can be seen in Figures 3.11 and 3.12. Both photographs are of sperm from the same sample at 1.5 hours after the initial introduction into supplemented medium, yet Figure 3.11 shows an acrosome-intact sperm head, while Figure 3.12 shows a sperm head that has acrosome-reacted. The fusion between the plasma membrane and the outer acrosomal membrane is clearly visible on Figure 3.12 and the partially empty acrosome indicates that the acrosome reaction had occurred.

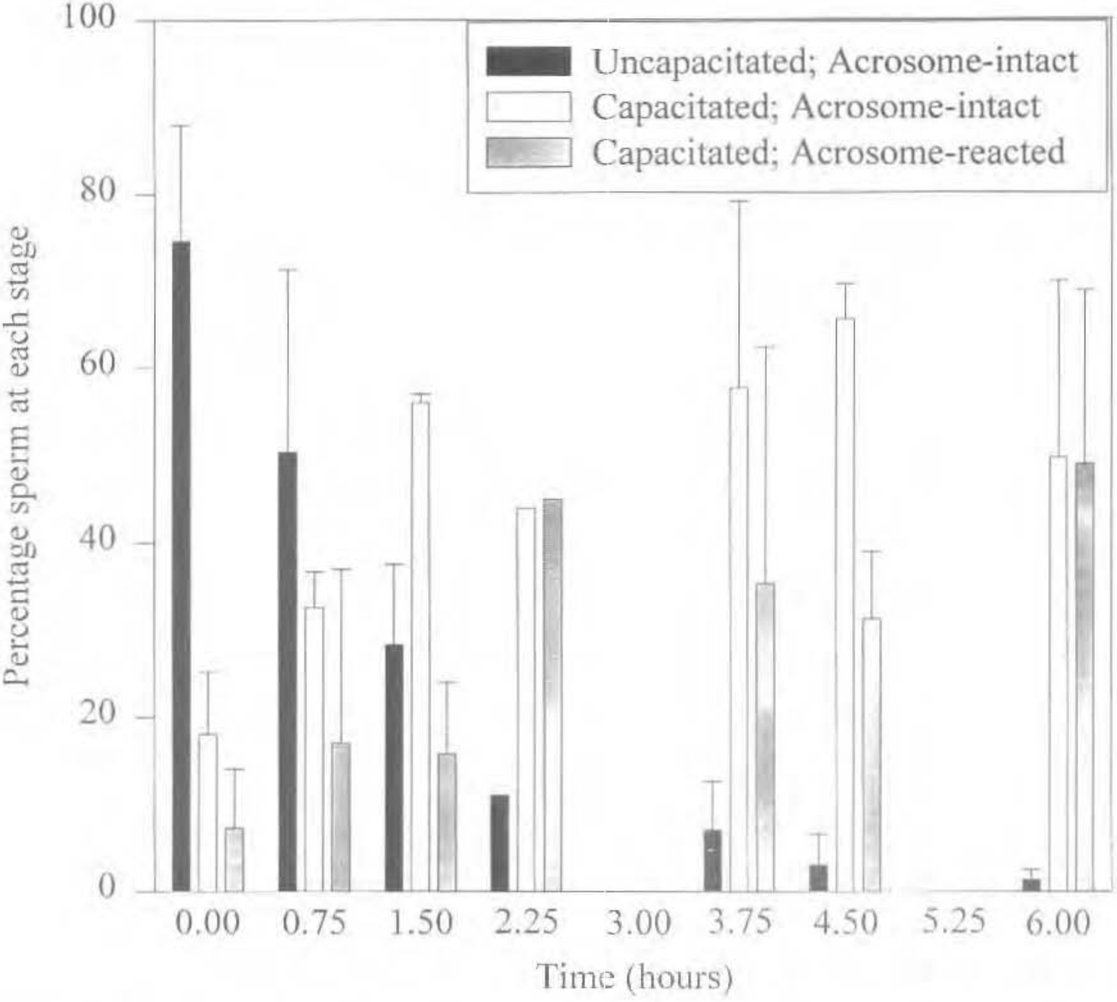


Figure 3.10 Change over time in the percentage occurrence of sperm in each class of capacitation.

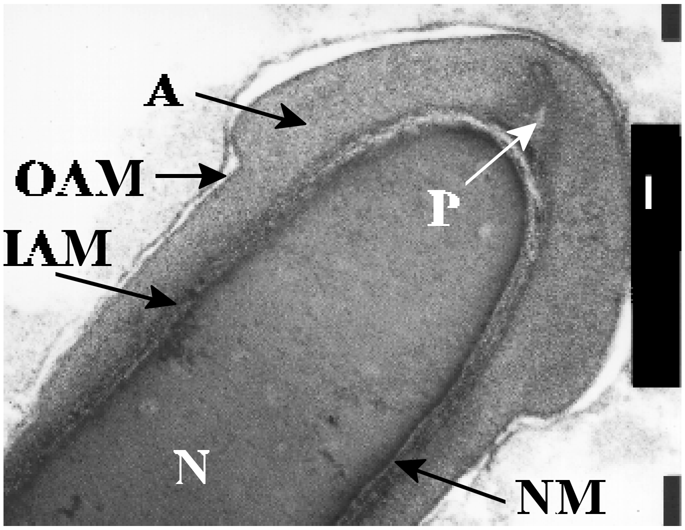


Figure 3.11. Longitudinal section through an acrosome-intact sperm head, after 1.5 hours in supplemented medium. There is no evidence of any physical change in sperm structure, with both outer (OAM) and inner acrosomal membranes (IAM) intact.
Labels: A, acrosome; N, nucleus; NM, nuclear membrane; P, perforatorium.
White scale bar represents 50nm.

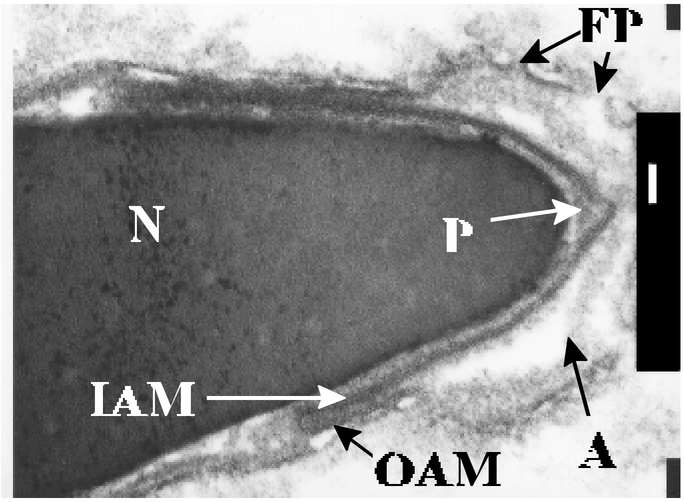


Figure 3.12. Longitudinal section through an acrosome-reacted sperm head after 1.5 hours in supplemented medium. Well-defined fusion pores (FP) between plasma membrane and outer acrosomal membrane (OAM) are evident. A substantial volume of the acrosomal contents has extruded.
Labels: A, acrosome; IAM, inner acrosomal membrane; N, nucleus; P, perforatorium.
White scale bar represents 50nm.

There was evidence of the acrosome reaction occurring even within the sample taken after only 0.75 hours. Figure 3.13 shows the initial formation of fusion pores between the plasma membrane and the outer acrosomal membrane, although the acrosomal content was still intact at this stage. Complete acrosome reactions were also noted.

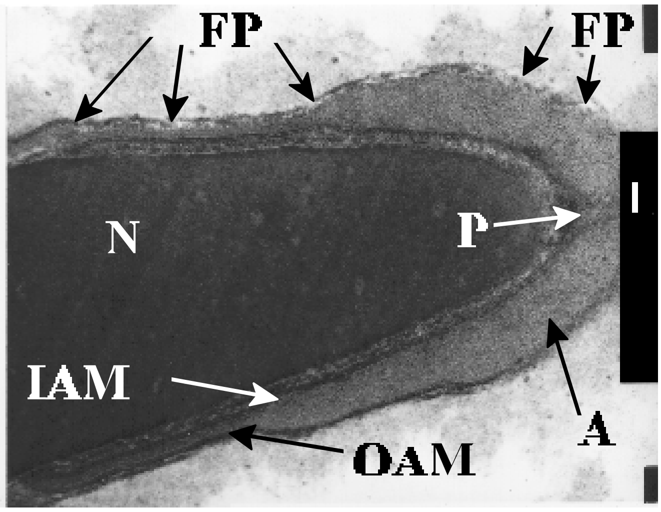


Figure 3.13 Longitudinal section through a sperm head after 0.75 hours in supplemented medium, showing the first signs of fusion pores (FP) between plasma membrane and outer acrosomal membrane (OAM).
Labels: A, acrosome; IAM, inner acrosomal membrane; N, nucleus; P, perforatorium.
White scale bar represents 50nm.

By 6.0 hours after the start of the experiment, at least half of the sperm had undergone the acrosome reaction (Fig. 3.10). Figures 3.14 and 3.15 show representative sections through such reacted sperm. In Figure 3.14 most of the acrosomal contents have been released, with just some acrosomal remnants remaining in contact with the head. In Figure 3.15 there is no evidence of the acrosome at all, with only the perforatorium remaining at the tip of the head.

Even though many of the sperm were acrosome-reacted, especially post-2.25 hours (Fig. 3.10), there

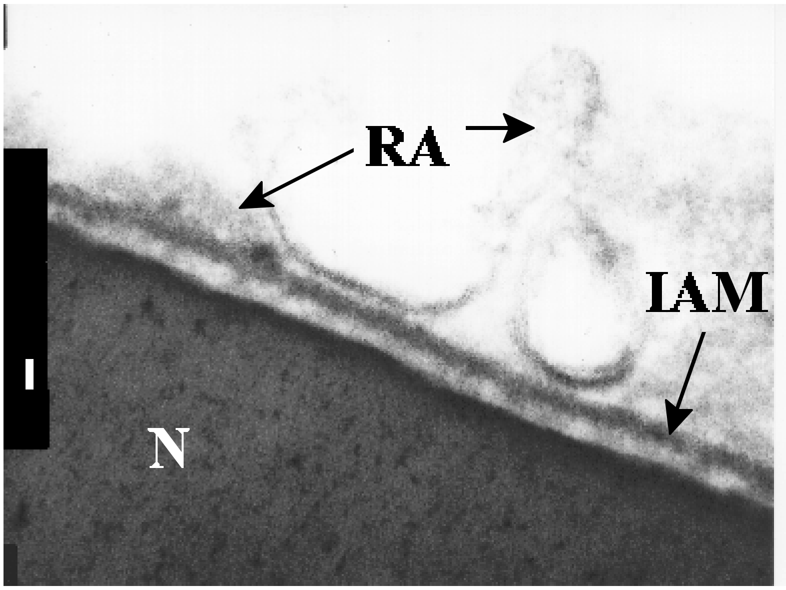


Figure 3.14. Longitudinal section through a sperm head after 6.0 hours in supplemented medium. The acrosome has reacted as evidenced by acrosomal remnants (RA) and absence of outer acrosomal membrane.
Labels: IAM, inner acrosomal membrane; N, nucleus.
White scale bar represents 20nm.

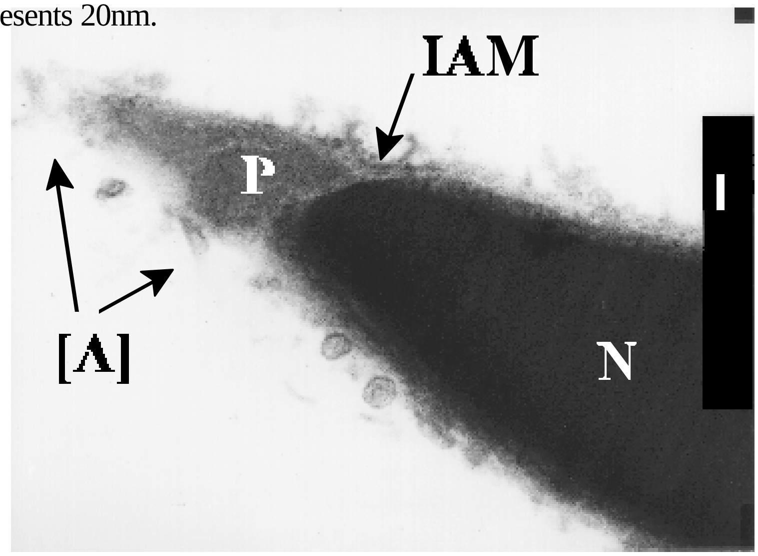


Figure 3.15. Longitudinal section through an acrosome-reacted sperm head after 6.0 hours in supplemented medium. [A] shows the previous position of the acrosome when it overlaid the perforatorium (P).
Labels: IAM, inner acrosomal membrane; N, nucleus.
White scale bar represents 100nm.

are sperm that remain acrosome-intact until the end of the experiment. The section in Figure 3.16 shows just such an acrosome-intact sperm after 4.5 hours in supplemented medium, with the membranes still intact and the acrosome still possessing its entire contents.

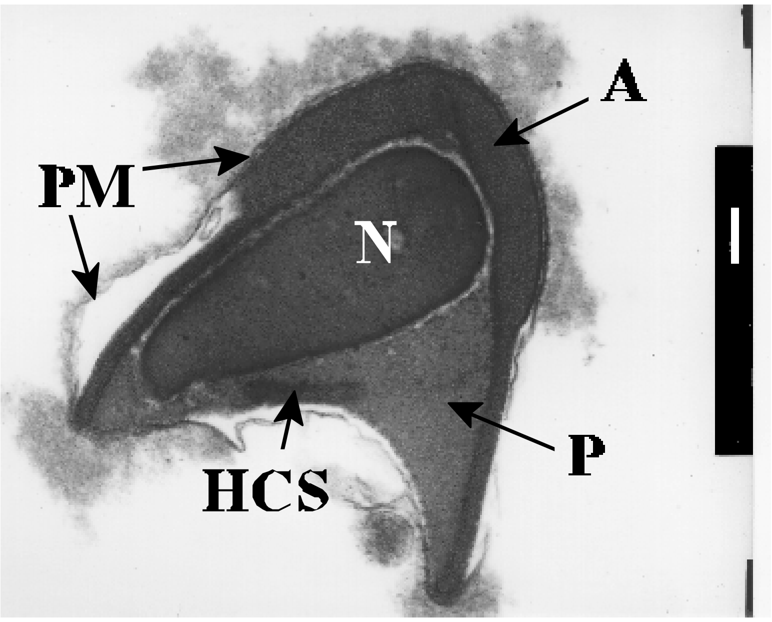


Figure 3.16 Transverse section through an acrosome-intact sperm head after 4.5 hours in supplemented medium.
Labels: A, acrosome; HCS, head cap segment; N, nucleus; P, perforatorium; PM, plasma membrane.
White scale bar represents 100nm.

3.5 DISCUSSION

The sperm of the male field mouse had a falciform structure, which is typical of many rodents such as *Otomys sloggeti* and *Parotomys brantsii* (Bernard *et al.*, 1991). The ventral spur, posterior nuclear bulge and displaced head-cap segment in the sperm head are unusual, but have previously been noted in other species (Oko & Clermont, 1988). The perforatorium which constitutes that portion of the head that is neither nucleus nor acrosomal apparatus, and is located in a narrow region between the inner acrosomal membrane and the outer leaflet of the nuclear membrane (Oura & Toshimori, 1990) is a characteristic of all rodent sperm (Millette, 1999; Oura & Toshimori, 1990).

Together the perforatorium and post acrosomal cytoskeleton form the perinuclear theca (Oko & Clermont, 1988). Although they are structurally continuous, the perforatorium and post acrosomal cytoskeleton have been shown to be biochemically distinct (Oko & Clermont, 1988). The function of the theca is unknown, but it is suggested that it serves to hold the nucleus and acrosome together (Millette, 1999) and may play a mechanical role during sperm penetration through the egg investments (Oura & Toshimori, 1990).

The sperm count of 3.75×10^6 sperm/ml is unusually low for a rodent. Cooper (1986) lists values of cauda epididymidal sperm counts for several species, including the rat and hamster, with averages of 1750×10^6 sperm/ml and 1870×10^6 sperm/ml respectively. The large difference in sperm numbers is not easily explained.

Sperm motility of the field mouse decreased over time and the observations revealed that the sperm in the initial samples had the highest percentage of forward progressive sperm, as well as the highest motility index. The greatest number of hyper activated, whiplash sperm were also present in the first two samples. From these results it might seem that the most functionally mature sperm were those from the initial samples and hence these might be the best gametes for use in fertilization investigations. However, the results of the CTC assay revealed that the highest numbers of capacitated sperm were from samples taken after 4.5 hours of incubation in supplemented medium. Hence, although the highest percentage of hyper activated sperm were found in the initial samples, these did not contain the most capacitated sperm and thus would not be the best sperm to use in attempts at fertilization. Rather, sperm from the samples incubated in supplemented medium for 4.5 hours were functionally most mature, with the majority having undergone capacitation and being functionally ready to achieve fertilization. Sidhu & Guraya (1989) report that there are no morphological changes associated with the process of capacitation *per se* and this is corroborated in this study. The onset of a whiplash swimming motion, or hyperactivation, has been used by several authors to be indicative of capacitated sperm (Bedford, 1970; Brackett & Oliphant, 1975). The temporal separation of sperm hyperactivity and the occurrence of capacitation in the present experiments either casts doubt on the general use of hyperactivity as a sign of capacitation, or on the ease of accurately interpreting the state of hyperactive swimming in sperm.

During *in vitro* fertilization, acrosome-intact mouse sperm initiate binding to the zona pellucida and then are induced to undergo the acrosome reaction by the zona pellucida glycoprotein ZP3 (Cross *et al.*, 1988). The ability to acrosome react in response to zona is known as the physiological acrosome reaction and is considered a distinguishing characteristic of fertile sperm (Biegler *et al.*, 1994). The acrosome reaction in the field mouse was shown to be no different to the acrosome reactions recorded in other species. Initial point fusions between the plasma membrane and outer acrosomal membrane develop as the initial steps of the reaction, which formed pores in the anterior sperm head whereby the acrosomal contents could escape. The zona pellucida is a major barrier to sperm penetration into the oocyte and it requires acrosomal enzymes and vigorous sperm motility (Ivani & Seidel, 1991). The enzymes leak from the acrosome and dissolve a path into the zona pellucida through which the sperm passes. In an *in vitro* situation the fused outer sperm membranes eventually disperse altogether leaving the nucleus exposed, covered anteriorly only by inner acrosomal membrane.

The acrosomal proteinase acrosin has been suggested as the agent that can help the spermatozoon in crossing the zona pellucida and the supporting evidence comes mainly from the fact that acrosin can digest the zona pellucida of the oocyte of several species (Barros *et al.*, 1984). However, it has been shown that purified ram sperm acrosin, while removing the zona pellucida of mouse oocytes, failed to remove the zona pellucida of pig, gerbil, or sheep oocytes (Barros *et al.*, 1984). These findings would throw some doubt on the sole contribution of acrosin on the whole process of sperm penetration through the zona pellucida (Barros *et al.*, 1984).

CHAPTER FOUR

OOCYTE STRUCTURE AND FUNCTION

4.1 INTRODUCTION

The oocyte is the mature female germ cell produced within the ovaries of an adult female animal (Wassarman, 1988a). Its development is arrested as a secondary oocyte in the first meiotic prophase, where it possesses a haploid chromosome set and is capable of initiating formation of a new individual by fusion with a male gamete (Stromstedt & Byskov, 1999). In mammals, oogenesis begins relatively early in fetal development and ends, months or years later, in the sexually mature adult. It begins with formation of the primordial germ cells and encompasses a series of cellular transformations, from primordial germ cells to mature oocytes (Wassarman, 1988a). Shortly after birth, therefore, the mouse ovary is populated with thousands of small, primary oocytes arrested in late prophase of meiosis I (Wassarman & Josefowicz, 1978). These oocytes represent a finite stock that is drawn upon throughout the female's reproductive life (Wassarman, 1988a).

In sexually mature mice, fully grown oocytes within the ovaries resume meiosis and complete the first meiotic division just prior to ovulation (Oura & Toshimori, 1990; Wassarman & Josefowicz, 1978). Ovulation of the oocytes is triggered by a surge of luteinizing hormone (LH), which not only leads to the reinitiation of meiosis and nuclear maturation, but also to cytoplasmic maturation and maturation of the zona pellucida (Kotsuji *et al.*, 1994; Stromstedt & Byskov, 1999). The oocytes undergo nuclear progression from the first meiotic prophase to metaphase II, and remain at this stage in the oviduct until stimulated to complete meiosis after fertilization (Wassarman, 1988a). This meiotic maturation is characterised by dissolution of the oocyte's nuclear membrane, condensation of chromatin into distinct bivalents, separation of homologous chromosomes, emission of the first polar body and arrest of meiosis with chromosomes aligned on the metaphase II spindle (Wassarman, 1988a).

The secondary oocyte is one of the largest cells in the body because, as well as providing for the embryo's protection and maintenance during development, it also provides stored cellular components to facilitate its early development (Robl & Fissore, 1999). The zygote inherits an extensive reserve of macromolecules and organelles from the egg that, to varying degrees, support the nutritional, synthetic, energetic, and regulatory requirements of the early embryo (Wassarman, 1988a). During its early history therefore, the oocyte is characterized by a predominance of anabolic metabolism. It prepares by extensive growth, accumulating a large amount of protoplasm, commonly laden with reserve food-matter and in many cases becoming surrounded by membranes or other protective envelopes, such as the cumulus oophorus (Robl & Fissore, 1999).

The oocyte is surrounded by two protective layers. The proximal is the plasma membrane or oolemma, which has a typical double membrane structure. The second layer, the zona pellucida, is composed of an amorphous polysaccharide-glycolipid matrix into which fine protein filaments are embedded (Hadek, 1969; Oura & Toshimori, 1990; Wassarman & Josefowicz, 1978; Wassarman & Mortillo, 1991). It is relatively thick, appearing during oocyte growth and increasing in width as the oocyte increases in diameter (Wassarman, 1988a; Wassarman & Mortillo, 1991; Yanagimachi, 1988). During the process of fertilization in mammals, sperm must bind to and penetrate through it in order to reach and fuse with the oolemma (Wassarman & Mortillo, 1991; Yanagimachi, 1988). It exhibits a 'Swiss cheese' appearance in scanning electron micrographs (Phillips & Shalgi, 1980). Whether or not the zona is structurally homogeneous throughout its width continues to be the subject of some debate (Wassarman, 1988b).

At ovulation the entire oocyte is also surrounded by layers of granulosa cells. Closely attached to the zona pellucida are the coronal cells, arranged radially and two to three cells thick (Oura & Toshimori, 1990). Surrounding these cells is the cumulus oophorus, a sticky gelatinous matrix containing many cells (Harper, 1988). The cumulus is composed of both cellular and acellular components. Carbohydrates and proteins are major constituents of the acellular matrix, with hyaluronic acid being the main component (Yanagimachi, 1988). During the process of transport through the oviduct, the oocytes lose the cumulus. This can occur mechanically within the oviduct of unmated animals or by the action of hyaluronidase released from acrosome-reacting sperm heads in mated animals (Harper, 1988). Hyaluronidase digests the cumulus matrix, while acrosin, carried on the surface of acrosome-reacted sperm, aids in the passage of sperm through the zona pellucida (Yanagimachi, 1988).

The association between the oocyte and cumulus cells is believed to be crucial for maintaining oocyte meiotic arrest (Buccione *et al.*, 1990; Kotsuji *et al.*, 1994; Oliver *et al.*, 1999), with follicle stimulating hormone (FSH) augmenting this meiosis-arresting activity (Kotsuji *et al.*, 1994). In mammals, cells of the cumulus oophorus communicate by means of intercellular gap junctions and interact with the oocyte by secretion of paracrine growth factors (Anderson, 1974; Oliver *et al.*, 1999).

Growth of the mammalian oocyte involves not just tremendous enlargement of the cell, but extensive alterations in its overall metabolism as reflected in the ultrastructure of the oocyte at various stages of growth (Wassarman & Josefowicz, 1978). The various organelles within the oocyte undergo ultrastructural transformations that are linked with oocyte maturation and growth.

As with the sperm cell, the structure and function of oocytes have been studied and reviewed for many species (Anderson, 1974; Oura & Toshimori, 1990; Wassarman, 1988a), however there are no data for the field mouse. The oocyte undergoes several physical and chemical changes after fertilization has occurred and it was thus essential to determine both the external and internal structure of the oocyte prior to any modification, in order that comparisons might be made with oocytes that had undergone alterations before, during or after fertilization.

4.2 *OOCYTE STRUCTURE*

4.2.1 METHODS AND MATERIALS

Female field mice were injected to induce superovulation as described in detail in Section 5.3. The animals were given 10IU gonadotropin from pregnant mare's serum (PMSG; Sigma, St. Louis) at 18:00 on day 1 and then 15IU human chorionic gonadotropin (hCG; *Pregnyl*, N.V. Organon Oss, Netherlands) at 19:30 on day 5 (97.5 hours post-PMSG).

Mice were sacrificed at 05:30 on day 6 (10 hours post-hCG) by intrathoracic injection of 0.35ml Euthenaze and the abdomen sterilized using 70% alcohol. The ovaries, oviducts and upper sections of the uteri were excised and placed into equilibrated, unsupplemented medium on a sterile petri dish. The ovarian bursae were dissected away and the oviducts isolated.

4.2.1.1 Light microscopy

In some experiments oviducts were immediately placed onto a glass microscope slide with four small spots of a wax:vaseline mixture (1:10) on it. A cover slip (24mm x 26mm) was then laid over the oviduct, with the corners resting on the wax-spots. Using forceps, the cover slip was then gently pushed down onto the wax-spots, squashing the oviduct slightly. The slide was then viewed and photographed under an Olympus BX50 microscope using Nomarski optics.

In a successfully superovulated mouse, the oviduct exhibits a translucent bulge at the site where the oocytes lie as a group. There is fluid pressure within the oviduct and, thus, it is possible to simply puncture the oviduct with watchmakers forceps at the site of the bulge, in order to allow for passive extrusion of the oocytes into medium. (If the oocytes did not extrude passively, they were gently extruded out into the medium using watchmakers forceps). Individual oocytes were collected from the medium using a ‘pulled’ glass pipette. These were made by heating a sterilized glass pipette (*Volac*, John Poulten Ltd., Essex) over a bunsen burner, at one point of the glass tip. When the glass had softened sufficiently, the pipette was removed from the flame and stretched (‘pulled’) to narrow out the pipette’s tip, from a 1mm diameter to approximately a 400µm diameter. These pipettes were prepared so as to be narrow enough to be able to pick up the microscopic oocytes from medium without losing them. Recovered oocytes were then placed individually onto wax-spot microscope slides, squashed gently, without disrupting the oolemma, and viewed and photographed under an Olympus BX50 microscope using Nomarski optics.

4.2.1.2 Transmission electron microscopy

For TEM work, oocytes were extruded into medium and collected as for light microscopy. However, they were then immediately placed into 2.5% glutaraldehyde in 0.2M cacodylate buffer. In initial experiments, the oocytes were then prepared for TEM as described in Appendix 1a. However, on sectioning, the oocytes pulled out of the resin and thus no sections could be obtained. Subsequently, before treatment for TEM work, the oocytes were first set into agarose

agar to allow for easier manipulation of the oocytes during chemical changes. Also, in the procedure for TEM preparation, the oocytes were left in the three propylene oxide:epoxy resin mixtures for 12 hours each, instead of a single hour as previously described in Appendix 1a. This allowed for sufficient penetration of the resin into the oocytes in order that adequate sections might be obtained. Sections were stained (Appendix 1b) and then viewed on a JEOL JEM 1210 transmission electron microscope.

4.2.2 RESULTS

In the oviduct of the field mouse, the spherical oocyte was surrounded by two layers: the proximal zona pellucida and the distal cumulus oophorus (Fig. 4.1). Once extruded, the investing layers were much clearer (Fig. 4.2). The oocyte in Figure 4.2.1 was immature, having been displaced from an ovarian follicle using forceps. It had a narrow band of densely packed cumulus cells and lacked a polar body, which were signs of the oocyte's immaturity. The second oocyte (Fig. 4.2.2) was one that has already been ovulated and collected from the oviduct. Here, the cumulus oophorus was less densely packed around the oocyte and would have continued to disperse as the oocyte was transported down the oviduct (Harper, 1988). The presence of the polar body in the perivitelline space was an indication of the oocyte's maturity.

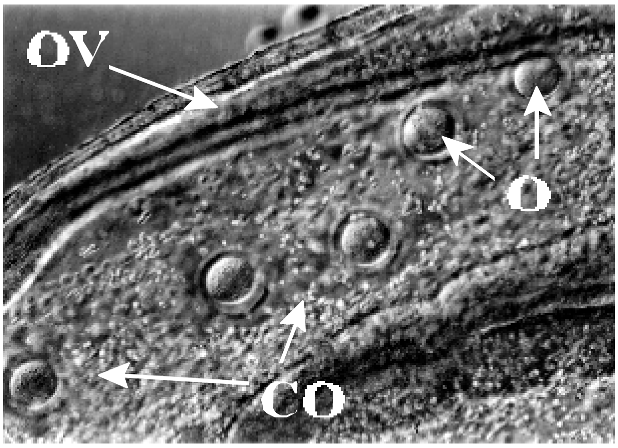


Figure 4.1. Oocytes (O) surrounded by cumulus oophorus (CO) lying in the oviduct (OV). 132 x magnification. Nomarski optics.

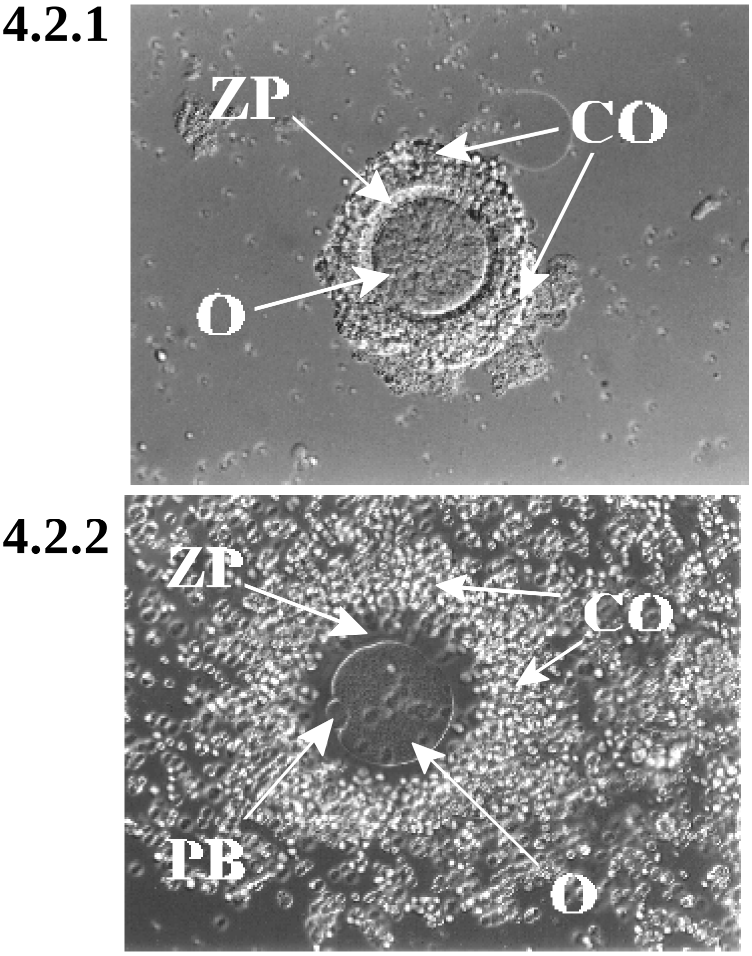


Figure 4.2 Oocytes of the field mouse. Figure 4.2.1 shows an oocyte (O) taken from the ovary. Note the dense cumulus oophorus (CO) and the lack of a polar body. Figure 4.2.2 is an ovulated oocyte with a polar body (PB). The cumulus oophorus is more dispersed than that from around the ovarian oocyte.

Labels: ZP, zona pellucida.

264 x magnification. Nomarski optics.

Ultrastructure of the cumulus oophorus

The cells of the cumulus oophorus (Fig. 4.3) were characterised by a great abundance of granular material evident in the cytoplasm (Fig. 4.3.5), as well as the presence of at least one active organelle of the endomembrane system, probably a Golgi complex in this case (Fig. 4.3.1). Invaginating vesicles were also observed on the plasma membrane (Fig. 4.3.4). The cells of the cumulus oophorus were in direct contact and communicate with one another, with gap junctions between adjoining cells (Fig. 4.3.2).

Ultrastructure of the zona pellucida

Surrounding the oocyte was a thick band of gelatinous material called the zona pellucida. Figure 4.4.1 clearly shows this layer lying between the cumulus oophorus and the oolemma. It had a relatively amorphous ultra-structure as is shown by the TEM section through it (Fig. 4.5).

Perivitelline space

There was a space between the zona pellucida and the oolemma of the oocyte, called the perivitelline space (Figs 4.4 & 4.5). Observations indicated that the width of the space varied depending on where measurements were taken in relation to the situation of the polar body. Measurements of the space taken from TEM micrographs, indicated that the width ranged from 0.5µm to 10.4µm (mean 5.8µm). There were also instances where the oolemma and zona pellucida were in direct contact and thus no perivitelline space was evident. Microvilli protruded from the oolemma into the perivitelline space (Figs 4.6 & 4.8.1), which was not empty but was filled by material of low electron density. When present, the polar body was located in the perivitelline space (Fig. 4.4).

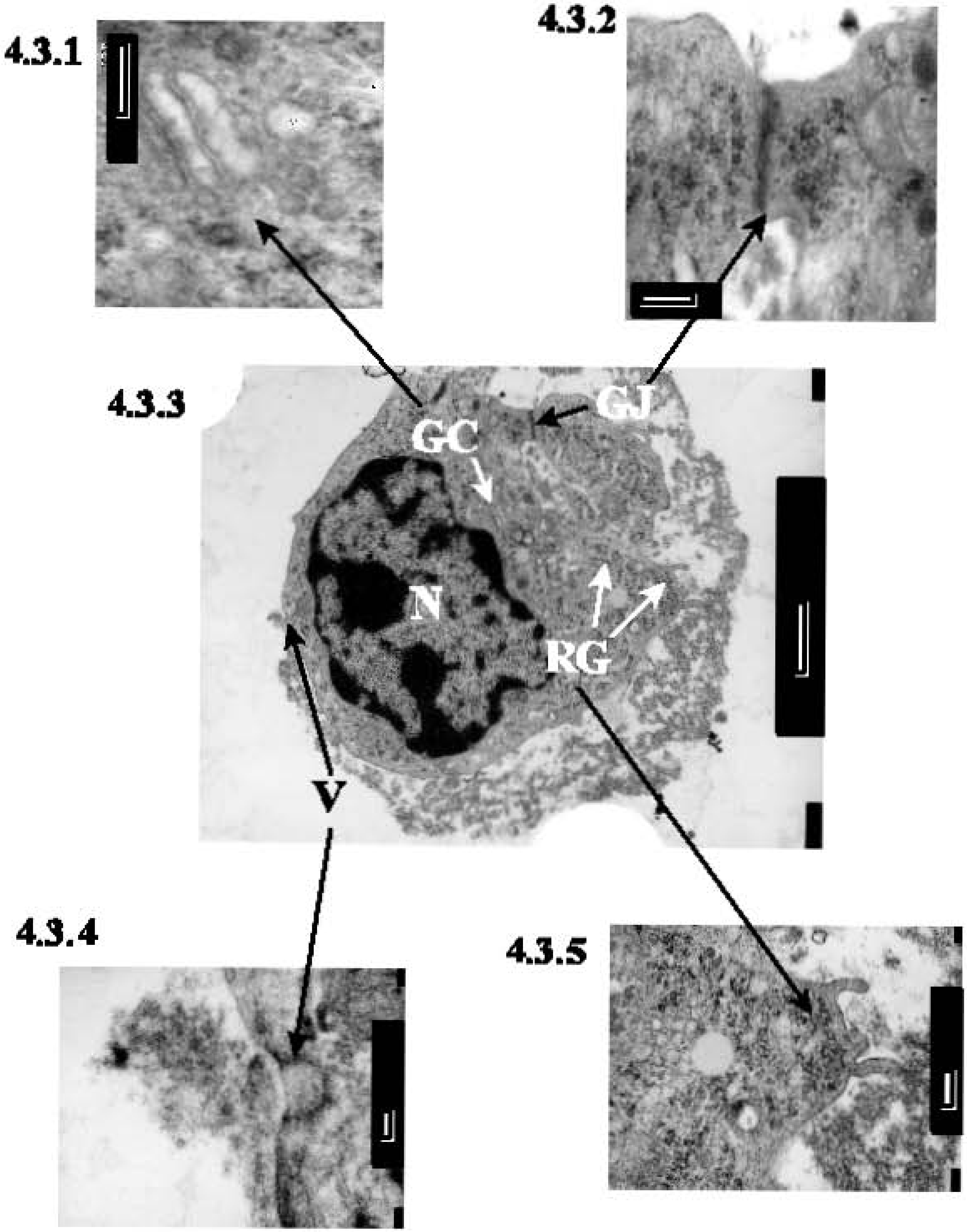
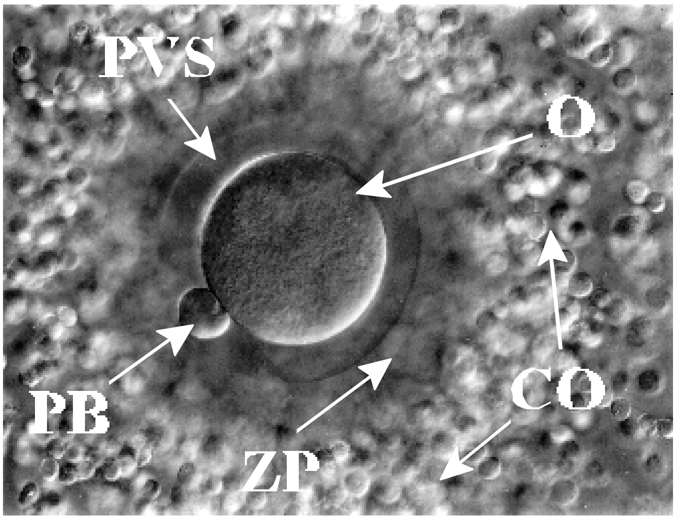


Figure 4.3 A section through a cell from the cumulus oophorus showing the characteristic endomembrane system (Fig. 4.3.1), intercellular junction (Fig. 4.3.2), endocytotic activity (Fig. 4.3.4) and ribosomal granules (RG) (Fig. 4.3.5).
Labels: GC, Golgi complex; GJ, gap junction; N, nucleus; V, vesicle.
White scale bars represent: Fig. 4.3.1= 300nm; Figs 4.3.2 & 4.3.5=200nm; Fig. 4.3.3=1µm;
Fig. 4.3.4=100nm.

4.4.1.



4.4.2.

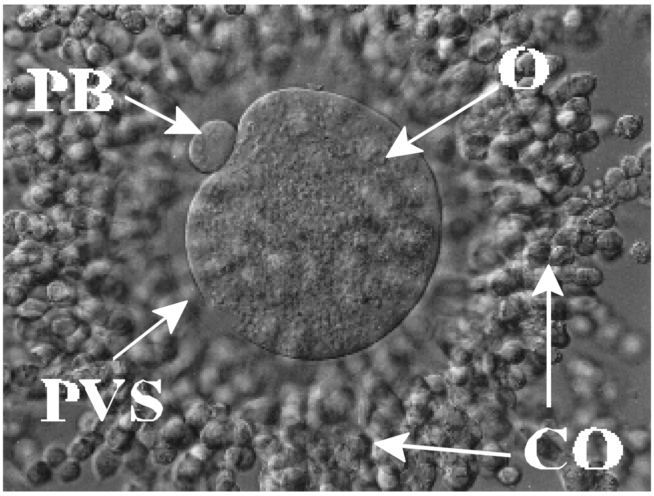


Figure 4.4. Oocytes of the field mouse. Figure 4.4.1 and Figure 4.4.2 both show the polar body (PB) lying in the perivitelline space (PVS). Figure 4.4.1 clearly shows the zona pellucida (ZP) around the oocyte (O).
Labels: *CO*, cumulus oophorus.
Fig. 4.4.1=176x magnification; Fig. 4.4.2= 600x magnification. Nomarski optics.

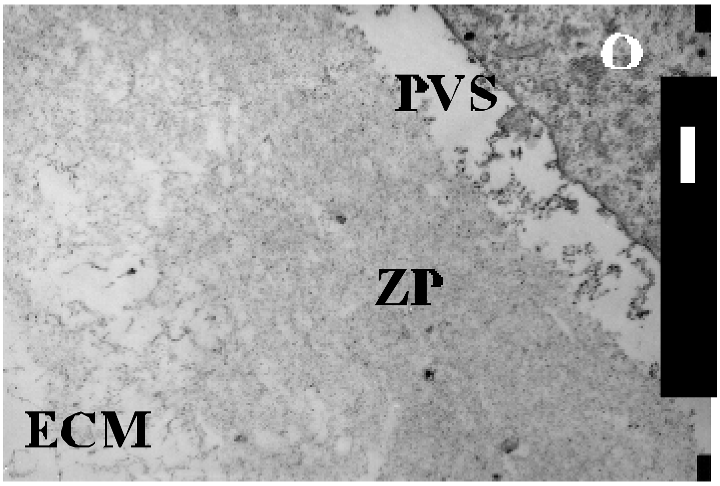


Figure 4.5 Section through an oocyte (O) of the field mouse showing the amorphous zona pellucida (ZP) and the perivitelline space (PVS).
Labels: ECM, extra-cellular material.
White scale bar represents 500nm.

Ultrastructure of the oocyte

The oocyte contained various organelles within the ooplasm including the nucleus, mitochondria, Golgi complexes, cytoplasmic lattices, ribosomes, cortical granules and vesicles (Fig. 4.6).

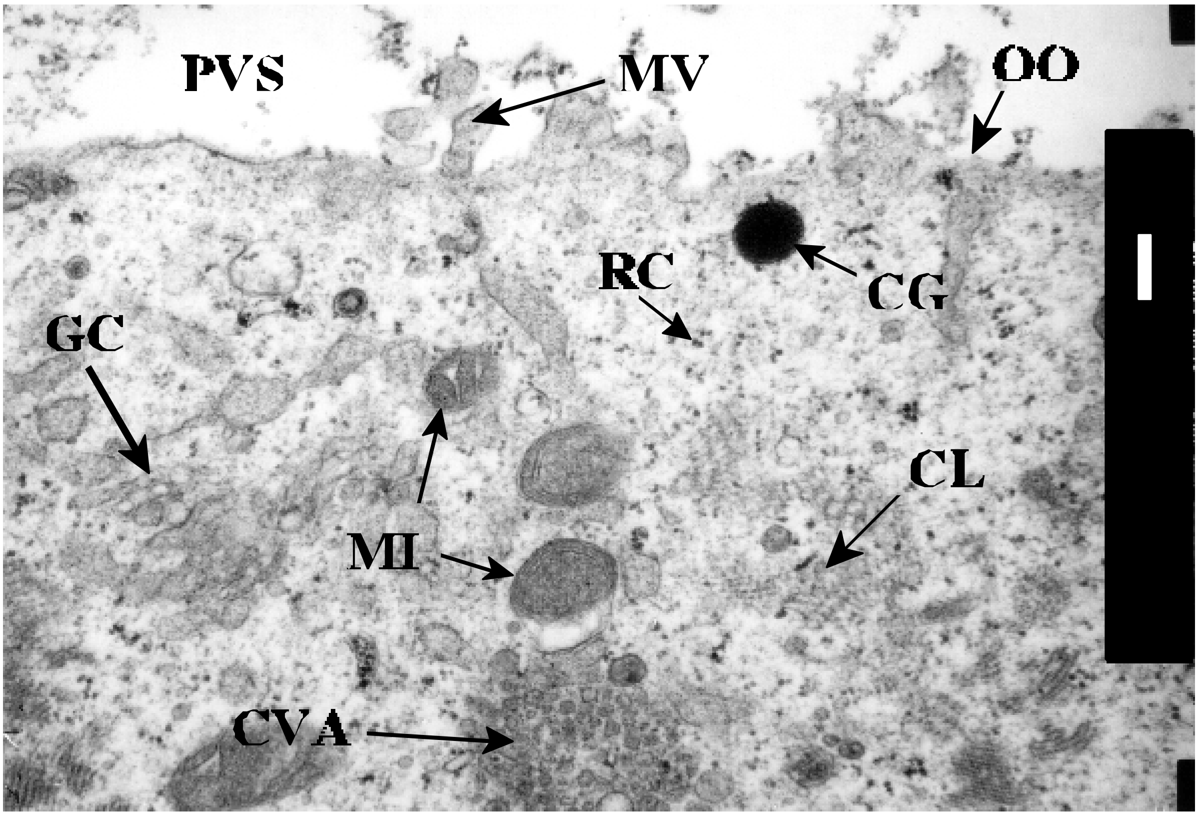


Figure 4.6 Overview of the organelles of an ovulated field mouse oocyte, showing a cortical granule (CG), cytoplasmic lattices (CL), mitochondria (MI), a Golgi complex (GC), a cytoplasmic vesicular aggregate (CVA) and ribosomal clusters (RC). The section also shows some of the perivitelline space (PVS).
Labels: MV, microvillus; OO, oolemma.
White scale bar represents 200nm.

One of the most abundant features distributed through the cytoplasm of the oocyte were lattice-like structures, referred to as crystalloids (Calarco & Brown, 1969), or cytoplasmic lattices (Hadek, 1969; Wassarman, 1988a) (Fig. 4.7). These lattices appeared as highly ordered aggregates of individual chains. The chains were composed of particles that were connected by bridges, giving rise to structural periodicity.

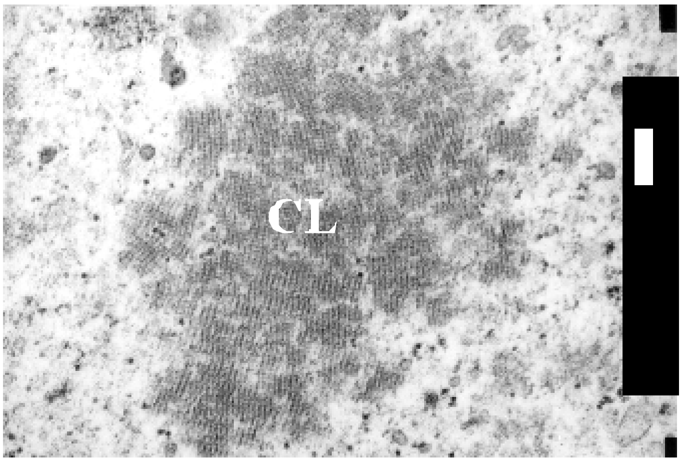


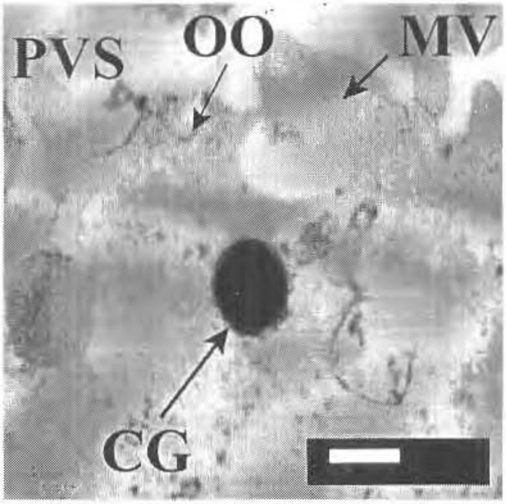
Figure 4.7 Section through the cytoplasmic lattices (CL) found in an ovulated oocyte of the field mouse.
White scale bar represents 200nm.

Other relatively common organelles found at the periphery of the oocyte, in close proximity to the oolemma, were small, spherical, membrane-bound cortical granules (Fig. 4.8).

Numerous mitochondria were also located throughout the cytoplasm of the oocyte (Fig. 4.9). Oocyte growth is accompanied not only by a substantial increase in the number of mitochondria present, but also by marked changes in mitochondrial ultrastructure (Wassarman, 1988a). Small oocytes contained elongated mitochondria with numerous transversely oriented cristae, in the so-called ‘orthodox’ configuration (Fig. 4.9.1). Oocyte growth was accompanied by accumulation of highly vacuolated, oval mitochondria that had concentrically arranged cristae (Fig. 4.9.2).

Few Golgi complexes were present within the ooplasm (Fig. 4.10). In small, immature oocytes the Golgi apparatus appeared as flattened stacks of lamellae. During oocyte growth the Golgi membranes became more active, as indicated by the appearance of numerous vacuoles and associated vesicles and the lamellae became very swollen and vacuolated (Fig. 4.10).

4.8.1



4.8.2

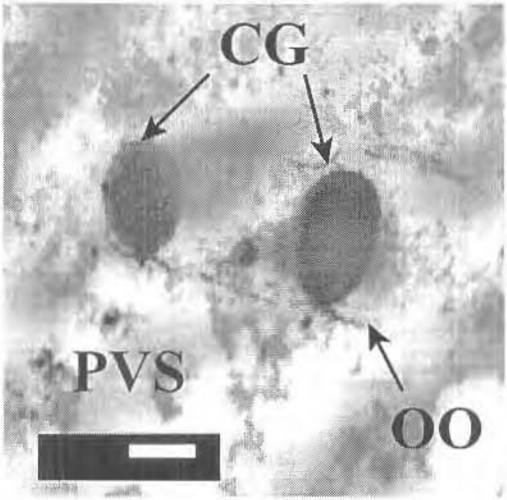
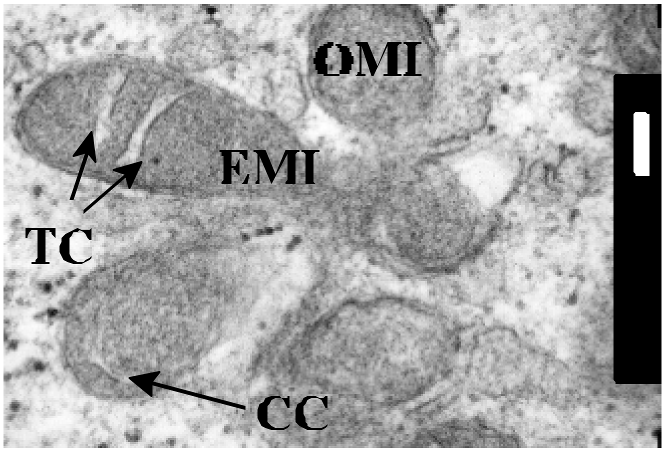


Figure 4.8 Cortical granules (CG) in the field mouse oocyte are shown in both Figures 4.8.1 and 4.8.2. The membrane around the cortical granule is visible in Figure 4.8.2.
Labels: MV, microvillus; OO, oolema; PVS, perivitelline space.
White scale bars represent 200nm.

4.9.1



4.9.2

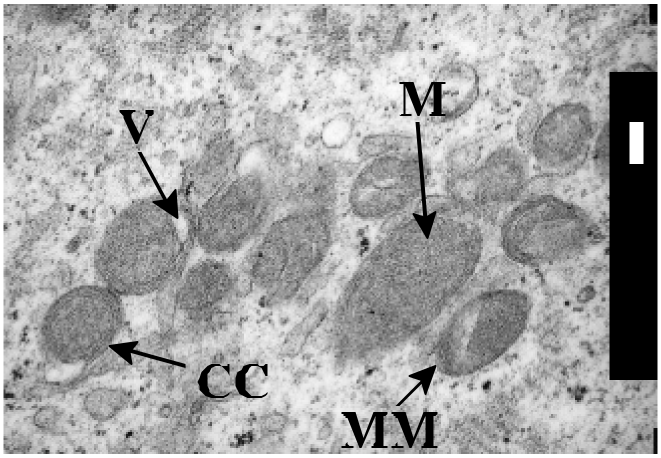
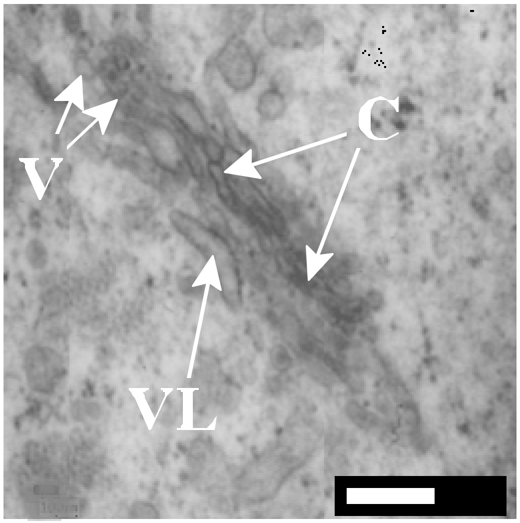


Figure 4.9 Mitochondria within the field mouse oocyte. Figure 4.9.1. shows both elongated (EMI) and oval mitochondria (OMI), while Figure 4.9.2 shows oval mitochondria with concentric cristae (CC).
Labels: M, matrix; MM, mitochondrial membrane; TC, transverse cristae.
White scale bars represent 100nm and 200nm in Figures 4.9.1 & 4.9.2 respectively.

4.10.1



4.10.2

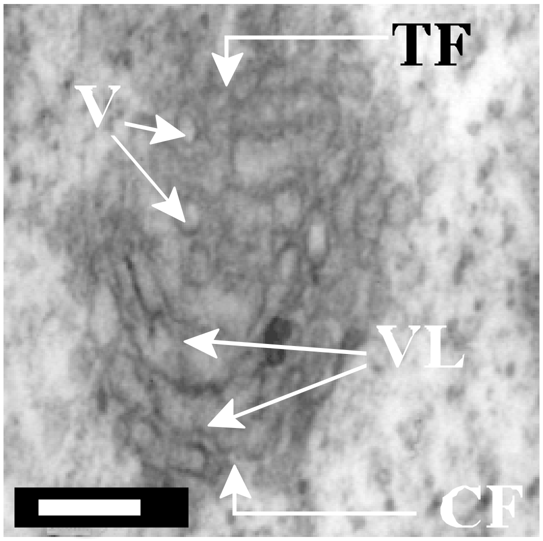


Figure 4.10 Golgi complexes of an ovulated oocyte of the field mouse. Figure 4.10.1 and 4.10.2 indicate the vacuolated lamellae (VL) and associated vesicles (V).
Labels: C, cristae; CF, cis face; TF, trans face.
White scale bars represent 300nm and 100nm in Figures 4.10.1 and 4.10.2 respectively.

Shortly after ovulation, accumulations of smooth-walled, cytoplasmic vesicles appeared in the periphery of the oocyte (Fig. 4.11). Vesicular aggregates occurred in different sizes and complexity, which may be related to the length of the postovulatory life of the oocyte (Hadek, 1969).

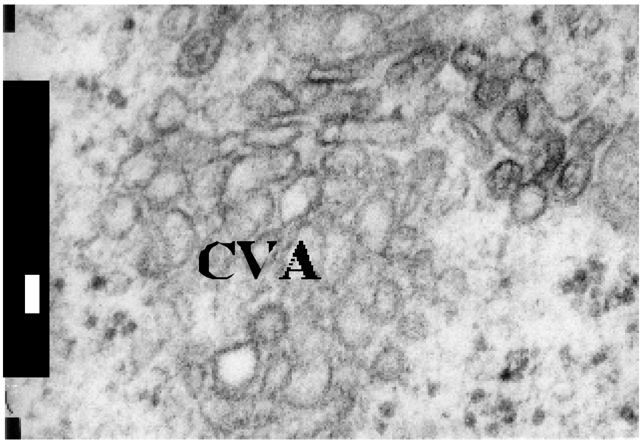


Figure 4.11 A cytoplasmic vesicular aggregate (CVA) in the field mouse oocyte.
White scale bar represents 50nm.

Finally, in two oocytes there were accumulations of material that looked like chromatin material (Fig. 4.12). However, the lack of a nuclear membrane surrounding these masses leads me to postulate that they may have been yolk bodies.

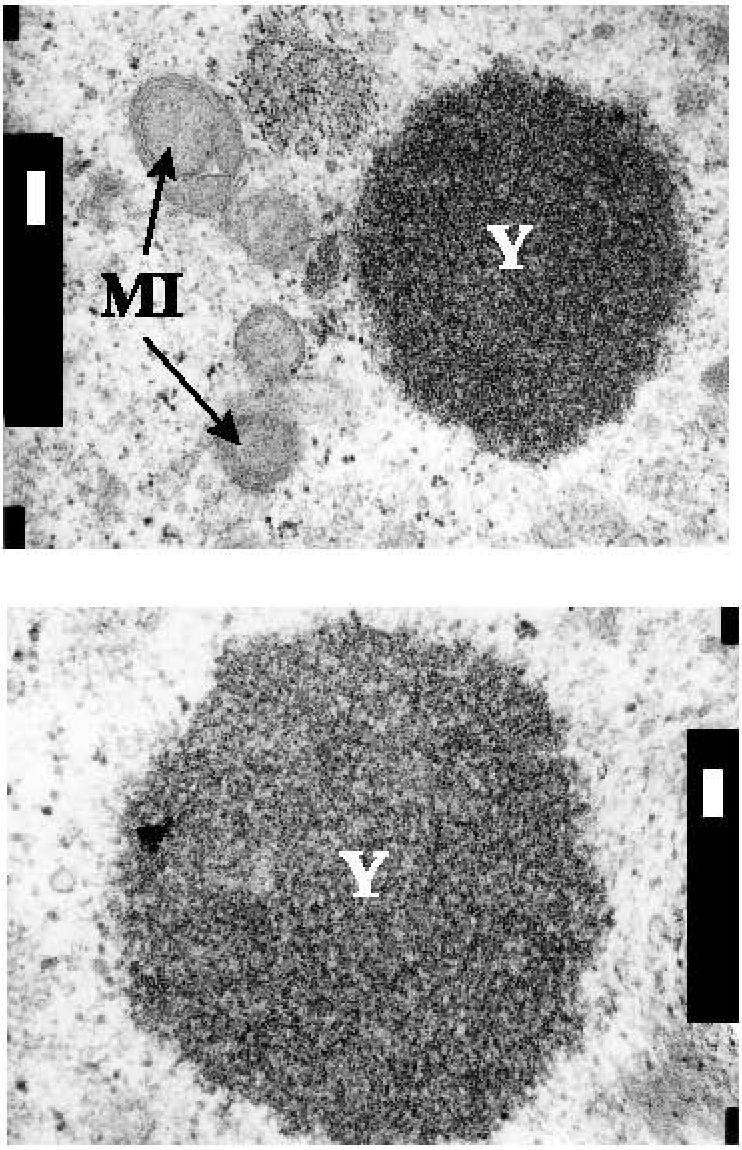


Figure 4.12 Yolk (Y) within the oocyte of the field mouse.
Labels: MI, mitochondria.
White scale bars represent 200nm and 100nm in Figures 4.12.1 and 4.12.2 respectively.

4.3. DISCUSSION

The results from the present studies on the field mouse oocyte show a typical mammalian female gamete. The field mouse oocyte is surrounded by a normal mammalian zona pellucida and cumulus oophorus and contains the same organelles as described for most mammalian oocytes (Anderson; 1974; Wassarman, 1988a).

The zona pellucida is responsible for species specificity of fertilization and is the site of the secondary block to polyspermy (Oura & Toshimori, 1990; Robl & Fissore, 1999; Wassarman & Mortillo, 1991). In many cases, removal of the zona pellucida from ovulated eggs (ie., removal of species-specific sperm receptors) eliminates the barrier to fertilization by sperm from heterologous species (Wassarman & Mortillo, 1991). Evidence suggests that specific sugar residues in the zona of mammalian oocytes are probably the key for this species specificity (Skutelsky *et al.*, 1994). The zona also contains sperm receptors that mediate sperm-egg interaction as a prelude to fertilization (Wassarman, 1988a) and in at least some mammals, the zona induces bound sperm to undergo the acrosome reaction (Wassarman & Mortillo, 1991).

In addition, the zona defines a micro-environment within which cleavage and blastocyst formation take place, as well as providing protection for the oocyte during transportation (Wassarman & Mortillo, 1991). It keeps the cells of the embryo from coming apart until they have developed sufficient junctional attachments to stay adhered together on their own (Robl & Fissore, 1999). There is also evidence that the zona presents the only physical barrier to the movement of molecules between embryonic and maternal tissue and acts as a protective barrier by excluding harmful compounds (Turner & Horobin, 1997).

Although the zona pellucida may seem totally lacking in structural complexity, it has been shown that it has a fibrous or filamentous nature (Wassarman & Josefowicz, 1978; Wassarman & Mortillo, 1991). It is a three-dimensional network of intricate, interconnecting filaments, with each filament consisting of 'beads' spaced regularly along the axis of the filament (Oura & Toshimori, 1990). The mouse zona pellucida is composed of three acid glycoproteins (ZP1, ZP2 and ZP3) unique to the zona (Stromstedt & Byskov, 1999; Wassarman & Mortillo, 1991). The ZP3 component appears to be the zona sperm receptor, and is responsible for inducing the acrosome reaction (Oura & Toshimori, 1990; Stromstedt & Byskov, 1999; Wassarman, 1999) and is required for assembly of the zona pellucida during oogenesis (Wassarman, 1999). About 80% of the zona mass has been shown to be protein and the remaining 20% almost all carbohydrate (Wassarman, 1988b).

In many species the properties of the zona pellucida change drastically upon fertilization such that excess sperm are unable to penetrate through it (Yanagimachi, 1988). This is the so-called 'zona reaction' and it is a cortical granule-mediated modification of zona material. In the mouse, partial hydrolysis of ZP2 and ZP3 glycoproteins by cortical granule proteinase is believed to be responsible for this reaction (Wassarman & Mortillo, 1991; Yanagimachi, 1988).

The protein components of the zona pellucida are synthesised and secreted by growing oocytes, representing a major metabolic activity in the oocyte during this period. As oocytes initiate growth, they secrete nascent zona pellucida nonuniformally into localized pockets between the oocyte surface and surrounding follicle cells (Wassarman & Mortillo, 1991). As the growing oocytes continue to increase in diameter, the pockets of zona pellucida filaments coalesce and form a uniform coat around the cell (Wassarman & Mortillo, 1991). The layer becomes a denser and thicker meshwork of interconnected filaments completely surrounding the oocyte and largely separating it from follicle cells. However, contact between the oocyte and the innermost layer of follicle cells continues via junctional complexes formed between oocyte microvilli and follicle cell extensions that penetrate the zona pellucida (Wassarman, 1988a). Besides forming a communication link with the surrounding cumulus, the microvilli apparently play the role of trapping and fusing with the sperm plasma membrane at the initial site of sperm-oocyte fusion (Oura & Toshimori, 1990). It is reported that microvilli are found in unfertilized oocytes, but become more abundant prior to the first cleavage (Calarco & Brown, 1969).

The second, distally situated layer surrounding the ovulated oocyte is the cumulus oophorus. This is composed of both cellular and acellular components, with carbohydrates and proteins being the major constituents of the acellular matrix. It has been indicated that the larger, more mature cumulus of naturally ovulated oocytes is successfully penetrated at lower sperm collision frequencies than the denser, less mature cumulus of precociously ovulated oocytes (Legendre & Stewart-Savage, 1993). It has also been shown that the presence of a normally developed cumulus oophorus is important for normal ovum pick-up at the infundibulum of the oviduct and may be important for the early stages of ovum transport (Harper, 1988).

An abundance of cortical granules was observed in the cortex of the field mouse oocyte. These are small, spherical, membrane-bound organelles found mostly beneath the oolemma of the unfertilized oocyte (Gulyas, 1980; Lessman, 1999; Perreault & Kirby, 1999). Mouse eggs contain approximately 4 500 cortical granules within about 2µm of the oolemma (Wassarman, 1988a). They first appear during oocyte growth, associated with expanding Golgi complexes that have moved to the subcortical region of growing oocytes (Calarco & Brown, 1969; Hadek, 1969; Hunter, 1999; Lessman, 1999;

Wassarman, 1988a). Cortical granules are considered as characteristic organelles of unpenetrated mammalian eggs which disappear after fertilization (Gulyas, 1980; Hadek, 1969). Numerous small, electron-dense vesicles are apparently pinched off from the saccules and interconnected tubules of the Golgi complex and fuse with one another. These subsequently fuse with vesicles derived from the rough endoplasmic reticulum to form mature cortical granules (Guraya, 1982). As the cortical granules start their development, they are placed within the peripheral ooplasm adjacent to the plasma membrane.

Cortical granules fuse with the oolemma at fertilization and, by releasing their contents into the perivitelline space, alter functional properties of the zona pellucida inducing a secondary block to polyspermy (Gulyas, 1980; Guraya, 1982; Hunter, 1999; Kalab *et al.*, 1991; Perreault & Kirby, 1999; Wassarman, 1988a). The precise biochemical properties of the cortical granules in the mammalian oocyte still remain obscure, although they are known to contain hydrolytic enzymes, polysaccharides and glycoproteins (Anderson, 1974; Gulyas, 1980; Guraya, 1982; Lessman, 1999). The exocytosis of cortical granule contents spreads from the point of the fertilizing sperm in a wave on the surface of the oocyte (Schatten, 1999; Shapiro & Eddy, 1980) and may possibly be carried out by actin-like microfilaments (Oura & Toshimori, 1990). The enzymes diffuse into the zona pellucida and modify ZP2 and ZP3 (Wassarman & Mortillo, 1991). As a result the zona becomes a more insoluble structure and the primary and secondary sperm receptor activities of the zona are destroyed (Oura & Toshimori, 1990; Shapiro & Eddy, 1980; Yanagimachi, 1988). The heads of supplementary sperm may continue to enter the outermost portion of the zona, but the inner portion is rendered impenetrable (Hunter, 1999). Structural changes in the zona have not been distinguished in the light or transmission electron microscope, so molecular modifications are inferred (Hunter, 1999). The large number of cortical granules seen in the oocytes of the field mouse are typical of unfertilized mammalian oocytes.

Much ribosomal material was noted in the oocytes of the field mouse and ribosomal RNA accumulates through much of mouse oocyte growth. It is estimated that, in the mouse, the number of ribosomes present in the cytoplasm increases three- to fourfold as the oocyte diameter increases from 20 to 65µm (Wassarman, 1988a). These changes in the ribosome population during oocyte growth are consistent with changes in overall rates of protein synthesis during this period.

Cytoplasmic lattices were observed in abundance within the oocyte of the field mouse. The function of these structures is not known, although the possibility that they serve either as yolk storage or as a storage form of ribosomes has been considered (Anderson, 1974; Wassarman, 1988a). These supramolecular complexes accumulate during oocyte growth and have been shown to contain ribosomal RNA, tRNA and poly(A)+ RNA (Brower & Schultz, 1982; Wassarman & Josefowicz, 1978).

The description of the oocyte of the field mouse is based on ovulated oocytes and thus the regular absence of a polar body is peculiar. The absence of the polar body is indicative of an immature oocyte that has remained at the stage of prophase I and has not undergone the meiotic division to metaphase II which normally occurs before ovulation and is an essential step in the process of fertilization and consequent development (Masui & Clarke, 1979). This will be discussed further in Chapter Five.

Observations suggest that growth of the oocyte is accompanied by significant changes in its metabolic activity. For example, at least four membranous components of the mouse oocyte undergo major ultrastructural changes during oocyte growth (Wassarman & Josefowicz, 1978). These are the smooth endoplasmic reticulum, the Golgi complex, the multivesicular bodies and the mitochondria. In the developing mammalian oocyte, there is a change in the ultrastructure of the mitochondria. These organelles have been observed to change their location, assumedly accommodating the areas in greater need of energy (Anderson, 1974; Hadek, 1969). Small oocytes contain elongated mitochondria with numerous transversely oriented cristae and, in most cases, contain a single vacuole (Hadek, 1969; Wassarman, 1988a). Continued oocyte growth is accompanied by accumulation of oval-shaped mitochondria, which are vacuolated display concentrically arranged, arched cristae. Throughout this growth period, mitochondria are closely associated with smooth endoplasmic reticulum (Wassarman, 1988a).

The Golgi complex also undergoes dramatic ultrastructural changes during oocyte growth. In small oocytes, Golgi membranes appear as flattened stacks of arched lamellae and are associated with few, if any, vacuoles or granules (Anderson, 1974; Wassarman, 1988a). During oocyte growth Golgi membranes become more active, as evidenced by lamellae that are spaced further apart, by the appearance of vacuoles and by the proximity of numerous lipid vesicles. These changes are consistent with increased participation of the Golgi in processing and concentration of secretory products (eg. zona pellucida glycoproteins), as well as cortical granule and yolk formation during oocyte growth (Anderson, 1974; Wassarman, 1988a). At the late stages of growth the Golgi consists of a large conglomerate of vacuoles, granules and lamellae (Wassarman & Josefowicz, 1978) and the typical aggregations of stacked cisternae and vesicles that normally characterise Golgi bodies are rarely encountered (Calarco & Brown, 1969). Shortly after ovulation the Golgi complex disappears (Hadek, 1969).

CHAPTER FIVE

OESTROUS CYCLE AND OVULATION

5.1 INTRODUCTION

The oestrous cycle is the sequence of reproductive events in mammalian species that are characterised by a cascade of hormonal and behavioural processes which are progressive, highly synchronized and repetitive (Kilen & Schwartz, 1999). The cycle itself is divided into four stages, centred around a period of oestrus, when ovulation occurs (Kilen & Schwartz, 1999; Vom Saal & Finch, 1988). The period prior to oestrus is called proestrus, which is the time of follicular growth and maturation in the ovary. The periods succeeding oestrus are metoestrus and dioestrus, where metoestrus is the period of recovery following oestrus and dioestrus is the period when the ovarian secretions from the corpus luteum prepare the uterus for implantation. Variation in gonadotropin levels occur in relation to the oestrous cycle (Tougard & Tixier-Videl, 1988).

Only oocytes that have successfully completed meiosis II are capable of being fertilized and developing normally. Female germ cells enter meiosis during foetal development and before the germ cells begin to grow, meiosis proceeds to the terminal stage of the first meiotic prophase. Growing primary oocytes have an enormously enlarged nucleus called the germinal vesicle. Towards the end of the growth period the chromosomes within the vesicle regress, after which the oocytes enter a quiescent state which persists until just prior to ovulation. The duration of this dormant state is consequently dependent on the age of sexual maturity of the animal and on the period of its reproductive cycle (Masui & Clarke, 1979; Wassarman, 1988a). The final maturation of oocytes occurs through conversion of fully grown oocytes present in antral follicles, into unfertilized eggs just prior to ovulation (Wassarman, 1988a). This maturation is interrupted by suspension of meiotic division in many species, and its resumption is later triggered by fertilization (Masui & Clarke, 1979).

During the follicular phase of the oestrous cycle, where several follicles in the ovary begin to grow, luteinising hormone (LH) and follicle stimulating hormone (FSH) are secreted in relatively small quantities in response to stimulation by gonadotropin releasing hormone (GnRH) (Karsch, 1984). The FSH stimulates the growth of follicles and the cells of these growing follicles secrete oestrogen. A high level of oestrogen stimulates greater secretion of LH and FSH by acting on the hypothalamus to increase GnRH output (Karsch,1984). The increase in LH induces final meiotic maturation of the follicle and ovulation occurs about a day after the LH surge (Mattheij *et al.*, 1994).

It is well known that the administration of gonadotropins, such as pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), to female mammals having fully grown oocytes in their ovaries, causes oocyte maturation with concomitant ovulation (Biggers *et al.*, 1971; Masui & Clarke, 1979). PMSG resembles LH more than FSH, but it has been reported to have dual FSH- and LH-like activities in immature rats and mice (Greenwald & Terranova, 1988; Talamantes & Ogren, 1988). The predominant function of hCG is the same as that of LH, namely the stimulation of steroidogenesis, with subsequent induction of ovulation (Talamantes & Ogren, 1988). Administration of these hormones can also induce superovulation, which is used as a routine procedure for producing greater yields of ova in developmental and genetic studies and to minimize the number of animals sacrificed (Garcia *et al.*, 1993; Lehtonen & Kankondi, 1987; Redina *et al.*, 1994). Young adult mice are superovulated by injections of PMSG and human chorionic gonadotropin (hCG) (Beaumont &

Smith, 1975; Biggers *et al.*, 1971; Hamilton & Armstrong, 1991; Nohara *et al.*, 1998).

Superovulation typically increases the number of oocytes produced by a factor of 5- to 10-fold, brought about by alteration of either exogenous or endogenous levels of gonadotropins (Greenwald & Terranova, 1988; Seidel, 1999). Superovulation is a low-cost, logical step and is almost always used in embryo transfer programmes. The protocol has been used in clinical, agricultural and experimental applications (Eppig *et al.*, 1992). In the livestock industry superovulation has become a well-established technique for increasing the supply of embryos from genetically superior animals (Miller & Armstrong, 1981; Walton & Armstrong, 1983).

The investigations described in this chapter were carried out in order to establish the character of the oestrous cycle in the field mouse, as well as the optimal regime for retrieval of the maximum number of oocytes, both through superovulation and natural ovulation methods.

5.2 *OESTROUS CYCLE*

5.2.1 METHODS AND MATERIALS

The vaginal smear technique was used to ascertain the presence or absence of an oestrous cycle in female mice (Anizoba, 1986; Hall[1], *Pers. comm.*; Redina *et al.*, 1994). Vaginal smears were taken every day for at least 7 days and up to 31 days, using a thin capillary tube. The tube was wiped with paper towelling, dipped in saline solution and inserted approximately 1.5cm into the vagina of the mouse. The tube was gently moved around within the animal to ensure that it touched the walls of the

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[1] Hall, J. Rhodes University; Grahamstown; South Africa.

vagina. Once removed, the tube was rolled over a small area on a glass microscope slide in order to wipe off the cells and mucus that were removed from the vagina.

The slide was initially placed into an ether:absolute alcohol fixative (1:1) and then stained in Harris' haematoxylin and Shorrs' stains (Appendix 2). DPX mountant (Saarchem, Krugersdorp) was then used to attach a cover slip to the slide in order to seal the smears from air, for storage and later reference.

The slides were observed under a Nikon light microscope in order to determine the various cell types present within the smear and their relative ratios. The stage of the oestrous cycle was determined by estimating the relative proportions of the leucocytes, epithelial cells and cornified cells on a scale of 0-3, with 0 being the total absence of a particular type of cell and 3 being an extreme abundance of a cell type (Anizoba, 1986).

The pouched mouse (*Saccostomus campestris*) was used as a control species for the method of detecting the stages of the oestrous cycle by vaginal smear. This species was selected as its oestrous cycle was known (Hall, *Pers. comm.; Pers. obs*).

5.2.2 RESULTS

The leucocytes, epithelial cells and cornified epithelial cells of the vaginal smears of the pouched mouse stained differently and were also recognizable by size and shape (Fig. 5.1). The leucocytes were the smallest cells within a smear. Much of a leucocyte cell was composed of nucleus, which stained a dark purple, while the minor cytoplasmic portion stained a light orange. The epithelial cells were larger than

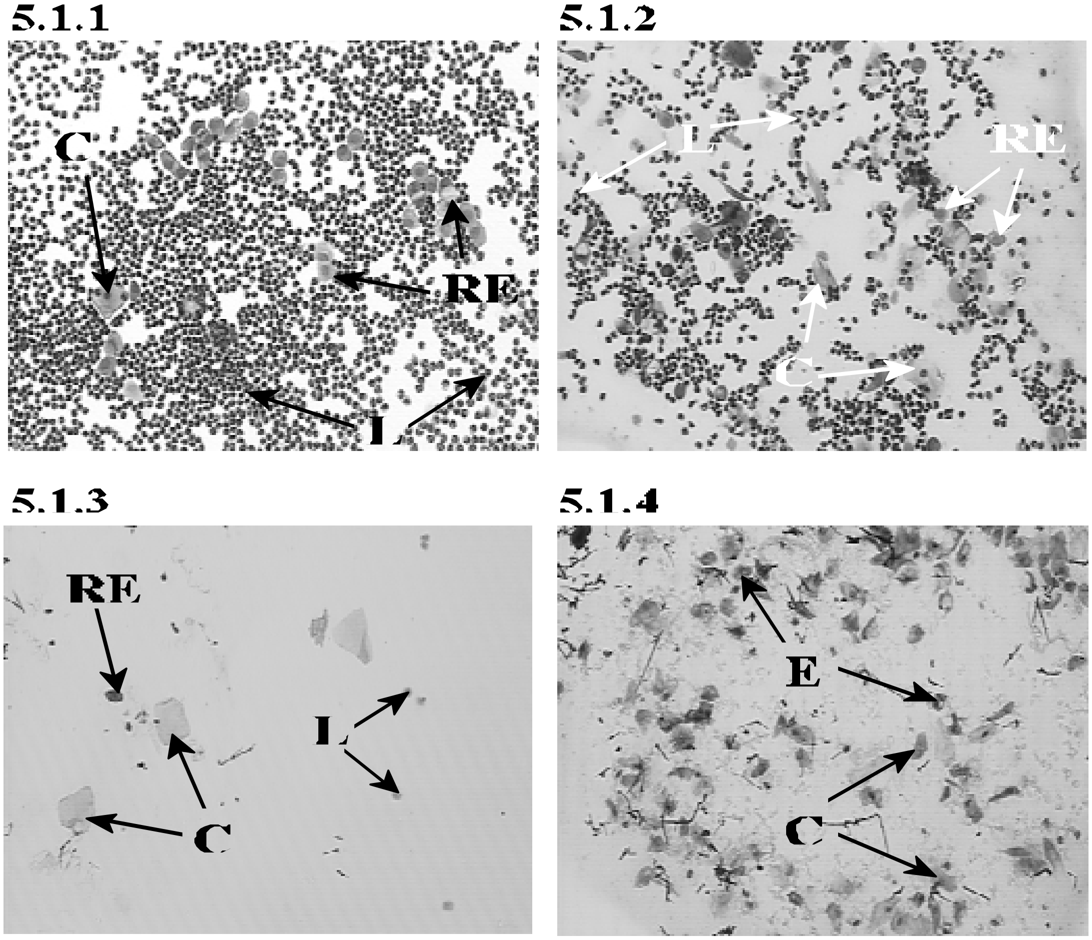

Figure 5.1 Cells constituting the vaginal smears of the pouched mouse (*Saccostomus campestris*), showing the stages of metoestrus (Fig. 5.1.1), dioestrus (Fig. 5.1.2), proestrus (Fig. 5.1.3) and oestrus (Fig. 5.1.4).

The straight dark lines in Figures 5.1.3 & 5.1.4 are hairs from the animal's fur that attached to the capillary tube during the smearing process.

Labels: C, cornified cells; E, non-round epithelial cells; L, leucocytes; RE, rounded epithelial cells. 132 x magnification.

the leucocytes and were usually easily recognizable due to their round shape. However, the epithelial cells' shape altered from being round during met-, di-, and proestrus, to being irregularly shaped with straight edges during oestrus. As with the leucocytes, the nuclei of the epithelial cells stained dark purple, but the cytoplasm stained a dark orange. The cornified cells stained dark red and were the largest cells within a smear. They were recognised by the lack of a nucleus within the cell, as well their straighter edges.

The metoestrous and dioestrous smears were difficult to differentiate from one another and were usually only categorised when the smear's position within a cycle was determined. The dioestrous smear sometimes had fewer leucocytes than the metoestrous smear (compare Figs 5.1.1 & 5.1.2). Both of these stages were characterised by an abundance of leucocytes and round epithelial cells, while the cornified cells were found in lower numbers. The proestrous smear usually exhibited fewer cells, with all three cell types typically scoring low on the rating scale (Fig. 5.1.3). An oestrous smear was characterised by a total lack of leucocytes and an abundance of epithelial and cornified cells (Fig. 5.1.4). The epithelial cells of an oestrous smear were not round however, but irregularly shaped with several straighter edges.

The results from the smears of the pouched mouse (*Saccostomus campestris*) consistently indicated a four-day oestrous cycle, with only one day for each stage. The field mouse, however, produced no evidence of such a repeating oestrous cycle (Fig. 5.2). Of the fifteen animals smeared during the experimental period, all exhibited at least two periods of oestrus. These, however, varied in length from one to seven days (mean 4 ± 4.1), with intervals between oestrous periods ranging in duration from one

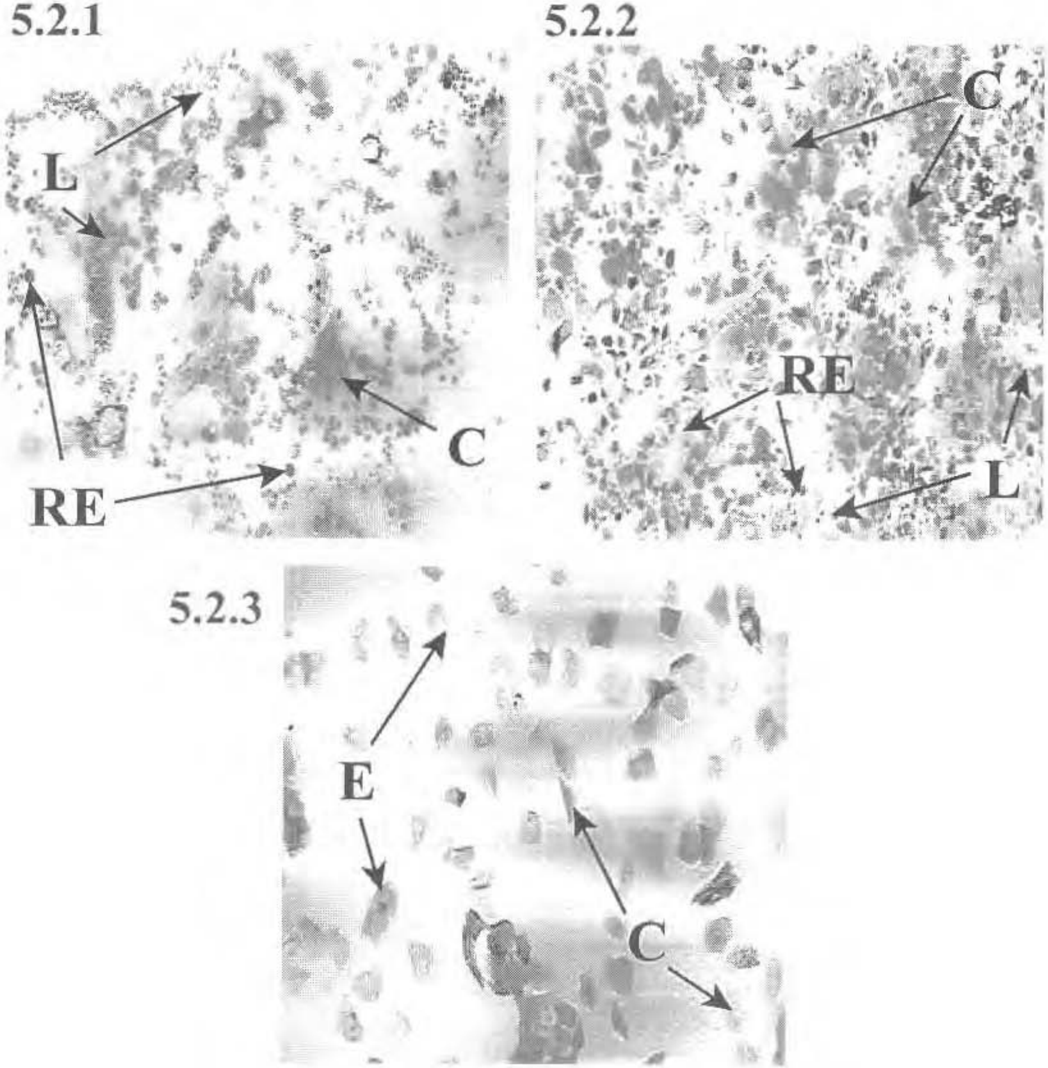

Figure 5.2 Cells constituting the vaginal smears of the field mouse, indicating the various stages observed. Figure 5.2.1 shows what could be either a metoestrous or a dioestrous smear, while Figure 5.2.2 shows a proestrous smear. The oestrous smear (Fig. 5.2.3) was the most commonly observed.
Labels: C, cornified cells; E, non-round epithelial cells; L, leucocytes; RE, rounded epithelial cells.
132 x magnification.

to twenty-two days (mean 2.4 ± 1.6). In each animal, all the

stages of a normal oestrous cycle were observed, although a day or two of a cycle were often not seen. The oestrous smear was the most commonly observed smear in the field mouse (Fig. 5.2.3).

5.3 *SUPEROVULATION*

5.3.1 METHODS AND MATERIALS

In order to determine the optimal procedure for superovulation in the field mouse, several females were subjected to different hormonal regimes, altering hormone dosage, time of sacrifice and time of hormone administration. Due to the absence of a typical oestrous cycle, hormones were not given on particular days of a cycle, but rather on random day, as is the practice of some authors (Lehtonen & Kankondi, 1987; Nohara *et al.*, 1998; Redina *et al.*, 1994).

Protocols 1 & 2

Female field mice were injected to superovulate by administering intramuscular injections at the base of the hind leg, ventrally and just anterior to the pelvic region, using 0.5ml insulin syringes. They were given 20IU PMSG at 11:00 on day 1 (lights on 06:00; lights off 20:00) and then 120IU hCG at 11:00 on day 3 (48 hours post-PMSG) (Barros *et al.*, 1984; Bavister, 1989; Bedford *et al.*, 1997; Cuasnicu & Bedford, 1991; Juetten & Bavister, 1983; Legendre & Stewart-Savage, 1993; Shalgi *et al.*, 1985; Wakayama *et al.*, 1995; Wood *et al.*, 1993). The mice were sacrificed at 14:00 on day 4 (27 hours post-hCG) with 0.35ml Euthenaze. The oviducts were removed and the contents extruded into 1ml of supplemented medium. Any oocytes were retrieved, placed onto wax-spot slides, covered with coverslips and viewed under an Olympus BX50 microscope with Nomarski optics. Based on the results obtained from this procedure, the protocol was modified and the time of sacrifice was changed to between 10:00 and 11:00 (23-24 hours post-hCG = protocol 2).

Protocols 3 & 4

Taking the results from the above experiments into consideration, as well as information from a number of papers (Cornwall *et al.*, 1991; Dumoulin *et al.*, 1992; Lee & Storey, 1986; Nasr-Esfahani *et al.*, 1992; Nohara *et al.*, 1998; Rayos *et al.*, 1994; Redina *et al.*, 1994; Sato, 1979; Schini & Bavister, 1988; Toshimori *et al.*, 1998) and personal communications (Bavister[2], *Pers. comm.*; Fraser[3], *Pers. comm.*), the hormone dosage and time of administration, as well as the time of sacrifice (Shaw[4], *Pers. comm.*) were altered again. In the revised protocol (protocol 3) the female mice received 10IU PMSG at 18:00 on day 1 and 15IU hCG at 18:00 on day 3 (48 hours post-PMSG). The mice were then sacrificed at 08:30, 14.5 hours post-hCG and approximately 10 hours earlier than in previous experiments. The animals were dissected as described before. A regime for a five-day oestrous cycle was also performed, where the same procedures as above were used, but with the hCG injection given on day 4 (72 hours post-PMSG = protocol 4)). On one occasion when no oocytes were retrieved from a treated female, the largest follicles of the ovaries were pricked with watchmakers forceps and the unovulated oocytes removed and observed.

Protocol 5

The results of the above experiments once again prompted a change in experimental practice. The

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[2] Bavister, B. University of Wisconsin; Madison; United States of America.

[3] Fraser, L. King’s College London; London; United Kingdom.

[4] Shaw, J. Monash University; Clayton; Australia.

hormones were now given according to a six-day oestrous cycle (PMSG day 1; hCG day 5) and on one occasion for a seven-day cycle (PMSG day 1; hCG day 6). The dosages of the hormones remained the same as in protocol 3 (10IU PMSG and 15IU hCG), while the times of hormone administration on each day and the time of sacrifice were altered. PMSG was now given between 18:00 and 20:00, hCG was given between 18:00 and 23:00 and sacrifice occurred between 04:15 and 09:00 .

5.3.2 RESULTS

The results obtained for any one protocol were highly variable in the number of oocytes yielded and their state of maturity.

Protocols 1 & 2

The experiments (n=10) using 20IU PMSG and 120IU hCG, with sacrifice at 14:00, yielded between 0 and 15 normally shaped oocytes (median 6.0), but none that possessed polar bodies or cumulus oophorus. When the mice were then sacrificed 3 hours earlier (n=8), between 10:00 and 11:00, 0 to 9 normally shaped oocytes were obtained (median 2.5) and at least two abnormal oocytes were also yielded per investigation. Many of these oocytes appeared to be squashed and had an uneven oval shape with irregular sides (Fig. 5.3.1). Several possessed distinct chromosomes which were clearly still in metaphase I (Figs 5.3.2 & 5.3.3), indicating that the oocytes were immature. Other oocytes obtained from the same hormonal treatment had an undamaged zona pellucida, but lacked an intact inner oocyte or cumulus oophorus (Fig. 5.4).

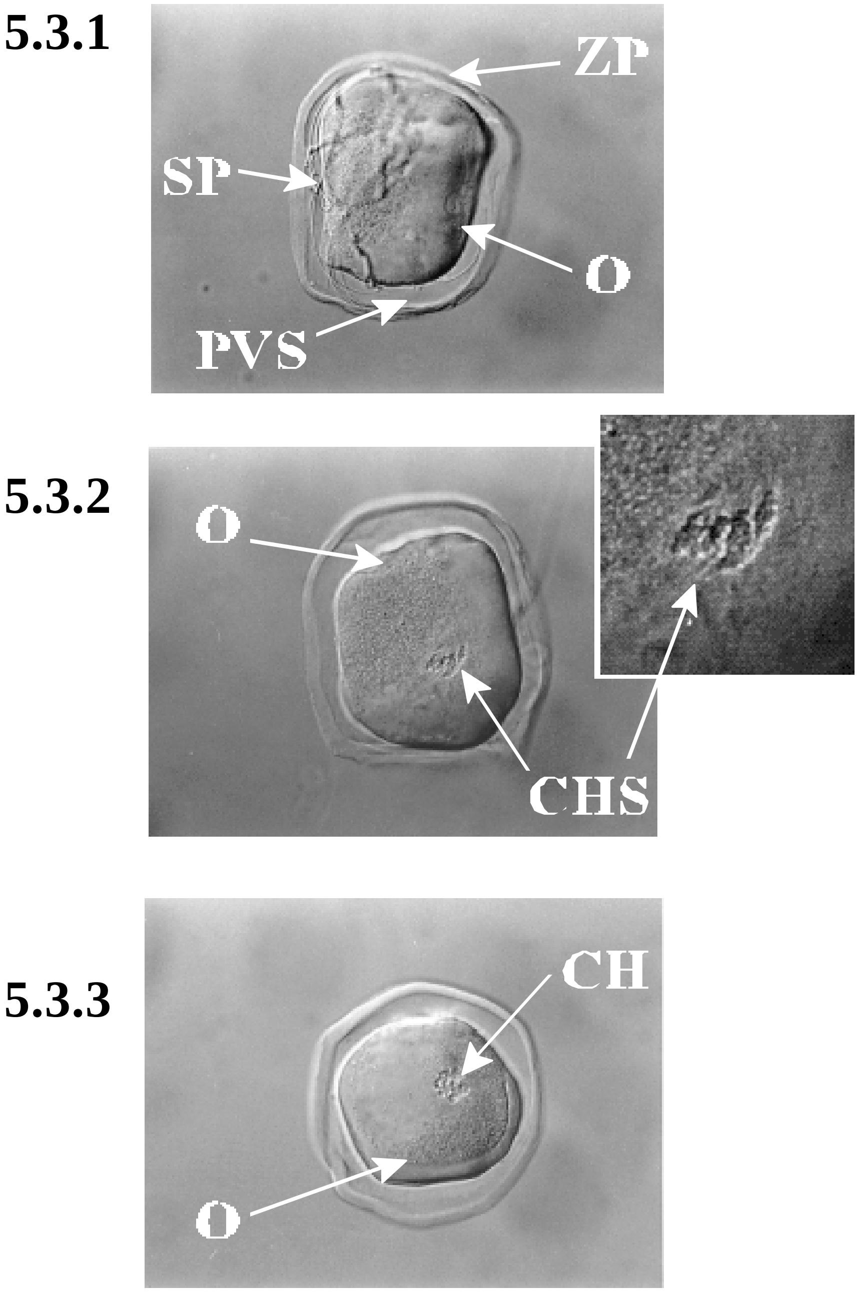


Figure 5.3 Oocytes obtained from field mice given 20IU PMSG and 120IU hCG according to a four-day oestrous cycle and sacrificed at 14:00. These oocytes were squashed. The oocyte (O) in Figure 5.3.1 has sperm (SP) lying on top of it from an attempt at *in vitro* fertilzation. Note the chromosomes (CH) lined up on the meiotic spindle in Figure 5.3.2. (including inset). Figure 5.3.3 shows similar spindle-chromosomes lieing on the metaphase plate, but from an end-on view.
Labels: CHS, chromosome spindle; PVS, perivitelline space; ZP, zona pellucida.
528 x magnification.

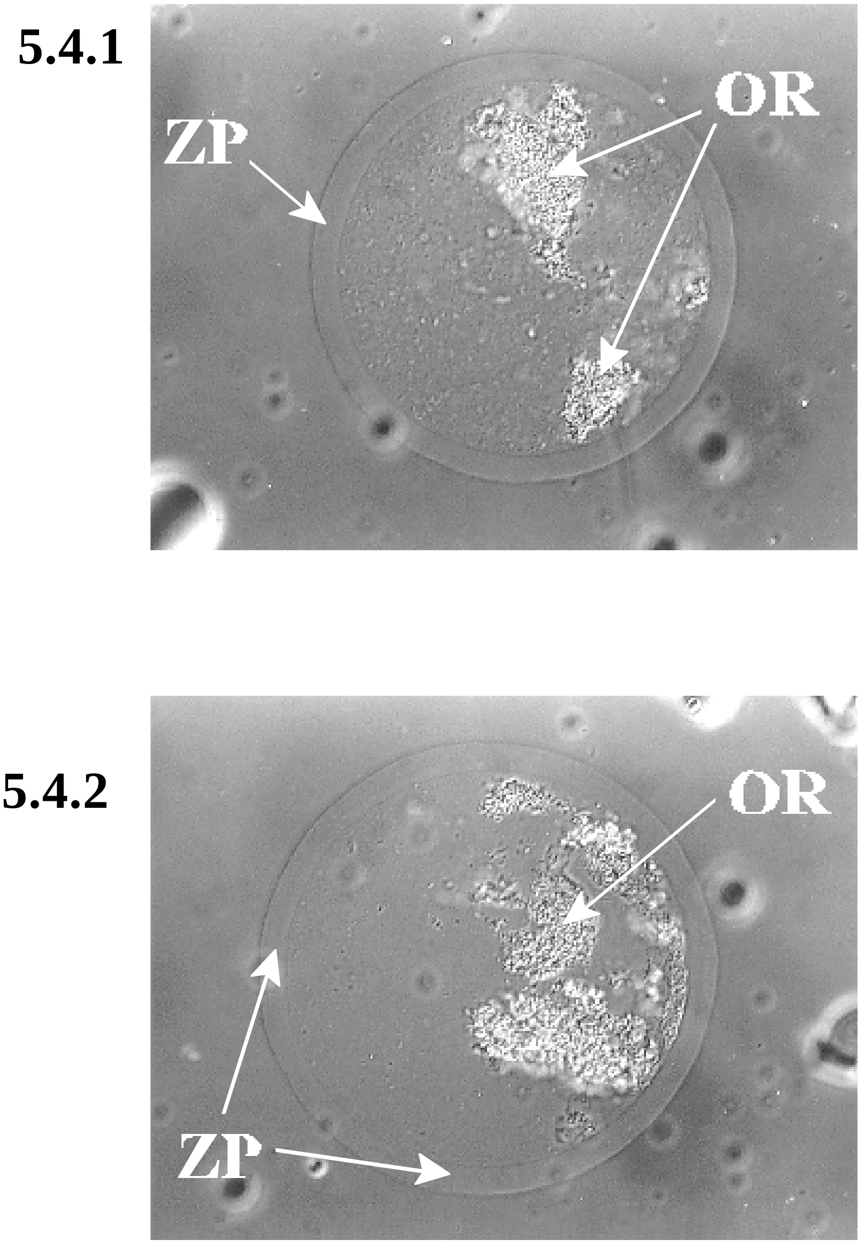
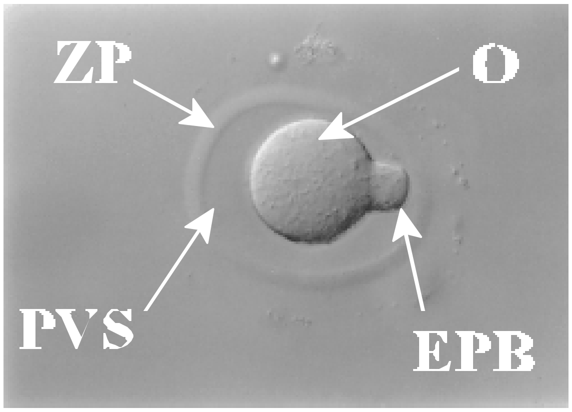
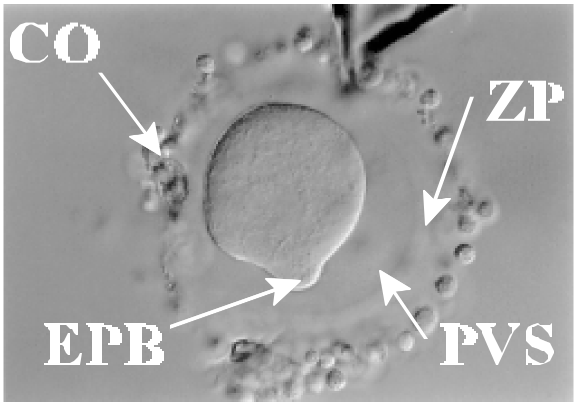


Figure 5.4 Degraded oocytes from a female treated with 20IU PMSG and 120IU hCG according to a four-day oestrous cycle and sacrificed at 14:00. The oocyte remnants (OR) are within the intact zona pellucida (ZP).
720 x magnification.

5.9.1



5.9.2



5.9.3

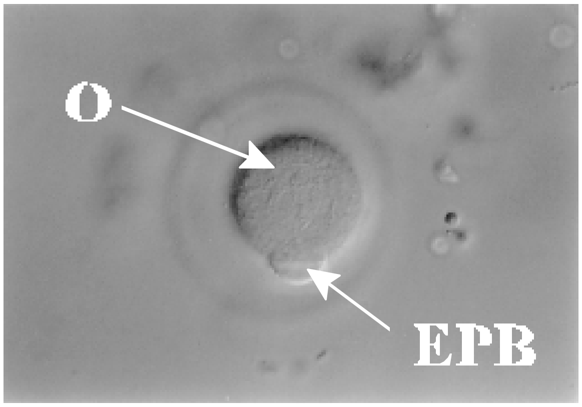


Figure 5.9 Oocytes (O) obtained from mice treated with 10IU PMSG and 15IU hCG according to a six-day oestrous cycle and sacrificed at 05:20. The extruding polar body (EPB) is visible within each oocyte.
Labels: CO, cumulus oophorus; EPB, extruding polar body; O, oocyte; PVS, perivitelline space; ZP, zona pellucida.
325 x magnification.

Protocol 3 & 4

In protocol 3, 10IU PMSG and 15IU hCG were administered according to a four-day oestrous cycle, with the hCG being injected later in the day than the first two protocols and with sacrifice at 08:30. The single experiment using a five-day oestrous cycle was also carried out using this procedure. All experiments (n=6) using these treatments resulted in normally shaped oocytes, with 86% of them surrounded by cumulus oophorus (Fig. 5.5). The yield ranged in number from 0 to 38 (median 10.0; n=6), with only a single experiment resulting in no oocytes. The occurrence of abnormally shaped, transparent oocytes was minimal, with only 2 deformed oocytes on a single occasion. However, none of the oocytes obtained from any of the experiments possessed polar bodies, indicating that they were probably immature.

The germinal vesicle was visible within the unovulated oocytes taken from the largest ovarian follicles (Fig. 5.6). In some oocytes the germinal vesicle was seen intact (Figs 5.6.1 & 5.6.2) and in others it was in the process of breakdown (Fig. 5.6.3). In the intact germinal vesicle the individual chromosomes were recognisable (Fig. 5.6.2). Ovarian oocytes would normally be immature and these observations, as well as the observation that ovulated oocytes lacked polar bodies, support the possibility that oocytes being ovulated were indeed immature.

Protocol 5

The above observations prompted the change to a regime where hormones were administered according to a six-day and once to a seven-day oestrous cycle, with sacrifice between 04:15 and 09:00. The number of oocytes yielded for the six-day cycle varied between 0 and 25 (median 3.5;

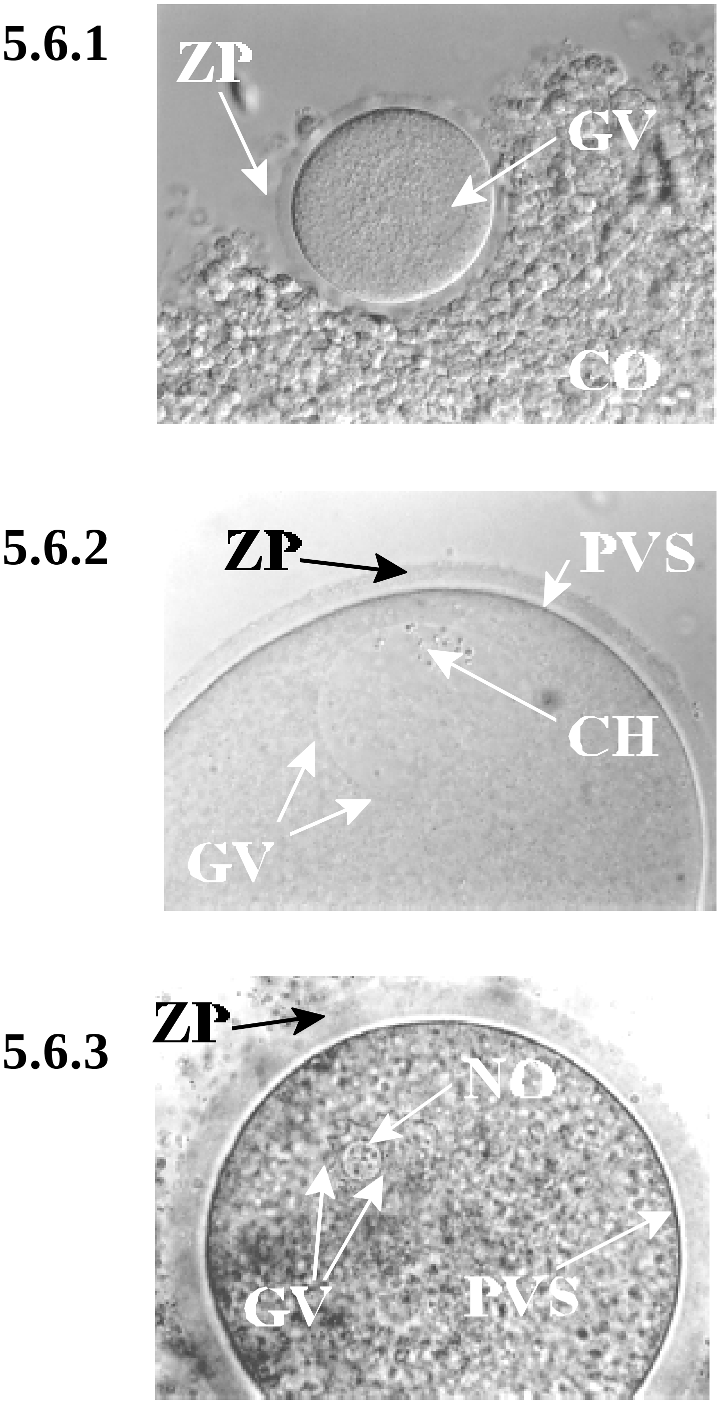


Figure 5.6 Oocytes retrieved from the largest ovarian follicles of mice treated with 10IU PMSG and 15IU hCG according to a four-day oestrous cycle and sacrificed at 08:30. The germinal vesicle (GV) is still intact in Figures 5.6.1 & 5.6.2, and is breaking down in Figure 5.6.3. (The oocyte in Figure 5.6.3 was stained with lacmoid stain).
Labels: CH, chromosomes; CO, cumulus oophorus; NO, nucleolus; PVS, perivitelline space; ZP, zona pellucida.
Fig. 5.6.1 =440 x magnification; Figs 5.6.2 & 5.6.3 =1090 x magnification.

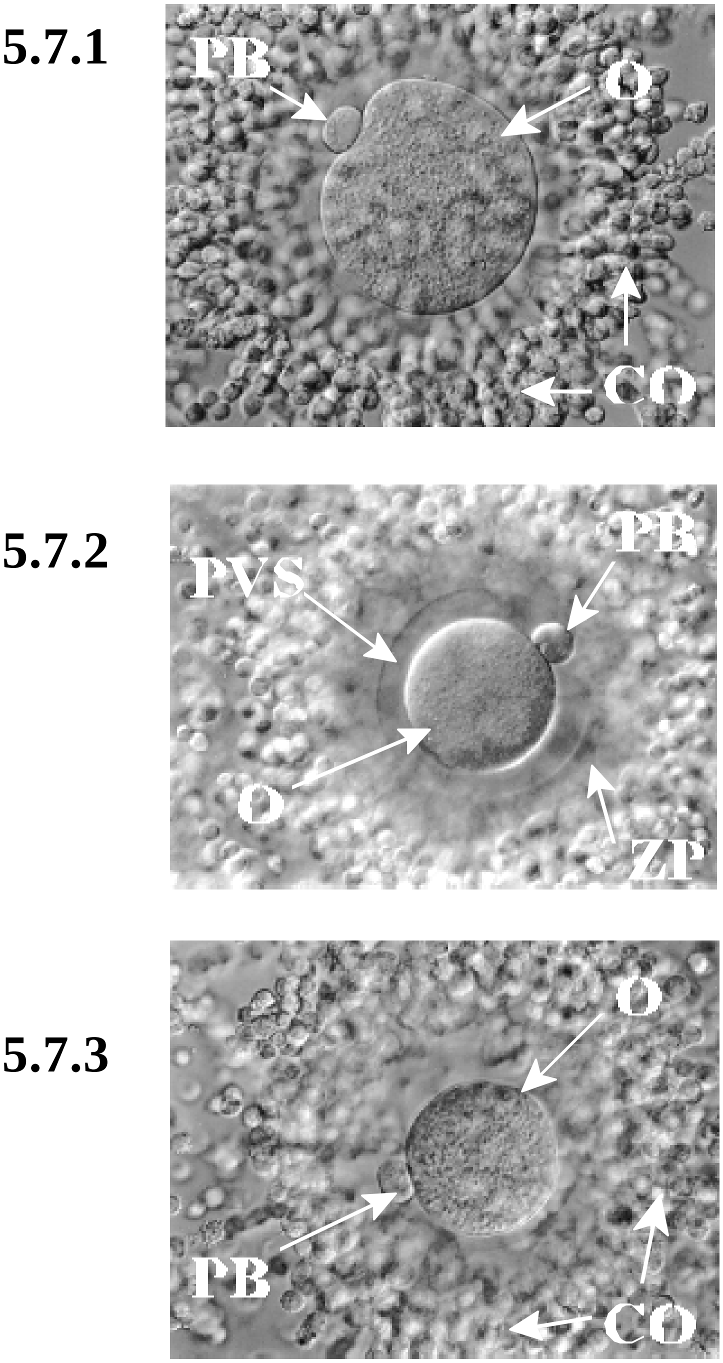
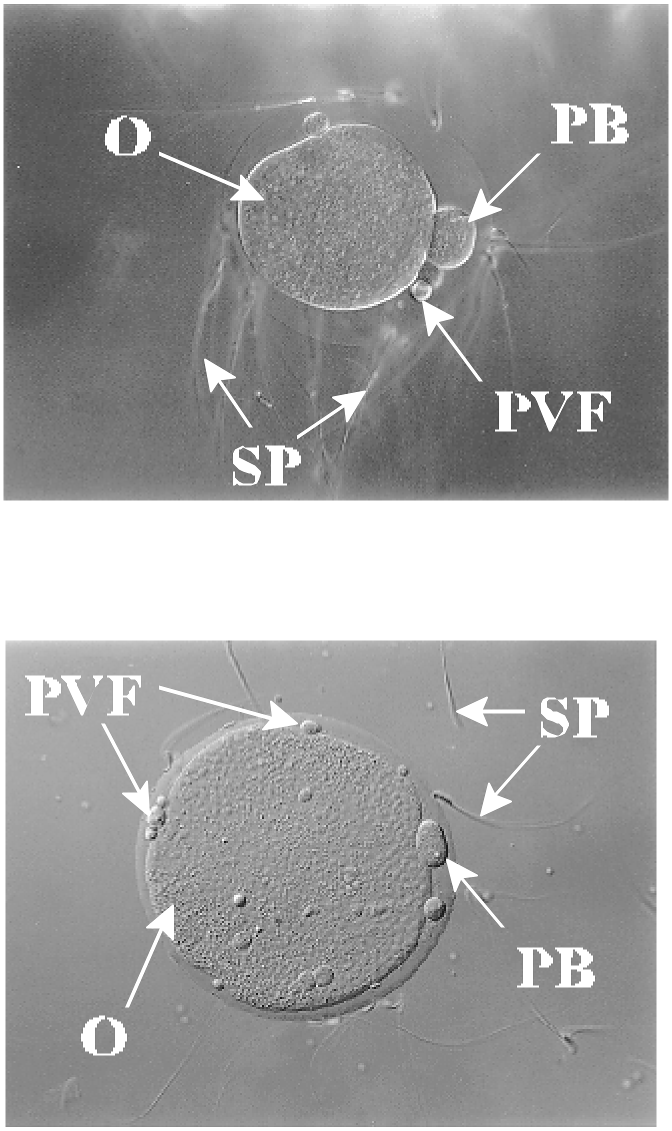


Figure 5.7 Oocytes (O) with polar bodies (PB) obtained from mice treated with 10IU PMSG and 15IU hCG according to a six-day oestrous cycle and sacrificed between 04:15 and 09:00. Labels: CO, cumulus oophorus; PVS, perivitelline space; ZP, zona pellucida.
Fig. 5.7.1 =528 x magnification; Figs 5.7.2 & 5.7.3 =350 x magnification.


5.8.1

5.8.2

Figure 5.8 Oocytes (O) obtained from mice treated with 10IU PMSG and 15IU hCG according to a six-day oestrous cycle and sacrificed at 05:30. The perivitelline space contains the polar body (PB) as well as several other perivitelline fragments (PVF).
Labels: O, oocyte; PB, polar body; SP, sperm.
Fig. 5.8.1 = 440 x magnification; Fig. 5.8.2 = 670 x magnification.

n=18). This hormonal treatment was successful in producing oocytes with polar bodies (Fig. 5.7), although their occurrence was rare and the results not repeatable. The presence of polar bodies varied within experiments, with a single female producing both oocytes with and without polar bodies. Of the nineteen experiments done, only two produced oocytes with fully formed and completely extruded polar bodies. Of the 2 and 9 oocytes yielded in these two experiments, only 1 and 2 oocytes possessed polar bodies respectively. On three other occasions using this treatment, polar bodies were produced in ovulated oocytes, however, the oocytes were atypical as they possessed several other perivitelline fragments (Fig. 5.8). 65% of the oocytes retrieved using protocol 5 were surrounded by cumulus oophorus. The treatment for the seven-day cycle resulted in 6 normal and 7 degraded, translucent oocytes. Only a single normal oocyte possessed a polar body. Due to the high incidence of abnormal oocytes and the low number of oocytes with polar bodies, the regime using the seven-day oestrous cycle was not repeated.

On one occasion using protocol 5, when the female was sacrificed at 05:20, the oocytes obtained seemed to be in the process of extruding polar bodies (Fig. 5.9), indicating that sacrifice was premature as the oocytes had not yet completed development.

5.4 *NATURAL OVULATION*

5.4.1 METHODS AND MATERIALS

Several natural matings were carried out to examine the possibility of obtaining oocytes without superovulation. A male was placed into the cage of an adult female between 19:00 and 19:45 (lights off 20:00). The animals were observed for approximately 15 minutes for any signs of aggression and,

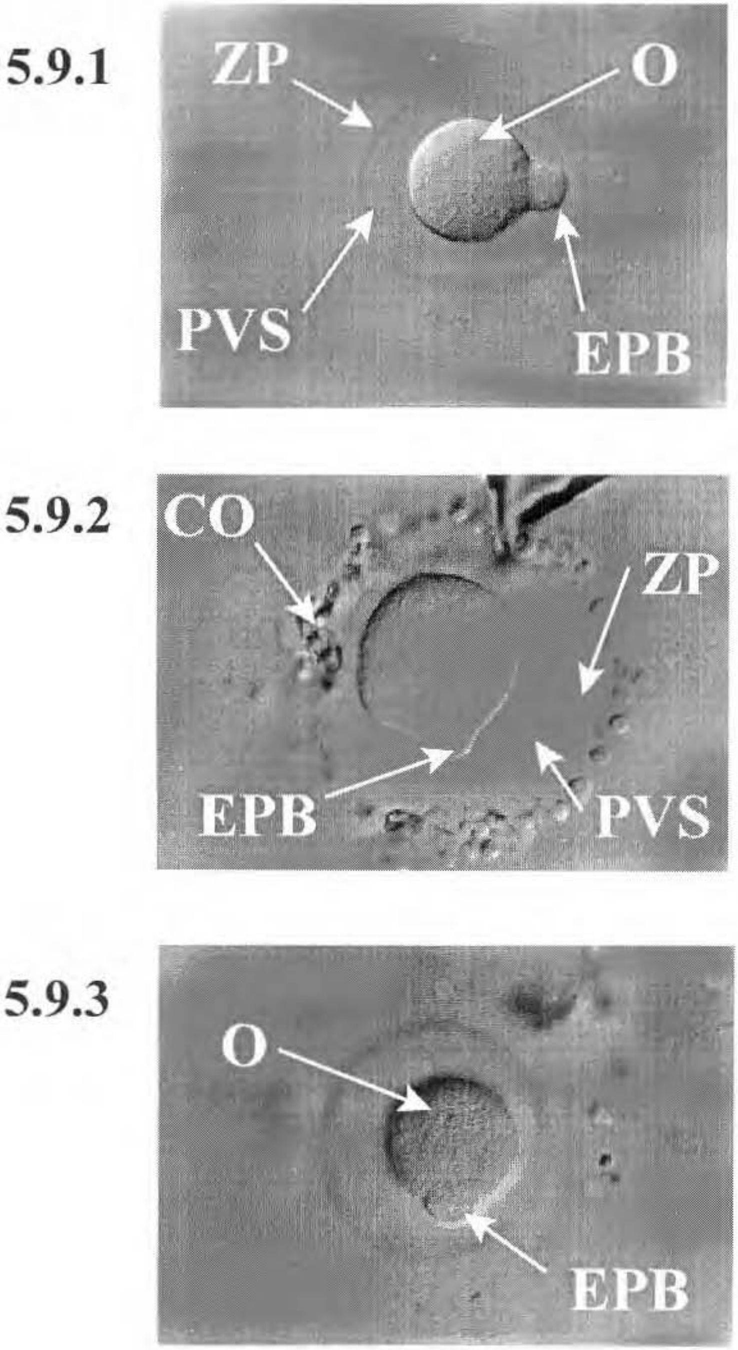


Figure 5.9 Oocytes (O) obtained from mice treated with 10IU PMSG and 15IU hCG according to a six-day oestrous cycle and sacrificed at 05:20. The extruding polar body (EPB) is visible within each oocyte.
Labels: CO, cumulus oophorus; EPB, extruding polar body; O, oocyte; PVS, perivitelline space; ZP, zona pellucida.
325 x magnification.

if observed, they were separated. In the absence of aggression, they were left together overnight. Between 07:00 and 07:30 of the following day the animals were separated and the female sacrificed with 0.35ml Euthenaze injected into the thoracic cavity using a 0.5ml insulin syringe. The oviducts were excised and the contents extruded into 1ml of supplemented medium in a petri dish. Any oocytes yielded were retrieved using a pulled glass pipette, placed onto a glass wax-spot microscope slide and examined under a Nikon light microscope.

In order to confirm that it was indeed the presence of a male and/or the occurrence of mating that induced ovulation, two control experiments were performed for the natural mating investigations. Without introduction of males into their cages, females were sacrificed between 07:00 and 07:30. The animals were then treated as described above.

Experiments (n=5) were also carried out where the female was given 10IU PMSG to induce follicular development prior to the introduction of a male (Ducibella *et al.*, 1988; Kalab *et al.*, 1991). The PMSG was administered as described in Section 5.3. and the male introduced two days later (no hCG was given). The females were sacrificed as before, between 07:00 and 07:30.

5.4.2 RESULTS

Of the twelve females that had spent the night prior to sacrifice with a male and that had not received hormone treatment, only two had ovulated. Both these animals exhibited oestrous-type smears at sacrifice. They had produced 7 and 2 oocytes respectively, however polar bodies were lacking in all of these. The two females used in the control experiments had not ovulated, even though their vaginal smears indicated that they were in oestrous.

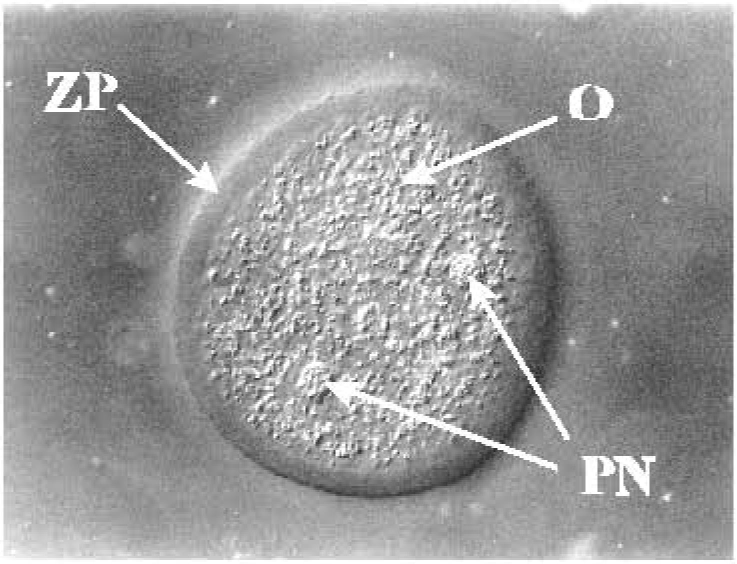


Figure 5.10 Fertilized oocyte from a naturally ovulated female, showing male and female pronuclei (PN).
Labels: O, oocyte; ZP, zona-pellucida.
810 x magnification.

On one occasion during the natural mating experiments without PMSG stimulation, one of the oocytes had already been fertilized and possessed both male and female pronuclei (Fig. 5.10).

Of the five females stimulated with PMSG prior to the introduction of a male, three had ovulated on the day after the introduction of the male. Two produced 8 and 2 normally shaped oocytes each, while the third yielded 5 degraded oocytes. None of these possessed polar bodies.

5.5 DISCUSSION

The oestrous cycle

The use of microscopic examination of cells from the vagina is one of the most commonly used measures of reproductive state in rats and mice (Vom Saal & Finch, 1988). Vaginal smears provide an indirect measure of the blood concentrations of ovarian hormones, since the vaginal epithelium exhibits changes in cytology in response to oestrogen and progesterone. The results from my investigations reveal that the field mouse did not exhibit a distinct oestrous cycle that was distinguishable through the vaginal smear technique. The periods of oestrus that were observed were variable in length within and between animals and were usually extended over several days, unlike the single days of oestrus described for other rodent species. Days of met-, di- and proestrus were observed, albeit relatively seldom. From the regular cycles observed in the pouched mouse, it is noted that the lack of a regular cycle in the field mouse is probably not due to incorrect experimental practice.

Bennett[7] (*Pers. comm.)* also found that the field mouse did not consistently exhibit a typical four-day oestrous cycle as found in many rodents. She indicates that from observations of regular vaginal smears from 5 females, 3 of them had regular four-day cycles and were in oestrus for only one of those days. However, one of the other females showed 3 cycles with 2 days of oestrus and 3 cycles with 3 days of oestrus, indicating that this female exhibited periods of extended oestrus. The smears of the fifth female were always made up of cornified epithelia, indicative of an extended or continuous oestrous period. Bennett suggests that the four-day oestrous cycle is the norm in the field mouse and mice with an extended oestrous are the exception. However this is a broad generalization based on a sample size of five mice. White[8] (*Pers. comm.*) also found that *Rhabdomys pumlio* did not exhibit the expected four day oestrous cycle. She found that they showed such atypical vaginal smears that she did not utilise the normal descriptions for the various stages of the cycle, but developed a new set of identifying parameters.

The characteristics of oestrous cycles in mice, rats and hamsters have revealed species differences, as well as intraspecific variability (Vom Saal & Finch, 1988). Variation in the length of oestrous cycles is due to a variety of factors, such as an animal's age and social and other environmental conditions (Vom

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[7] Bennet, L. University of the Witwatersrand; Johannesburg; South Africa.

[8] White, R.H.M. University of the Transkei; Umtata; South Africa.

Saal & Finch, 1988). In C57BL/6J mice the mean oestrous cycle length decreases and cycles become more regular between 3 and 5 months of age (Vom Saal & Finch, 1988). During the period between 5 and 8 months of age, most females exhibit oestrous cycles that are 4 days long, but after 10 months of age, the modal cycle length shifts to 5 days and many females exhibit irregular and lengthened cycles of 6 days or longer (Vom Saal & Finch, 1988).

Housing female rodents to maximise continuous oestrous cycles for many months may represent an unnatural condition that ordinarily would not be encountered outside of the laboratory by these animals (Vom Saal & Finch, 1988). This may be true for the current investigations. The female field mice housed in the constant environment (C.E.) room as described in Section 2.1, experienced unnatural conditions, even though the environment within the room was set to an optimum. This may well result in unnatural reproductive cycles and a change in responsiveness of the mice to exogenous and endogenous hormones. It should be noted however, that the pouched mouse showed regular oestrous cycles under these same conditions. It would be interesting to determine whether wild-caught mice, that had not been exposed to laboratory conditions and the high concentration of mice within the C.E. room, could be superovulated.

Vom Saal & Finch (1988) indicate that between puberty and 3 months of age, the presence of a male is an absolute requirement for the occurrence of regular oestrous cycles in female mice. This requirement is lost by 5 months of age and females will exhibit regular oestrous cycles when housed individually away from males. This male presence factor was probably not a problem in the present experiments, due to the presence of male mice within the same room as the females. The presence was, however, only olfactory and no physical or visual contact was possible between mice. However, regardless of this fact, the female mice that were used in experiments were over 5 months old and thus, as indicated by Vom Saal & Finch (1988), should be exhibiting regular oestrous cycles without the presence of a male. Thus, this does not shed any light on the lack of a regular oestrous cycle in this species.

Superovulation

The experiments performed to induce superovulation in the four-striped field mouse and to ascertain the ideal regime for doing so, did not result in a repeatable protocol for this purpose. The four-striped field mouse responded differently, both between protocols and within protocols, in terms of the number of oocytes released and their stage of development.

The mean number of oocytes obtained from females treated according to what seems to be the optimal hormonal treatment (protocol 5) determined in this project, was 3.5. This is far fewer than values reported in previous experiments [40 -60 hamster (Barnett & Bavister, 1992); 20-30 mouse (Biggers *et al.*, 1971); 15-20 hamster (Cuasnicu & Bedford, 1991); 19-49 rat (Shalgi *et al.*, 1985); 30-60 hamster (Yanagimachi & Chang, 1964)]. Redina *et al.* (1994) report that the yield of ova within the same strain of mouse may vary considerably. These differences may be due to different concentrations of endogenous hormones in females; a different responsiveness of the ovaries to exogenous gonadotropins, which could influence the number of eggs superovulated; or due to genetic factors (Biggers, *et al.*, 1971; Redina *et al.*, 1994).

Redina *et al.* (1994) showed that synchronizing PMSG treatment with the endogenous hormonal cycle provides optimal conditions for the development of superovulated oocytes. They showed that the greatest number of viable oocytes were found after PMSG injection at oestrus and that the proportion of abnormal embryos was highest after PMSG injection at dioestrus. Beaumont & Smith (1975) synchronized the administration of gonadotropins with the innate oestrous cycle of mice, by timing the administration of hCG to coincide with the endogenous LH surge. However, this did not result in any better embryo survival. The practice of injecting animals on any day within their reproductive cycle has been shown to be successful in certain species (Lehtonen & Kankondi, 1987; Nohara *et al.,* 1998). In the case of the field mouse, however, this is not the case. This practice was however necessary due to the lack of a regular oestrous cycle in the species and the presence of extended periods of oestrous, making the recognition of a particular day of the oestrous cycle difficult.

Oocyte maturity

The variation in the development of the oocytes produced by superovulation treatments was most notable through the presence or absence of the first polar body. Under normal conditions the extrusion of the first polar body in the mouse occurs before ovulation and the best developmental capacity is expected among oocytes that are ovulated with a formed polar body, as these are synchronized with normal development (Redina *et al.*, 1994).

In mice, the pre-ovulatory follicle is prepared for ovulation by a gonadotropin surge (Harper, 1988). In response, the oocyte's nucleus, the germinal vesicle, breaks down and the oocyte enters metaphase I. The chromosomes condense and become arranged in pairs in the middle of a spindle (Masui &

Clarke, 1979; Wassarman, 1988a). Separation of paired homologous chromosomes is followed by formation of a membranous ring around the central region of the spindle and pinching off of the first polar body (Wassarman, 1988a). This asymmetric cleavage of oocyte cytoplasm results in the polar body containing one-half of the original chromosomal complement, allowing for later fertilization.

Bedford[9] (*Pers. comm.*) suggests two possible reasons why the majority of the oocytes from *Rhabdomys pumilio* females were retrieved without polar bodies after ovulation. Firstly, there is the possibility that the first polar body could degenerate rapidly after ovulation. Bedford (1971) states that in rats and often in mice, the first polar body disappears soon after its extrusion and Wassarman (1988a) says that polar body chromosomes begin to degenerate in late telophase I. Although Bavister (1989) indicates that, in the hamster, the polar bodies do not degenerate or fragment soon after fertilization, he does indicate that this occurs in other species. Some authors report that degenerative changes occur in some ova as a result of fertilization failure (Evans & Armstrong, 1984; Lian[10], *Pers. comm*). Thus, it seems that there is the possibility that the first polar body in field mouse oocytes could have deteriorated completely by the time they were recovered, however it seems unlikely due to the early sacrifice times that were used in experiments.

A second possible explanation put forward by Bedford (*Pers. comm.*) is that the oocytes were ovulated at metaphase I, or at the germinal vesicle stage and thus had not yet extruded the polar body. Oocyte maturation would thus occur within the oviduct. Redina *et al.* (1994) points to the possibility

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[9] Bedford, J.M. Cornell University; New York; United States of America.

[10] Lian, L.S. National University of Singapore; Singapore; China.

that not all oocytes have time to complete the first meiotic division before ovulation and that some oocytes without a polar body could have a chance to complete the first meiotic division in the ampulla of the oviduct and to be fertilized later than others. This explanation also seems unlikely as the germinal vesicle stage was only ever observed in oocytes taken directly from the ovary and never in oocytes recovered post-ovulation.

Problems with superovulation

There are conflicting opinions and results concerning the normality of superovulated oocytes and their subsequent ability to develop normally. Pinkert[11] (*Pers. comm.*) states that the results obtained for follicular development induced by gonadotropin administration vary widely and are dependent upon specific superovulatory regimes, source of hormones, animal breed and specific populations.

Superovulation has been shown to produce normal ova and offspring in several species (Greenwald, 1976; Juetten & Bavister, 1983). Gates (1971) believes that the ova produced by mice which have superovulated are genetically normal and capable of normal development. He reported that there was no difference between zygotes produced by mice which superovulated and controls which ovulated spontaneously, with respect to the proportion which developed and gave rise to normal viable embryos. The results obtained by Walton and Armstrong (1983) also suggest that oocytes recovered from superovulated or control rats are equally able to develop and the failure to maintain pregnancy that has previously been reported for superovulated rats is not attributable to defects in the oocytes.

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[11] Pinkert, C. University of Alabama at Birmingham; Alabama; United States of America.

However, several authors have reported that superovulation results in an increase in fragmented, degenerate and denuded oocytes (Beaumont & Smith, 1975; Moor *et al.*, 1985; Lehtonen & Kankondi, 1987; Walton & Armstrong, 1983). Beaumont & Smith (1975) and Redina *et al.* (1994) also report that, when compared with spontaneous ovulation, superovulation is associated with a greater loss of embryos during pregnancy. Almost half of the oocytes produced by treated mice (LACA strain) were lost before implantation, whereas only about 10% of those ovulated spontaneously failed to implant (Beaumont & Smith, 1975). It has further been shown that treatment with gonadotropins produces genetic defects in the ova *in vivo* and *in vitro* (Fujimoto *et al.*, 1974; Luckett & Mukherjee, 1986; Maudlin & Fraser, 1977). The mechanisms by which gonadotropins increase the incidence of abnormal ova are not known (Miller & Armstrong, 1981).

Moor *et al.* (1985) showed that exogenous gonadotropins, administered at high levels, will grossly disturb the biochemical function of the oocyte. This premature activation of oocytes causes abnormalities both because the follicular environment is likely to be unsuitable for maturation at this early stage and because the oocyte will already be aged at ovulation.

Natural ovulation

The results obtained from the experiments involving natural ovulation varied and some females yielded oocytes on the morning after a male had been placed in the cage with them. These results were surprising as they suggested that *Rhabdomys pumilio* might be an induced ovulator. Species can be separated into induced and spontaneous ovulators (Kilen & Shwartz, 1999). Induced ovulators require coitus to elicit the preovulatory surge of the gonadotropins followed by ovulation. Spontaneous ovulation is also preceded by the preovulatory gonadotropin surge but takes place in the absence of a male and coitus. Induced ovulation requires the continuous presence of growing and mature follicles ready for ovulation and thus these species demonstrate a continuous oestrus characterised by follicular growth and oestrogen secretion. Although some of the results obtained in the present experiments suggest that the field mouse may be an induced ovulator, it is unlikely. No other mouse is known to possess this characteristic and the number of mice in the present study that exhibited this trait was very low. It might also be that the female mice showing this trait were fortuitously placed with a male on the evening of proestrus, with oestrus and associated ovulation occurring naturally the following day.

Experimental practice

The mice used in the experimental procedures were given hormones intraperitoneally. Lehtonen & Kankondi (1981) report that a few authors found that administration of gonadotropins i.p. increases the proportion of abnormal ova more than if they are administered subcutaneously. Lehtonen & Kankondi (1981) found that, regardless of the site of gonadotropin injections, the numbers of oocytes were equal, but the proportion of abnormal oocytes in mice injected intraperitoneally was significantly higher than in mice injected subcutaneously. This factor may possibly be responsible for the yield of abnormal, immature oocytes.

Another possible source of error in the experimental procedures is the use of Euthenaze for sacrifice. Several authors sacrifice their experimental animals by cervical dislocation. This, however, was not possible in the field mouse due to the fact that the skin covering the field mouse's tail slips off when pulled abruptly. This causes the animal much discomfort and stress. It was thus decided not to use cervical dislocation and, instead, to use a dose of Euthenaze to sacrifice the animals. It is suggested that the use of this chemical could affect the oocytes (Coates[12], *Pers. comm.*; White[13], *Pers. comm.*), but this does seem highly unlikely, as the oocytes were removed rapidly from the sacrificed female, thus making the likelihood of such rapid deterioration of an oocyte or an accompanying polar body, highly questionable.

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[12] Coates, A. Leeds General Infirmary; Leeds; United Kingdom.

[13] White, D. University of Ottawa; Ottawa; Canada.

CHAPTER SIX

IN VITRO FERTILIZATION

6.1 INTRODUCTION

Fertilization is the process that culminates in the union of one sperm nucleus with an oocyte nucleus within the activated oocyte cytoplasm, to form a new diploid fertilized zygote (Harper, 1988; Schatten, 1999). The process of *in vitro* fertilization involves the laboratory manipulation of gametes within an environment other than the natural *in vivo* situation.

The final steps of mammalian oogenesis and spermatogenesis prepare oocytes and sperm, respectively, for fertilization (Wassarman, 1987). During the process of ovulation, fully grown primary oocytes from follicles undergo meiotic maturation and become unfertilized secondary oocytes that are ready to interact with sperm. Similarly, after deposition into and migration up, the female reproductive tract, mature sperm undergo capacitation, a process that enables them to bind to oocytes and undergo the acrosome reaction (Yanagimachi, 1988).

The process of fertilization involves a series of coordinated events involving both sperm and the oocyte, including events like the sperm acrosome reaction, penetration of the oocyte zona pellucida and the oocyte cortical reaction and zona pellucida reactions (Legendre & Stewart-Savage, 1993; Saling & Storey, 1979; Wassarman, 1999). The latter reactions result in alteration of the zona pellucida such that free-swimming sperm are unable to bind to fertilized oocytes. Before they can successfully interact with the oocyte, the sperm must undergo capacitation (Section 3.1). They gain this fertilizing ability through maturation in the female genital tract (Yanagimachi, 1988). Uncapacitated sperm may attach to the surface of the cumulus oophorus, but they fail to penetrate into it. After capacitation, the sperm must penetrate the cumulus and the zona pellucida of the oocyte before they can fuse with the oocyte (Yanagimachi, 1988).

In most mammals that have been studied, an acrosome-intact sperm usually makes contact with the oocyte to undergo a specific gamete recognition process (Brewis & Wong, 1999). However, various authors have reported interactions of the oocyte zona pellucida with both acrosome intact and acrosome reacted sperm (Bedford, 1983; Cherr *et al.*, 1986; Hadek, 1969; Kuzan *et al.*, 1984; Saling *et al.*, 1979; Yanagimachi, 1988).

Sperm-zona pellucida interactions exhibit species specificity (Oura & Toshimori, 1990) and the locus on the oocyte for interaction with sperm varies from species to species (Shapiro & Eddy, 1980). Gamete recognition is mediated by complementary molecules associated with the zona pellucida and the plasma membrane over the sperm head. ZP3 has been shown to be the main oocyte protein involved in initial sperm-zona binding in most species (Brewis & Wong, 1999; Yanagimachi, 1988), but it is recognized that multiple receptors on the sperm are involved in ZP3-binding.

Sperm pass through the cumulus layer, recognize and attach to the zona pellucida and then firmly bind to the zona to start penetration (Oura & Toshimori, 1990). Immediately after passing through the zona pellucida, the single sperm passes through the perivitelline space and then almost instantly fuses with the oolemma (Oura & Toshimori, 1990; Schatten, 1982; Yanagimachi, 1988). Subsequently the sperm head is gradually incorporated into the vitellus.

Upon fusion with the sperm, the oocyte is activated to initiate a series of morphological and biochemical events that lead to differentiation and the formation of a new individual (Schatten, 1982; Yanagimachi, 1988). The most easily recognizable visible indication of oocyte activation in mammals is the exocytosis of cortical granules (Section 4.3) and the resumption of meiosis.

At ovulation the oocyte nucleus is arrested at metaphase II and the oocyte is metabolically quiescent before fertilization. Upon sperm-oocyte fusion, the arrested nucleus resumes meiosis and transforms into the female pronucleus (Oura & Toshimori, 1990). The abstriction of the second polar body also commences. The decondensation of the sperm nucleus starts soon after sperm-oocyte fusion (Oura & Toshimori, 1990). As soon as the sperm nuclear membrane breakdown begins, the sperm chromatin becomes directly exposed to the oocyte cytoplasm and the chromatin becomes decondensed forming the male pronucleus. The two pronuclei migrate and eventually intermingle their haploid nucleic substances to form a diploid zygote (Oura & Toshimori, 1990).

Once fertilization has been successfully completed, signalled by the proper fusion of the two pronuclei, the zygote initiates the cell divisions that will permit the morphogenetic motions leading to cleavage, differentiation and eventual development into an adult organism (Longo, 1988).

The experiments described below were performed to determine whether *in vitro* fertilization could be carried out routinely in the field mouse and to determine the optimal procedure for this technique in this species.

6.2 *IN VITRO FERTILIZATION*

6.2.1 METHODS AND MATERIALS

For each *in vitro* fertilization experiment a male was sacrificed at 02:30, approximately 3.5h before sacrifice of a female, using 0.35ml Euthenaze injected i.p. as previously described (Section 3.2). The cauda epididymides were excised (Section 3.2.1) and washed in supplemented medium. They were placed into separate wells of a multi-well plate each containing 1ml of equilibrated, supplemented medium. They were punctured several times with watchmakers forceps and left in the incubator for 10 minutes to allow motile sperm to swim out. The tissue was then removed and discarded.

10µl samples of the two sperm solutions were removed using an autopipette and placed onto a heated glass microscope slide. The sperm were observed using a Nikon light microscope and the percentage of forwardly motile sperm estimated. If the sample exhibited less than 80% forward motility it was discarded and another male was sacrificed. The sperm solutions were pipetted into two warmed 1.5ml eppendorf tubes and overlaid with 100µl of pre-equilibrated paraffin oil. These were placed into a tube rack and set at a 45° angle in the desiccator that had previously been put into the incubator (Section 2.2). The lid of the desiccator was left slightly ajar to allow for free-flow of air into and out of the desiccator. This prevented a sudden drop of temperature and CO_2 in the air surrounding the samples.

Female mice were induced to superovulate by administration of 10IU PMSG and 15IU hCG according to a six day oestrous cycle, 97.5h apart, as described in detail in Section 4.2.1. The super-stimulated female was sacrificed at 05:30 on day 6, approximately 10h post-hCG injection, using 0.35ml Euthenaze injected i.p. as previously described (Section 4.2). The ovaries and oviducts were removed from the female and placed into equilibrated, supplemented medium where they were washed. The oviducts were separated from the ovaries and were placed individually into 100µl drops of medium on two petri dishes. If the female had been successfully stimulated by the hormonal treatment, the oviducts showed a translucent bulge at the area where the oocytes lay within the oviduct. The oviducts were pricked at this point and the oocytes passively extruded into the medium under the fluid pressure within the oviduct. Often, however, oocytes were not produced in great enough numbers by the hormonal treatment to result in the indicative bulging of the oviduct. In these cases the entire contents of the oviduct had to be extruded into the medium using watchmakers forceps.

On the night before an experimental procedure, a 100µl drop of supplemented medium was placed onto a petri dish and was overlaid with equilibrated paraffin oil. Oocytes obtained from a stimulated female on the day of an experiment, were recovered from the medium using warmed pulled glass pipettes and were then released into the pre-equilibrated drop of medium under the oil.

After 4 hours of incubation in supplemented medium, 10µl samples were once again taken from the top of the sperm solutions. These were placed onto heated glass microscope slides and the sperm motility observed. If there were sperm that were showing hyperactive motility, indicative of the capacitated state (Section 3.1), the sperm were assumed to be capable of accomplishing fertilization and were used in *in vitro* fertilization trials. 100µl of the capacitated sperm solution was pipetted off the top of the solution from one of the eppendorf tubes, ensuring that only motile sperm were selected. This was then placed into the drop of supplemented medium containing the oocytes.

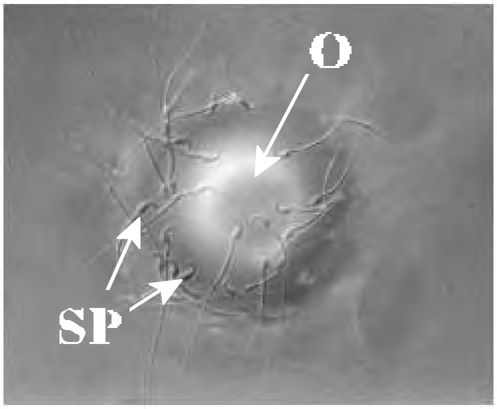
The petri dish was returned to the incubator by approximately 06:30 and left for approximately six hours. After this period, the oocytes were retrieved from the drop of medium using pulled glass pipettes and placed individually onto wax-spot slides. A coverslip was placed onto the slide and gently pushed down over the oocyte. The slide was then examined using an Olympus BX50 microscope with Nomarski optics. Often, after initial examination for polar bodies and pronuclei, an acetic acid:alcohol (1:3) fixative was utilised to remove the zona pellucida from the oocyte and 1% orcein stain in saline was then used to stain any nuclear material within the oocyte.

6.2.2 RESULTS

Six *in vitro* fertilization experiments were carried out. The number of oocytes yielded from females treated for superovulation ranged from 1 to 21 (median 8.0). The oocytes obtained from five of the females used in these investigations possessed no polar bodies (Fig. 6.1). The one female that did yield oocytes with polar bodies, produced 1 oocyte lacking a polar body, 1 oocyte with a normal, intact polar body and 2 with a polar body plus several perivitelline fragments (Fig. 6.2).

In the *in vitro* fertilization experiments, sperm were regularly observed bound to the cumulus oophorus or the zona pellucida of the oocyte (Fig. 6.1 & 6.2). However, none of the trials resulted in fertilization. Sperm were never seen within the perivitelline space, the second polar body was never noted and pronuclei were never observed, with or without the use of orcein stain.

6.1.1



6.1.2

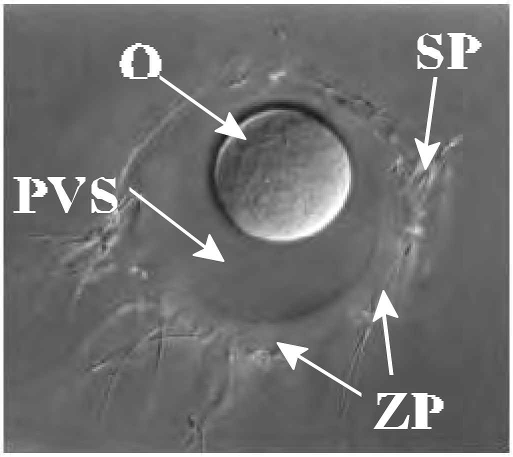


Figure 6.1 Sperm (SP) bound to oocytes (O) from superovulated females. Note the lack of polar bodies.
Labels: PVS, perivitelline space; ZP, zona pellucida.
317 x magnification.

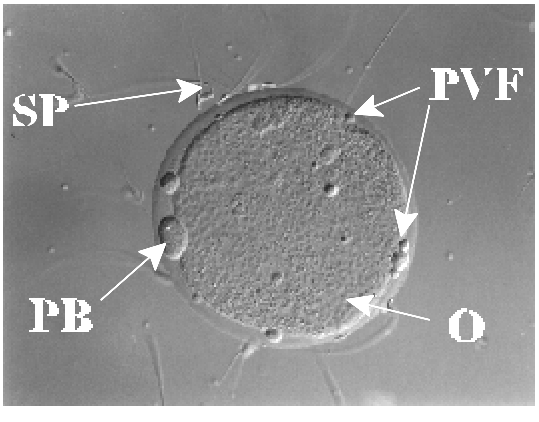


Figure 6.2 Sperm (SP) bound to an oocyte (O) from a superovulated female. Note the single polar body (PB) and several perivitelline fragments (PVF).
563 x magnification.

6.3 DISCUSSION

The complications in obtaining a repeatable regime for obtaining mature oocytes through superovulation reported in this and the previous chapter, combined with the results from the present investigations, indicate that obtaining fertilization *in vitro* in the field mouse is indeed difficult and has not been possible in this project. With the accomplishment of an ideal superovulatory hormonal treatment for this species and the consequent collection of a large number of mature superovulated oocytes, the *in vitro* fertilization might be possible.

Several authors have reported consistently successful superovulatory regimes for various species (Beaumont & Smith, 1975; Bedford *et al.*, 1997; Hamilton & Armstrong, 1991; Walton & Armstrong, 1983). The procedures for superovulating and *in vitro* fertilization with hamsters has often been reported (Cuasnicu & Bedford, 1991; Legendre & Stewart-Savage, 1993; Yanagimachi & Chang, 1964), with Bavister (1989) giving highly detailed methods in his paper.
Although these authors used slightly different regimes, the general procedures were the same throughout. The animals were given between 20-30IU PMSG approximately two days prior to an injection of 20-40IU hCG, depending on the study species. The length of time between PMSG and hCG injections in the present study was about 48 hours longer than that used in other studies (Section 5.3.1).

Authors sometimes synchronised the hormonal injections with the endogenous oestrous cycle (Beaumont & Smith, 1975; Hamilton & Armstrong, 1991; Yanagimachi & Chang, 1964), however this was not possible with the field mouse (Section 5.3.1). Authors that did not use the oestrous cycle as a cue for the time of hormonal administration reported successful superovualtion (Bedford *et al.*, 1997; Cuasnicu & Bedford, 1991; Nohara *et al.*, 1998; Walton & Armstrong, 1983).

The reasons for the failure in accomplishing *in vitro* fertilization in the field mouse are unclear, yet the possibilities numerous. The medium used in the culture of the gametes might not be conducive to creating the optimum conditions for the gametes of this species. However, the medium contains the necessary components for gamete culture and previously has been successfully used by Bedford *et al.* (1997; 1998). It was also shown in the present study that this medium successfully induced capacitation in the sperm of the present study animal and maintained motility of this sperm for long periods. Therefore, as far as the male gametes are concerned, the medium did not seem to pose any problem in terms of fertilization. The problem could in fact lie with the oocyte and its intolerance of culture conditions. However, it is difficult, if not impossible, to determine this unequivocally.

Other problems could lie with the CO_2 content and temperature conditions within the incubator. These were however set at an optimum and the sperm, which are reportedly much more sensitive to unfavourable changes in these factors than the oocyte, were able to undergo the capacitation process and acrosome reaction, as well as maintain motility for long periods of time. Although the pH of the medium was never actually tested, the medium contained an indicator that changed colour with pH fluctuation and this was never observed in any experiments. Thus it seems unlikely that the problem lies with either CO_2 levels or temperature.

The most likely source of problems in the failure to accomplish *in vitro* fertilization lies with the inability to obtain normal, mature oocytes with a single polar body and female pronucleus. Fertilization will never be attained if the oocyte is not meiotically ready to be fused with the nuclear material of sperm. As indicated in Section 5.2.2, the field mouse lacks a definite oestrous cycle and has consequently proved difficult to superovulate and obtain the desired oocytes. Thus, unless the oocytes are matured *in vitro* prior to coincubation with sperm, it seems that fertilization, other than in the *in vivo* situation, will not be possible.

CHAPTER SEVEN

SUMMARY AND GENERAL CONCLUSIONS

The investigations and experiments carried out in the present study on the four-striped field mouse (*Rhabdomys pumilio*) produced observations and results indicating both a typical and an atypical rodent species.

The gametes of *Rhabdomys pumilio* were shown to be similar in several respects to many rodent species, including the falciform structure of the sperm head. The induction of the capacitated state in the sperm, including hyperactivated motility, through incubation in supplemented medium, was readily achieved as has been reported by other authors. The acrosome reaction was also noted to have occurred once the sperm had completed capacitation.

The structure of the mature oocyte of the field mouse was the same as has been reported in other mammalian species. However, the success of retrieving a completely matured oocyte from this species was very low indeed and the occurrence of a polar body within the perivitelline space of an oocyte was rare. The problems accompanying the unsuccessful retrieval of mature oocytes are probably related to either the lack of a regular oestrous cycle, or to the protocol used to induce superovulation.

The oestrous cycle of a rodent normally consists of four consecutive days arranged around a day of oestrus. Through use of the vaginal smear technique it was possible to gain insight into the state of the experimental animals in terms of their position in an oestrous cycle. The field mouse, however, lacks this regularly repeating cycle, with the oestrous-type smear often observed, but the expected subsequent days seldom noted.

Due to the lack of a regular oestrous cycle, the accomplishment of a superovulatory regime for the field mouse was largely unsuccessful. Several protocols were used in attempts to induce superovulation and these yielded varied results. The regime that gave the best results was utilized in later experiments, although the number of oocytes produced and their maturity varied throughout the duration of the investigations.

In vitro fertilization (IVF) experiments were also unsuccessful. Although sperm were observed to bind to both cumulus oophorus and the zona pellucida and the sperm were known to be capacitated and able to acrosome react, penetration of the oocyte was never noted. The failure to achieve IVF is most probably related to the immaturity of the oocytes obtained for use in IVF trials. If an oocyte is meiotically immature it will never undergo successful fertilization.

This project was carried out in order to introduce *Rhabdomys pumilio* into the field of assisted reproductive technolgies. In order for this, a complete knowledge of both the male and female gametes was necessary, as well as a repeatedly successful IVF protocol. This project has resulted in a detailed description of both structure and some physiological processes of the gametes. However, a repeatable IVF protocol was not achieved. Consequently, without further studies on the field mouse and further attempts at achieving IVF, it would seem that *Rhabdomys pumilio* is not a species that could easily be introduced into the discipline of assisted reproduction.

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APPENDICES

Appendix 1: Preparation for transmission electron microscopy.

1a. Chemical fixation

2.5% glutaraldehyde in 0.1M cacodylate buffer - Minimum 4 hours

Cacodylate buffer - 2 x 10 minutes

Osmium tetroxide - 90 minutes

Ethanol series (30, 50, 70, 80, 90, 100, 100 %) - 5 minutes each

Propylene oxide: epoxy resin 75:25 - 1 hour each
50:50 -
25:75 -

100% epoxy resin - Overnight

Put into block-moulds containing 100% epoxy resin and placed into an oven at 60°C for 36 hours.

1b. Staining

5% Aqueous uranyl acetate - 30 minutes

Reynold's lead citrate - 5 minutes

<u>Appendix 2</u>: Procedure for staining vaginal smears.

1:1 ether:absolute alcohol fixative - 2 minutes

Absolute alchohol - 2 x 2 minutes
90% alcohol - 2 minutes
70% alcohol - 2 minutes

Harris' hematoxylin stain - 2 minutes
Running water - 5 minutes
Shorr's stain - 2-3 minutes

70% alcohol - 2 minutes
90% alcohol - 2 minutes
Absolute alcohol - 2 x 2 minutes
Xylene - 2 minutes
Xylene - 3 minutes