CHAPTER 6

Fertilization in Monotreme, Marsupial and Eutherian Mammals

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1 Introduction

1.1 The Three Groups of Mammals

There are three quite distinct evolutionary lineages of mammals which have been separated for many millions of years by time and place. The northern-origin (European, Asian, African and North American) eutherian mammals – often called placental mammals – and the southern-origin monotremes (Australasian) and marsupials (Australasian and American).

1.2 Monotremes

There are only three living species of monotreme – the Australian platypus and echidna and the larger New Guinean echidna (echidnas, or spiny anteaters, externally resemble porcupines). Monotremes are hair-bearing animals with a true cloaca for faeces, urine and reproduction exiting via a single external opening (monotreme means one hole). Monotremes lay relatively large yolky eggs approximately 3.5–4.0 mm in diameter (Fig. 1) and cleavage is meroblastic as in birds and reptiles. However, there is a small component of uterine support of embryo development and once hatched the embryo-like young is supported by milk as in all mammals. Male monotremes have abdominal testes and relatively simple sperm more similar to those of reptiles than the other mammals. For further information on monotreme biology see Grant (1989) and Griffiths (1989).

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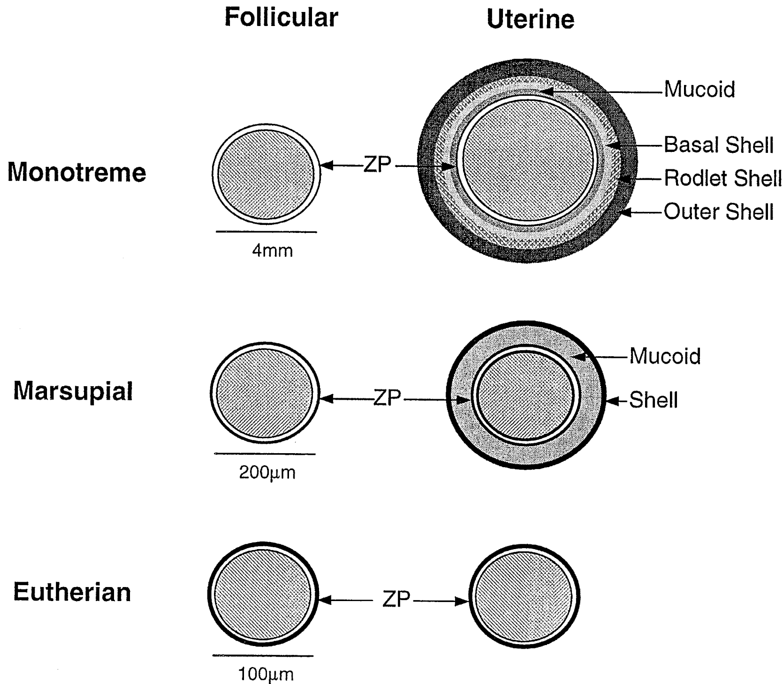

Fig. 1. Comparison of egg structure and vestments in monotreme, marsupial and eutherian mammals. *ZP* Zona pellucida

1.3 Marsupials

Marsupials are hair-bearing animals that give birth to embryo-like young which complete development supported by milk usually in a pouch or marsupium. Marsupials are placental but the period of placental support is remarkably short – only a few days towards the end of a 2- to 4-week gestation. A choriovitelline (yolk sac) placenta is the norm, although some develop a chorioallantoic placenta shortly before birth. Marsupial reproduction is otherwise similar to that of eutherian mammals in character and is regulated by the same suite of reproductive hormones. The marsupial ovary and oocyte are very similar to that of eutherians and even an expert would be hard pressed to distinguish between the two on basic morphology (Fig. 1). Marsupial sperm also share many structural features with those of eutherians although the manner of spermiogenesis means that the sperm head and acrosome are quite different to those of eutherians and are easily distinguishable. The reproductive tract of the female marsupial develops quite differently to that of eutherian mammals due to the relative positions of the embryonic ureters and Müllerian (female) and Wolffian (male) ducts (Fig. 2). In marsupials (plus

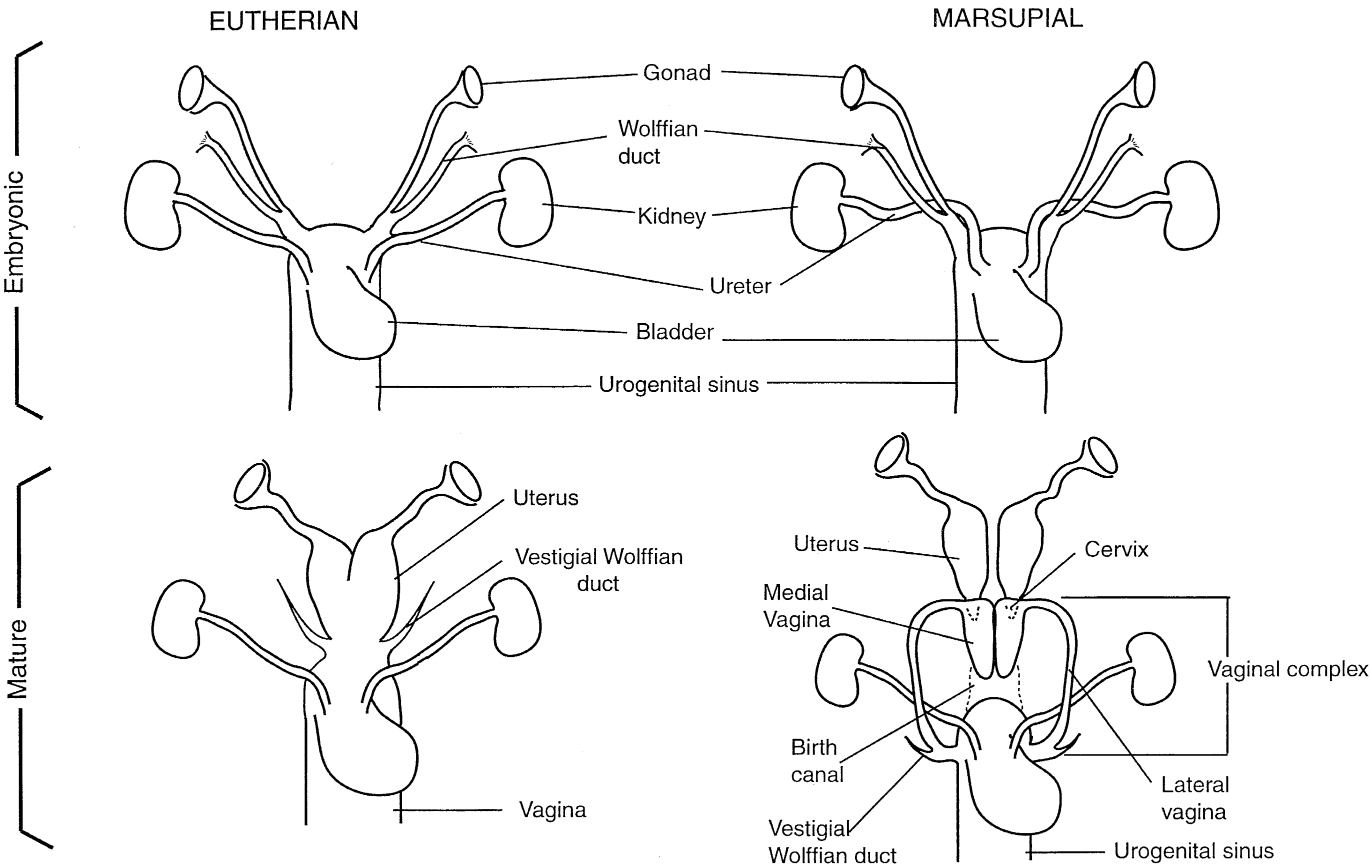

Fig. 2. Urogenital tract at the indifferent stage during embryonic development and in its mature female form in eutherian and marsupial mammals. (Redrawn from Tyndale-Biscoe 1973)

monotremes and other amniotes) the ureters develop in the midline between the developing female ducts. In eutherians Müllerian ducts are central and the ureters develop lateral to the reproductive ducts. This means that the two sides of the female reproductive tract develop as separate left and right systems – vagina, cervix, uterus and oviduct (Fig. 2). As a result, marsupials have a vaginal complex made up of two lateral vaginae for sperm transport and a central sac from which a usually transient central birth canal forms for the newborn to pass to the urogenital sinus.

The male tract of marsupials is very similar to that of eutherians with usually well-developed scrotal testis and highly differentiated regionalized epididymis. Marsupials lack seminal vesicles and coagulating glands and have only a disseminate prostate; see Tyndale-Biscoe and Renfree (1987) for a detailed treatment of marsupial reproduction; Tyndale-Biscoe (1973) provides an excellent although now dated overview of marsupial biology.

Marsupials are more numerous, diverse and widely distributed than monotremes, but there are a relatively small number of species (around 300) compared with the some 6000 eutherians. There are no whale or bat-like marsupials, but homologues of most eutherian forms are found, including aquatic and gliding specializations. Roughly three-quarters of marsupial species are found in Australia, on the island of New Guinea, eastern islands of Indonesia and West Pacific islands adjacent to New Guinea. The remaining species are found in Central and South America and one species, the Virginia opossum, is a relatively recent immigrant into North America. Feral populations of marsupials are found in Europe including the UK, Hawaii and New Zealand, where one, the common brushtail possum, has become the latter's major vertebrate pest.

1.3.1 Marsupial Diversity

Marsupials were originally thought to be equivalent to one eutherian order such as Carnivora or Rodentia. Current taxonomy recognizes seven orders of living marsupials, four in Australasia and three in the Americas (Kirsch et al. 1997). Further, some of the Australian orders are themselves diverse, containing many species. The order Diprotodontia which includes most of the more familiar Australian marsupials (kangaroos, possums and the koala) has been divided into three suborders, and these into five superfamilies containing ten families in all. The diversity of marsupials is emphasized here because there is a tendency to think that marsupials will display common biology. In this chapter we will thus be discussing and comparing not marsupial and eutherian fertilization but fertilization in diverse groups of marsupials and eutherians.

1.4 Eutherians

The eutherian mammals, which include all familiar domestic and laboratory animals and humans, are hair-bearing animals that give birth to well-developed often essentially independent young after a period of uterine development supported by a usually chorioallantoic placenta. We will not go into detail on the eutherians except to emphasize their remarkable diversity (111 families within 17 orders) and their success in essentially all habitats except the terrestrial Antarctic. Thus, as with the diverse marsupials, when considering the processes of fertilization one cannot assume that this will be the same in all species.

1.5 Mammalian Relationships and Evolution

The relationship between living and extinct mammal groups remains an area of considerable debate. A very important early and continuing influence is the Prototheria (monotreme), Metatheria (marsupial), Eutheria system of T.H. Huxley. This lead to a popular conception which saw the three groups of mammals as successive steps to 'true' therian (mammal) status and that marsupials represented a halfway position. As marsupial biology became better understood biologists working with marsupials have strongly argued that marsupials should be regarded as alternative rather than inferior therians. Currently, views are divided on whether marsupials are phylogenetically linked to monotremes (Marsupionta) or eutherians (Theria) (Janke et al. 1997; Kirsch et al. 1997; Kirsch and Mayer 1998). The renewed support for a marsupial monotreme link is not a return to stages of 'therianness' as in Huxley's system. Rather it argues that the placental support of embryo development in utero arose independently in marsupials and eutherians.

1.6 Fertilization in Mammals

Unfortunately the available information on Monotreme gametes is very limited and nothing is known about fertilization. Where some knowledge of gametes, or relevant reproductive processes, is available this will be mentioned. However, a current synthesis of fertilization in mammals must lack a significant monotreme perspective. It is encouraging that only three decades ago the situation was little better for marsupials; thus, provided appropriate access to monotreme gametes and early embryo stages can be gained, increased understanding of monotreme gametes and fertilization can be expected. Since the early 1970s there has been a rapid growth in knowledge of marsupial gametes and fertilization which would now equal, and in many cases exceed, that known about eutherians which are not laboratory or domestic animals. In this chapter,

where possible, we will draw on what is known about a range of eutherian mammals not simply the best studied groups. An excellent survey of fertilization phenomena across the therian mammals is found in Dunbar and O'Rand (1991). One feature of reproduction which would appear to unite all mammals is the absolute dependence on fertilization as the start of a new individual. All mammalian oocytes require a sperm to initiate successful development. Parthenogenetic development can be initiated experimentally, or may occur naturally, but it does not result in full development and rarely proceeds beyond the blastocyst stage. Eutherian mammals, and presumably marsupials, do occasionally have a form of asexual reproduction where post-fertilization events result in the single fertilized oocyte giving rise to partially, or completely, separated individuals which are genetically identical clones. An unusual example of a mammal where post-fertilization asexual reproduction is the norm is the armadillo which routinely undergoes embryo splitting to produce a litter of up to eight identical individuals derived from a single fertilized oocyte.

2 Sperm Production

2.1 Marsupials and Eutherians

Although the processes of spermatogenesis are essentially similar in marsupials and eutherians, the spermatid nuclei flatten in different planes (Fig. 3). In eutherians nuclear flattening is lateral, parallel to the forming tail. As a result the acrosome and tail come to lie at opposite ends of the flattened head and the acrosome forms a cap over the leading edge of the head. In marsupials flattening occurs at 90° to the forming tail. The result is a sperm with a distinct dorsal ventral structure with the acrosome, which never forms a cap, restricted to the dorsal side of the head, and the tail on the ventral side attached opposite the acrosome. As discussed in more detail later, in many marsupials the sperm leaves the testis in a quite immature morphological form – the head and tail are not aligned and the acrosome is only partly formed (Fig. 3).

2.2 Monotremes

Although marsupial and eutherian spermatozoa show differences in the manner and timing of sperm formation the final product is very similar, with a compact head and acrosome, well-developed midpiece with mitochondrial spiral and a tail with a complex of accessory fibres. Indeed, it would require expertise to differentiate between marsupial and eutherian sperm at the ultrastructural level if one only examined the midpiece and tail. Monotreme sperm

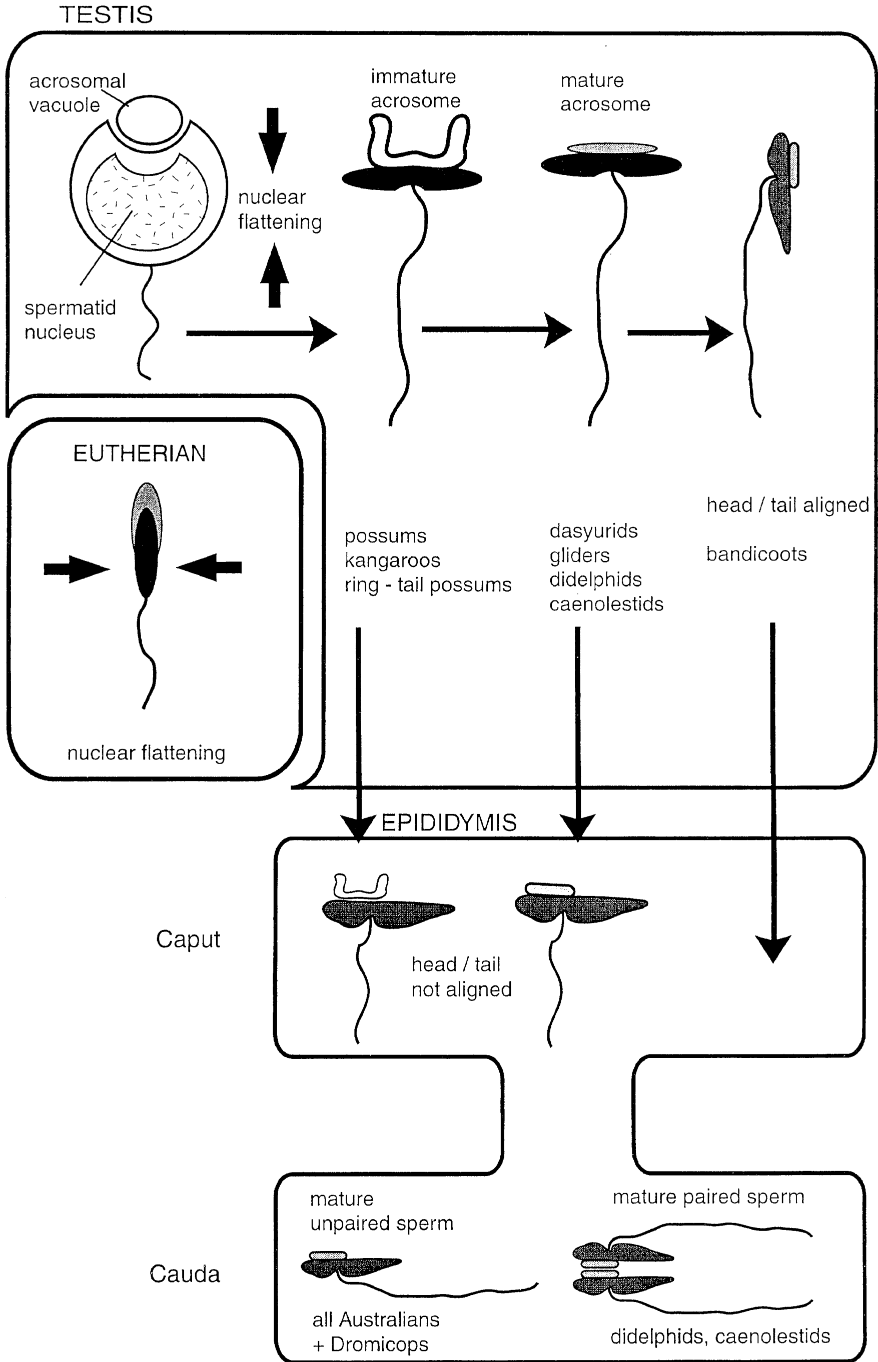

Fig. 3. Spermiogenesis in the testis and epididymal maturation in marsupials. (Redrawn from Rodger 1991)

in contrast are non-mammalian in character (Fig. 4) and resemble sperm of birds and reptiles. The sperm head is filamentous, the acrosome and midpiece are simple and the accessory fibres of the tail are poorly developed (Bedford and Rifkin 1979; Carrick and Hughes 1982).

3 Epididymal Maturation of Spermatozoa

Testicular spermatozoa, although fully differentiated, are not progressively motile and are unable to fertilize the oocyte. They gain these abilities as they pass through the epididymis via a series of modifications collectively termed epididymal maturation (reviewed in Yanagimachi 1994). The epididymis and post-testicular sperm maturation seen in higher vertebrates are thought to have evolved as an adaptation for internal fertilization and sperm storage (Jones 1998b). However, the structure and function of the epididymis are more complex in mammals compared to other vertebrates, and distinct differences also exist between the different mammalian subclasses. The structure of the mammalian epididymis can be divided into a number of regions based on function and epithelial morphology (Fig. 4).

3.1 Eutherians

3.1.1 Morphological Maturation

The morphological changes seen in eutherian sperm during epididymal transit are generally less dramatic than those in marsupials – ranging from minimal in humans and rodents, moderate in the rabbit, elephant and some primates, to substantial in the guinea pig and chinchilla (Bedford 1979). The cytoplasmic droplet, containing the residual spermatid cytoplasm, is initially located in the neck region of eutherian sperm and migrates distally to the posterior midpiece during epididymal transit. Loss of the cytoplasmic droplet accompanies ejaculation. Migration of the droplet is accompanied by midpiece modifications, whilst the sequestering of redundant enzymes in these vesicles may also protect the sperm from oxidative damage (Moore 1995).

3.1.2 Development of Progressive Motility

The development of the capacity for progressive motility is perhaps the most conspicuous change associated with epididymal maturation in all mammalian spermatozoa. Spermatozoa released from the testis and head of the mammalian epididymis are immotile or only very weakly motile. In contrast,

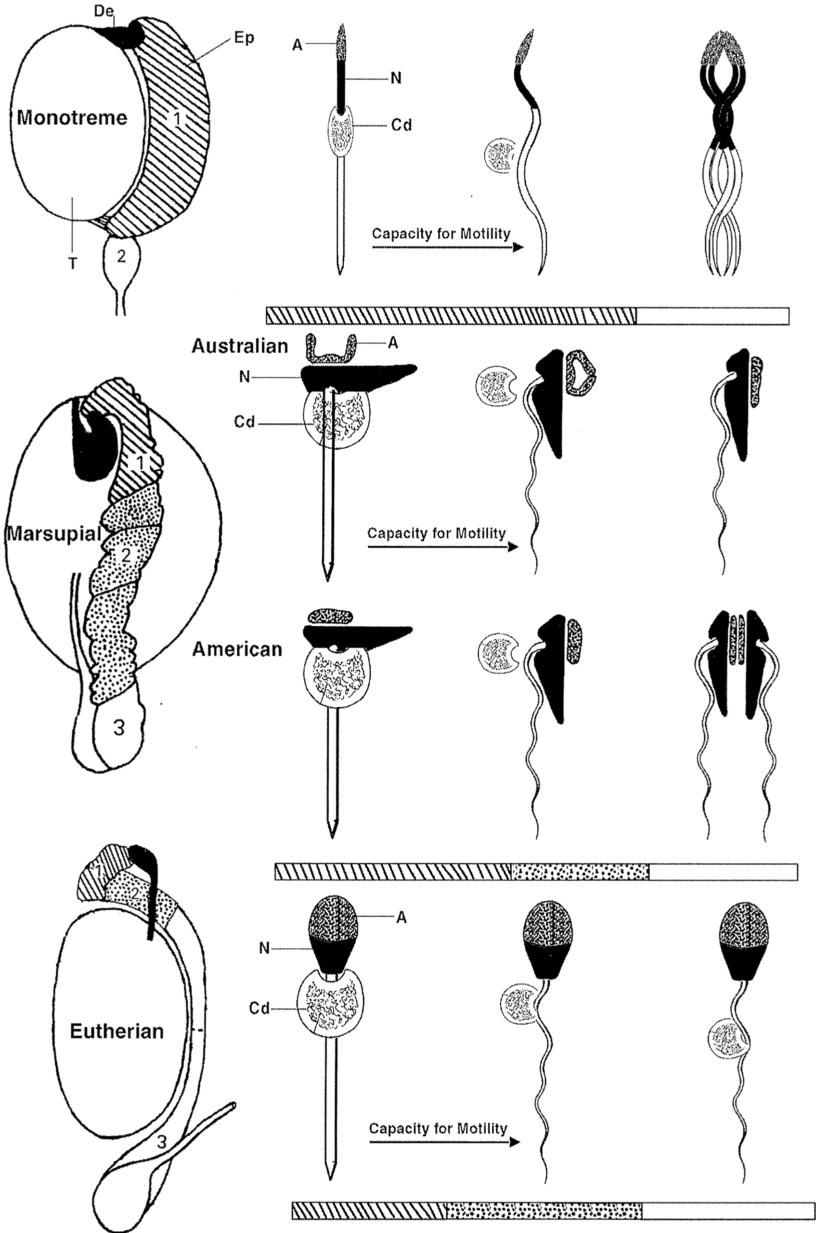

Fig. 4. Structural differentiation of efferent ducts and epididymis of monotreme, marsupial and eutherian mammals, showing relationship between histologically distinct zones and sperm maturation. The efferent duct is *black*, initial segment is *striped*, distal initial segment is *speckled* and terminal segment is *white*. *A* Acrosome ; *N* nucleus; *Cd* cytoplasmic droplet; *T* testis; *De* efferent duct; *Ep* epididymis. (Redrawn from Jones 1998b and Rodger 1991)

the majority of spermatozoa from the body and tail of the epididymis in marsupials and eutherians display strong forward motility when released into physiological salt solutions, though they are maintained in an inactive state *in situ*. Whilst the capacity for progressive motility is of obvious importance for transport to the site of fertilization, its development is associated with the maturation of the plasmalemma. The acquisition of several factors, including forward motility, protein, carnitine and glycerophosphocholine, from the epididymal fluid to the sperm plasmalemma may mediate the development of progressive motility (Cooper 1992).

3.1.3
Maturation of Sperm Surface

The sperm plasmalemma is extensively remodelled during epididymal maturation by a variety of mechanisms, culminating in the development of sperm fertility (Kirchhoff and Hale 1996; Jones 1998a). Changes in the configuration of the eutherian sperm surface are effected by the continually changing epididymal milieu of the luminal fluid. This milieu is in turn regulated by the androgen-dependent activity of the epididymal epithelium through selective and/or non-selective absorption, active secretion and accumulation from and in the blood. This results in the alteration of both the protein and lipid components of the sperm surface.

Selective changes in the amount and composition of lipids in the sperm membrane during epididymal maturation are thought to affect membrane fluidity and thus allow the reorganization of some membrane components (reviewed in Jones 1998a). There are differences between species, and much conflicting data. The situation is complex because the relative fluidity of each membrane domain also varies significantly as a result of differential densities of some phospholipids within the domains (Parkes and Hammerstedt 1985). The establishment of such lipid domains may precede, and perhaps direct, the reorganization of integral membrane components during epididymal transit.

Remodelling of the protein components of the sperm surface appears to be mediated by the movement and modification of existing proteins, the addition of proteins from the luminal secretions, or the loss of proteins from the spermatozoon. Approximately 50% of the glycoproteins added to the sperm surface are actively synthesized and secreted by the epithelium in different regions of the epididymis. Roles for these 'epididymal maturation antigens' have been proposed in the development of sperm fertility and the fertilization process itself (Martinez et al. 1995), acrosome stabilization, decapacitation and as motility regulators (Jones 1989; Cooper 1992). Some of these maturation antigens are only weakly associated with the sperm surface via ionic bonds. However, others are bound strongly to the membrane by a number of mechanisms (Jones 1998a) including direct covalent binding by enzymes such as transferases, and direct membrane interchange mediated

by glycosylphosphatidylinositol lipid anchors (Kirchhoff and Hale 1996; Moore 1996).

Both pre-existing and epididymal maturation proteins on the sperm surface are also modified by post-translational processing and/or a move to different surface domains during epididymal maturation. Post-translational processing of sperm proteins is known to include glycosylation, methylation, disulphide bonding, transglutaminase-mediated cross-linking and phosphorylation (Jones 1998a). A number of proteins involved in fertilization such as fertilin and PH-20 undergo site-specific endoproteolytic cleavage during epididymal maturation. Such processing of these proteins may be a prerequisite for their movement from the plasmalemma from the whole sperm head to the post-acrosomal domain (Phelps et al. 1990). However, epididymal remodelling is not restricted to the sperm surface. Growing evidence suggests that significant processing of intracellular acrosomal and flagellar antigens also occurs (Phillips et al. 1991). For example, stabilization of a number of sperm tail and head structures, including the nucleus, during epididymal transit by increased disulphide bonding is likely to lead to more rigid tail movement for motility and stabilization of the head structures for zona penetration (Bedford 1996).

Together these epididymal modifications to the form and location of sperm components produce mature spermatozoa that have the ability to survive prolonged storage in the epididymis and are capable of both progressive motility and undergoing capacitation prior to fertilization. In most mammals this period of storage lasts days to a few weeks, but in certain bats fertile sperm may be stored in the tail of the epididymis for many months (Krutzsch and Crichton 1991).

3.2 Marsupials

3.2.1 Morphological Changes

Whilst eutherian spermatozoa undergo predominantly functional modifications during epididymal transit, marsupial spermatozoa also undergo significant morphological alterations (Figs. 3 and 4). By far the most extensive post-testicular reorganization of mammalian acrosomes occurs in phalangerid (possums), macropodid (kangaroos) and petaurid marsupials (Harding et al. 1979; Setiadi et al. 1997). The most detailed information on the process is available for the tammar wallaby and common brushtail possum. On leaving the testis the immature acrosome is a 'scoop'-shaped structure projecting from the sperm head which bends in on itself and fuses during transit through the body of the epididymis to produce the mature button-shaped acrosome (Fig. 5; Lin et al. 1997; Setiadi et al. 1997; Lin and Rodger 1999). The acrosome of American marsupial species does not undergo such marked morphological

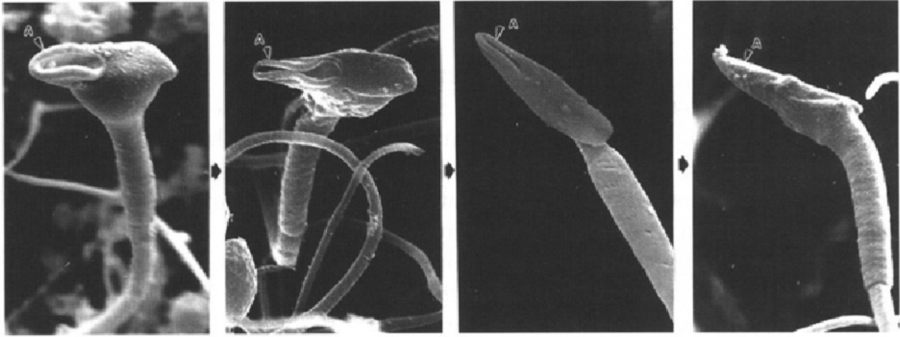

Fig. 5. Ultrastructure of extratesticular acrosome formation in the tammar wallaby. *A* Acrosome

modification but is instead involved in a pairing process that begins in the body of the epididymis. This pairing occurs via an association between the plasma membranes overlying the acrosome of two randomly selected spermatozoa. Sperm pairing does not occur in Australian marsupials nor in the American marsupial *Dromiciops australis* (Temple-Smith 1987).

The nucleus of the majority of marsupial species rotates during epididymal transit, to produce a streamlined spermatozoon (Fig. 3 and 4; Temple-Smith and Bedford 1980). In marsupials, the cytoplasmic droplet is initially located around the head and neck region, it condenses around the neck region of the spermatozoa and is lost directly into the lumen in the proximal epididymis (Temple-Smith and Bedford 1980). Other features of epididymal maturation in marsupials include the development of a unique cytoskeletal structure called the midpiece fibre network and, in phalangerids and macropodids, membrane stacks in the neck region (Temple-Smith and Bedford 1976; Harding et al. 1979).

3.2.2 Molecular Changes

Molecular modifications of the marsupial sperm surface are likely to rival those seen in eutherian species, but the evidence for such modification has been obtained using relatively non-specific probes. Freeze-fracture studies of marsupial spermatozoa have demonstrated the existence, and modification, of plasmalemma domains during epididymal transit (Olsen 1980; Cooper et al. 1998). Differences in the lectin-binding characteristics of sperm from the head and tail of the marsupial epididymis suggest modification of carbohydrate moieties during epididymal transit (Temple-Smith and Bedford 1976; Cooper et al. 1998). Electrophoretic studies provide some evidence for the acquisition and loss of proteins from the marsupial sperm surface during epididymal transit (Chaturapanich et al. 1992; Cooper et al. 1998). Recently it

was found that tammar wallaby sperm acquired a 31-kDa maturation antigen during epididymal transit (Harris and Rodger unpublished observation). The protein was secreted by the epithelium of the epididymis and associated with the whole plasmalemma as sperm passed into region 2 (Fig. 4). Some evidence also exists for the maturation of intracellular antigens during epididymal transit in the marsupial. Sperm surface and intracellular structures are stabilized by disulphide bonding as in eutherians (Setiadi et al. 1997) and new antigenic epitopes are generated by the formation of the midpiece fibre network (Harris and Rodger 1998).

3.3 Monotremes

Epididymal maturation of monotreme spermatozoa involves the development of motility in the initial segment and movement of the cytoplasmic droplet, and there is evidence of changes in surface properties (Bedford and Rifkin 1979; Djakiew and Jones 1983). However, there are some notable differences. Monotreme sperm form into bundles with their heads embedded in electron-dense material, adsorbed to the periacrosomal plasmalemma, as they enter the terminal segment of the epididymis; formation of sperm bundles is associated with an increase in sperm velocity in vitro, which may be comparable to the change in the pattern of motility seen in other mammals (Djakiew and Jones 1983).

Whilst there is some evidence that the initial segment of the monotreme epididymis secretes specific proteins (Djakiew and Jones 1983), the molecular modification of the sperm plasmalemma has not been meaningfully examined. However, sperm maturation in these species does not appear to involve disulphide stabilization (Bedford and Rifkin 1979). The cytoplasmic droplet is initially located around the head of monotreme spermatozoa and migrates to the distal end of the midpiece in the initial segment. It is not discarded until sperm enter the terminal segment (Fig. 4; region 2) of the epididymis (Djakiew and Jones 1983). The terminal segment of the epididymis is involved in sperm storage, but the period of storage may be relatively short compared to that of other mammals (Djakiew and Jones 1983).

3.4 Common Themes and General Overview

In all mammalian species, epididymal maturation of spermatozoa results in the development of progressive motility, fertilizing capacity, and prepares the sperm for storage in the distal epididymis. These maturation changes are associated with morphological modifications that range from being minimal in monotremes, moderate (but variable) in eutherians to considerable in marsupials. A change in the location and loss of the cytoplasmic droplet is a consistent feature of epididymal maturation in all mammalian species,

though there are differences in the location, migration and loss in the reproductive tract.

Molecular modification of the sperm surface and intracellular components, through the addition of proteins secreted by the epididymal epithelium and the alteration of others, is likely to occur in all mammals. Disulphide stabilization of sperm surface and intracellular organelles occurs in the epididymis of marsupials and eutherians, but apparently not in monotremes. Numerous studies on eutherian spermatozoa indicate that extensive molecular modification occurs during epididymal transit. Some evidence for a similar complexity of molecular maturation of marsupial sperm exists.

4 Sperm Deposition, Storage and Transport in the Female Tract

4.1 Marsupials and Eutherians

In the interval between mating and ovulation sperm are stored in the female reproductive tract awaiting the oocyte. Fertilization in most mammals occurs within 24 h of mating (most eutherians, possums, kangaroos and opossums). Nevertheless, in a number of species sperm are stored for several days (dasyurid and didelphid marsupials, canid eutherians) to several months in insectivorous bats (see Mahi-Brown 1991; Taggart 1994). In most species the caudal isthmic oviduct serves as the sperm storage reservoir (Suarez 1998). Within this structure the spermatozoa are maintained either by interactions with the oviduct epithelial lining (e.g. pig, wallaby and possum) or in subluminal oviductal crypts lined with a specialized epithelium (reviewed by Taggart 1994). Oviductal storage crypts are peculiar to dasyurid and didelphid marsupials and only one group of eutherians, the insectivorous shrews (Bedford 1996). These storage crypts may allow a reduction in the number of sperm deposited into the female reproductive tract whilst retaining maximum reproductive efficiency. In comparison, other marsupials and eutherians deposit high concentrations of sperm into the female tract, far in excess of the number required for fertilization (Bedford 1996).

Sperm association with the oviduct epithelium is quite strong since repeated flushing is required to release the sperm in vivo, and enzymatic treatment of sperm bound to oviductal explants in vitro does not release the sperm (Raychoudhury and Suarez 1991; Smith and Yanagimachi 1991). Indeed, the sperm remain associated with the oviduct epithelium until ovulation, when a small number are released to meet the oocyte. There is evidence in both eutherian and marsupial species that association with the epithelium prolongs sperm motility and viability (Smith and Yanagimachi 1991; Sidhu et al. 1998). This interaction may also have a role in mediating capacitation-related changes

in head orientation (marsupials) and hyperactivation (eutherians) which coincide with sperm release from the epithelium (Rodger and Bedford 1982; Yanagimachi 1994; Sidhu et al. 1998). Indeed, a change in the sperm surface at capacitation is proposed to trigger the release of spermatozoa from the epithelium (Smith and Yanagimachi 1991). Sperm transport from the lower isthmus to the site of fertilization in the upper ampulla is then achieved by the combined action of cilia-mediated fluid flow, oviductal contraction and sperm motility (Taggart 1994). Thus, sperm storage is ultimately responsible for the temporal coordination of fertilization by ensuring that the appropriate number of sperm arrive at the site of fertilization in the appropriate condition.

4.2 Monotremes

There is circumstantial evidence, based on the apparent variability of the gestation period, that monotremes may also store sperm in the oviduct for long periods of time (Griffiths 1978). A female echidna examined 34 days after segregation from males, had a 4.5-mm fertilized oocyte of normal appearance in one of her uteri and a recently formed corpus luteum, indicating that ovulation had occurred recently and that the oocyte had been apparently fertilized by a stored spermatozoon. Further evidence for sperm storage in monotremes is the presence of sperm in the lumina of the uterus and uterine glands of a platypus with oocytes that were not fully grown (Flynn and Hill 1939). These observations suggest that viable spermatozoa may be stored for up to 30 days in the female monotreme reproductive tract prior to fertilization.

5 Capacitation

Eutherian spermatozoa must undergo a period of 'capacitation' in the female reproductive tract before they can fertilize the oocyte. The capacitation of eutherian spermatozoa is a reversible phenomenon involving a complex array of post-ejaculatory functional modifications in the female reproductive tract. Only capacitated spermatozoa are able to undergo a regulated acrosome reaction in response to zona binding and subsequently fertilize the oocyte (reviewed in Yanagimachi 1994).

5.1 Eutherians

Capacitation is achieved in vivo during sperm interactions with the female reproductive tract, and takes from 6 h in the rabbit to as little as 0.5 h in the mouse (Yanagimachi 1994). Capacitation has also been achieved in vitro in a number of eutherian species, in defined synthetic medium containing energy

sources, serum albumin, bicarbonate and calcium ions. In eutherians capacitation is associated with modifications of the sperm surface, metabolism and motility (hyperactivation – discussed separately) which provide the spermatozoon with the reaction pathways necessary to undergo a regulated acrosome reaction in response to signal induction by the zona pellucida (ZP; the extracellular coat surrounding the oocyte; Storey 1995).

Sperm surface modifications include the redistribution and removal of membrane proteins. For example, in the rabbit a surface-coating protein termed acrosome stabilizing factor added during epididymal maturation in preparation for sperm storage is removed during capacitation (Eng and Oliphant 1978). Cholesterol removal from the plasmalemma by oviductal fluid proteins leads to a concomitant decrease in the cholesterol/phospholipid ratio of the membrane (reviewed in Yanagimachi 1994; Cross 1998; Visconti et al. 1998).

Capacitation is also associated with a submicromolar rise in intracellular calcium ions, pH and cAMP, as well as hyperpolarization of the sperm plasmalemma. Though the mechanisms are poorly understood these changes are likely to be involved in transmembrane and intracellular signal transduction pathways which ultimately lead to the tyrosine phosphorylation of a specific subset of proteins essential for the attainment of capacitation (reviewed in Visconti and Kopf 1998).

5.1.1 Molecular Basis

The molecular basis of sperm capacitation is complex and controversial, though some consensus on a number of the controlling mechanisms is beginning to emerge (Aitken et al. 1998; Visconti and Kopf 1998; Visconti et al. 1998). Elevation of intracellular ions such as bicarbonate and calcium through ion channels, whose activity could be mediated by cholesterol efflux, may serve as transmembrane signals initiating intracellular signal transduction cascades. These reaction pathways involve the elevation of intracellular cAMP, brought about through bicarbonate (oviductal fluid), activation of sperm adenyl cyclase or redox regulation by reactive oxygen species. The elevation of cAMP activates protein kinase A (PKA) which acts on tyrosine kinases to induce protein tyrosine phosphorylation.

5.2 Marsupials

In the simplest sense, the fact that sperm flushed from the oviduct of the opossum after mating readily penetrated the ZP in vitro, whereas sperm from the vas deferens did not (Rodger and Bedford 1982), indicates that capacitation of marsupial spermatozoa in the female reproductive tract is a prerequisite for fertilization. The fact that many marsupial spermatozoa reside in the

female tract for moderate to long periods (up to several days) prior to fertilization also suggests a requirement for capacitation (Selwood and McCullum 1987; Taggart and Temple-Smith 1991).

5.2.1 Morphological Correlates

To date there have been no definitive timing studies of marsupial capacitation, nor have any molecular markers of possible capacitation-related changes been identified. However, morphological changes that accompany sperm transit in the female tract have been suggested to be indicative of functional maturation in these mammals (see Fig. 6). Unpairing of American marsupial spermatozoa and reversion to a T-shaped (thumb tack) configuration in the female reproductive tract appear to be prerequisites for fertilization in these species, and are thus interpreted as being analogous to capacitation-associated changes in eutherian species (Rodger and Bedford 1982; Moore and Taggart 1993). Indeed, the spermatozoa of a number of Australian marsupials are also T-shaped after incubation in the female reproductive tract and approach the oocyte in this orientation, supporting the hypothesis of an involvement in capacitation (Mate and Rodger 1991; Bedford and Breed 1994). However, marsupial spermatozoa do not display hyperactivated motility as a result of capacitation.

5.2.2 Differences Between Marsupials

There also appear to be differences in the requirements for achieving capacitation between Australian and American marsupials. Capacitation in vitro is easily achieved in *Monodelphis domestica* by the incubation of epididymal spermatozoa in a simple culture medium containing bovine serum albumin (BSA). During incubation the spermatozoa unpair and are subsequently capable of fertilizing (Moore and Taggart 1993). In contrast, capacitation in Australian marsupials, such as the tammar wallaby and common brushtail possum, is not achieved in vitro in media conditions that capacitate monodelphid and many eutherian sperm, including addition of protein sources and increased temperature (Mate and Rodger 1996). *Sminthopsis crassicaudata* spermatozoa flushed from the isthmus of the oviduct displaying T-shape orientation (Bedford and Breed 1994) were still incapable of fertilizing oocytes in vitro, suggesting that further capacitative modifications were necessary (Bedford and Breed 1994). Thus, different mechanisms may regulate capacitation in Australian and American marsupials.

Recent experiments using various oviduct epithelial cell culture and co-culture systems using possum and wallaby sperm are providing insights into capacitation in Australian marsupials. Sperm–oviduct epithelial cell monolayer cultures and culture supernatants have been shown to prolong the viability and

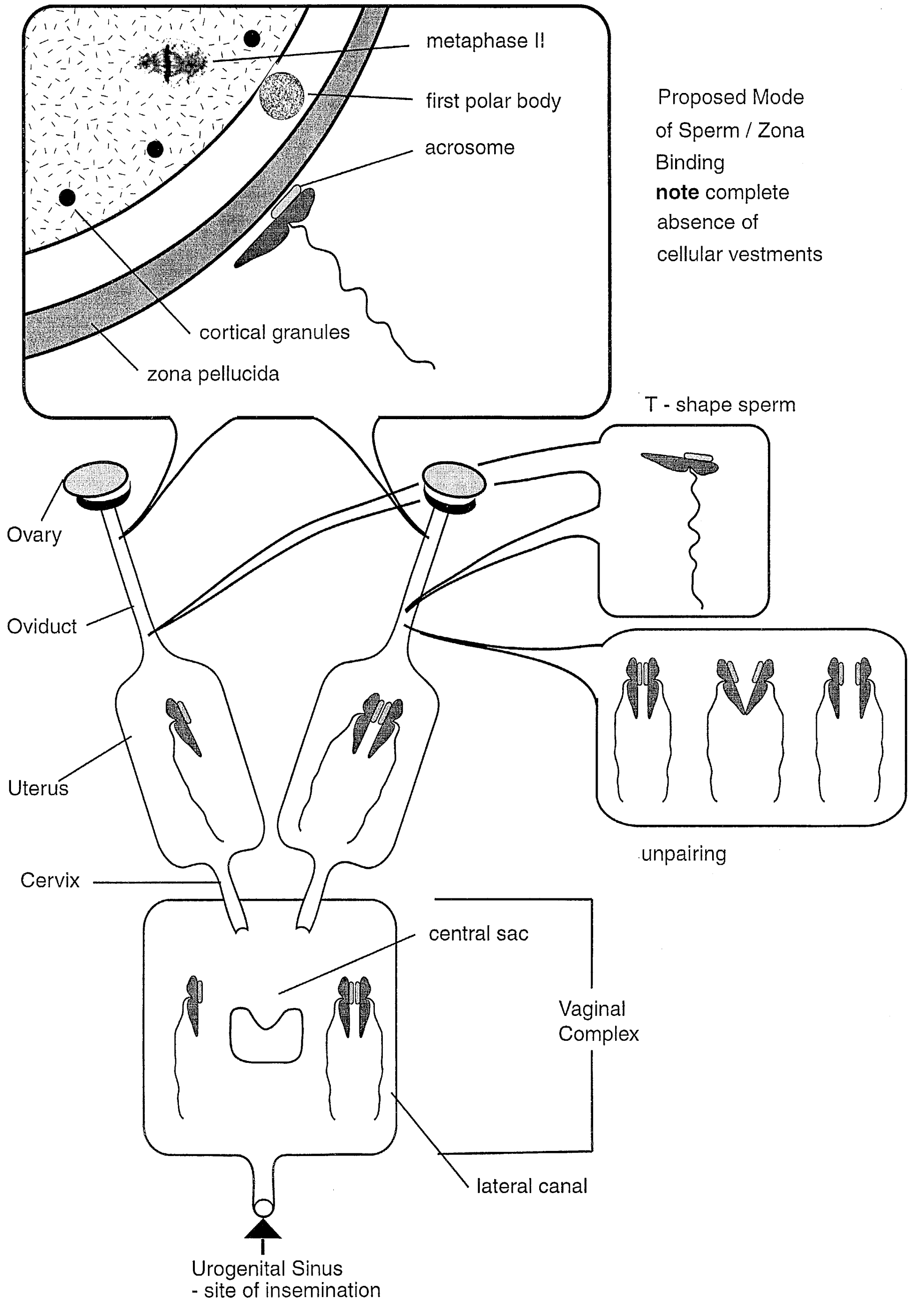

Fig. 6. Sperm transport, function and fertilization events in the marsupial female tract

motility of tammar wallaby and brushtail possum spermatozoa (Sidhu et al. 1998, 2000). Co-cultured spermatozoa attached to the ampulla and isthmus epithelial cells. Significantly, a percentage of these spermatozoa were transformed into the T-shape orientation upon release from the epithelium (10–15% wallaby, 60–70% possum). In IVF experiments using these in-vitro-produced T-shaped sperm, and in-vivo-capacitated T-shaped sperm flushed from the oviduct as a control, resulted in sperm binding to and penetration of the ZP (Mate et al. 2000). Thus, this study provides preliminary evidence for the necessity of sperm–oviduct epithelial cell interaction for capacitation in Australian species and lends further support to the suggestion that the T-shape head orientation is indicative of capacitation. Despite the occurrence of sperm–ZP binding and penetration, sperm–oocyte membrane fusion and oocyte activation were not observed in the IVF studies. Although it is possible that capacitation in these marsupials also involves sperm membrane alterations which facilitate sperm–oocyte membrane fusion it is more likely that cytoplasmic maturation and development of zona receptivity of the oocyte (discussed in ensuing sections) is a major factor.

Although the sperm of many eutherian species have been able to be capacitated in vitro using defined media and supplements, this has not been achieved for bats (Krutzsch and Crichton 1991). Perhaps some eutherian species also require unique interactions between the sperm and the oviduct and its products to achieve capacitation.

5.3 Monotremes

There have been no studies of sperm capacitation in monotremes. Phenotypically, monotreme spermatozoa more closely resemble those of reptiles and birds than their mammalian counterparts. As reptile and bird sperm do not require capacitation it is possible that monotreme spermatozoa also do not need to undergo maturational changes in the female reproductive tract (Bedford 1991). It is not known whether stabilizing factors/proteins are added to the monotreme sperm surface during epididymal maturation or ejaculation. However, prolonged storage of sperm in the female tract of preovulatory monotremes has been observed, suggesting that sperm are in some arrested state awaiting ovulation and thus presumably require a reactivation step equivalent to eutherian capacitation.

5.4 Common Themes and General Overview

The caudal isthmic oviduct is a site of sperm storage prior to fertilization in most eutherians and marsupials and probably monotremes. Sperm are stored in association with the oviduct epithelium in the majority of eutherians and in most marsupials other than dasyurids and didelphids. In these marsupials

specialized oviductal crypts store sperm, perhaps reducing the number of sperm lost in the female tract prior to fertilization.

Coincident with release from the oviduct epithelium, both marsupial and eutherian sperm display capacitation associated changes. Capacitation is a prerequisite of fertilization in both marsupials and eutherians and enables sperm to undergo a regulated acrosome reaction in response to oocyte signals and thus fertilize. In eutherians, but not marsupials, capacitation is also associated with hyperactivated motility. However, reorientation of the sperm head to a T-shape appears to be a unique characteristic of capacitated marsupial sperm. The sperm of many eutherians and monodelphid marsupials can be capacitated in vitro under relatively simple media and conditions. In contrast, capacitation in Australian marsupials apparently requires interaction with the oviduct epithelium. Whilst it is likely that monotreme spermatozoa are stored for prolonged periods in the female tract it is not known if they require capacitation prior to fertilization. It is possible, given their similarity to avian spermatozoa that they may be fertile after epididymal maturation.

The molecular modifications associated with capacitation have only been investigated in eutherian species but appear to involve modification of sperm surface proteins and lipids, including tyrosine phosphorylation of specific proteins.

6 Hyperactivation of Spermatozoa

6.1 Eutherians

Capacitation in eutherian species is accompanied by a distinct change in the pattern of sperm motility, termed hyperactivity (reviewed in Yanagimachi 1994). In vivo, hyperactivation occurs in the oviduct and sperm approaching and interacting with the oocyte display the hyperactive pattern of motility. Hyperactivity has been observed in vitro in a wide variety of eutherian species. Hyperactivated motility is characterized by high-amplitude flagellar bending and permits increased penetration of viscous fluids, such as those encountered in the oviduct. However, when placed into low-viscosity medium, hyperactivated sperm, although vigorous, are erratic and generally non-progressive. A number of biological roles have been proposed for hyperactivated motility including aiding sperm detachment from the oviduct epithelium (Demott and Suarez 1992; Suarez 1996). However, the relative roles of the extra force generated by hyperactivated motility, and membrane changes associated with capacitation, in mediating sperm detachment have not been assessed. The ability of the hyperactivated spermatozoon to better penetrate viscous oviductal fluid increases the cell's chance of reaching the oocyte. The frequent direction changes associated with this asymmetric flagellar bending pattern may also

help the sperm escape the grooves of the oviductal mucosa and increase its probability of encountering the oocyte (Suarez 1996). Hyperactivated sperm have also been observed to penetrate the zona far more effectively than progressively motile sperm, even when both had undergone the acrosome reaction (Stauss et al. 1995). Thus, the extra thrust generated by hyperactivated motility appears to be important for sperm penetration of the cumulus cells and zona in eutherian mammals (Bedford 1996).

In vitro studies have indicated that hyperactivity is a reversible event requiring a number of physiological factors including metabolic substrates, BSA and calcium, bicarbonate and potassium ions. Like other capacitation-related changes which enable the sperm to undergo a regulated acrosome reaction, hyperactivation appears to be activated via a signal transduction pathway involving cAMP-dependent phosphorylation of specific proteins via PKA (Si and Okuno 1999). Some of the proteins that are phosphorylated during capacitation and hyperactivation have been identified as A kinase anchor proteins which bind protein kinase A to the fibrous sheath (Carrera et al. 1996). The number of hyperactivated spermatozoa in the eutherian oviduct rises near the time of ovulation, suggesting that a specific oviductal signal may also be associated with hyperactivation in vivo (Cooper et al. 1979).

6.2 Marsupials and Monotremes

The resolution of whether marsupial or monotreme spermatozoa undergo any changes in motility associated with transport in the female reproductive tract or capacitation awaits thorough investigation of these processes in these species. Hyperactivity was not observed by Rodger and Bedford (1982) during in vitro fertilization in *Didelphis virginiana*, but Moore and Taggart (1993) suggested that hyperactivity was evident in unpaired *Monodelphis domestica* spermatozoa capacitated in vitro. However, these unpaired sperm exhibited only periods of erratic 'whiplash' tail movement with non-progressive motility after 4 h of incubation and may therefore represent degenerative effects. More recently, Taggart (1994) proposed that the sinusoidal or 'snake-like' sperm motility observed in the ejaculatory fluid of the dasyurid marsupial *Antechinus stuartii* (Taggart and Temple-Smith 1990) may be the marsupial equivalent of eutherian hyperactivation since it permits sperm penetration of viscous fluids.

6.3 Common Themes and General Overview

Marsupial sperm approaching and interacting with the oocyte have a T-shaped head, but no obvious change in motility or flagellar bending pattern similar to the hyperactivation observed in eutherian species. The extraflagellar force generated by hyperactivated motility appears to assist sperm to escape the

oviduct epithelium and to possibly aid penetration of the relatively thick eutherian zona. Such a mechanism would seem unnecessary in marsupial species in which large holes in the zona are induced by the acrosomal contents (Taggart et al. 1993).

7 The Acrosome

7.1 Mammalian Acrosome Morphology and Enzymes

The acrosome is the sperm organelle specialized for sperm binding to, and penetration of, the ZP (Fig. 7). In eutherians and monotremes the acrosome forms a cap-like structure over the anterior portion of the head. However, in marsupial spermatozoa the acrosome is restricted to the dorsal side of the nucleus (Rodger 1991). Nevertheless, in all species the acrosome is a species-specific, membrane-enclosed organelle containing an array of hydrolytic enzymes (Yanagimachi 1994). The eutherian sperm acrosome is a relatively fragile structure (Dudenhausen and Talbot 1982), while in marsupial sperm it is remarkably stable and resists a variety of disruptive treatments (Cummins 1980; Mate and Rodger 1991). This stability is apparently explained by disulphide stabilization of the acrosomal membranes and/or matrix (Mate et al. 1994; Setiadi et al. 1997).

7.2 Acrosome Reaction in Eutherians

7.2.1 Morphology and Functional Significance in Vivo

Only capacitated spermatozoa are capable of undergoing a regulated, physiological acrosome reaction in response to ZP binding, and only acrosome-reacted sperm can penetrate the zona and fuse with the oocyte. In eutherian sperm the acrosome reaction (AR) involves multiple-point fusions between the outer acrosomal membrane and the overlying plasmalemma, vesiculation and the exocytosis of the acrosomal contents (Fig. 7; Yanagimachi 1994). The hydrolytic acrosomal contents released by the AR have long been thought to facilitate the enzymatic digestion and subsequent penetration of the eutherian ZP. However, recent contradictory evidence such as the fertility of 'knockout' mice which do not produce the putative zona protease, acrosin, has brought into question the relative importance of zona digestion in eutherian fertilization (reviewed in Bedford 1999). The physical thrust generated by the hyperactivated sperm tail may be more important for ZP penetration in eutherian species (Bedford 1991). The acrosomal contents have been implicated in other roles including the development of the AR (Meizel 1984)

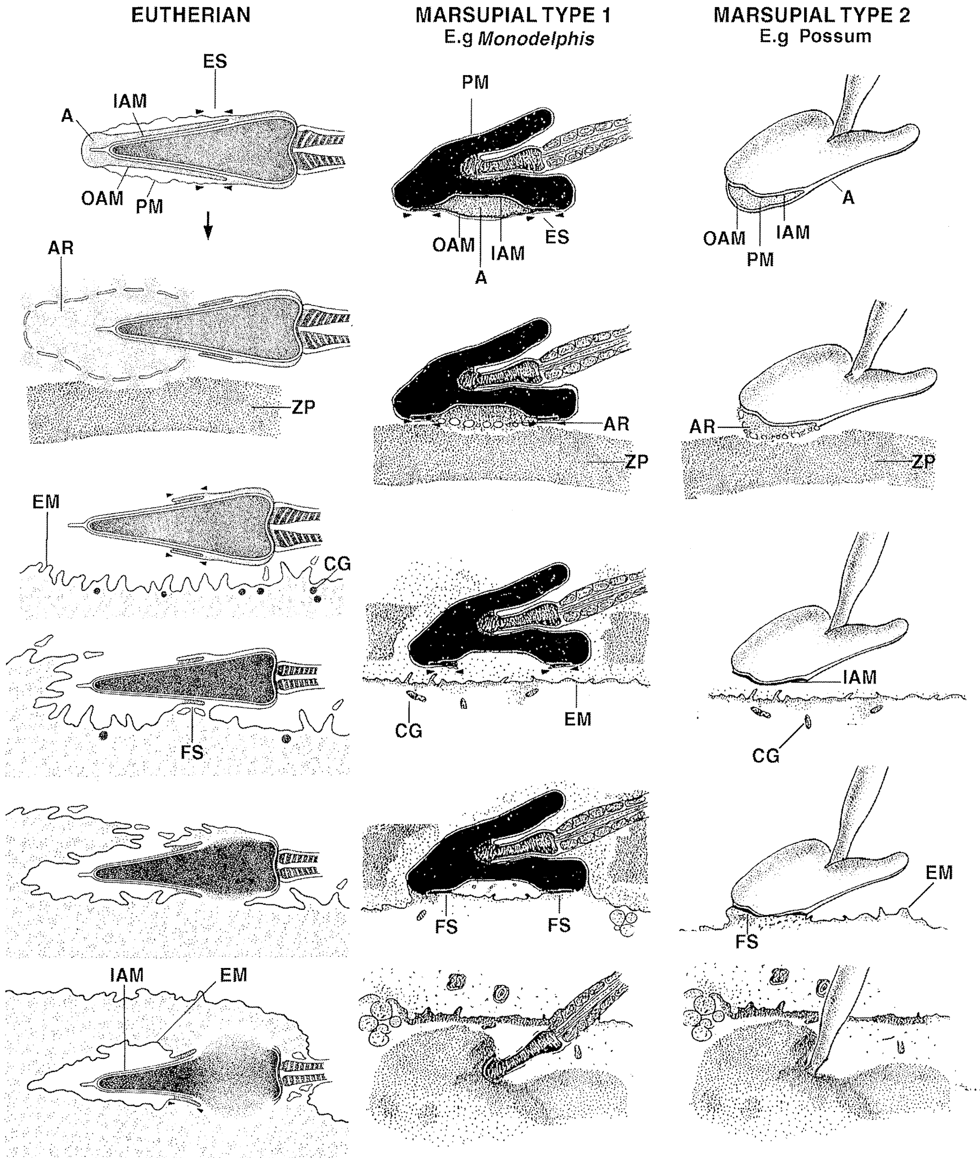

Fig. 7. Morphology of the mammalian acrosome reaction and sperm–egg fusion. *A* Acrosome; *PM* plasma membrane; *OAM* outer acrosomal membrane; *IAM* inner acrosomal membrane; *ES* equatorial segment; *ZP* zona pellucida; *EM* egg plasma membrane; *CG* cortical granules; *FS* sperm–egg fusion. (Adapted from Moore 1996 and Bedford 1982)

and mediating sperm secondary binding to the ZP (Topfer-Pederson and Henschen 1988).

In eutherians a specialized region of the plasma membrane (the equatorial domain) and outer acrosomal membrane are excluded from the acrosome reaction (Fig. 7). This equatorial region of the sperm surface subsequently mediates sperm interaction with the oocyte plasma membrane (Yanagimachi 1994). However, the acrosome reaction modifies the equatorial domain, inner acrosomal membrane and/or posterior head of the spermatozoa which facilitates attachment to the oocyte membrane (Sidhu and Guraya 1989). Mouse sperm PH-20, for example, migrates from the posterior head to the inner acrosomal membrane (Myles and Primakoff 1984).

7.2.2 Mechanism of the Acrosome Reaction in Vivo

A physiological acrosome reaction is induced by ZP3 when acrosome-intact sperm bind to the zona. ZP3 binding triggers the elevation of intracellular calcium ions essential for initiation of the AR. A number of signal transduction mechanisms have been proposed to mediate the AR, which may turn out to be dependent on multivalent interactions. Interaction of the sperm surface receptors with GI-proteins (ligand-receptor–GI-protein second messenger system) activates an Na^+/H^+ exchanger or Na^+-dependent Cl^-/HCO_3 exchanger leading to an increase in internal pH. Zona binding also depolarizes the membrane by activating a selective cation channel which produces a transient and focal elevation of intracellular calcium levels. Calcium channels on the outer acrosomal membrane are also opened by cAMP and contribute to the increase in Ca^{2+} concentration.

7.2.3 Mechanism of the Acrosome Reaction in Vitro

In addition to ZP3, a long list of physiological (e.g. progesterone, prostaglandins) and non-physiological (calcium ionophore, diacylglycerol) agents has been used to induce AR-like events in vitro in both epididymal and ejaculated eutherian spermatozoa (reviewed in Breitbart and Spungin 1997). However, the mechanisms underlying AR induction by the physiological inducers are not clearly understood and the putative sperm receptors are unidentified. Non-physiological inducers such as calcium ionophore and diacylglycerols (DAG) can be used to effectively bypass the receptor-mediated signalling pathways described above and explore the down stream mechanisms involved in AR induction.

7.3 Acrosome Reaction in Marsupials and Monotremes

There is no information about the acrosome reaction in monotreme spermatozoa, though it is undoubtedly necessary to facilitate ZP penetration. The physiological acrosome reaction in *Monodelphis* has been shown to involve multiple point fusions as in eutherians (Taggart et al. 1993). Similar multiple point fusions are induced by the diacylglycerol DiC8 and arachidonic acid in brushtail possum and tammar wallaby spermatozoa (Sistina et al. 1993; Sistina and Rodger 1998) suggesting that similar acrosome reaction mechanisms function in marsupials and eutherians. A major difference between the acrosomes of marsupial and eutherian sperm is that marsupial sperm are unresponsive to calcium ionophores and that diacylglycerol-induced AR occurs in the absence of extracellular calcium, suggesting a role for internal calcium stores (Mate and Rodger 1993). A region of the acrosome equivalent to the eutherian equatorial segment has been observed to persist in *Monodelphis domestica* and *Sminthopsis crassicaudata* sperm penetrating the zona (Taggart et al. 1993; Breed 1994b). This equatorial segment-like region subsequently appeared to participate in sperm–oocyte interaction. However, no region of the brushtail possum or tammar wallaby acrosome remained intact after AR induction by diacylglycerol or arachidonic acid treatment in vitro (Sistina et al. 1993; Sistina and Rodger 1998) or in possum sperm interacting with oocytes in vivo (Jungnickel et al. 1999).

7.4 Common Themes and General Overview

The acrosome is similar in all mammalian species in that it is a membrane-bounded organelle containing a variety of hydrolytic enzymes. However, characteristic differences in the stability of the marsupial versus the eutherian acrosome exist. The physiological significance of this difference is not known. The acrosome reaction facilitates sperm penetration of the zona in all mammalian spermatozoa. In eutherian species, and some marsupials, an unreacted equatorial region persists after the AR to participate in sperm–oocyte interaction, but no equivalent equatorial region has been observed in diacylglycerol-treated wallaby and possum spermatozoa or during in vivo fertilization in the possum. It is possible that two different AR systems exist in marsupials (Fig. 7; Mate and Rodger 1996).

8 Sperm Interaction with the Zona Pellucida

8.1 Eutherians

8.1.1 Structure and Function of the ZP

All growing oocytes, ovulated oocytes and pre-implantation embryos are surrounded by a ZP, an extracellular coat responsible for vital functions during the fertilization process including specific binding of spermatozoa, induction of the acrosome reaction, prevention of polyspermy and physical protection of the embryo.

The ZP of eutherian mammals consists of three sulphated glycoproteins (designated ZP1, ZP2 and ZP3) that serve specific functions during fertilization and early development, which are well defined in the mouse. ZP1 is a structural protein that cross-links filaments made of ZP2 and ZP3 heterodimers (Greve and Wassarman 1985; reviewed by Green 1997). During fertilization sperm initially bind to ZP3 (Bleil and Wassarman 1980), which induces the sperm acrosome reaction (Leyton and Saling 1989; Ward and Kopf 1993; Tomes et al. 1996). After induction of the acrosome reaction, ZP2 acts as a secondary sperm receptor (Bleil et al. 1988) which is necessary for penetration of the sperm through the ZP. Following fertilization the proteolytic cleavage of ZP2 and modification of ZP3 are thought to be involved in the prevention of polyspermy and protection of the early embryo.

Eutherian *ZP* genes are expressed primarily in the oocytes (reviewed by Liang and Dean 1993) and also in the granulosa cells of some species (Grootenhuis et al. 1996). The ZP genes are conserved among diverse vertebrate species (Fig. 8). The *ZP3* gene is the most highly conserved of the three with amino acid identities between eutherian mammals (human, mouse, rabbit, pig) ranging from 66 to 74% (Fig. 9; reviewed by Epifano and Dean 1994). The *ZP2* gene is also well conserved with amino acid identities between eutherian mammals (human, mouse, rabbit, pig) ranging from 54 to 72% (Fig. 9; Epifano and Dean 1994). Although the amino acid sequence of the ZP proteins is conserved between species, variations in the post-translational modifications of the ZP proteins result in significant differences in the molecular weights of the proteins between species (reviewed by Dunbar et al. 1991; Castle and Dean 1996; McLeskey 1998). These extensive post-translational modifications, which include glycosylation and sulphation, are critical to the structural and functional integrity of the ZP (Greve et al. 1982; Salzmann et al. 1983). In addition to the conservation of ZP proteins between species, specific protein domains are conserved among the three classes of zona proteins. A 277-amino acid domain exists in mouse ZP1 and ZP2 in which the two polypeptides are 47% similar and 34% identical (Epifano et al. 1995).

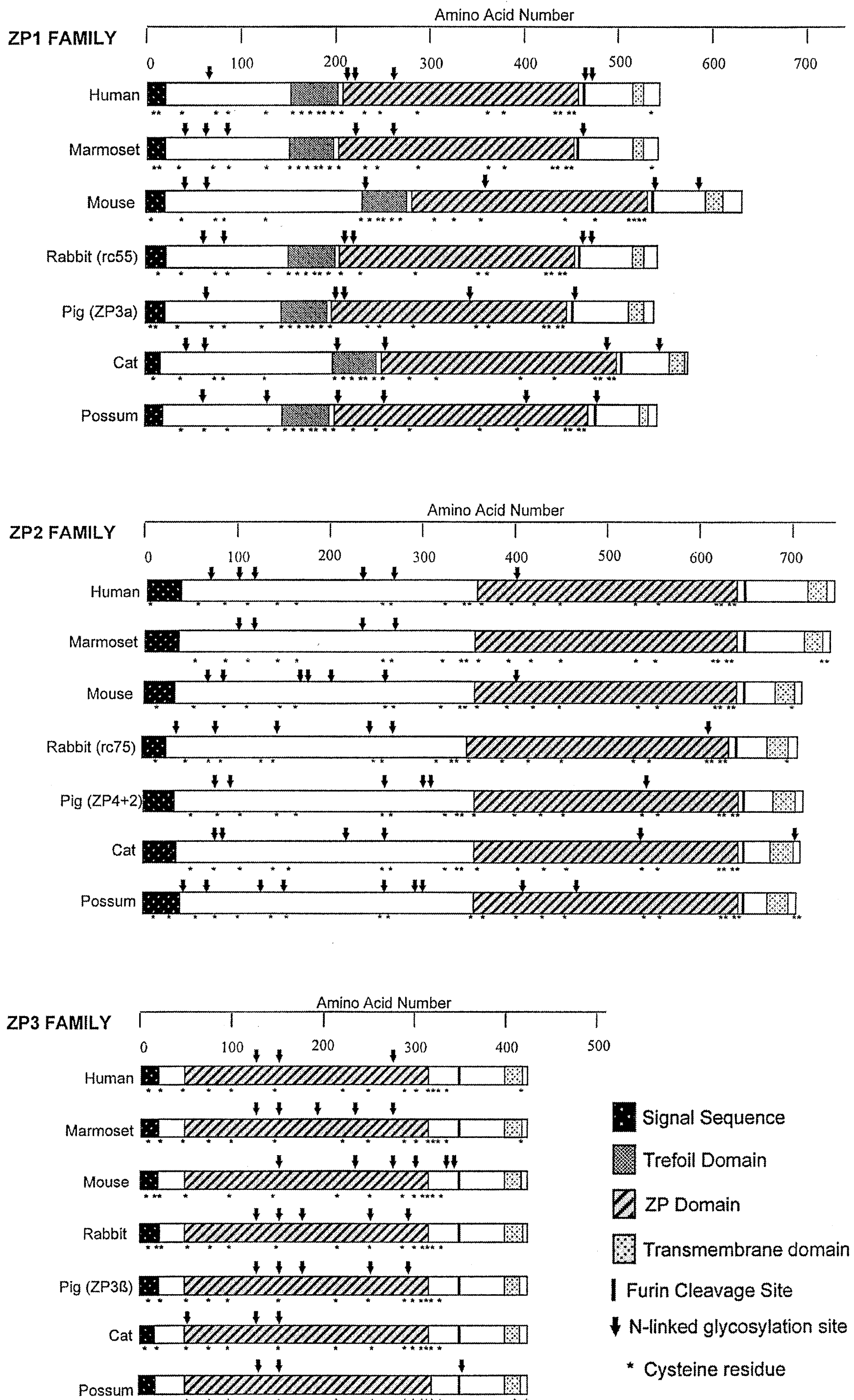

Fig. 8. Structure of the zona pellucida 1 (*ZP1*), 2 (*ZP2*) and 3 (*ZP3*) protein families from a representative group of mammals. (Redrawn from McLesky et al. 1998)

ZP1 FAMILY		% Nucleic Acid Identity						
		Human	Marmoset	Mouse	Rabbit	Pig	Cat	Possum
% Amino Acid Identity	Human		91	40	79	79	74	64
	Marmoset	87		48	77	75	76	64
	Mouse	32	39		39	41	43	48
	Rabbit	73	68	30		78	79	64
	Pig	72	70	33	71		75	46
	Cat	67	68	33	64	67		62
	Possum	54	53	40	54	30	53	

ZP2 FAMILY		% Nucleic Acid Identity						
		Human	Marmoset	Mouse	Rabbit	Pig	Cat	Possum
% Amino Acid Identity	Human		92	69	77	75	78	63
	Marmoset	86		72	77	77	78	62
	Mouse	57	59		73	68	70	60
	Rabbit	68	68	62		76	79	65
	Pig	63	63	55	64		82	62
	Cat	67	67	58	66	72		63
	Possum	51	50	48	54	50	51	

ZP3 FAMILY		% Nucleic Acid Identity						
		Human	Marmoset	Mouse	Rabbit	Pig	Cat	Possum
% Amino Acid Identity	Human		92	72	70	78	78	52
	Marmoset	91		71	70	76	76	54
	Mouse	67	67		64	69	71	52
	Rabbit	69	68	65		69	69	56
	Pig	74	72	66	68		80	55
	Cat	64	68	64	65	73		54
	Possum	47	46	46	47	47	46	

Fig. 9. Zona pellucida family cDNA and amino acid identities

8.1.2 Primary Sperm–ZP Binding

Ultrastructural Observations. Primary sperm–ZP binding is a rapid transitional event, and it is very rare to observe the fertilizing spermatozoon at the point of initial contact with the ZP, as penetration follows almost immediately. Saling et al. (1979) observed that mouse sperm bound to the outside of the ZP were acrosome intact; however, changes observed in the structure of the acrosomal cap of rat and rabbit sperm bound to the ZP (Esaguy et al. 1988; Shalgi et al. 1989) suggest that there may be some leakage of acrosomal enzymes to aid progress through the cumulus (a complex of former follicular cells in an acellular matrix) enclosing the ZP and oocyte.

Molecular Mechanisms. Acrosome-intact mouse sperm bind to the ZP via O-linked oligosaccharide residues that are located on a cluster of serine residues near the carboxy terminus of the ZP3 glycoprotein (Litscher and Wassarman 1996). Evidence suggests that a terminal α-galactose and β-N-acetylglucosamine, as well as the presence of a fucosyl residue, are important functional components of these oligosaccharides (Bleil and Wassarman 1988; Miller et al. 1993; Johnston et al. 1998). The importance of heterogeneity in the post-translational glycosylation of the ZP proteins for specificity of sperm binding is well illustrated by the restoration of fertility in Zp3 null mice by breeding with transgenic mice expressing human ZP3 (Rankin et al. 1998). The resultant chimeric ZP consisting of mouse ZP1, mouse ZP2 and human ZP3 did not bind human sperm but bound mouse sperm, resulting in restoration of fertility.

Identification of a single complementary ZP3-binding protein on the sperm surface has been more difficult and remains controversial, but candidates include (1) zonadhesin, a large membrane protein containing multiple types of domains (Gao and Garbers 1998), and (2) spermadhesins, a family of small sperm-associated proteins, which bind solubilized ZP (Topfer-Petersen and Calvete 1995). Reviews of the molecules involved in sperm–oocyte interaction in eutherian mammals can be found in McLesky et al. (1998) and Wassarman (1999).

8.1.3 Penetration of the ZP and Secondary Sperm–ZP Binding

Ultrastructural Observations. The eutherian spermatozoon undergoes the acrosome reaction soon after binding to the ZP, releasing its acrosomal contents at the ZP surface. The penetrating sperm leaves a narrow, sharply defined penetration slit in the ZP which appears more indicative of a mechanical cutting action than enzymatic digestion (Bedford 1998). Also supporting this view are ultrastructural observations of distortion and displacement of the zona's fibrous matrix around the head of the penetrating sperm (Phillips 1991). The relative contribution of mechanical versus enzymatic penetration of the

ZP in eutherian mammals was reviewed by Yanagimachi (1994), who suggested that acrosin and perhaps other acrosomal enzymes may be important for binding of the acrosome-reacted sperm and insertion of the sperm head into the ZP matrix. When the sperm head is within the zona, acrosomal enzymes may no longer be required and mechanical forces may be sufficient for progression of the sperm through the ZP.

Molecular Mechanisms. Sperm binding to ZP3 initiates the acrosome reaction, a fusion of the sperm plasma membrane and outer acrosomal membrane resulting in the release of hydrolytic enzymes. Following the AR, a secondary binding event takes place which again adheres the sperm to the ZP and facilitates transit through the ZP. Candidate secondary ZP binding proteins on sperm have been reviewed recently by McLesky et al. (1998) and include: (1) PH-20, a glycosyl phosphatidylinositol-anchored membrane protein located on the post-acrosomal surface of the sperm head as well as within the acrosome (Phelps et al. 1988), which has both ZP-binding and hyaluronidase activity (Primakoff et al. 1985; Hunnicutt et al. 1996); (2) proacrosin, which is located within the acrosome of all mammalian sperm and has ZP-binding and proteolytic properties (Jones and Brown 1987); and (3) Sp17, a 17-kDa protein that becomes exposed during the AR and migrates posteriorly to the equatorial region of the head of acrosome-reacted sperm (Richardson et al. 1994).

8.2 Monotremes

The ZP of the platypus reaches a maximum thickness of approximately 11 μm at the onset of vitellogenesis but is less than 0.5 μm in the preovulatory oocyte (Hughes 1977). There is no information about sperm–ZP interaction in monotremes. It has been suggested that the filiform sperm head and modest acrosome of echidna sperm may be related to the fragile nature of the ZP (Bedford and Rifken 1979).

8.3 Marsupials

8.3.1 Structure and Function of the ZP

The marsupial ZP is thought to perform similar functions to that of eutherian mammals but is considerably thinner, varying from 6 to 8 μm in the brushtail possum (*Trichosurus vulpecula*) and tammar wallaby (*Macropus eugenii*) (Tyndale-Biscoe and Renfree 1987; Mate 1998) to only 1–2 μm in the didelphid marsupials (Talbot and DiCarlantonio 1984; Falconnier and Kress 1992). The marsupial ZP also appears to be more easily digested than the zona of eutherian oocytes (Rodger and Young 1981; Baggott and Moore 1990; Rodger

1991). Like that of eutherians, the marsupial ZP is composed of a matrix of three proteins which have approximately 50% homology to eutherian ZP1, ZP2 and ZP3 (Fig. 9; Mate and McCartney 1998; Haines et al. 1999; McCartney and Mate 1999) and are antigenically related to porcine ZP (Mate 1998). Although the homology of the marsupial ZP genes to their eutherian counterparts is relatively low, the structure of the ZP proteins is highly conserved (Fig. 8).

There is evidence of maturational changes to the ZP, affecting both structure and function, during the latter stages of follicular development and even following ovulation in some marsupial species. There are prominent structural differences between the ZP of follicular and ovulated oocytes from the brushtail possum and *Monodelphis* (Mate 1996). The ZP surrounding follicular oocytes in these species is a wide diffuse structure, but in ovulated oocytes it is narrow and compact (Mate 1996). These periovulatory changes are suggestive of a physical compression of the ZP, perhaps as a consequence of late preovulatory growth of the oocyte "stretching" the ZP. An in vitro fertilization study of *Monodelphis* suggests that the ZP also undergoes maturational changes vital for acquisition of sperm-binding ability; germinal vesicle (GV)-stage oocytes collected within 15 h after mating were incapable of binding sperm; however , PB1-stage oocytes collected 15–18 h after mating were capable of sperm–ZP binding and penetration (Moore and Taggart 1993). As secretion of the ZP proteins and formation of the ZP is essentially complete by this stage of follicular development, it is likely that these functional changes in the ZP are due to modifications to the carbohydrate residues on the ZP proteins.

8.3.2
Sperm Interaction with the ZP

Marsupial oocytes are ovulated free of any surrounding granulosa cells; thus a naked ZP forms the only barrier to the fertilizing spermatozoon. Our current knowledge of sperm–ZP interaction in marsupials is limited to ultrastructural observations, but suggests that quite different modes of zona penetration may operate. Prior to binding to the ZP, marsupial sperm undergo reorientation of the sperm head to form a T-shape so that the flat surface of the intact acrosome can be applied to the ZP (Bedford and Breed 1994; Molinia et al. 1998) perhaps to maximize the subsequent effects of acrosomal enzymes on the ZP. *Monodelphis* spermatozoa have been observed to undergo the acrosome reaction at the zona surface, suggesting that it may be triggered by zona components (Taggart et al. 1993).

In the opossums *Didelphis* and *Monodelphis* (Rodger and Bedford 1982; Taggart et al. 1993) and the brushtail possum (Jungnickel et al. 1999; Mate et al. 2000), a large hole is digested in the ZP as a result of the AR. However, in *Sminthopsis*, a narrow penetration slit is formed by the penetrating sperm, and electron micrographs suggest the involvement of both physical pressure and

localized enzymic digestion in penetration of the ZP (Breed and Leigh 1988, 1992; Breed 1994a).

8.4 Common Themes and General Overview

Despite significant variation in the thickness of the ZP amongst the three mammalian groups, its structural components are well conserved. The eutherian and marsupial ZP proteins share several structural features including short 5′ and 3′ untranslated regions, N-terminal hydrophobic signal peptides, potential and O-linked glycosylation, a hydrophobic transmembrane domain near the C terminus, a furin or furin-related cleavage site upstream of the transmembrane domain and a ZP signature domain. As most of these structural features are also present in non-mammalian vertebrate ZP proteins, it is likely that they are also conserved in monotremes. However, our current understanding of the function of the ZP glycoproteins is based almost entirely on the mouse and may not be representative of gamete interactions in all eutherian mammals, marsupials or monotremes.

It appears that in some marsupials at least, the completion of oocyte maturation and acquisition of fertilizability, including changes to the ZP, may not occur until late in the periovulatory period. In contrast, the ZP appears penetrable in eutherian GV oocytes as well as in subsequent stages of preovulatory maturation (reviewed by Bedford 1991).

9 Sperm–Oocyte Fusion

9.1 Eutherians

Only acrosome-reacted spermatozoa are capable of fusing with the oocyte. Sperm binding and fusion with the oocyte plasma membrane are independent events which, together, result in the formation of the zygote. In eutherian mammals, sperm binding and subsequent membrane fusion is initiated in the equatorial region (Yanagimachi 1994). The stabilized inner acrosomal membrane is excluded from the fusion process and is instead encapsulated in a vacuole within the oocyte cytoplasm (Bedford 1991). In species in which the tail is incorporated into the vitellus, membrane fusion spreads posteriorly to the flagellar membrane and the sperm–oocyte membranes form a continuum.

The sperm proteins proposed to participate in sperm–oocyte binding and fusion include DE/AEG (Rochwerger at al. 1992) and fertilin (PH-30) (Primakoff et al. 1987). DE/AEG is an epididymal maturation antigen that migrates from the post-acrosomal region to the equatorial region during capacitation (Rochwerger and Cuasnicu 1992). Antibodies raised against

DE/AEG inhibit sperm–oocyte fusion (Rochwerger at al. 1992). Fertilin arises in the testis and undergoes endoproteolytic cleavage during testicular and epididymal maturation. Both the a and b subunits of this heterodimeric protein belong to a family of proteins containing a disintegrin and metalloprotease domain (ADAM) (Wolfsberg et al. 1995). Both the a and b subunits bind to the oocyte plasmalemma via their disintegrin domains through an interaction with integrins on the oolema (Myles et al. 1994). A second ADAM protein, cyritestin, functions with fertilin b in mediating sperm–oocyte plasma membrane adhesion leading to fusion (Yuan et al. 1997).

9.2 Monotremes

The only relevant information concerning monotremes is that the oolemma of monotreme oocytes has an abundance of microvilli (Hughes and Carrick 1978).

9.3 Marsupials

As discussed earlier, a narrow peripheral region of the *Monodelphis* sperm acrosome remains intact following the acrosome reaction, suggesting that it may be structurally and functionally similar to the stabilized equatorial region of eutherian spermatozoa (Taggart et al. 1993). Ultrastructural analysis of fertilization in vitro in *Monodelphis* indicates that sperm fusion with the oolemma occurs via this persistent region of the acrosome (Taggart et al. 1993), suggesting that it is the sperm plasma membrane overlying this region of the acrosome that actually fuses with the oolemma. An earlier study of fertilization in *Didelphis* suggested that it was the inner acrosomal membrane of the acrosome-reacted spermatozoon that fused with the oolemma. This conclusion was based on light microscopy observations and electron microscopy which indicated that there was no evidence of incorporated oocyte or sperm membranes surrounding the decondensing sperm head (Rodger and Bedford 1982). This is an important point because fertilization in eutherians is not only distinguished by the role of the equatorial segment as the site of sperm–oocyte membrane fusion but by the unusual phagocytic-like mode of sperm engulfment by the oocyte. Although an equatorial segment-like region may be present in the acrosomes of some marsupials it does not necessarily follow that the sperm is incorporated in a manner similar to that of eutherians.

9.4 Common Themes and General Overview

Until recently it was thought that the mechanism of sperm–oocyte fusion in eutherian mammals, involving the plasma membrane over the equatorial

segment of the acrosome, was unique to this group. It was hypothesized that this departure from the conventional mode of fusion (via the inner acrosomal membrane of the sperm head), observed in all non-mammalian vertebrates and invertebrates, evolved in eutherian mammals from the need to stabilize the inner acrosomal membrane to withstand penetration through a relatively thick and resilient ZP (Moore and Bedford 1983; Bedford 1991). The observation that sperm–oocyte fusion in *Monodelphis* also occurs via the plasma membrane over an equatorial segment-like region (Taggart et al. 1993) indicates that this process in marsupials may be more akin to the eutherian situation (Fig. 7) than the lower vertebrates.

In contrast to *Monodelphis*, there is no evidence that a region of the acrosome persists after the acrosome reaction in brushtail possum and tammar wallaby sperm (Sistina et al. 1993; Sistina and Rodger 1998; Jungnickel et al. 1999), suggesting that there may be significant differences between marsupial species in the sperm membranes involved in fusion with the oolemma (see types 1 and 2 in Fig. 7). Observations of sperm–oocyte binding, fusion and incorporation in macropods and possums are required to establish definitively which region of the spermatozoon fuses with the oocyte so that a representative model(s) of marsupial sperm–oocyte fusion can be established.

10 Oocyte Activation

10.1 Eutherians

The fusion of sperm and oocyte triggers a cascade of cellular events in the oocyte that result in the exocytosis of cortical granules, recruitment and translation of maternal mRNAs and the resumption of meiosis known as activation. Many of the events of oocyte activation are triggered by a transient increase in intracellular calcium – $[Ca^{2+}]_I$ – at the time of fertilization. In the mouse there is a large initial increase in $[Ca^{2+}]_I$ followed by a series of cytosolic Ca^{2+} oscillations which last for several hours after sperm–oocyte interaction (reviewed by Ben-Yosef and Shalgi 1998). Unlike fish and amphibians, the intracellular pH of eutherian oocytes is not affected by fertilization and thus is not associated with activation (Kline and Zagray 1995; Ben-Yosef et al. 1996). Two pathways in the cascade of events leading to oocyte activation can be segregated: early events (including the cortical reaction) can be triggered by PKC which is activated by the initial rise in Ca^{2+}, whereas later events (such as resumption of meiosis) require higher Ca^{2+} elevation but are PKC independent (Raz et al. 1998a).

The increase in $[Ca^{2+}]_I$ is generated by IP_3-mediated release of Ca from intracellular stores (Miyazaki et al. 1992) but also requires Ca^{2+} entry from outside the oocyte to refill the Ca^{2+} pools and maintain Ca^{2+} oscillations (Miyazaki 1995). It is therefore likely that PLC is activated during fertilization as it is the

enzyme responsible for hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) to IP_3 and DAG, leading to activation of PKC. One of the ways that the fertilizing sperm could initiate these events is via binding of a sperm ligand with a receptor on the oolemma and subsequent activation of PLC. It has been demonstrated in the mouse that this pathway does not involve G proteins (Williams et al. 1998) and therefore most probably involves receptor tyrosine kinases which are known to be involved in invertebrate oocyte activation. Another possibility is that activation of the oocyte may be initiated by a sperm factor(s) that is introduced into the oocyte during sperm–oocyte fusion. The candidates for this role include a component of the sperm cytosol (Wolny et al. 1999), and a relatively insoluble sperm head component(s) known as SOAF (Perry et al. 1999).

Development of activation competence by the oocyte occurs gradually after GV breakdown (Ducibella et al. 1990; Ducibella and Buetow 1994; Wang et al. 1997; Raz et al. 1998b) concomitant with migration of cortical granules from a subcortical to cortical location (Cran and Cheng 1985), accumulation of clusters of endoplasmic reticulum in the oocyte cortex (Ducibella et al. 1988) and a four-fold increase in the intracellular stores of Ca^{2+} (Tombes et al. 1992). Maximal activation competence is not fully developed until MII (Wang et al. 1997), and this suggests that there is likely to be a temporal window in which normal oocytes have the highest likelihood of undergoing a normal cortical reaction and activation of development (reviewed by Ducibella 1996). The general pattern for eutherian mammals is that oocytes are ovulated and fertilized at MII of meiosis. Fertilization reactivates meiosis and a second polar body is produced. A major exception to this rule is found in wild and domestic canids. Canid oocytes are ovulated with a germinal vesicle in meiotic arrest. The first meiotic division occurs in the oviduct around 2 days later and then meiosis arrests, awaiting fertilization (Mahi-Brown 1991).

10.2 Monotremes

The fully grown monotreme oocyte is surrounded by a follicular epithelium that is two cell layers thick. The follicle cells contain secretion granules that contain the precursors of a fluid which forms a continuous layer around the oocyte at the time of maturation. Flynn and Hill (1939) suggested that this secretion is the homologue of the follicular fluid of the Graafian follicle of the eutherians and marsupials. The first polar body is extruded from the monotreme oocyte before ovulation and like most of the mammals it is fertilized at MII of meiosis. Activation of the monotreme oocyte following fertilization results in the resumption of meiosis and the extrusion of the second polar body (reviewed by Griffiths 1968, 1978).

10.3
Marsupials

Activation of marsupial oocytes following fertilization is marked by the resumption of meiosis and extrusion of the second polar body (Rodger and Bedford 1982; Selwood 1982; Breed and Leigh 1990; Taggart et al. 1993). A protrusion of oocyte cytoplasm through the hole created in the ZP by the fertilizing sperm, somewhat reminiscent of a fertilization cone in invertebrates, has been observed in vitro in *Didelphis* and *Monodelphis* (Rodger and Bedford 1982; Moore and Taggart 1993; Taggart et al. 1993). However, the cytoplasmic protrusion in these oocytes did not display the organization of filamentous actin typical of fertilization cones in lower vertebrates (Schatten and Schatten 1987), suggesting that it is most likely an artefact of in vitro fertilization and the absence of the oviduct-deposited mucoid coat.

There is some ambiguity regarding the association of cortical granule exocytosis with activation in marsupials as an absence of cortical granules has been observed in both unfertilized and fertilized oocytes recovered from the oviducts of *Monodelphis* (Taggart et al. 1993) and brushtail possum (Jungnickel et al. 1999).

10.4
Common Themes and General Overview

Fusion of the membranes of sperm and oocyte sets off a chain of events which has a profoundly stimulatory effect on the oocyte. Although little is known about these events and their regulation in monotremes and marsupials the general pattern appears likely to be similar in all mammals. In eutherians activation is associated with a transient increase in intracellular calcium and it will be very surprising if similar events do not occur in the oocytes of marsupials and monotremes. A common feature of eutherian and marsupial, and presumably monotreme, oocytes is that the immature oocyte is not competent to undergo activation. This competence appears to develop gradually in the hours following GV breakdown. Even in canid oocytes which are ovulated at the GV stage fertilization appears not to occur until these cytoplasmic maturation events and first meiotic division are completed.

11
Cortical Reaction and Block to Polyspermy

11.1
Eutherians

The major block to polyspermy is provided by alterations to the ZP although there is at least some role played by the plasma membrane in some species (e.g.

rabbit; O'Rand and Nikolajczyk 1991). The ZP of eutherian oocytes becomes "hardened" following cortical granule release induced by sperm–oocyte fusion (reviewed by Ducibella 1996).

11.1.1 Cortical Granules

Cortical granules (CG) are small membrane-bound vesicles (0.2–0.6 µm in diameter, depending upon the species) located directly beneath the plasma membrane of mature unfertilized eutherian oocytes. Their formation in eutherian mammals is related to the onset of follicular growth (Gulyas 1980) and is associated with many active Golgi complexes in the peripheral cytoplasm of the oocyte (Zamboni 1970). Condensation and accumulation of the luminal contents of the fusing vesicles results in formation of the mature dense granules (Cran and Moor 1990) Migration of cortical granules to their final position just below the oocyte membrane occurs around the same time as the resumption of meiosis (Cran and Moor 1990). A marked polarity develops in the distribution of cortical granules after extrusion of the first polar body and formation of the second meiotic spindle; in the mouse and hamster up to 24% of the total cortex of the oocyte in the area of the second meiotic spindle is totally devoid of cortical granules (Cran 1989; Ducibella et al. 1990).

11.1.2 Cortical Reaction

Initiation of the cortical reaction is correlated with an increase in intracellular Ca^{2+} concentration and activation of protein kinase C, resulting in a block to polyspermy within 10 min of fertilization (reviewed by Ducibella 1996). A cortical granule protease released into the perivitelline space during the cortical reaction partially hydrolyses ZP2 (120 kDa), the secondary ZP ligand that preferentially binds acrosome-reacted spermatozoa to a 90-kDa form known as $ZP2_f$ (Bleil et al. 1981). The smaller peptides generated during this process remain covalently linked to the glycoprotein by intramolecular disulphide bonds (Bleil et al. 1981). ZP3 is also altered so that sperm are no longer able to bind or penetrate the ZP, thus establishing a barrier to supernumerary spermatozoa (Wassarman et al. 1985). Although the physical and chemical characteristics of ZP3 and $ZP3_f$ are virtually indistinguishable, $ZP3_f$ represents an inactive form of ZP3 perhaps due to modification of terminal carbohydrate residues (Aviles et al. 1997).

11.2 Monotremes

Shortly after fertilization, during passage through the oviduct, monotreme oocytes become surrounded by two additional coats. The mucoid coat and

oocyte shell of monotremes are tertiary oocyte membranes, typical of the cleidoic oocytes of sauropsid vertebrates. The thickness of the mucoid coat of the echidna oocyte varies from 17 to 30 μm (Flynn 1930) and is composed of sulphated acidic glycoprotein derived from membrane-bound precursors within the cytoplasm of non-ciliated oviduct epithelial cells (reviewed by Hughes and Carrick 1978). There is no information on the functional role of the mucoid layer in monotremes; however, sperm have been observed trapped in the mucoid (Griffiths 1968) suggesting that it may play a role in the block to polyspermy. Cortical granules have not been observed in monotreme oocytes.

11.3 Marsupials

11.3.1 Cortical Granules

The cortical cytoplasm of marsupial oocytes contains spherical membrane-bound organelles with similar morphology to eutherian cortical granules (CG) (Breed and Leigh 1992; Mate et al. 1992). Their size of 0.2–0.4 μm diameter is comparable to pig and rabbit CG (Oh and Brackett 1975; Cran and Cheng 1985) but slightly smaller than most eutherian CG which generally range from 0.2 to 0.8 μm in diameter (Sathananthan and Trounson 1982; Cherr et al. 1988; Ducibella et al. 1988).

Formation of CG in the marsupial species studied thus far is closely associated with the Golgi apparatus and smooth endoplasmic reticulum (SER) (Mate et al. 1992), similar to that described in long-cycling domestic species (Cran and Cheng 1985; Hyttel et al. 1986). The timing of CG formation in some marsupial species represents a significant departure from the timing of CG formation in eutherian mammals. An apparently full complement of mature peripherally positioned CG is only observed in ovulated oocytes which have undergone the first meiotic division in the brushtail possum, tammar wallaby and grey short-tailed opossum (Falconnier and Kress 1992; Mate et al. 1992). In contrast, preovulatory oocytes from *Didelphis virginiana* contain a large number of CG indicating that the timing of their formation differs among different marsupial species. Cortical granules have also been observed in germinal vesicle stage oocytes from *Sminthopsis crassicaudata* (Breed and Leigh 1990, 1992; Breed 1994a), the brush-tailed bettong and northern short-nosed bandicoot (Breed 1994b), although there was no indication of their number or distribution.

11.3.2 Cortical Reaction

The efficiency of the cortical granule-induced block to polyspermy in marsupials has been questioned because of the large hole that the penetrating spermatozoa leaves in the zona in the opossum (Rodger and Bedford 1982); the occasional observation of polyspermy in *Antechinus* (Selwood 1982) and *Sminthopsis* (Breed and Leigh 1990) *in vivo*; and multiple sperm penetration of *Didelphis* and *Monodelphis* oocytes during *in vitro* culture (Rodger and Bedford 1982; Moore and Taggart 1993). The block to polyspermy in both *Monodelphis* and *Didelphis* appears to be efficient *in vivo*, with all supernumerary sperm restricted to the surface of the ZP or within the mucoid layer, secreted by the oviduct. Mucoid deposition prevents sperm from making contact with the ZP and may form the most important block to polyspermy *in vivo* as it is closely correlated temporally with the early stages of gamete interaction and penetration (Rodger and Bedford 1982; Baggott et al. 1987; Breed and Leigh 1990). Certainly, the mucoid layer traps a large number of sperm, which are clearly visible under phase contrast.

There is evidence, however, that the cortical reaction does play a role in the block to polyspermy in marsupials. The fusion of the membrane of cortical granules with the oolemma and exocytosis of the cortical granule contents have been described in *Sminthopsis* (Breed 1994b). Also, fertilized oviductal oocytes from the laboratory opossum lack cortical granules and exhibit a new coat within the perivitelline space between the ZP and the tips of the oocyte microvilli (Dandekar et al. 1995). This new coat, termed the cortical granule envelope, is more electron dense than the ZP and causes a significant increase in the width of the perivitelline space. A similar matrix has been described in the perivitelline space of fertilized oocytes from naturally mated females of the fat-tailed dunnart (Breed and Leigh 1992) as well as in eutherian species such as the human, mouse and hamster (Talbot and DiCarlantonio 1984; Dandekar and Talbot 1992) and is thought to arise, at least in part, from the cortical granule exudate. The function(s) of the cortical granule envelope is not known but may include a block to polyspermy at the level of the perivitelline space or oolemma.

11.4 Common Themes and General Overview

It remains uncertain whether the cortical reaction functions as an efficient block to polyspermy in all mammals, or whether it has evolved eutherian mammals concomitant with the disappearance of the mucoid layer. The study of cortical granule formation and function in marsupial oocytes remains limited as they cannot be visualized at the light microscope level using the lectin *Lens culinaris*, used to specifically label cortical granules in eutherian species (e.g. Ducibella et al. 1988).

12 Decondensation of Sperm Nucleus

12.1 Eutherians

The nuclear envelope rapidly disintegrates when the sperm nucleus is incorporated into the cytoplasm of eutherian oocytes, regardless of their maturational status (Usui et al. 1997). Factors within the oocyte cytoplasm then begin decondensation of the disulphide-stabilized "sperm-specific" protamines in the sperm nucleus. The reduced form of glutathione (GSH) has been proposed as one of the factors in the oocyte cytoplasm involved in decondensation by mediating the reduction of disulphide bonds (Calvin et al. 1986). Following reduction of SS bonds, sperm protamines are replaced by oocyte histones and other proteins allowing packaging of the sperm DNA into nucleosomal chromatin. The removal of sperm protamines may be mediated by high-affinity binding of the reduced protamines for a cytoplasmic nucleoplasmin-like protein (reviewed by Yanagimachi 1994).

Nucleoplasmin is released from the GV into the ooplasm of frog oocytes at the time of GV breakdown; it then must undergo massive hyperphosphorylation before it can replace sperm protamines with histones and enable chromatin decondensation (Leno et al. 1996). Frog nucleoplasmin can also bind human protamines and decondense DTT-treated human spermatozoa in vitro (Ito et al. 1993). A nucleoplasmin-like protein may also be involved in decondensation of eutherian spermatozoa, and its release into the ooplasm during GV breakdown, and a requirement for hyperphosphorylation, dependent upon oocyte maturation, would correlate well with the acquisition of the oocytes' ability to decondense sperm nuclei (McLay and Clarke 1997).

12.2 Monotremes

There is no information on the process of decondensation of the sperm nucleus in monotremes.

12.3 Marsupials

Sperm incorporation in marsupials seems to follow the conventional mammalian pattern. The sperm nucleus begins decondensation rapidly after entering the oocyte cytoplasm in *Monodelphis*, *Didelphis* and the brushtail possum (Rodger and Bedford 1982; Taggart et al. 1993; Jungnickel et al. 1999); however,

in *Sminthopsis* the sperm head appears to travel a considerable distance within the oocyte cytoplasm before decondensation is initiated (Breed and Leigh 1988, 1992). The longer time taken for decondensation in *Sminthopsis* may be related to the large size of the sperm tail (approx. 100 μm), which is visible by phase contrast microscopy in the cytoplasm of recently fertilized oocytes (Rodger 1991). Rapid decondensation of the marsupial sperm nucleus is not surprising considering its lack of disulphide stabilization. The protamines of all marsupials were believed to lack cysteine and thus disulphide stabilization (Calvin and Bedford 1971; Balhorn et al. 1988; Fifis et al. 1990). Recently it has been demonstrated that in one genus of Australian dasyurid marsupial – *Planigale* – but not other dasyurids, sperm protamines contain cysteine and presumably the sperm heads are disulphide stabilized (Retief et al. 1995). Fertilization events in planigales have not been studied.

In *Sminthopsis*, expansion and decondensation of chromatin began in the ventral region of the sperm head, whilst chromatin along the dorsal region of the nucleus remained condensed for a longer period (Breed 1996). Membranous material was found associated with the decondensing sperm head in recently fertilized *Monodelphis* and *Sminthopsis* oocytes (Breed and Leigh 1988; Taggart et al. 1993) but not *Didelphis* oocytes (Rodger and Bedford 1982). As discussed earlier, the precise manner of sperm incorporation by the oocyte awaits direct evidence.

12.4 Common Themes and Overview

In both eutherians and marsupials (there is no information for monotremes), the sperm head begins to decondense rapidly on incorporation into the oocyte cytoplasm. In some cases this is extremely rapid and is quite advanced before the sperm tail is fully incorporated. Once decondensed the protamines of the sperm nucleus are transformed into the histones of somatic cell nuclei. An intriguing but as yet poorly understood difference between the sperm of apparently all but one genus of marsupials and all eutherians is the absence of cysteine in the protamines, the result being a lack of disulphide stabilization of the marsupial sperm head, which is far more fragile than those of eutherians and more similar to monotremes and non-mammalian vertebrates. Despite this in vitro fragility there is no evidence that the sperm head is particularly fragile in vivo because the heads of sperm injected into immature tammar wallaby GV oocytes remain condensed for many hours in the oocyte cytoplasm (K.E. Mate and G. Magarey, unpubl. observ.).

13 Completion of Meiosis, Pronuclei Development and Syngamy

13.1 Eutherians

13.1.1 Completion of Oocyte Meiosis

Meiotic maturation in eutherian oocytes involves the activation and inactivation of maturation-promoting factor (MPF) which is a serine/threonine protein kinase composed of a catalytic subunit, $p34^{cdc2}$, and a regulatory subunit, cyclin B. In order to be activated, MPF requires dephosphorylation of Thr14 and Tyr15 and phosphorylation of Thr161. The activity of MPF is low in the GV-intact oocyte, increases to a peak level by MI, transiently decreases following exit from MI, and then increases and remains elevated during MII (Choi et al. 1991). Activation of MPF induces chromosomal condensation and GV breakdown during the first meiotic division (Motlik and Kubelka 1990). The arrest of oocytes at MII of the second meiotic division is thought to be due to maintenance of elevated MPF levels by a cytostatic factor (CSF or p39mos). The rise in intracellular Ca^{2+} following sperm–oocyte fusion leads to inactivation of MPF due to the destruction of the cyclin component, which correlates with the resumption of meiosis and the emission of the second polar body (Moos et al. 1996).

Mitogen-activated protein kinase (MAP kinase) is another protein kinase involved in signalling pathways regulating the cell cycle of the oocyte (Moos et al. 1995; Sun et al. 1999). MAP kinase activation increases subsequent to that of MPF, and remains elevated during the transition from MI to MII and in the MII-arrested oocyte. Following fertilization there is a time-dependent decline in the activity of MAP kinase that is temporally correlated with the formation of the male and female pronuclear envelopes (Moos et al. 1995).

13.1.2 Pronuclei Development and Syngamy

A new nuclear envelope forms around the decondensed sperm head when the oocyte reaches telophase II. In contrast to disassembly of the sperm nuclear membrane which is independent of the cell cycle stage of the oocyte, the ability of the oocyte cytoplasm to assemble this new envelope is restricted to telophase of meiosis, correlated with the absence of active MAP kinase (Moos et al. 1996) and MPF (Usui et al. 1997) in the ooplasm. After incorporation, the structures of the sperm tail connecting piece are disassembled, exposing the single sperm centriole to the zygotic cytoplasm. Following accumulation

of maternal centrosomal components around the sperm, cytoplasm-derived components around the sperm centriolar complex form the microtubule-based sperm aster, a structure responsible for union of the male and female pronuclei (Schatten 1994). With the exception of rodents, this scheme of centrosome assembly seems valid for all eutherian mammals. Concomitantly, the major structure of the sperm principal piece, the fibrous sheath, disappears. Clusters of sperm-derived mitochondria can be detected in early stage four-cell cow embryos (Sutovsky et al. 1996) but not by the beginning of the third mitotic cycle.

Integrated assembly of microtubules and microfilaments is required for migration of the male and female pronuclei towards the centre of the oocyte where they become closely apposed in preparation for the first mitotic division (Schatten et al. 1985; Kim et al. 1997; Palermo et al. 1997). Microtubules are primarily associated with the arrested meiotic spindle in the unfertilized oocyte. In most animals, the microtubules of the sperm aster are responsible for moving the male and female pronuclei from the oocyte cortex to the centre of the oocyte (Le Guen et al. 1989; Long et al. 1993). However, in the mouse numerous cytoplasmic asters assemble after incorporation of the sperm, and begin to associate with the developing pronuclei (Schatten et al. 1985). As the pronuclei migrate to the centre of the oocyte, these asters develop into a dense array, which is subsequently replaced by sheaths of microtubules surrounding the adjacent pronuclei, which give rise to the mitotic apparatus. Pronuclear fusion is not observed, but rather the envelopes of the sperm and oocyte pronuclei disintegrate and the maternal and paternal chromosome sets meet at metaphase.

13.2 Completion of Fertilization in Monotremes

The second polar body is extruded from the monotreme oocyte in response to fertilization. The male pronucleus comes to lie in the germinal disc approximately 1 mm away from the female pronucleus and the two polar bodies migrate to the margin of the disc (Griffiths 1968). The two pronuclei migrate towards each other and become closely associated, forming an almost spherical body divided at the equator by the limiting membrane. Shortly after, it is not possible to detect the membrane between the two pronuclei (Griffiths 1968).

13.3 Completion of Fertilization in Marsupials

Following sperm incorporation in both *Monodelphis* and *Sminthopsis*, a microtubule-based sperm aster is formed, indicating that the centrosome is paternally inherited in marsupials (Breed et al. 1994; Merry et al. 1995). The sperm aster is restricted to the non-yolky region of the oocyte cytoplasm, resulting

in a cytoplasmic polarization consisting of a microtubule-rich region and an eccentrically located microtubule-free yolk mass (Breed et al. 1994; Merry et al. 1995). Once formed, the male and female pronuclei move toward each other and lie in close apposition (Breed and Leigh 1990), surrounded by abundant microtubules (Breed et al. 1994). The manner of syngamy has not been directly observed for any marsupial.

13.4
Common Themes and Overview

The cytoskeleton plays an integral role during the latter stages of fertilization in all mammals; the assembly of microtubules and microfilaments is vital for pronuclear formation and migration, as well as cleavage after fertilization. In those marsupials examined, as in most eutherian mammals, it is the microtubules of the sperm aster that are responsible for moving the male and female pronuclei from the inner face of the oocyte cortex in the oocyte cytoplasm. Thus, it appears that the observation of numerous cytoplasmic microtubule foci in the cytoplasm of fertilized mouse oocytes is atypical of all other mammals.

14
Concluding Remarks

We hope that it is now clear that despite many common themes running through fertilization in mammals, there are major differences between the three groups – monotremes, marsupials and eutherians. The greatest differences are between monotremes and the other two groups based on the character of the oocyte and sperm; however, our knowledge of monotreme fertilization is so limited that it is probably unwise to speculate more. Eutherian and marsupial fertilization clearly have much in common. Indeed, if there are two distinct AR and sperm–oocyte fusion systems in the American and Australian marsupials the major comparison perhaps is between marsupials rather than between marsupials and eutherians. Major issues that require closer study are the process of capacitation in marsupials, regulation of the AR, the manner of sperm–oocyte fusion and sperm incorporation. A better understanding of these dynamic aspects of marsupial fertilization and their molecular biology, together with the large body of knowledge of eutherian fertilization, will no doubt allow the development of a broad understanding of and perhaps a unified conceptual analysis of mammalian fertilization. The place of monotremes in this scenario is intriguing but awaits a new generation of intrepid gamete biologists.

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