



The Marsupial Zona Pellucida: Its Structure and Glycoconjugate Content

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July 2003

**A thesis submitted as fulfilment of the requirements for the Degree of
Doctor of Philosophy**

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Abstract

The zona pellucida (ZP) is an extracellular matrix that surrounds the oocytes of all higher vertebrates. Whilst there is considerable data on the ZP of eutherian mammals, the structure and function(s) of the ZP of marsupials is poorly known.

In this thesis the structure and glycoconjugate composition of the ZP surrounding marsupial oocytes and the changes that occur during ovarian development, following ovulation, and following cortical granule exocytosis was investigated. In addition, the glycoconjugates of the oviduct epithelial lining of the brushtail possum around the time of ovulation were also examined in order to determine if there was any contribution of the oviductal secretions to the post-ovulatory ZP.

The ultrastructural organisation of ZP and its development during oocyte maturation in five marsupial species was found to differ in the relative thickness around mature oocytes. The thicknesses of the ZP of the marsupials ranged from 3.9 μ m in the fat-tailed dunnart, 4.6 μ m in the western grey kangaroo, 5.2 μ m in the brushtail possum, 6.6 μ m in the wombat and 8.6 μ m in the koala.

The glycoconjugates of the ZP surrounding antral follicular oocytes of seven marsupial species, were determined by differential lectin histochemistry, before and after either removal of sialic acids with neuraminidase, or *O*-acetyl groups on sialic acids by mild alkali hydrolysis. Interspecific variation in the glycoconjugate composition of the ZP was found, especially that of the mannose-containing glycoconjugates. The presence of terminal sialic acids was indicated since increases in binding of lectins to galactose- and *N*-acetylgalactosamine-(GalNAc)-containing glycoconjugates occurred after desialylation. Removal of *O*-acetyl groups also resulted in the demonstration of the presence of *N*-acetylglucosamine (GlcNAc) in the ZP of fat-tailed dunnarts, brushtail possums and ringtail possums, and mannose in the ZP of fat-tailed dunnarts, brushtail possums, grey, short-tailed opossums and eastern grey kangaroos. Furthermore, localisation of *N*-acetylglucosamine residues in all species changed following de-*O*-acetylation which suggests that a higher proportion of *O*-acetylated sialic acids occur within the core of the ZP than near the inner and outer surfaces.

Ultrastructural changes to the ZP were found to occur following ovulation, with the ZP of ovarian oocytes appearing dense and disorganised, whilst that surrounding ovulated oocytes was highly filamentous and had an ordered structure. A greater abundance of four glycoconjugates (mannose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine) occurred in the ZP of ovulated, compared to ovarian, oocytes.

Artificial induction of cortical granule exocytosis of possum oocytes was carried out to investigate the role of the egg coats following egg activation. Artificial activation showed that *N*-acetylgalactosamine residues decreased significantly within the ZP, whilst after staining with ruthenium red, the perivitelline space matrix (PVM) appeared to be largely oviductal in origin, and not a “cortical granule envelope” as previously claimed.

To determine whether the increase in glycoconjugates of the ZP following ovulation occurs as a result of the incorporation of oviductal secretions into its matrix, the glycoconjugate composition of the oviductal epithelium was also investigated. The epithelium of the oviduct was shown by lectin histochemistry to have an abundance of β -galactose and *N*-acetylglucosamine residues, with *N*-acetylgalactosamine being localised to the secretory cells of the ampulla, and absent in the isthmus; a finding that was also supported by lectin immunocytochemistry. Furthermore, lectin immunocytochemistry showed greater *N*-acetylgalactosamine and mannose residues in the ampulla than in the isthmus, whereas, by contrast, *N*-acetylglucosamine residues were in higher concentration within the isthmus. Gold labelling of β -galactose residues was equally distributed within the secretory granules between the two regions.

Acknowledgments

Firstly, I would like to thank my supervisor Bill Breed for many years of patience and persistence. Thankyou for all the encouragement, the constructive criticisms and experience that you have shared.

To my other supervisor Ole Wiebkin. Thankyou for helping me see the story of the zona pellucida from another perspective– the carbohydrate's point of view.

To Chris Leigh. Your friendship, patience and bottomless depth of practical knowledge have been invaluable to me over the years.

To everyone else who played a role in the practical component of my thesis: Nadia Gagliardi and Gail Hermanis for their help and patience in all the facets of light microscopy; Marilyn Henderson and Lyn Waterhouse for help with the cryo-electron microscopy techniques; Craig Noble for his help with analySiS; the rest of the staff at CEMMSA (now Adelaide Microscopy) for their help and friendship over the years; Tavik Morgenstern for his help in the printing of the plates.

Thanks to Mario Ricci for his friendship over the years of our PhDs: I'm very fortunate to have gone through both Honours and a PhD with such a great mate.; Thanks also to all of the other members (past and present) of the Reproductive Biology group for your friendship and constructive criticism.

To my family, Kerry, Roger and Stacy Chapman. Thankyou for your love and patience while I went off and did whatever it was I did at Uni.

And lastly, to my beautiful wife Michelle. Your love, commitment, patience and understanding have been amazing and I couldn't have finished without you. You kept me sane when I thought my head would explode and you helped me more than you will ever know. Thankyou so much for everything, I love you very much.

Research arising from this thesis has been published and/or presented at conferences as follows, examples of which can be found at the rear of this thesis:

Publications:

Breed, W. G., Hope, R. M., Wiebkin, O. W., Spargo, S. C. and **Chapman, J. A.** (2002). The marsupial zona pellucida: its structural organization and evolution. *Reproduction* **123**: 13-21.

Chapman, J. A., Wiebkin, O. W. and Breed, W. G. (2000). Interspecific variation of zona pellucida glycoconjugates in several species of marsupial. *Journal of Reproduction and Fertility* **119**: 111-120.

Chapman, J. A. and Breed, W. G. (in press). The egg coats of the brushtail possum (*Trichosurus vulpecula*) following ovulation and artificial activation with particular reference to the origin of the Perivitelline Space Matrix. In 'The Biology of Australian Possums and Gliders' (Eds. Goldingay, R. L. & Jackson, S. M.) Surrey Beatty & Sons: Chipping Norton.

Conference Presentations:

Chapman, J. A. and Breed, W. G. (2003). The oviduct of the brushtail possum, *Trichosurus vulpecula*: the glycoconjugate environment at ovulation and its probable contribution to the post-ovulatory zona pellucida. *21st New Zealand Conference on Microscopy, Wellington, February 7-12.*

Chapman, J. A., Wiebkin, O. W., and Breed, W. G. (2002). Structure of the zona pellucida of a marsupial, the brushtail possum *Trichosurus vulpecula*, following ovulation and egg activation. *The 17th Australian Conference for Electron Microscopy (ACEM17), Adelaide Convention Centre, South Australia, February 4-8.*

Chapman, J. A., and Breed, W. G. (2001). What prevents polyspermy in a marsupial egg? A study in the brushtail possum. *The 47th Scientific Meeting and Possum and Glider Symposium of the Australian Mammal Society, Queensland Museum, Brisbane, July 2-6.*

Hope, R. M., **Chapman, J. A.**, Wiebkin, O. W. and Breed, W. G. (2000). How similar is the molecular structural organization of the marsupial zona pellucida to that of eutherians? *The Third International Symposium on the Molecular and Cellular Biology of the Egg and Embryo Extracellular Matrix, Grandlibakken Resort and Conference Center, Lake Tahoe, California, August 27-September 1.*

Chapman, J. A., Wiebkin, O. W., and Breed, W. G. (2000). Structure of the zona pellucida of a model marsupial, the brushtail possum (*Trichosurus vulpecula*: Family, Phalangeridae). *Fertility 2000: EICC Edinburgh, 2nd Joint Meeting of BAS/BFS/SSF, July 31-August 2.*

Chapman, J. A., Wiebkin, O. W., and Breed, W. G. (1999). Interspecific variation in glycoconjugates of the zonae pellucidae of several marsupial species. *31st Annual Conference of the Australian Society for Reproductive Biology, Melbourne, September 26-29.*

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Chapter 1 Literature Review

1.1 Introduction

Fertilization is the process by which the male and female gametes, the spermatozoon and egg respectively, fuse to form a new individual. This mode of sexual reproduction provides that half of each parent's genetic makeup is passed on to the next generation. In mammals, in order for successful fertilization to occur there are a number of very complex and relatively poorly understood pre-fertilization events that must first take place. These include:

- 1) binding of sperm to the zona pellucida;
- 2) penetration of the zona pellucida by the sperm; and
- 3) binding and then fusion of the sperm with the oocyte's plasma membrane, the oolemma.

These events all take place within the upper reaches of the female reproductive tract, the oviduct. While these events have been extensively studied in eutherian mammals, particularly with the aid of *in vitro* fertilization, relatively little research has been performed on marsupials. Similarly, *in vitro* fertilization has not yet been repeatedly achieved in Australian marsupials, therefore, little is known about these events in these species.

1.2 Evolution of the Oocyte Vestments

The mature ovulated oocytes of all vertebrate species studied appear to be surrounded by an extracellular matrix (ECM), composed of at least one, but sometimes more, vestments. Moreover, the oocytes of a number of invertebrates, such as those of some marine bivalve molluscs (e.g. *Unio elongatulus*; Focarelli *et al.*, 2001), ascidians (Fukomoto, 1990; Litscher & Honegger, 1991), and sea urchins (Nomura & Osaka, 1985; Vacquier *et al.*, 1989; Ohlendiek & Lennarz, 1995) are also surrounded by an ECM. In fact, much more information appears to be known about the molecules involved in sperm-oocyte interaction in a few species of invertebrates than is known for mammals.

For example, in the starfish *Asterias amurensis* three components of the outermost layers of the ECM of the oocyte, the jelly coat, have been found to act in concert to induce the acrosome reaction in sperm (Nishigaki *et al.*, 1996; Koyota *et al.*, 1997; Hoshi *et al.*, 2000). Sperm of this species bind specifically to the complex and highly ordered saccharide chains of an acrosome-reaction inducing substance (ARIS) in the jelly coat while another jelly substance, Co-ARIS, is adsorbed onto the sperm surface to modify the ARIS receptor. These events, in turn, bring about an increase in intracellular Ca^{2+} , while the third jelly substance, called asterosaps, binds to a guanylate cyclase sperm receptor, which induces a dramatic increase in the level of cGMP, leading to an increase in intracellular pH. When coupled together, the calcium and pH rise, induce acrosomal exocytosis or, more specifically, the acrosome reaction (Hoshi *et al.*, 2000). Such specifics in the mammalian system are only now being understood and described (Florman *et al.*, 1998; Arnoult *et al.*,

1999; Visconti *et al.*, 1999; O'Toole *et al.*, 2000; Sato *et al.*, 2000; Revelli *et al.*, 2001), with much more still to be discovered.

In non-mammalian vertebrates, the extracellular matrices that surround oocytes display a high degree of homology with those surrounding mammalian oocytes, both in structure and composition (a more detailed comparison of zona pellucida gene and protein sequences will be discussed later in section 1.2.3 Glycoproteins of the ZP), although a number of differences exist. In teleost fishes, oocytes are surrounded by an ECM known as the chorion (although it is sometimes also referred to as the zona pellucida: Iino & Inoue, 1982; Hamazaki *et al.*, 1989). As one of the most ancient groups of vertebrates, the chorion has a number of unique features that are not found in the egg coats of more recently evolved vertebrates. One unique feature is the presence of a single opening within the chorion, known as the micropyle, through which sperm must traverse to gain access to the oocyte for fertilisation to occur (Kudo, 1988; Amanze & Iyengar, 1990). Following sperm-oolemma fusion, a wave of calcium ions spreads out from the point of fusion and organelles known as cortical alveoli (homologous to cortical granules in most other species) release their contents into the micropyle blocking entry of other sperm, thereby preventing the fatal occurrence of more than one sperm from fertilising the egg, known as polyspermy (Gilkey, 1981; Kudo, 1988).

Unique to some teleost fishes too, is the site of production of one or more of the glycoproteins that comprise the chorion. While in most vertebrates the ovary (either the oocyte and/or granulosa cells) is the organ responsible for production of egg coat glycoproteins, in the teleosts the winter flounder, medaka, turbot, and cod, chorion

glycoproteins are synthesised in the liver and transported to the ovary through the bloodstream (Hamazaki *et al.*, 1989; Lyons *et al.*, 1993; Larsson *et al.*, 1994; Epifano *et al.*, 1995a; Murata *et al.*, 1997; Hyllner *et al.*, 2001). In other species of teleosts, however, such as the carp, goldfish, sea horse and eel, chorion glycoproteins have an ovary-specific site of synthesis (Larsson *et al.*, 1994; Chang *et al.*, 1997).

In anuran amphibians, oocytes are surrounded by an ECM known as the vitelline envelope (VE), which has been a major focus of study in one of the oldest animal models of reproduction, the South African clawed toad, *Xenopus laevis*. Like the eggs of many non-mammalian vertebrates, the eggs of *X. laevis* display two hemispheres, a vegetative pole which contains those organelles and inclusions (particularly yolk) important in embryo nutrition, and an animal pole, which contains the genetic material and is the site of fertilisation. Only sperm that bind to the VE overlying the animal pole are able to subsequently fertilise the egg and result in development (Elinson, 1986; Stewart-Savage *et al.*, 1991).

The VE of *X. laevis* transforms through four distinct forms related to its maturational state as defined by ultrastructure, composition, function and site of origin (for review see Hedrick & Nishihara, 1991). The first maturational form of the envelope surrounds oocytes within the ovary, and is known as the ovarian envelope; following ovulation into the coelomic cavity and prior to entry into the oviduct, meiotically-mature, yet unfertilisable, oocytes are surrounded by the second maturational state of the envelope known as the coelomic envelope; upon entering the first part of the oviduct, known as the pars recta, the envelope displays its third maturational form, the VE, and it is only at this stage that eggs

become potentially fertilisable; and lastly, following fertilisation, the zygote is surrounded by the fertilisation envelope. The latter two maturational stages of the envelope consist of the most dramatic changes in ultrastructure and biochemistry from the original forms, while major modifications to the function of the envelopes also occur - the first change from the coelomic envelope to the VE makes the envelope penetrable by sperm and the egg therefore fertilisable, while the second modification from the VE to the fertilisation envelope, in response to the release of cortical granules, produces the opposite effect, making the coat impenetrable to further sperm thereby preventing polyspermy (Larabell & Chandler, 1991; Quill & Hedrick, 1994; Lindsay & Hedrick, 1998). Similar modifications have been described in several other species of frogs including *Bufo japonicus* (Takamune *et al.*, 1986; Takamune & Katagiri, 1987), and the painted frog, *Discoglossus pictus* (Caputo *et al.*, 2001).

In *D. pictus*, however, not only is the site of fertilisation restricted to animal pole of the eggs, but there is also a further compartmentalisation of the site of fertilisation to an area known as the dimple. The dimple is a glycoprotein-filled depression at the animal pole that keeps the surface of the egg from the VE to a depth of around 300µm (Denis-Donini & Campanella, 1977). Studies have demonstrated that sperm that enter the dimple-less region of the egg do not activate development, even when intra-cytoplasmic spermatozoon injection is performed (Talevi & Campanella, 1988; Maturi *et al.*, 1998). Furthermore, there also appears to be further compartmentalisation with respect to the plasma membrane of the dimple, with only one region of the dimple, known as D1 (with surrounding areas known as D2 and D3) capable of binding sperm and inducing normal development (Talevi & Campanella, 1988). In support of this, differences in the oolemma glycoproteins have

been demonstrated to occur at the three regions, with only D1 containing fucose, which is then subsequently lost following fertilisation (Denis-Donini & Campanella, 1977; Maturi *et al.*, 1998). Fucose, therefore, is suggested to be the glycoconjugate responsible for sperm-egg binding, with its loss after fertilisation possibly related to the prevention of polyspermy.

In order for successful fertilisation to occur in anurans, another feature is essential, the formation of the tertiary oocyte membranes known as the jelly coats (Hedrick & Nishihara, 1991). Following ovulation and entry into the oviduct, the oocyte of frogs become encased within one or more (depending on the species) layers of mucin, known as the jelly coat. In *X. laevis*, each of the three jelly coat layers, termed J1, J2 and J3 from the layer closest to the egg to the outermost layer respectively, is ultrastructurally, biochemically and functionally distinct - J1 is around 150µm thick, is the only layer to contain sulphate, and is thought to be composed of 3 glycoproteins; J2 is only 15µm thick and composed of 2 glycoproteins; and, J3 is around 200µm thick and composed of 4-5 glycoproteins (Bonnell & Chandler, 1996). Each layer is also composed of differing glycoconjugates, as indicated through lectin binding, while functionally they also differ in the ability to bind sperm, with J3 commonly reported as being responsible for capacitation and/or induction of the acrosome reaction in sperm, while J1 is thought to play a role in the block to polyspermy (Hedrick & Katagiri, 1988, 1991; Bonnell & Chandler, 1996). In *D. pictus*, the jelly coats play a vital role in fertilisation by forming a plug surrounding the depression of the dimple, which is thought to direct sperm towards the site of fertilisation, while the outer jelly layer (J3) also appears responsible for the induction of the acrosome reaction in sperm (Campanella *et al.*, 1997). The jelly coats in *B. japonicus*, however, are suggested as

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The proteins of the albumen and PM of estuarine crocodile eggs have also been described in comparison with those of chicken eggs (Burley *et al.*, 1987). The outer layer of the crocodile PM, like that of the chicken, appeared to be composed of two non-ovarian glycoproteins: namely, lysozyme and vitelline membrane outer protein I (or VMO1). No proteins were found in the inner layer, however, unlike in the chicken (which has two glycoproteins), although the authors note that the fragility of the crocodilian PM may have resulted in its loss during experimental manipulation (Burley *et al.*, 1987).

functioning in binding Ca^{2+} and/or Mg^{2+} , thereby providing a suitable environment for inducing the acrosome reaction and penetration by sperm (Ishihara *et al.*, 1984; Shimoda *et al.*, 1994). Whatever its function, the jelly coats, therefore, play a vital role in successful sperm-egg interaction.

The egg coats of reptiles and birds, known as the perivitelline membrane (PM), are perhaps the least understood egg coats of all non-mammalian vertebrate groups. There have in particular been very few investigations into the reptilian PM with few papers published on gene sequences or protein weights. Recently, incidental observations were made on the PM as part of the ultrastructural features of oogenesis the American Alligator *Alligator mississippiensis* (Uribe & Guillette Jr., 2000), while the glycoconjugates of the developing follicular oocytes of the squamate reptile *Podarcis sicula* were determined using lectin histochemistry (Andreuccetti *et al.*, 2001). The PM glycoproteins of this lizard appeared to contain *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), mannose (Man) and galactose (Gal), and the glycoconjugates, and therefore the glycoproteins, appeared to be secreted initially by the oocyte, and then by the follicle cells (Andreuccetti *et al.*, 2001). *Note: see erratum – opposite page

The PM of birds have recently been the subject of investigations, with most research being performed on the chicken and the Japanese quail. The PM of the chicken is formed of two layers, an inner layer that is secreted during the preovulatory phase, and an outer layer which appears to originate following ovulation (Bellairs *et al.*, 1963). Ultrastructurally, the PM is composed predominantly by flocculent material interspersed between irregularly arranged bundles of filaments (Bakst & Howarth Jr, 1977a,b), while biochemically it is

composed of two major glycoproteins gp97 and gp42 (Takeuchi *et al.*, 2001). At fertilisation, sperm bind to N-linked oligosaccharides, such that sperm binding can be inhibited by incubating PMs in N-glycanase, the enzyme which removes N-linked oligosaccharides, but not O-glycanase, the enzyme which removes O-linked oligosaccharides (Robertson *et al.*, 2000). Sperm penetration of the PM, like that in anurans, occurs more frequently at the animal pole of the egg (Bakst & Howarth Jr, 1977b), and appears to primarily involve enzymatic dissolution of the matrix, with large holes being formed in the PM due to degradation of gp97 by acrosomal enzymes, mainly acrosin (Takeuchi *et al.*, 2001).

While only two major glycoproteins were found when PM were run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), four genes, homologous to those of the mammalian zonae pellucidae, have been found to exist (Waclawek *et al.*, 1998; Takeuchi *et al.*, 1999; Bausek *et al.*, 2000; Tsuda *et al.*, 2000). Investigations into the egg coats of non-mammalian vertebrates such as the fish, frogs and birds have demonstrated a high degree of homology, in terms of structure, composition and function, among many vertebrates, and have provided insight into the evolution of the egg coat that surrounds the eggs of eutherian and metatherian (marsupial) mammals, known as the zona pellucida.

ERRATUM

There are exceptions, however, and cross-species sperm-ZP binding, penetration and/or fertilisation have been shown in a number of species (e.g. primates: Bedford, 1977; VandeVoort *et al.*, 1992; rodents: Fukuda *et al.*, 1979; Roldan & Yanagimachi, 1989; Wakayama *et al.*, 1996; bovine, ovine, porcine and equine: Slavik *et al.*, 1990; Sinowatz *et al.*, 2003).

1.3 The Metatherian and Eutherian Zona Pellucida (ZP)

1.3.1 Functions of the ZP

The ZP of marsupials and eutherians is a relatively thick extracellular coat that is formed around the oocyte during early growth. Its structure is composed primarily of glycoproteins and it has a number of important functions, not the least of which involves the initial interactions between the sperm and egg. As already stated, in order for the sperm to reach the egg, sperm must first bind to, and then penetrate, the ZP. This has been suggested to occur in a species-specific manner, such that it has even been suggested that the variations in gamete adhesion molecules involved in sperm-zona interactions drive speciation (Swanson & Vacquier, 1995; Swanson *et al.*, 2001). Under *in vitro* conditions suitable for fertilization, it is often cited that sperm from one species will not fertilize eggs of another, unless the ZP is first removed (Gwatkin & Williams, 1977; Bedford, 1982; Wassarman, 1987b; Kouba *et al.*, 2001). Therefore, like the vitelline envelope of echinoderms, the mammalian ZP is said to function as a post-mating, but pre-zygotic, isolating mechanism (Mayr, 1963) restricting interspecies fertilization (Wassarman & Litscher, 1995). * Note: see erratum – opposite page

Once bound, the sperm undergo a form of exocytosis, the acrosome reaction (AR), that is a prerequisite for fertilization (Bleil & Wassarman, 1980; Yanagimachi, 1994). The acrosome is a secretory granule-like organelle overlying the sperm head and nucleus that contains hydrolytic enzymes such as hyaluronidase and acrosin (Yanagimachi, 1994; Abou-Haila & Tulsiani, 2000). Upon binding, the plasma membrane overlying the acrosome and

the outer acrosomal membrane fuse in a number of places, these vesiculations enabling the acrosomal contents to be released (Barros *et al.*, 1967; Bedford, 1982; Yanagimachi, 1994). While the exact function of the acrosomal enzymes released during the AR in relation to zona penetration in mammals is controversial, there are two main views: one view suggests that the acrosomal enzymes are involved in the initial hydrolysis and entry by sperm into the ZP, while the second view favours that the mechanical forward thrust of the sperm tail allows penetration of the zona to be completed without the need for enzymatic digestion of the ZP matrix (for review see Yanagimachi, 1994; Bedford, 1998). For example, initially pro-acrosin/acrosin was thought to be the major acrosomal enzyme responsible in the penetration of the ZP (Müller-Esterl & Fritz, 1981; Fraser, 1982; Brown, 1983). Recent work, however, in which male mice have carried a targeted mutation for acrosin (i.e. acrosin knock-out mice) has shown that sperm can still penetrate the ZP, albeit at a reduced rate, and fertilise eggs (Baba *et al.*, 1994; Adham *et al.*, 1997), while other acrosomal proteases have been implicated in aiding ZP penetration (Yamagata *et al.*, 1998a; Yudin *et al.*, 1999). Acrosin, at least in lab mice, is now thought to play more of a role in accelerating the dispersal of the other acrosomal contents and/or a partial role in egg activation (Yamagata *et al.*, 1998b; Smith *et al.*, 2000). What is known about the function of the AR in mammals, however, with the exception of field vole spermatozoa (Wakayama *et al.*, 1996), is that only acrosome-reacted sperm can penetrate the ZP and fuse with the oolemma (Saling, 1996). The binding of a spermatozoon to the oolemma induces what is known as the "cortical reaction" leading to another of the ZP functions, that is, as a secondary block to polyspermy (Gulyas & Schmell, 1980; Guraya, 1982).

Cortical granules (CG) are small, membrane-bound vesicles located in the cortical region of unfertilized eggs. Upon sperm-oolemma fusion, a wave of calcium release spreads out from the internal stores underlying the area of fusion and then throughout the whole egg causing the membranes of the CG to fuse with the oolemma. The contents of the CG are then released into the space between the ZP and oocyte called the perivitelline space (PVS) (Cran, 1989; Cran & Esper, 1990; Hoodbhoy & Talbot, 1994). This process is the cortical reaction and the CG exudate, which is released into the PVS, contains proteolytic enzymes that alter the functional and structural properties of the ZP making it impenetrable to other sperm, and thereby preventing polyspermy from occurring (Schmell & Gulyas, 1980; Bleil *et al.*, 1981; Wassarman, 1987*a,b*, 1988, 1990; Wassarman & Mortillo, 1991).

In marsupials, it has been reported that the cortical reaction produces a new coat in the PVS, the cortical granule envelope, visible only after staining with ruthenium red (Dandekar *et al.*, 1995). Exactly how much of this material within the PVS is CG exudate and how much may be the result of the incorporation of oviductal secretions or material within the PVS prior to ovulation has not been addressed. While Breed and Leigh (1988) were cited by Dandekar *et al.* (1995) as demonstrating the cortical granule envelope in *Sminthopsis crassicaudata*, they also clearly showed material in the PVS of mature ovarian eggs.

In eutherians, following fertilization, the ZP also plays an important role in protecting and holding the early embryonic cells together so that the blastomeres do not separate. In marsupials, the role of the ZP in embryonic development appears to be unique with evidence that, at least in some dasyurid marsupials, blastomeres attach to the ZP prior to attaching to each other, a stage vital for normal embryonic development (Selwood, 1992,

2000). In eutherians, the ZP also plays a role in preventing premature attachment of the developing embryo to the oviductal epithelium (Dunbar *et al.*, 1991), while this role of the ZP is nullified in marsupials by the acquisition of the tertiary egg envelopes, the mucoid coat and, less so, the shell membrane, external to the egg and ZP within the early part of the oviduct and uterotubal junction, respectively. It is only once an embryo has reached the expanded blastocyst stage of development that the ZP is no longer needed and the embryo "hatches" from the ZP and implants in the uterus (Bergstrom, 1972; Wassarman, 1987b; O'Sullivan *et al.*, 2001).

1.3.2 Morphology of the ZP

The morphology of the mammalian ZP also varies between species. Probably the most distinctive feature of different mammalian ZP is that of differences in relative thickness (Table 1.1). In eutherians this variation in thickness ranges from 5-7 μm in the mouse to 13 μm in the human and up to 27 μm in the cow (Dunbar *et al.*, 1991). In marsupials, however, the range has not been reported to be as great with ZP thickness ranging from 1 μm in the Virginian opossum, *Didelphis virginiana*, to apparently 6.3 μm in the Tammar wallaby, *Macropus eugenii* (see references in Tyndale-Biscoe & Renfree, 1987). It can be assumed that in general, however, the ZP of marsupials is somewhat thinner than that of most eutherian zonae.

The general appearance of mammalian ZP at the light and electron microscopical levels appears to be similar suggesting a similar three-dimensional structure (Green, 1997). In a number of eutherian species, the ZP has been described as heterogeneous, consisting of two

Table 1.1. Summary of the relative sizes of zonae pellucidae from a variety of mammalian species (marsupial data from Tyndale-Biscoe & Renfree, 1987 and Selwood, 2000; eutherian data from Dunbar *et al.*, 1991).

	SPECIES	ZONA PELLUCIDA (μM)
Marsupial	Virginian opossum	1-2
	Southern brown bandicoot	1.3
	Northern quoll	1.6
	Tasmanian devil	4
	Brown antechinus	5.5 $\pm$ 0.4
	Brushtail possum	5.9 $\pm$ 3.1
	Tammar wallaby	6.3 $\pm$ 1.4
Eutherian	Mouse	5-7
	Hamster	8
	Human	13
	Dog	13
	Baboon	13
	Sheep	14.5
	Cat	15
	Rabbit	15
	Pig	16
	Cow	27

morphologically distinct layers: an inner, densely packed layer closest to the oolemma; and an outer, less dense layer, closer to the cumulus cells (Dietl, 1989; Yanagimachi, 1994;

Keefe *et al.*, 1997). The appearance of the inner layer has been suggested as resulting from the secretion of ZP proteins into a compacted space, under a stretched ZP (Green, 1997).

Scanning electron micrographs show the outer surface of the zona to be fenestrated and sponge-like in appearance, probably as a result of extensions of the cumulus cell traversing the ZP to the oocyte (Phillips & Shalgi, 1980*a,b*; von Weymarn *et al.*, 1980). Using various disruptive treatments and electron microscopical techniques, Greve and Wassarman (1985) demonstrated that the mouse ZP was composed of a matrix of interconnected filaments which possessed a structural repeat. The mouse ZP filaments resembled "beads-on-a-string" with each bead measuring 9.5nm in diameter and located every 17nm along the filament (Greve & Wassarman, 1985). A similar appearance of the "beads-on-a-string" was also demonstrated using scanning electron microscopy in the human ZP after treatment with the detergent saponin and fixation in the presence of ruthenium red (Familiari *et al.*, 1992).

The composition of marsupial zonae, however, have been little investigated, although they have been cited as undergoing late maturational changes in their appearance as viewed by transmission electron microscopy. According to Rodger and Mate (1993) the ZP of the brushtail possum changes from "broad and diffuse" prior to ovulation, to "thin and compact" after ovulation. Breed (1996), however, questioned this observation and cited no observable difference in consistency or thickness of the ZP before or after ovulation in the fat-tailed dunnart, *Sminthopsis crassicaudata*.

1.3.3 Glycoproteins of the ZP

Despite the differences in size and appearance of the different mammalian zonae, the number of individual glycoproteins which make up the ZP is very limited, originally thought to number between 2 and 5, depending on the species, resolution of protein separation and detection methods used (Bleil & Wassarman, 1980; Dunbar & Raynor, 1980; Dunbar *et al.*, 1981, 1991, 1994), it is now generally thought that all mammalian ZP's consist of 3 glycoproteins. For example, the mouse ZP was shown to be composed of three glycoproteins (mZP1-mZP3) (Bleil & Wassarman, 1980; Wassarman, 1987*b*, 1988), as was the pig ZP (pZP1, pZP3 α , pZP3 β) (Yamasaki *et al.*, 1996), and in the human, horse, rabbit, and rat (Yanagimachi, 1994). The bovine ZP, however, was thought to be composed of 3-4 ZP families (Topper *et al.*, 1997), while the cat ZP was thought to be only two (Maresh & Dunbar, 1987). It has been established, however, by cDNA cloning techniques that the ZP of all mammalian species investigated are composed of three glycoproteins, encoded from three genes (glycoprotein families): ZPA, ZPB, ZPC (Harris *et al.*, 1994). To add confusion to the nomenclature of the three glycoproteins, the terminology differs between species, as do the apparent functions of the different glycoproteins amongst the mammalian species (Table 1.2) (Prasad *et al.*, 1996). Recently, even more confusion has been added by the discovery of a fourth ZP gene, so far in the human and chicken, which corresponds to the mouse ZP1 and is separate from the rest of the mammalian ZPB gene families (Hughes & Barratt, 1999, 2000; Bausek *et al.*, 2000; Spargo & Hope, 2003). Hughes & Barratt (1999) found that there was relatively low sequence similarity between mouse ZP1 and the other mammalian ZPBs because it was, in fact, not orthologous to ZPB. In fact, to date, no ZPB gene has been found in mice or rats. The human ZPB gene is

Table 1.2. Summary of mammalian ZP glycoprotein nomenclature, molecular weights and proposed functions (partly from Hedrick, 1996 and Prasad *et al.*, 1996)

Glycoprotein ^a	Species	ZP protein ^b	Mw (x10 ³ kDa) ^c	Proposed function ^d	References
A	Mouse	ZP2	120 ^e	secondary sperm receptor	Wassarman, 1990
	Rat	ZP2	119	unknown	Akatsuka <i>et al.</i> , 1998
	Human	ZP2	64-78	unknown	Shabanowitz & O'Rand, 1988
	Pig	ZP1	49-119	structural maintenance of ZP	Dunbar <i>et al.</i> , 1981; Maresh & Dunbar, 1987
	Rabbit	rc75	75	unknown	Lee & Dunbar, 1993; Lee <i>et al.</i> , 1993
	Possum	ZP2/ZPA	92 ^e	unknown	Rodger, 1997; Mate & McCartney, 1998; Voyle <i>et al.</i> , 1999
B	Human	ZP1	90-110	unknown	Shabanowitz & O'Rand, 1988
	Pig	ZP3 α	55	primary sperm receptor	Yurewicz <i>et al.</i> , 1993
	Rabbit	rc55	55	sperm receptor	Prasad <i>et al.</i> , 1995
	Possum	ZP1/ZPB	137 ^e	unknown	Rodger, 1997; Mate & McCartney, 1998; Haines <i>et al.</i> , 1998
C	Mouse	ZP3	83 ^e	primary sperm receptor; induces acrosome reaction	Bleil & Wassarman, 1980; Florman & Wassarman, 1985; Wassarman, 1990
	Rat	ZP3	115	unknown	Akatsuka <i>et al.</i> , 1998
	Human	ZP3	57-73	<i>in vitro</i> sperm receptor	Shabanowitz & O'Rand, 1988; van Duin <i>et al.</i> , 1994
	Pig	ZP3 β	55	potentiates sperm binding to ZP3 α	Yurewicz <i>et al.</i> , 1993b
	Marmoset	ZP3	N/A	<i>in vitro</i> sperm receptor	Thillai-Koothan <i>et al.</i> , 1993
	Rabbit	rc45	45	sperm receptor	Yamasaki <i>et al.</i> , 1995
	Possum	ZP3/ZPC	62 ^e	unknown	Rodger, 1997; McCartney & Mate, 1999
ZP1	Mouse	ZP1	200 ^e	crosslinking ZP2 & ZP3 filaments	Greve & Wassarman, 1985
	Rat	ZP1	205	unknown	Akatsuka <i>et al.</i> , 1998

^aFour major ZP glycoprotein families as described by Harris *et al.* (1994) and Hughes & Barratt (1999)

^bZP protein nomenclature as described by Hedrick (1996)

^cValues as determined by 2D-SDS-PAGE under reducing conditions

^dProposed functions of ZP proteins as described by Prasad *et al.* (1996)

^eValues represent non-reducing conditions on 1D-PAGE

found on a region of chromosome 1 which no longer exists in rodents (due to a break in conservation of synteny with the human genome during their evolutionary past which

produced the rodent chromosomes 8 and 13) and it is argued that another ZP gene evolved in an early rodent ancestor (ZP1) to replace the loss of ZPB (Hughes & Barratt, 2000).

Using simple non-reducing, one-dimensional SDS-PAGE, Bleil and Wassarman (1980) determined the first mammalian ZP protein's average molecular weights and molar ratios. The lab mouse ZP was found to be composed of mZP1 (200kDa), mZP2 (120kDa) and mZP3 (83kDa) in the molar ratio of 0.2:1:1, respectively. Typically, mouse ZP glycoproteins exhibited considerable heterogeneity on SDS-PAGE as a result of their highly charged oligosaccharide side chains (Wassarman, 1987*c*, 1988).

While the three mouse ZP glycoproteins can be easily resolved using this method, the ZP glycoproteins of other mammalian species cannot due to the problems in isolating large quantities of protein, and the large amount of heterogeneous glycosylation of the ZP proteins (Harris *et al.*, 1994). While early work required one-dimensional and, more often, the more sensitive two-dimensional SDS-PAGE to resolve native ZP proteins in mammals (Dunbar *et al.*, 1981; Shabanowitz & O'Rand, 1988; Miller *et al.*, 1992*a*), recent advances in molecular biology have enabled a more detailed investigation into the composition of the ZP, by cloning the genes which encode for the ZP proteins (Harris *et al.*, 1994). Using these methods, a number of ZPs of other eutherian mammalian species have been characterized (see Table 1.2). The ZPs show a large diversity in molecular weights of the different glycoprotein families. In marsupials, however, the molecular weights of the ZP constituents have only been described for the brushtail possum (137kDa, 92kDa and 62kDa; Rodger, 1997; Mate & McCartney, 1998), while the cDNA for the three ZP genes

have only recently been described (Mate & McCartney, 1998; Haines *et al.*, 1999; McCartney & Mate, 1999; Voyle *et al.*, 1999).

Comparisons between the ZP cDNA and putative amino acid sequences of mammals and other vertebrates have shown them to be highly conserved molecules. For example, hamster, human and pig ZPC proteins share 81%, 67% and 66-75% identity respectively with the mouse ZPC amino acid sequence (see references in Dunbar *et al.*, 1994). Not only is there evidence of a high level of conservation amongst eutherian mammal's ZP proteins, but recent work on the brushtail possum ZP genes has shown that sequence similarities between possum ZPB and that of seven eutherian species range from 71% (possum ZPB vs. human ZPB) to 51% (possum ZPB vs. mouse ZP1) (Haines *et al.*, 1998). Possum ZPA sequence similarities to eutherians range between 65% to 60% (Mate & McCartney, 1998). Additionally, sequence and deduced amino acid comparisons with other non-mammalian vertebrates including birds, amphibians (*Xenopus*) and teleost fishes have displayed remarkable conservation of these molecules over time. In chickens, there appear to be four genes and these molecules display significant similarity to mammalian and other vertebrate ZP sequences (e.g. gp42 shows 40-50% identity to ZP3 of other species such as mouse, human and frog [Takeuchi *et al.*, 1999]). Certain sequences within mammalian ZPB and ZPA have also been found to have homologs in the proteins which make up the vitelline envelope in teleost fish, which is particularly interesting because the vitelline envelope proteins in some teleost species are derived from the liver and not from the oocyte or granulosa cells, as suggested for mammals (Lyons *et al.*, 1993; Epifano *et al.*, 1995a).

In addition to conservation amongst vertebrates within each glycoprotein family, there is evidence of common ancestry between the families. The mouse ZP1 protein contains a 348 amino acid sequence that is 47% similar to mouse ZPA (Epifano *et al.*, 1995*a*). With a number of mammalian ZP gene and protein sequences established, an increased amount of information such as the potential post-translational modifications and methods of secretion of the mammalian ZP could then be obtained.

Most early work on the mammalian ZP had been performed on the lab mouse and it is from this species that the first model about the structure and function of the ZP arose. Greve and Wassarman (1985), as discussed previously, first described that ultrastructurally, the mouse ZP was composed of filaments. Using the molar ratios described for each glycoprotein, Wassarman (1988) proposed the arrangement of the glycoproteins in mouse zona filaments. In this model, the ZP filaments are constructed of repeating ZPA-ZPC heterodimeric units that are, in turn, cross-linked by ZPB dimers (Fig. 1.1). While supported by the facts that mZPA and mZPC are found in near equimolar amounts, and that they are synthesized co-ordinately (Epifano *et al.*, 1995*b*), the molar ratios of the different glycoproteins in other species are much different, therefore questioning the validity of the model for all species of eutherian mammals (Green, 1997).

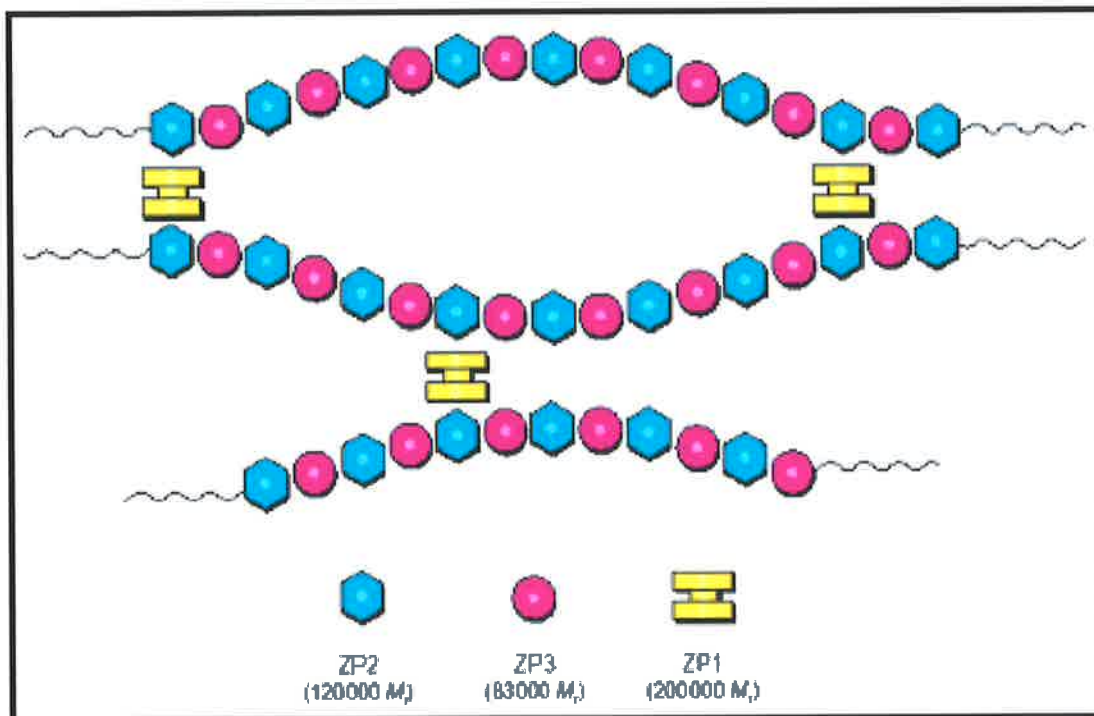


Figure 1.1 The model of the structure of the zona pellucida filaments as proposed by Wassarman and redrawn from Wassarman (1988). Filaments are constructed of repeating ZP2-ZP3 units and are cross-linked by ZP1. Taken from the review by Green (1997).

Early research by Bleil and Wassarman (1980, 1986) established through a competition assay that mouse ZPC contained the primary sperm receptor. Subsequently, it was found that it was in fact the O-linked oligosaccharide side-chains alone that accounted for the sperm receptor activity of ZPC (Florman & Wassarman, 1985). While ZPC and its homologs in other mammalian species such as rats, hamsters, baboons and humans have also been implicated as key adhesion molecules, ZPB homologs have also been suggested as playing a major role in sperm-zona interactions in some species (Snell & White, 1996). For example, the pig (Yurewicz *et al.*, 1993) and rabbit (Prasad *et al.*, 1995; Yamasaki *et al.*, 1995) ZPB molecule appears to be the primary sperm receptor in these species (see Table 2), or a complex of ZPB-ZPC (Yurewicz *et al.*, 1998).

In addition to its sperm receptor activity, however, recent work focusing on the targeted mutation of the ZPC gene in the mouse has shown that ZPC has a major structural role so that in its absence (mZPC-/mZPC-), no ZP develops (Liu *et al.*, 1996; Wassarman *et al.*, 1996). When mutant female mice carried a single mZPC allele (mZPC+/mZPC-), however, a ZP still developed, but was only half the thickness of the normal ZP (Wassarman *et al.*, 1997). Also, when infertile ZPC gene knockout mice had the human ZPC gene inserted into their genome, they produced chimeric ZP with mouse ZPB and ZPA and human ZPC, and became fertile again (Rankin *et al.*, 1998). Human sperm, however, did not bind to the ZP raising questions about the contention of human ZPC acting as a primary sperm receptor, as well as questioning the species-specificity of the ZP.

ZPA has also been shown to be a secondary sperm receptor in mice, binding strongly to acrosome-reacted sperm and, with the primary sperm receptor, it is modified during the cortical reaction to become ZPA_f so that its receptor function is lost (Bleil *et al.*, 1981, 1988; Bleil & Wassarman, 1986; Moller & Wassarman, 1989, cited by Saling, 1996; Yanagimachi, 1994). The role of this molecule, and its homologs, is still to be elucidated for other species.

1.3.4 Carbohydrate composition of the ZP

While initial studies focussed primarily on the proteins of the ZP, much of the structural and functional complexity of mammalian ZP was found to be due, in part, to the carbohydrate component of the glycoprotein moiety. The importance of carbohydrates in mammalian gamete interaction is supported by a variety of experimental evidence, including

the ability of various lectins and saccharides to inhibit the binding of sperm to ZP *in vitro* (Wassarman, 1988; Litscher *et al.*, 1995).

Carbohydrate analysis of the mammalian ZP glycoproteins indicated that all three glycoproteins contain oligosaccharides of both the asparagine- (*N*)-linked type as well as the serine/threonine- (*O*)-linked type (Wassarman, 1987c; for review see Benoff, 1997; Shalgi & Raz, 1997; Tulsiani *et al.*, 1997). *N*-linked oligosaccharides are added to protein backbones co-translationally in the endoplasmic reticulum and then are further glycosylated post-translationally in the Golgi apparatus to generate high mannose, hybrid or complex oligosaccharide chains which have a common pentasaccharide core (Man α 1-6(Man α 1-3)Man α 1-4GlcNAc α 1-4GlcNAc)-(Asn) (Kobata, 1992). Potential *N*-linked glycosylation sites on a polypeptide can be deduced from the amino acid sequence Asn-*X*-Ser/Thr, where *X* is any amino acid other than proline and asparagine, although it appears that a sequence which has threonine in the hydroxy position is three times more likely to be glycosylated than one which has serine (Kasturi *et al.*, 1997). It has been shown that *N*-linked glycans have an important role in the regulation of protein conformation by aiding in the folding of the protein and stabilization of mature glycoproteins, as well as having many other non-structural roles (Benoff, 1997).

O-linked oligosaccharides, on the other hand, are glycosylated to polypeptides exclusively in the Golgi apparatus via an attachment of *N*-acetylgalactosamine to either serine or threonine in a α 1 linkage (Benoff, 1997). Therefore, *O*-linked oligosaccharides may be attached at any serine or threonine residue, of which there are generally over 100 in each ZP polypeptide sequence studied so far. As mentioned previously, it was the *O*-linked

oligosaccharides of ZPC in lab mice, which sperm recognize and to which they bind (Florman & Wassarman, 1985; Wassarman *et al.*, 1989). More specifically, it appears that it is the monosaccharide at the non-reducing end of the *O*-linked oligosaccharide that is essential for sperm binding (Wassarman *et al.*, 1989; Miller *et al.*, 1992*b*). However, there is some controversy as to whether this terminal monosaccharide is α -galactose or *N*-acetylglucosamine (Bleil & Wassarman, 1988; Miller *et al.*, 1992*b*), although recent evidence suggests the former (Litscher *et al.*, 1995).

Unlike in the mouse, however, it has been shown that in the pig it is the *N*-linked oligosaccharides of pZP3 α (ZPB), and more specifically an *N*-linked oligosaccharide attached to Asn220 of ZPB, that binds sperm (Yonezawa *et al.*, 1995, 1997; Nakano *et al.*, 1996; Kudo *et al.*, 1998; Yurewicz *et al.*, 1998). In humans α -*L*-fucose is suggested to be the terminal monosaccharide to which sperm bind, not α -galactose (Patankar *et al.*, 1993; Lucas *et al.*, 1994; Clark *et al.*, 1996*a*).

Skutelsky *et al.* (1994) have also demonstrated, with the use of lectins, that ZPs of 7 different eutherian mammalian species show variations in their ZP carbohydrate distribution patterns. Lectins are (glyco)proteins of non-immune origin that agglutinate cells or precipitate glycoconjugates having saccharides of appropriate complementarity (Goldstein *et al.*, 1980). Lectins, therefore, are powerful tools in the localization of sugar residues at both the light and electron microscopical levels in various tissues and as such have been used to characterize the glycoconjugates of various eutherian mammalian ZPs (Nicolson *et al.*, 1975; Wu *et al.*, 1984; Shalgi *et al.*, 1991; Skutelsky *et al.*, 1994). Not only were there interspecific differences in the carbohydrates within the ZP, for example, only

rodent ZP bound to soybean agglutinin (Skutelsky *et al.*, 1994), there was also variation in the distribution of the carbohydrates. For example, Avilés *et al.* (1999, 2000a,b) demonstrated that α -galactose, GalNAc and GalNAc β 1,4Gal β 1,4 were localized in the ZP of mouse, human and hamster on the inner surface and therefore, by implication, could not be involved in primary sperm reception.

The differences seen in the features of sperm receptors and carbohydrate composition of mammalian ZP have been suggested to result in the species-specificity of gamete interactions. However, the exact nature and characterization of the ZP receptor(s) will probably only be resolved once the corresponding sperm ligands have been determined (Snell & White, 1996; Brewis & Moore, 1997).

1.3.5 Development of the ZP

During the growth phase of the oocyte, the ZP develops between the oocyte and its surrounding granulosa cells. The early stages of this process are poorly understood and over the years the exact cell type of the ZP glycoproteins has remained controversial. Recently, however, the oocyte has been shown to be the sole source of the ZP proteins in the mouse (Kimura *et al.*, 1994; Epifano *et al.*, 1995b), while its origin in other species remains under debate. The oocyte has been favoured as the source by some for the ZP proteins in the hamster (Léveillé *et al.*, 1987b) and pig (Takagi *et al.*, 1989), although this has recently been challenged (Sinowatz *et al.*, 1995). On the other hand, in the rabbit, pig, human, rhesus and cynomolgus monkey, dog, cat and horse both the oocyte and granulosa cells are suggested as producing the constituents of the ZP (Lee & Dunbar, 1993; Sinowatz

et al., 1995; Grootenhuis *et al.*, 1996; Kölle *et al.*, 1996; Martinez *et al.*, 1996; Barber *et al.*, 2001). With the differences in size, appearance, biochemical composition, and function it may be that there are differences in the origin of the ZP molecules for the different mammalian species.

The ultrastructural development of the ZP has been documented by a number of researchers for a number of species, including the rat (Kang, 1974), mouse (Chiquione, 1960; Wassarman & Josefowicz, 1978) and rabbit (Dietl, 1989), while few studies had been performed solely on marsupial ZP (Mate, 1998). In the tammar wallaby and brushtail possum, however, ZP construction seems to follow a similar plan: the ZP is deposited at separate sites as islands of zona material, which over the course of development, eventually combine to form a uniform extracellular matrix.

In the mouse, ZP1, A and C are synthesized as precursors in the oocyte as 75kDa, 81kDa, and 44kDa polypeptide chains, respectively (Wassarman, 1987c). *N*-linked, high mannose-type oligosaccharides are then added cotranslationally, presumably in the endoplasmic reticulum, and prior to secretion *N*-linked oligosaccharides are converted to complex-type and *O*-linked oligosaccharides are added within the Golgi complex (Wassarman *et al.*, 1996). While construction of the ZP2:ZP3 filaments may begin within the secretory granules prior to exocytosis (Wassarman *et al.*, 1996), the exact mechanisms of the three-dimensional (3-D) construction of the ZP are not known, however, two main models have recently been proposed (Green, 1997).

In the first model, the 3-D construction of the ZP occurs extracellularly in the gap between

the oocyte and granulosa cells. The ZP glycoproteins are transported to the surface of the oocyte in Golgi vesicles whose membrane fuses with the oolemma and the products released by exocytosis. The glycoproteins are, in fact, attached to the plasma membrane during this exocytosis and thus have to be cleaved from the surface by proteolysis at a furin cleavage site (Rankin *et al.*, 1996; Litscher *et al.*, 1999). While the early filament construction may occur within the Golgi vesicles, the entire 3-D construction of the filaments does not occur until the zona proteins move away from the oocyte surface. Green (1997) suggests that this prevents any self-assembly of the zona proteins and a zona matrix forming within the oocyte itself. In the second model the ZP is constructed on the surface of the oocyte, prior to the secretion into the extracellular space.

In the latter case, sheets of two-dimensional zona material are released onto the surface of the oocyte in the same manner as in the first model but, in this case, the proteins are not cleaved until the 3-D assembly of the filaments is completed. The construction of the ZP on the surface of the oolemma is thereby thought to prevent the self-assembly within the oocyte by releasing only two-dimensional zona material to the surface, and having no early filament construction occurring within the oocyte whatsoever (Green, 1997). Immunocytochemical and mRNA studies have demonstrated that the synthesis of the ZP proteins ceases either around the end of oocyte growth (before follicular antrum formation), or not long after, well before the oocyte is ovulated and picked up by the fimbriae of the oviduct (Epifano *et al.*, 1995b; Sinowatz *et al.*, 1995; Liang *et al.*, 1997).

Within the oviduct a number of important events occur which are required to take place if successful sperm-egg interaction and fertilization are to result. These include the changes

that occur to the oviduct during the oestrous cycle, the storage, maintenance and capacitation of sperm, as well as the possible modification of the ZP and/or the oocyte after ovulation. This review will provide a brief overview of the structure and function of the oviduct, as well as the potential modifications of the egg and its ZP after ovulation.

1.4 The Oviduct

1.4.1 Overview

In order for fertilization and normal early embryonic development to occur, the environment in which these processes take place, and the events leading up to them, must be precise. Although many of these events are poorly understood, they are known to involve a series of complex and highly specific interactions with, and within, the microenvironment of the oviduct. The establishment and maintenance of this environment is seen as the primary function of the oviduct, but whether this environment is passively or actively produced is still under debate (Gandolfi, 1995). On the one hand, the oviduct is thought to passively provide an optimal environment “in terms of temperature, pH, osmotic pressure, nutrients, oxygen tension and other factors” (Buhi *et al.*, 1997, p.285; Hunter, 1988; Leese, 1988). On the other hand, it may be actively producing certain proteins and factors which maintain and enhance the events leading up to, and following fertilization and early embryonic development (Buhi *et al.*, 1997, 2000).

1.4.2 General morphology of the Oviduct

The oviducts of many mammals are classically divided into 3 basic regions: the infundibulum, the ampulla and the isthmus. The infundibulum, and in particular its finger-like processes the fimbria, the most distal part of the oviduct, generally comes to surround the ovary and functions to pick up ovulated eggs. The isthmus, the most proximal part is considered the narrowest part of the oviduct and is the site for sperm storage prior to ovulation in many species of mammals. The ampulla, the connecting region between the isthmus and infundibulum is generally the widest region of the oviduct, at least in eutherians, hence its name (roughly meaning "wide bottle"), and is where fertilization occurs.

In marsupials, however, there are some differences. Rodger and Bedford (1982) were unable to distinguish any morphologically-distinct regions of the oviduct, except for the fimbriated infundibulum, in the Virginian opossum *Didelphis virginiana*. In Australian marsupials, however, it appears that the ampulla is the narrowest part of the oviduct, not the isthmus as in eutherians, and it has therefore been re-termed the "ovarian segment" by some authors (Bedford & Breed, 1994), due to its proximity to the ovary.

It has been established that with the division of the different oviductal regions

morphologically, each region also displays its own unique ultrastructural and histological features that relate to their biosynthetic and functional properties (Buhi *et al.*, 1997).

1.4.3 Functional Histology of the Oviduct

The epithelium of the oviduct consists of two main cell types: ciliated cells (CC) and non-ciliated (secretory) (NCC) cells. The number, height and synthetic activity of each cell type within the epithelium has been shown to depend on the hormonal levels during the different stages of the oestrous cycle (Hunter, 1988).

In most mammals, in which the ultrastructural changes of the oviductal epithelium during the oestrous cycle have been studied, ovariectomized (or in humans, post-menopausal) females form the control group. In these, the oviductal epithelium reverts from a normally active columnar or pseudo-stratified epithelium with the two cell types, to a single-layered cuboidal epithelium with a cell type that is often neither CC or NCC. If oestradiol is then administered to these females, however, the epithelium differentiates into the two structurally and functionally distinct cell types and reverts to the active form (for reviews see Hunter, 1988; Leese, 1988; Buhi *et al.*, 2000).

Similar findings have been found in studies in which both oestradiol and progesterone treatments were administered to ovariectomized females. Verhage *et al.* (1990) demonstrated that in the ampulla of ovariectomized baboons, the addition of oestradiol induces hypertrophy, hyperplasia, ciliogenesis and increased secretory activity, while progesterone causes atrophy, deciliation, apoptosis and loss of secretory activity. These

changes were not seen to the same degree in the isthmus, however, highlighting differences between different regions of the oviduct in this regard.

The importance of the oestradiol/progesterone antagonism in oviductal histology and function has been known for quite a while, at least in rabbits. Greenwald (1958) identified 2 cell types in the rabbit oviduct - ciliated non-mucin forming cells (or CC), and non-ciliated mucigenous cells (NCC) - which changed in response to oestradiol and progesterone. The non-ciliated mucigenous cells synthesized and stored mucin in response to oestrogen, while progesterone caused the mucin to be discharged into the lumen of the oviduct. He also observed that ovariectomized rabbits had oviductal epithelium that was one half the height of the epithelium during oestrus.

Like many mammals, the rabbit also displays considerable differences between the different regions of the oviduct. The NCC of the oviductal epithelium has been found to be rare in the infundibulum but the cell type increases in abundance toward the isthmus where it reaches its maximum (Greenwald, 1958; Jansen & Bajpai, 1982). Ultrastructurally, the NCC also differ between the regions of the oviduct. The NCC of the ampulla contain few compact, homogeneous electron-dense granules, while the isthmic NCC contain numerous large, non-homogeneous, electron-lucent granules, which are Alcian blue (pH 1.0) positive and which appear to be responsible for the mucoid coat which comes to surround the oocytes.

Marsupial eggs also become surrounded by a mucoid coat after entering the oviduct. Unlike in rabbits, however, the deposition of the mucoid coat around marsupial eggs is almost

immediate after ovulation and pick up by the infundibulum, whereas the rabbit oviduct does not release mucin until 10-12 hours after ovulation (Greenwald, 1958). This may reflect the temporal differences in the transit of the eggs through the oviducts, which is very short in marsupials (15-20h: Rodger & Bedford, 1982), but takes much longer in rabbits (60h: Harper, 1982). Like eutherian mammals, marsupial oviducts also display morphological and ultrastructural changes to hormonal levels and regional histological variation.

Despite the relative paucity of research on the oviducts of marsupials, some unique and interesting results have been obtained. One such result involves the regional variation of the oviducts, at least in the opossums and dasyurids, in which crypts are found only in the region of the isthmus. These small out-pockets of the oviduct are connected to the oviductal lumen via narrow openings and appear to have a unique epithelium.

The crypts are lined by cuboidal to low-columnar cells with few, if any, ciliated and mucoid-secreting cells (Rodger & Bedford, 1982; Breed *et al.*, 1989; Taggart & Temple-Smith, 1991; Roberts *et al.*, 1994; Taggart *et al.*, 1998). These were first described in the Virginian opossum, *Didelphis virginiana* (Rodger & Bedford, 1982; Bedford *et al.*, 1984) and then later described in a number of species of dasyurids (Selwood & McCallum, 1987; Breed *et al.*, 1989; Taggart & Temple-Smith, 1991). They appear, however, to be unique amongst marsupials, although it appears that, so far, little work on the histology and ultrastructure of the oviducts of other marsupials has been carried out. Crypts also appear relatively unique amongst eutherian mammals, with perhaps the exception of the shrews (in which there appear deep isthmus crypts in the genus *Crocidura*, as well as ciliated

ampullary crypts in the genera *Blarina*, *Cryptotis*, *Sorex*, and *Myosorex* [Bedford *et al.*, 1998]) and cows (which have crypts in the utero-tubal junction [Hunter, 1995, cited by Bedford *et al.*, 1998]). The isthmic crypts in marsupials seem to function as sperm storage sites.

Marsupial oviducts also appear to undergo similar changes in response to the changing hormonal levels during the oestrous cycle. Arnold and Shorey (1985) found regional variation and changes occurred in the oviductal epithelium of the phalangerid, the brushtail possum *Trichosurus vulpecula*. In this species, like that of many eutherian species, the NCC were significantly fewer in the infundibulum than in the ampulla and isthmus, while CC were found equally distributed throughout these two regions. Ciliogenesis was found to occur mostly in the infundibulum between oestrus and day 3 of the oestrous cycle and only in the rest of the oviduct when levels of oestrogen were increasing. Ovariectomized animals had oviducts with 99% of their epithelium composed of NCC (Arnold & Shorey, 1985), supporting the role of oestrogen in the maintenance of CC and normal biosynthetic activity of the oviductal epithelium in both marsupials and eutherians.

1.4.4 Biosynthetic activity – Oviductal-specific proteins

The oviduct has been demonstrated to be a highly synthetically active structure, despite the suggestion of its passive role in the production of a suitable environment for sperm-egg interaction. High biosynthetic activity includes the uptake, synthesis and release of a number of macromolecules including various proteins, lipids and energy substrates. (In this section, however, only the oviduct-specific proteins will be discussed).

Many of the proteins secreted by the oviduct are derived from the serum as a transudate, including albumin and transferrin (Hunter, 1988). A number of proteins found in oviductal fluid, however, are not derived from the serum but originate from the oviduct itself. These oviduct-specific glycoproteins (OGPs), which have also been collectively termed "oviductins" for those which have been shown to modify the oocyte after ovulation (Bleau & St-Jacques, 1989; Malette *et al.*, 1995), have been found in a number of mammalian species, the summary of which can be found in Table 1.3. Many of these OGPs have been found to be stage- and/or region-specific, that is, occurring only at a certain stage of oestrous cycle and/or in a specific region of the oviduct (for review see Gandolfi, 1995).

In the mouse, 25 oviductal proteins were found to show differential expression during the oestrous cycle using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Horvat *et al.*, 1992). In the oviduct, most proteins were found to be expressed during dioestrus, with two OGP restricted to oestrus and 7 restricted to dioestrus. Kapur and Johnson (1986, 1988) localized an OGP of 215kDa, which they termed GP215, to the secretory granules of the apical cytoplasm of NCC, but specifically only in the ampulla.

The ampulla appears to be the major source of OGP in many mammals. A 66kDa OGP from the rabbit is predominantly secreted by the ampulla (Hyde & Black, 1986), while an 85kDa OGP from pigs, which has been resolved by 2D-PAGE into two subunits (75 and 85kDa), is also found almost entirely in the NCC of the ampulla (Buhi *et al.*, 1989, 1990, 1992). Similarly, in sheep a 90-92kDa OGP was localized to NCC of the ampulla and fimbriae (Murray, 1993), while a 95-97kDa OGP (estrus-associated protein [EAP])

Table 1.3. Summary of the biological characteristics of mammalian oviduct-specific glycoproteins

Species	Mw (kDa)	Description	Localisation & Association	Function?	References
Rat	>330	Oviduct-specific	Secretory granules of NCC of oviduct	Unknown	Abe & Abe (1993)
Mouse	215	Oviduct-specific (GP215)	Secretory granules of NCC of oviduct (only in ampulla); PVS	Unknown	Kapur & Johnson (1986, 1988)
Rabbit	74	Sulphated protein (in vitro culture) – oviduct specific	N/A	Unknown	Barr & Oliphant (1981)
	>200; 71 & 32kDa subunits	Only 71kDa is sulphated (in vitro culture) – oviduct specific	N/A	Unknown	Oliphant & Ross (1982)
	66	Oviduct specific	Mostly ampulla	Unknown	Hyde & Black (1996)
Equine	106, 50(x3), 24(>1)	106kDa and several 24kDa proteins found at non-estrus; 3 x 50kDa proteins at estrus – oviduct specific	N/A	Unknown	Willis <i>et al.</i> (1994)
Hamster	200-240	Isolated from oviductal ZP (ZP0)	ZP; secretory granules and golgi of NCC of oviduct (specifically, > binding in isthmus)	Unknown	Abe & Oikawa (1990a,b, 1991)
	200	Hamster oviductin-I	As above; PVS; blastomeres of 2-8 cell stage embryos	Sperm-egg interaction? Uterine implantation?	Roux & Kan (1995); Martoglio & Kan (1996)
	α - 160-210 β - 210-350	α -acidic isoform; β -basic isoform – oviduct specific	ZP	Protective; influence on early fertilisation steps	Malette & Bleau (1993)
	?	Oviduct specific glycoprotein (OGP)	ZP	Species-specificity of sperm-ZP interaction	Schmidt <i>et al.</i> (1997)
Bovine	97	Estrus-associated protein (EAP)	ZP	Fertilisation/early embryonic development	Wegner & Killian (1991, 1992)
	95	EAP	N/A	Facilitates capacitation + sperm fertilising ability	King <i>et al.</i> (1994)

Bovine (cont.)	60	Catalase specific to oviduct	Sperm (acrosomal cap)	Protection of sperm + sperm survival	Lapointe <i>et al.</i> (1998)
	80, 74, 60, 45, 30 (+95)	Oviductal specific	ZP	Unknown	Staros & Killian (1998)
Baboon	130, 160, 88	130 – 1 basic + 2 acidic proteins; 160kDa + 88kDa – oviduct-specific	Oviduct	Unknown	Fazleabas & Verhage (1986)
	100-130	Family of oviduct-specific proteins	NCC of oviduct; ZP; PVS; blastomeric cytoplasm	Fertilisation and embryonic development	Verhage <i>et al.</i> (1989) Boice <i>et al.</i> (1990)
Human	110-130	mid-cycle group of human oviduct-specific glycoproteins (huOGP)	Apical granules of NCC of oviduct; ZP	Unknown	Verhage <i>et al.</i> (1988); Rapisarda <i>et al.</i> (1993); O'Day-Bowman <i>et al.</i> (1995)
Macaque	120	Oviduct-specific glycoprotein (OGP)	Oviduct	Unknown	Verhage <i>et al.</i> (1997)
Porcine	335, 115, 85; 60, 20	335, 115, 85kDa – pro-estrus, estrus, metoestrus; 60 + 20kDa – luteal – oviduct specific	Ampulla>isthmus	Unknown	Buhi <i>et al.</i> (1989)
	115 (100 x 2), 85 (75 + 85)	115kDa – 100kDa acidic and basic proteins; 85-75 + 85kDa proteins – estrus associated protein (EAP)	Ampulla	Unknown	Buhi <i>et al.</i> (1990, 1992)
	75-85	Acidic EAP	Ampulla; PVS; ZP; microvilli and plasma membrane of oocyte	Unknown	Buhi <i>et al.</i> (1993)
Sheep	90-92	Oviduct specific	Ampulla and fimbriae	Unknown	Murray (1993)
Brushtail Possum	61	Oviduct secretory protein	Sperm	Prolong sperm survival	Sidhu <i>et al.</i> (1999)

from cows was found to be produced by the epithelial cells of the ampulla in greater quantities than those of any other oviductal region (Wegner & Killian, 1992).

The hamster, on the other hand, produces an OGP (variously termed "ZP0" [Abe & Oikawa, 1990*a*, 1990*b*, 1991] or "Hamster oviductin-I" [Roux & Kan, 1995; Martoglio & Kan, 1996]) that is produced by the NCC of the entire oviduct, which was immunolocalised predominantly to the caudal isthmus (Abe & Oikawa, 1990*a*, 1990*b*, 1991). The isthmus is also responsible for the secretion of an OGP in the baboon (Verhage *et al.*, 1990).

Secretion of many of the OGP are oestrogen-dependent while others are synthesised only during the luteal phase of the oestrous cycle. For example, the secretion of a family of pig OGPs, termed porcine oviductal secretory proteins (POGP) 1-3 (75kDa-85kDa, pI <4; 100kDa, pI 4-5.5; 100kDa, pI >8) are oestrogen-dependent and this dependency has been shown to be antagonized by progesterone (Buhi *et al.*, 1992).

Three 50kDa OGP have been found within the oviductal secretions of horses during oestrus, while a 106kDa and several 24kDa OGP were found during non-oestrus (Willis *et al.*, 1994).

A number of mammals have also had the cDNA of their OGPs partially or completely sequenced including the pig (Buhi *et al.*, 1996), cow (Sendai *et al.*, 1994), baboon (Donnelly *et al.*, 1991), human (Arias *et al.*, 1994), macaque (Verhage *et al.*, 1997*b*), hamster (Suzuki *et al.*, 1995) and mouse (Sendai *et al.*, 1995). Comparisons between species of the deduced

amino acid sequences shows a high level of conservation amongst mammalian OGP, especially between closely related species. For example, the amino acid sequence of the macaque OGP is 97.8% homologous to the baboon OGP and 92% homologous to the human OGP (Verhage *et al.*, 1997a). Less closely related species, however, have only slightly lower figures with the protein sequence of porcine OGP exhibiting a significant identity (65-78%) and similarity (78-87%) to OGPs from the cow, sheep, human, mouse and hamster (Buhi *et al.*, 1996).

While there appears considerable conservation of protein sequences of mammalian OGPs, most variation and species-specificity is afforded through their carbohydrate components. The number of potential *N*-linked (Asn-*X*-Ser/Thr) glycosylation sites, as deduced by amino acid sequences, varies widely between species ranging from only 1 in the cow, baboon and macaque to 8 potential sites in the golden hamster. A number of lectin-binding experiments have demonstrated that oligosaccharides of the OGP of the mouse, cow and hamster were WGA-reactive providing evidence of terminal α -D-NeuNAc (sialic acid) and/or non-terminal β -D-(GlcNAc)₂ residues (Malette & Bleau, 1993). Wegner and Killian (1992) found that bovine OGP bound peanut lectin, which stains for Gal(β 1-3)GalNAc residues, indicating that the 1 potential *N*-linked glycosylation site deduced from its amino acid sequence is indeed utilized. Interestingly, however, potential *N*-glycosylation sites do not always indicate the *N*-linked glycosylation of the mature protein. In the hamster, as mentioned previously, the OGP has 8 potential *N*-linked glycosylation sites, but in vitro metabolic labelling studies suggest the presence of only one or two *N*-glycan chains (around 10kDa) (Malette & Bleau, 1993). This also suggests that at least 120kDa of the mature post-translated hamster OGP is formed by sulphated *O*-linked glycans.

Other post-translational modifications, besides the addition of glycans, which may occur to OGP include phosphorylation and sulphation. Buhi *et al.* (1990) reported that porcine OGP is phosphorylated, while the deduced amino acid sequence of human OGP indicates that it may also be phosphorylated (Arias *et al.*, 1994). Hamster OGP, as previously mentioned, is sulphated but did not incorporate inorganic phosphate during *in vitro* studies (Malette & Bleau, 1993), while other sulphated OGPs include those from the rabbit (Oliphant & Ross, 1982), and pig (Buhi *et al.*, 1990).

Research on the oviductal secretions of marsupials has arisen of late by the investigations into the origin and composition of the tertiary egg membranes (Roberts *et al.*, 1994, 1997; Roberts & Breed, 1996*a,b*). Through immunocytochemical and histological work, it has been determined that in the brushtail possum and fat-tailed dunnart, the mucoid material secreted by the oviduct is a sulphated and acidic glycoprotein (after staining with Alcian blue, pH 1.0 - pH 2.5 and Periodic Acid-Schiff's Reagent). Recently, however, more detailed investigations into the oviductal secretions of marsupials have been undertaken. These studies have demonstrated that, when co-cultured, sperm from the tammar wallaby *Macropus eugenii* bind to oviductal monolayers and, after 6h, undergo morphological and functional changes thought to be associated with capacitation (Sidhu *et al.*, 1998). Several proteins from the oviduct have also been found to associate with sperm *in vitro*, when sperm are incubated in oviduct-conditioned media, which also appears to increase the viability of sperm and induce capacitation-like changes (see Table 1.3) (Sidhu *et al.*, 1999). For the first time in an Australian marsupial, sperm cultured in oviduct-conditioned media have been found to bind to, and penetrate, the ZP *in vitro* in the brushtail possum (Mate *et*

al., 2000), although sperm-oolemma binding and fusion did not take place. To date, *in vitro* fertilisation has not been achieved in an Australian marsupial, and it appears that this might relate to the maturational changes to the gametes induced by oviductal glycoproteins.

1.4.5 Functions of Oviductal-Specific Glycoproteins (OGPs)

A number of functions for the OGPs have been proposed ranging from general maintenance of the oviductal microenvironment to acting as a fourth element within the ZP which aids in sperm binding. This latter function, in fact, is commonly proposed for many of the OGPs due to a large number of them being found to associate with the ZP (see Table 1.3 for OGP associations).

Early work by L  veill   *et al.* (1987*a*) on the hamster ZP found that antibodies raised against oviductal ZP glycoproteins cross-reacted with the oviduct, indicating that the ovulated ZP consisted of 2 groups of immunogenic components, one from the ovary and one from the oviduct. Later work in this species isolated the hamster OGP and was termed ZP0 because it was so intimately associated with the other three components of the ZP, and because it was not, in fact, isolated from the oviduct but from ovulated ZP itself (Abe & Oikawa, 1990*a*, 1990*b*, 1991). To investigate its effect on sperm-ZP interactions, hamster OGP was added to *in vitro* culture media and significantly increased binding of sperm to both oviductal and follicular eggs (Schmidt *et al.*, 1997). When other species OGPs were added to the culture media, however, sperm binding decreased, indicating, according to the authors, that OGPs play a role in the species-specificity of sperm-ZP interactions (Schmidt *et al.*, 1997).

Like the ZP, and as previously mentioned, the protein cores of OGPs appear highly conserved between species, while there are great interspecific differences in glycosylation, relating, perhaps, to the species-specificity of the OGPs. This is plausible and supported by the fact that *in vitro*, sperm from one species will bind to the ZP of another in spite of suggestions to the contrary. For example, mice and hamster sperm readily bind to the ZP of the other species *in vitro* (Schmell & Gulyas, 1980a; Moller *et al.*, 1990). Missing from this *in vitro* environment is, of course, the secretions of the oviduct, which may, therefore, afford the ZP with its species-specificity. It appears then, that OGP and ZP glycoproteins are extremely similar in terms of their roles in sperm-egg interactions, their high degree of proteinaceous conservation and therefore the species-specificity afforded by their glycosylation differences.

An interesting OGP has been discovered which is not an "oviductin", and therefore unrelated to the others, but which functions to protect sperm within the oviduct. This 60kDa OGP, isolated in cows, has been found to be an oviduct-specific catalase that is related to the common catalases found in liver (99% amino acid homology with bovine liver catalase), and has similar functions (Lapointe *et al.*, 1998b). Catalases activate the decomposition of hydrogen peroxide, which has been found to be toxic to sperm and inhibits the acrosome reaction, sperm-egg binding and oocyte penetration. In cows, the oviduct-specific catalase was detected in oviductal fluid, its activity peaking around the time of ovulation, and was found to bind to bovine, boar and human sperm (Lapointe *et al.*, 1998a). Bovine liver catalase did not bind to sperm, however, which may relate to the 20 amino acid extended carboxyl terminus, found only in the oviductal catalase. It is suggested,

therefore, that the oviductal fluid catalase plays an important role in sperm survival within the female reproductive tract, however, similar molecules remain to be identified in other species (Lapointe *et al.*, 1998a,b).

OGPs may play a role in early embryonic development due to their association with embryos in a number of species. The addition of OGPs to culture media has been found to significantly increase the cleavage rates and developmental competence of embryos in sheep (Hill *et al.*, 1996), pigs (Archibong *et al.*, 1989) and cows (Ellington *et al.*, 1990; Eyestone *et al.*, 1991), while in hamsters, Oviductin-I appears to bind to uterine luminal epithelial cells but decreases in binding around implantation suggesting its function as a modulator of uterine receptivity (Martoglio & Kan, 1996; Roux *et al.*, 1997).

Information on OGPs in marsupials is scant. With the large number of OGPs being identified in numerous eutherian species, and the demonstration of their high degree of conservation, it seems likely that the marsupial oviduct produces some form of OGP. With the considerable evidence supporting the importance of OGPs in normal sperm-ZP interactions, the inability of researchers to achieve successful and repeatable *in vitro* fertilization in Australian marsupials supports the importance of an, as yet, uncharacterised marsupial OGP. This substance may associate and modify the ZP within the oviduct, allowing sperm-ZP binding to occur, while the absence of it within the *in vitro* media prevents this interaction.

1.5 Summary and Project Proposal

Within eutherian mammals, sperm-ZP interactions are one of the most studied areas of gamete biology, but the molecular structure of the zona pellucida still largely remains an enigma. Despite the fact that it is over 20 years since the initial identification of the zona pellucida glycoproteins of the lab mouse (Bleil & Wassarman, 1980), numerous questions still exist, such as what is the cellular site of origin of the glycoproteins and how are they secreted? Also, it is still not understood how the three-dimensional structure around the oocyte forms, nor which carbohydrates are important for sperm-zona pellucida interaction. Confounding these questions is the fact that, within eutherians, there are a number of interspecific differences in size, composition, extent and type of glycosylation, as well as the function(s) of the individual zona pellucida glycoproteins.

In marsupials, even less is known about the structure and function of the ZP. Until recently, there had been relatively few studies which had focussed solely on the ZP of marsupials, with most information largely drawn from incidental observations from morphological studies on the oocytes and eggs of different species, and particularly in didelphid and dasyurid species (Philips & Fadem, 1987; Breed & Leigh, 1988, 1990; Breed, 1996). With very few species of marsupials existing as laboratory animals, it has been extremely difficult to obtain enough material to perform similar studies to those undertaken for some eutherian species. Additionally, until the recent success by Mate *et al.* (2000), repeatable and reliable sperm-ZP binding and penetration *in vitro* had not been achieved in any Australian marsupial. As such, prior to this project, very little was known about the

method of secretion and origin of the ZP in marsupials, its basic structure and composition, or even some of its basic functions.

The first part of this thesis, therefore, has involved a comparative ultrastructural study of the formation and secretion of the ZP in several marsupial species. This has involved a detailed transmission electron microscopical (TEM) study of the formation of the ZP around developing ovarian oocytes of the fat-tailed dunnart (Family *Dasyuridae*), brushtail possum (Family *Phalangeridae*), koala (Family *Phascolarctidae*), southern hairy-nosed wombat (Family *Vombatidae*) and western grey kangaroo (Family *Macropodidae*) with an emphasis on timing of formation, method and origin of secretion and change in morphology during oocyte maturation.

There is a high degree of similarity in the ZP genes and their putative proteins, with the greatest degree of homology occurring between members of the same family or order (e.g. lab mouse and lab rat in Rodentia; humans and baboons in Primates, etc). With such high homology, much of the heterogeneity of ZP glycoproteins amongst mammalian species is likely to relate to the glycosylation patterns. Similarly, the aforementioned claimed species-specificity of sperm-ZP binding may be due to this heterogeneity, as there is an almost infinite possible combinations of glycoconjugates of the ZP glycoproteins (Skutelsky *et al.*, 1994; Shalgi & Raz, 1997; Tulsiani *et al.*, 1997; Takasaki *et al.*, 1999).

In marsupials, other than a note that the ZP of marsupials is periodic acid-Schiff's reagent (PAS)- (in *Trichosurus vulpecula* and *Sminthopsis crassicaudata*) and Alcian blue- (pH 0.2 and 2.6 in *T. vulpecula*) positive (Hughes, 1974; Breed, 1996), no work has been performed

on the glycoconjugate content of marsupial ZP. Consequently, the second part of this project involved a comparative study of the glycoconjugate content of the ZP of ovarian oocytes from seven marsupial species, each from a different family - the fat-tailed dunnart, southern brown bandicoot (Family Peramelidae), grey short-tailed opossum (Family Didelphidae), brushtail possum, ringtail possum (Family Pseudocheiridae), koala and eastern grey kangaroo. This was carried out by differential lectin histochemistry using ten fluorescently-conjugated lectins on paraffin sections of marsupial ovaries, before and after sections had been subjected to enzymatic or saponification treatments to visualise glycoconjugates masked by sialic acids.

The inability to achieve spermatozoa-ZP binding and fertilisation *in vitro* in Australian marsupials has hampered efforts to determine the molecules involved in this process, while also frustrating attempts to develop assisted reproduction. This inability suggests that a component from the *in vivo* environment may be missing in the *in vitro* system that is critical for successful fertilisation to occur. The lack of sperm binding, and the observation that the ZP of the brushtail possum changes in consistency from 'broad and diffuse' prior to ovulation to 'thin and compact' after (Rodger & Mate, 1993), suggests that following ovulation some changes are occurring to the ZP within the oviduct that enables fertilisation to proceed. Thus, the next aim of this project was to investigate the changes that occur to the ZP of the brushtail possum following ovulation, including the possibility that additional material is incorporated from the oviduct that facilitates sperm-ZP binding. Firstly, lectin histochemistry and immunocytochemistry were used to investigate the glycoconjugates secreted by the oviduct around the time of ovulation that might bind to the ZP, to facilitate sperm-ZP interactions in the oviduct. Secondly, several methods of ZP matrix preparation

were employed including: routine TEM and scanning electron microscopy (SEM), cryo-SEM, and rapid freezing and freeze substitution followed by TEM to determine its detailed structural organisation around ovarian and ovulated oocytes. Ovarian and ovulated oocytes were then subjected to lectin immunocytochemistry in order to determine if any change in glycoconjugate content of the ZP occurs following ovulation.

One of the major functions of the egg envelope, including that of the ZP of eutherian mammals, is to prevent more than one sperm from fertilising an egg. The ZP block to polyspermy occurs very soon after sperm-oolemma fusion and involves a morphological and functional modification of the ZP due to the exocytosis of the cortical granules of the egg (for reviews see Cran & Esper, 1990; Ducibella, 1991). In *M. domestica*, it has been suggested that, as a result of the cortical granule exocytosis (cortical reaction), a new coat forms within the perivitelline space (PVS), termed the 'cortical granule envelope' (Dandekar *et al.*, 1995). It is unknown if a similar process occurs in the eggs of Australian marsupials, nor the exact origin of this coat as granular material has been noted in the PVS of ovulated, and even ovarian, eggs of *S. crassicaudata* (Breed & Leigh, 1990; Breed, 1996). It is also unclear how important the cortical reaction in marsupials is in preventing polyspermy since, soon after ovulation, the egg becomes encased within a tertiary membrane, the mucoid coat, which may act as a barrier to polyspermy (Rodger & Bedford, 1982; Selwood, 1982, 1992). The final component of this study then, was to develop an artificial activation system of brushtail possum eggs using the calcium ionophore A23187 that were then processed for examination of the structural organisation of the egg coats. The glycoconjugate content of the ZP following artificial activation was also investigated using

lectin immunocytochemistry to determine what biochemical changes occur to the ZP following cortical granule exocytosis.

This project, therefore, attempts to characterise some of the fundamental, yet poorly understood or unknown, features of the marsupial ZP. It follows the formation of the ZP, determining, in part, its basic biochemistry surrounding ovarian and ovulated oocytes. Finally, its structure and the related biochemical changes associated with egg activation are determined. In carrying out these observations, comparative investigations of ovarian oocytes of several marsupial species have been performed, while those involving the basic biochemical and/or maturational changes of ovulated oocytes focussed upon one species, the brushtail possum. This species was selected for several reasons - firstly, it is an abundant species and is relatively readily available in the Adelaide region of South Australia; secondly, it is one of a very few marsupial species in which a superovulation regime has been devised (Rodger & Mate, 1988; Glazier & Molinia, 1998); and thirdly, it is the only marsupial species which, during the course of this project, has had the full cDNA and putative protein sequences of all three ZP genes characterised (Mate & McCartney, 1998; Haines *et al.*, 1999; McCartney & Mate, 1999; Voyle *et al.*, 1999). The brushtail possum, therefore, served as the marsupial model for study of the ZP in this project.

Chapter 2 Research Project

2.1 Animals

The Animal Ethics Committee of the University of Adelaide approved all aspects of this project and work was carried out under the approval numbers: S/45/95 and S/003/96; and from National Parks and Wildlife Service under the approval numbers of K23749-02 and M23766-02.

2.1.1 Brushtail possums (*Trichosurus vulpecula*)

The common brushtail possum (*Trichosurus vulpecula*; Family: Phalangeridae) used in this study were obtained from the Division of Animal Services of The University of Adelaide which, in turn, were obtained from professional catchers in the Adelaide Metropolitan area. Animals were housed at the Division of Animal Services central animal facility at the University of Adelaide's Waite campus until needed, whereupon they were transported and housed at the University of Adelaide's Medical School Animal House.

All brushtail possums, which are a mono-ovulatory species, were housed alone and maintained in a lighting regime of 14h of light and 10h of darkness with lights out at 7pm. Food consisting of slices of fruit and carrot were provided once a day and water was supplied *ad libitum*.

To enhance egg production, brushtail possums were primed with gonadotrophins (see section

2.2) to induce ovulation. In order to study the zona pellucida of periovulatory ovarian eggs, brushtail possums were also primed with gonadotrophins, but collected prior to ovulation.

2.1.2 Fat-tailed dunnarts (*Sminthopsis crassicaudata*)

Fat-tailed dunnarts (*Sminthopsis crassicaudata*; Family: Dasyuridae) were obtained from a colony housed in the Division of Animal Services of the University of Adelaide. This small nocturnal marsupial, weighing around 15g, is found widely in southern Australia, particularly in arid regions.

All animals were maintained in a lighting regime of 14h of light and 10h of darkness with lights out at 7pm. Food and water were supplied *ad libitum*, as described by Bennett *et al.* (1990).

For this project, only the ovaries of naturally cycling females were used and all ovaries were obtained opportunistically through the culling of excess females from the colony's stock.

2.1.3 Koalas (*Phascolarctos cinereus*)

Koalas (*Phascolarctos cinereus*; Family: Phascolarctidae) were obtained from Cleland Wildlife Park in the Mount Lofty Ranges, South Australia and/or from wild animals brought to the contract veterinarian of Cleland Wildlife Park, Dr Ian Hough. Koala pouch young were obtained from the contract veterinarian for the sterilisation program of koalas on Kangaroo Island, South Australia, Dr Greg Johnsson.

Animals were obtained opportunistically for this project from individuals either brought in and housed and/or raised at Cleland Wildlife Park, and which were due to be euthanased due to injuries (predominantly from vehicle accidents and/or dog attacks) or health problems not related to reproduction, the most common of which was kidney failure. Only ovaries and reproductive tracts from naturally cycling females were used in this study.

2.1.4 Southern hairy-nosed wombats (*Lasiorhinus latifrons*)

Southern hairy-nosed wombats (*Lasiorhinus latifrons*; Family: Vombatidae) were obtained from near Swan Reach, South Australia.

Animals were obtained opportunistically from culls of a wild population near Swan Reach, some prior to the commencement of this project for other research. Ovaries obtained, therefore, were taken from naturally cycling females throughout different times of the year.

2.1.5 Eastern Grey Kangaroos (*Macropus giganteus*)

Eastern grey kangaroos (*Macropus giganteus*; Family: Macropodidae) were obtained from Cleland Wildlife Park in the Mount Lofty Ranges, South Australia.

Animals were obtained opportunistically from a cull of excess stock from Cleland Wildlife Park. Ovaries and reproductive tracts were obtained from naturally cycling females in spring.

2.1.6 Western Grey Kangaroos (*Macropus fuliginosus fuliginosus*)

Western grey kangaroos (*Macropus fuliginosus*; Macropodidae) were obtained from Kangaroo Island, South Australia.

Ovaries were obtained opportunistically from the Kangaroo Island subspecies from a cull of wild populations. Ovaries and reproductive tracts were obtained from naturally cycling females during summer, spring and winter.

2.1.7 Ringtail Possums (*Pseudocheirus peregrinus*)

Ringtail possums (*Pseudocheirus peregrinus*; Family: Pseudocheiridae) were obtained from Cleland Wildlife Park's contract veterinarian Dr Ian Hough.

Ringtail possums were obtained opportunistically from animals due to be euthanased due to injury or health problems. Ovaries and reproductive tracts were obtained from naturally cycling females during summer.

2.1.8 Southern Brown Bandicoot (*Isodon obesulus*)

A southern brown bandicoot (*Isodon obesulus*; Family: Peramelidae) was obtained from Warrawong Wildlife Sanctuary in the Adelaide hills, South Australia.

A female southern brown bandicoot was obtained opportunistically from a cull of excess animals. The ovaries and reproductive tract were obtained from an adult female which had four pouch young in winter.

2.1.9 Grey Short-tailed Opossum (*Monodelphis domestica*)

Grey short-tailed opossums (*Monodelphis domestica*; Family: Didelphidae) were obtained from a colony bred at the South West Foundation for Biomedical Research, San Antonio, Texas.

Grey short-tailed opossums were donated from the colony of Professor John VandeBerg for use in this study. Ovaries were obtained from naturally cycling females at various stages of their reproductive cycle.

2.2 Superovulation by priming with Gonadotrophins

For studies requiring investigation of the zonae pellucidae of ovulated oocytes, female brushtail possums were superovulated using a modified regime as described by Rodger and Mate (1988). Pouch young were removed 24-48h prior to the first intraperitoneal injection of 15 i.u. of pregnant mare's serum gonadotrophin (PMSG) (Folligon, 50 i.u./ml, Intervet, New South Wales). Seventy-two hours later, animals received 4 intraperitoneal or intramuscular injections of 60µg of gonadotrophin releasing hormone (GnRH) (Fertagyl, 100µg/ml, Intervet, New

South Wales) spaced at 90 mins apart, with ovulation usually occurring around 28h following the first injection. Females were sacrificed by anaesthesia with CO₂ and then an intracardiac overdose of Lethabarb (sodium pentobarbitone 325mg/ml, Virbac Australia Pty Ltd, New South Wales). Following a mid-ventral incision, their uteri, oviducts and ovaries were dissected free and placed into a culture dish of pre-warmed (35-37°C) Tyrode's culture media (CSL Limited, Victoria, Australia). Excess tissue was removed with the aid of a dissecting microscope, the ovaries removed and checked for recent corpora lutea indicative of ovulation. A blunted 23G needle on a 3ml syringe filled with pre-warmed Tyrode's culture media was then inserted retrograde into the uterus and first part of the oviduct which was then flushed and any ovulated oocytes expelled into the culture dish. This was also repeated for the other uterine horn and oviduct. Ovulated eggs were then collected using a mouth operated hand-drawn fine glass pipette and kept in a drop of Tyrode's culture media under oil at 35-37°C until required for experimentation.

2.3 Dissections and Processing for routine Light and Electron Microscopy

Immediately following death, all animals were dissected mid-ventrally, usually through the lower pelvic (pouch) region. Reproductive tracts, including the ovaries, oviducts and uteri were removed, trimmed free of peritoneum and immersed into a fixative of choice.

For light microscopy (LM), and for lectin histochemistry in particular, the reproductive tracts were fixed by immersion into Rossman's fluid (90ml of 100% alcohol, 10ml of 10% buffered formalin, saturated with picric acid) for 24-48h. Following fixation, the ovaries and oviducts

were isolated and washed for 72h in 95% alcohol prior to being processed for LM. Processing for LM was performed in a Shandon Duplex Processor which involved dehydration in a series of increasing alcoholic solutions, clearing in Safsolv and infiltration into paraffin wax. The tissues were then infiltrated under vacuum to remove air bubbles and finally embedded into paraffin wax using a Tissue Tek II wax dispenser.

For electron microscopy (EM), reproductive tracts were placed into the routine EM fixative, composed of 3% glutaraldehyde/3% paraformaldehyde made up in 0.2M phosphate buffer, pH 7.2, and the ovaries and oviducts isolated under a dissecting microscope. The tissues were then sliced into small pieces ($\sim 1\text{mm}^2$) and fixed for 2h at room temperature or overnight at 4°C. Following fixation, tissues were washed in 0.2M phosphate buffer, pH 7.2 and then post-fixed in 1% osmium tetroxide (OsO_4) for 2h at room temperature and washed in buffer again. For TEM, tissues were dehydrated by passing them through a graded series of alcohols, cleared in propylene oxide, embedded into TAAB TK3 epoxy resin (TAAB Laboratories, Berkshire) and polymerised for 72h in a 60°C oven. For SEM, tissues were processed as above but dehydrated by passing them through a graded series of acetones, followed by complete dehydration with liquid CO_2 in a BAL-TEC CPD 030 critical point dryer before being coated with carbon and palladium and viewed under a Philips XL30 Field Emission Gun SEM.

2.4 Sectioning and Staining

For LM, paraffin blocks were trimmed of excess wax before being serially sectioned at $7\mu\text{m}$ using a Leitz Lab Tek rotary microtome and floated onto a warm water bath. Sections were

then placed onto aminopropyltriethoxysilane- (APES-) coated slides, one section at either end of the slide, and dried in an oven at 40°C for 24h. Sections on every fifth slide were then routinely stained with haematoxylin and eosin for survey of structure and orientation. Unstained sections with the correct structure and orientation were then used for further studies (see Chapter 4).

For EM, semi-thin plastic survey sections (0.5-1µm) were cut using a Reichert-Jung Ultracut ultramicrotome with a glass knife and stained with 0.025% toluidine blue in 0.5% sodium tetraborate and viewed under a compound light microscope. Once areas of interest were found, the excess resin and tissue were trimmed into a trapezoidal shape, and thin sections (70-90nm) with silver/gold interference colours cut with a diamond knife (Diatome). Sections were picked up onto acetone-cleaned copper/palladium grids (200 mesh), left to air dry for 24h, and then stained with alcoholic (70%) uranyl acetate and then lead citrate (Reynolds, 1963). Thin sections were then viewed using a Philips CM 100 TEM at 80kV and images captured either on Kodak black and white 3^{1/4}"x4" film (Kodak Eastman Company, Atlanta) or using an analySIS Mega-View II digital image capture system (Soft Imaging System, GmbH, Germany).

Chapter 3 Comparative ultrastructure of the secretion and formation of the zona pellucida during oocyte growth in marsupials

3.1 Introduction

The mammalian zona pellucida (ZP) forms between the oocyte and the surrounding granulosa cells during the growth phase of the oocyte. Exactly how the three glycoproteins (ZPA, ZPB/ZP1 and ZPC) are secreted and interact to form the relatively thick transparent coat is poorly understood, although these phenomena have been most studied in eutherians and several hypotheses have been suggested (for reviews see Wassarman, 1988; Green, 1997).

The ZP of marsupials have been reported to be much thinner than those of eutherians (for reviews see Bedford, 1991, 1996; Dunbar *et al.*, 1991, 1994; Selwood, 2000). The relatively thin ZP of marsupials has been suggested to have co-evolved with the structure and method of zona penetration of marsupial spermatozoa (Bedford, 1991, 1996). The spermatozoon from most marsupials has an acrosome which forms a flattened cap on the dorsal surface of the head of the sperm and appears highly stable, perhaps due to disulphide bonding between acrosomal membranes, while the underlying nucleus appears less stable (Cummins, 1980; Mate & Rodger, 1991; Sistina *et al.*, 1993; Lin *et al.*, 1995). These features have been postulated as suggesting a primarily enzymatic method of penetration of the relatively thin marsupial ZP (Bedford, 1991).

The spermatozoa of two groups of marsupials, however, the wombats and koalas, have a very different structure. Spermatozoa from wombats and koalas have a highly curved nucleus,

while the acrosome lies within the concavity upon its ventral surface (Hughes, 1965; Harding *et al.*, 1987; Temple-Smith & Taggart, 1990; Breed *et al.*, 2001). The reason for such a different sperm structure from other marsupials is unclear, however very little work has been performed on the structure of their eggs to determine if there is any correlation with the structure of the ZP. While to date, only one ultrastructural study has specifically detailed the formation of the ZP in marsupials (Mate, 1998), a number of studies have reported on its formation during oogenesis (*Didelphis virginiana*: Guraya, 1968; *Sminthopsis macroura*: Kress *et al.*, 2001; *Trichosurus vulpecula*; Frankenberg & Selwood, 2001) and oocyte maturation (*Monodelphis domestica*: Falconnier & Kress, 1992). While Mate (1998) reported the localisation of ZP antigens within the follicle cells of primary oocytes of the tammar wallaby and brushtail possum using immunohistochemistry, a result not supported by immunocytochemistry in the same study, and although not specifically investigated by any other study, little evidence has been found for any contribution to the ZP by the follicle cells in marsupials.

This study, therefore, in describing the ultrastructural features of ZP secretion and formation in five species of marsupials, attempted to answer the following questions: 1) is there any ultrastructural evidence for the contribution by the follicle cells to ZP formation? 2) Are there any differences in ZP formation and structure from that reported in eutherians? And 3) Are there any differences in ZP formation and structure in the wombats and koalas from those of the other marsupials that may account for the unique morphology of their spermatozoa?

3.2 Materials and Methods

3.2.1 Animals

Ovaries of adult female brushtail possums *T. vulpecula*, western grey kangaroos (Kangaroo Island subspecies) *Macropus fuliginosus fuliginosus*, koalas *Phascolarctos cinereus*, southern hairy-nosed wombats *Lasiorhinus latifrons*, and fat-tailed dunnarts *S. crassicaudata* were used for this investigation. Animals were housed and/or obtained as previously described (see section 2.1) and only naturally cycling animals that had never been subjected to treatments that could affect natural follicular development were used. In the case of brushtail possums, any females carrying PY had their young removed to facilitate resumption of follicular development. The presence of suckling PY results in lactational anoestrus and, following removal of young, female brushtail possums are expected to ovulate between 7-18 days later, although the success rate for ovulation following removal of PY has recently been shown to be highly variable (Pilton & Sharman, 1962; Rodger & Mate, 1988; Crawford *et al.*, 1997). In order to obtain a range of follicular stages of development, female possums were euthanased between 4-8 days following removal of PY.

Ovaries from several koala pouch young (PY) (aged approximately 60 [n=2], 100 [n=1], 160 [n=1] and 180-250 [n=2] days-old, as determined from head length according to Smith [1979]) were also investigated (see section 2.1).

3.2.2 Collection and processing of ovarian tissue for electron microscopy

Ovaries were obtained and processed routinely for transmission electron microscopy as described previously in sections 2.3 and 2.4. Due to the density of the ovarian stroma in adult koala ovaries, and thereby practical problems with adequate fixation, the ovarian tissue of one adult koala were stained *en bloc* with 1% uranyl acetate in 0.1M maleate buffer, pH 5.5, in the dark at 4°C for 2h following aldehyde (primary) and osmium tetroxide (secondary) fixation to maximise preservation of cell membranes. The ovarian tissue was then processed as normal as described in sections 2.3 and 2.4. The ovaries of at least 3 individuals for each species were used for these ultrastructural investigations, while only thick plastic sections were used to investigate the follicular stage of development within the ovaries of koala PY. All attempts were made to ensure only viable follicles were used in this study while those displaying any ultrastructural features of atresia (e.g. pyknotic nuclei, vacuolated cytoplasms) were omitted.

3.3 Results

3.3.1 Description of the follicular stages

As expected, the ovaries of the adult animals contained follicles at various developmental stages ranging from primordial follicles up to large antral follicles within which oocytes and their ZP were observed. For classification purposes, oocytes and their ZP (if present) within the follicles of the adult animals were grouped according to following criteria, based on observations on oocyte growth in *M. domestica* (Falconnier & Kress, 1992): *Primordial follicles* were primary oocytes surrounded by a simple squamous follicular epithelium; *Primary follicles* were oocytes surrounded a simple cuboidal follicular epithelium; *Secondary follicles* were primary oocytes surrounded initially by two, and then several, layers of

follicular cells prior to the formation of the follicular antrum; *Tertiary follicles* were oocytes surrounded by several layers of follicular cells within which fluid-filled spaces had begun to appear and which would eventually fuse at a later stage to form the follicular antrum; *Graafian follicles* were oocytes within a completely formed antrum which increased in size due to fluid accumulation. Only general distinguishing and unique ultrastructural features for the various follicular stages, and those specifically related to the formation of the ZP, have been described here for all species. Following the development of the ZP into a uniform structure surrounding the oocyte, measurements of the ZP at the various stages of follicular development were made on the electron micrographs for each species and can be found summarised in Table 3.1.

Basic observations on the stages of follicular development found within the ovaries of the koala pouch young have been described separately.

3.3.2 Koala pouch young ovaries

The ovaries of the 60 day-old koala only contained oocytes within primordial follicles, while the ovaries of the 100 and 160 day-old PY contained numerous oocytes within primordial follicles and a number within primary follicles (Figs. 3.1 & 3.2). Many of the primordial and primary follicular oocytes contained large lipid droplets within their cytoplasm, adjacent to the nucleus (Fig. 3.1 & 3.2). The ovaries of the 180-250 day-old PY contained follicles ranging from primordial to small antral.

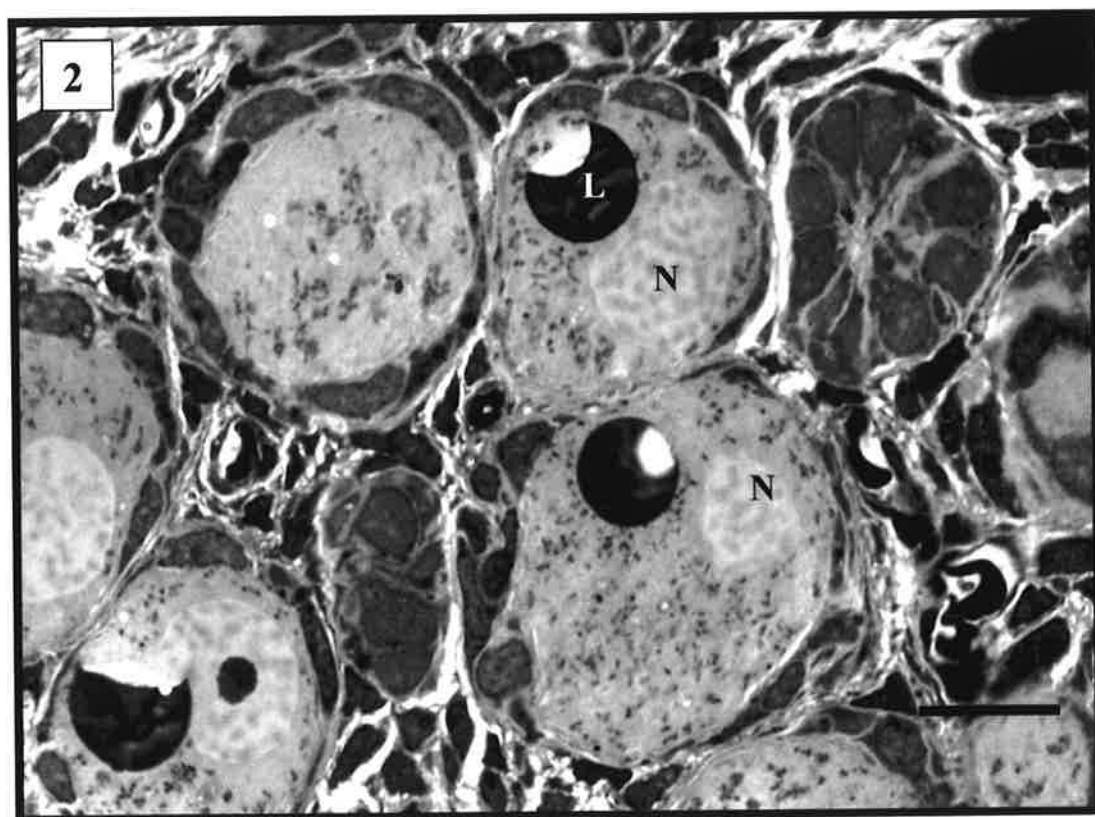
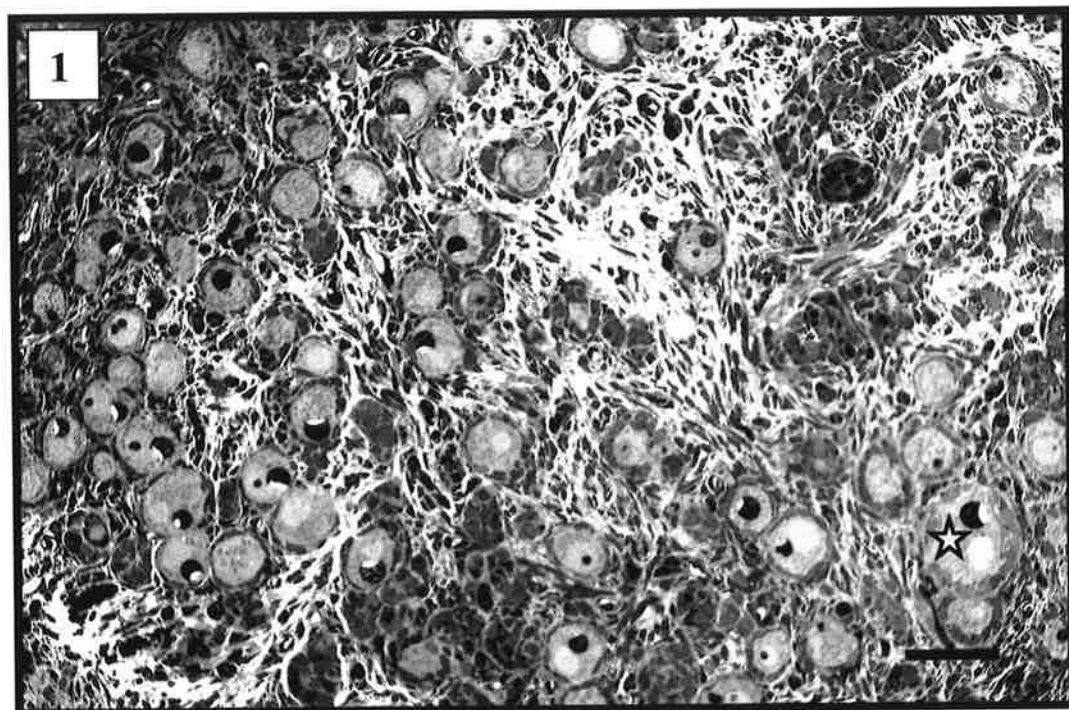
Table 3.1. Size of the zona pellucida around oocytes of follicles of different developmental stages in five species of marsupial*

	Stage of Follicular Development			
	Primary (μm)	Secondary (μm)	Tertiary (μm)	Graafian (μm)
Fat-tailed dunnart	1.1 - 1.6 (1.3); n=3	1.8 - 2.7 (2.3); n=3	2.2 - 5.0 (4.0); n=5	3.0 - 6.0 (3.9); n=4
Brushtail possum	1.3 - 1.6 (1.4); n=5	1.9 - 2.5 (2.2); n=4	2.7 - 4.2 (3.4); n=3	4.6 - 6.1 (5.2); n=3
Southern hairy-nosed wombat	1.3 - 3.0 (2.2); n=2	2.1 - 5.0 (3.1); n=4	4.2 - 4.8 (4.6); n=3	4.8 - 10.0 (6.6); n=3
Koala	1.9 - 2.3 (2.1); n=3	5.3 - 6.0 (5.6); n=4	6.6 - 8.6 (7.7); n=4	6.4 - 10.0 (8.6); n=3
Western grey kangaroo	1.8 - 2.0 (1.9); n=2	1.6 - 2.9 (2.1); n=4	3.3 - 4.6 (3.9); n=4	4.3 - 5.0 (4.6); n=3

* Zona pellucida measurements represent the range of measurements found (mean values in parentheses and bolded) and the number of ZP measured for each stage of development

Figure 3.1 Koala pouch young ovary. Thick plastic section of the ovary of a 160 day-old koala pouch young demonstrating numerous primordial follicles and several primary follicles (star). Note the large crescent-shaped lipid droplet within the cytoplasm of many of the oocytes. Bar = 100 μ m.

Figure 3.2 Koala pouch young ovary. Higher magnification of part of Fig. 3.1 showing koala pouch young primordial follicles with prominent nuclei (N) and nucleoli of the oocytes. Note round osmophilic lipid droplets (L) and mitochondria within, and a few flattened follicle cells around, the oocytes. Bar = 25 μ m.



3.3.3 Primordial follicles

The primordial follicles of all animals were typically elliptical in shape, consisting of a primary oocyte surrounded by a flattened layer of follicle cells. In koalas and wombats, as noted above in the ovaries of the koala pouch young (Figs. 3.1 & 3.2), many oocytes within primordial follicles contained large spherical lipid droplets that filled a large portion of the cytoplasm, a feature not observed in the other species (Fig. 3.3). A major portion of the oocyte in all species, however, was taken up by a large vesicular nucleus, typically with a prominent nucleolus.

The oocytes of primordial follicles of all species showed a lack of microvilli on the oolemma, with the oocyte surface relatively tightly opposed against the surrounding squamous follicle cells (Fig. 3.4). Occasional interdigitation of the membranes between the oocyte and follicle cells was also seen in all species, with numerous tight and/or desmosome-like junctions present between the oolemma and plasma membranes of the follicle cells (Fig. 3.4). No ZP was present surrounding oocytes of primordial follicles in any species.

Unique to the western grey kangaroo and noted in the ovaries of all of the kangaroo individuals studied ($n=3$) was the relatively frequent presence of polyovular primordial follicles (Fig. 3.5). These polyovular primordial follicles were composed of several tightly opposed oocytes, ranging in number from two to seven, surrounded externally by flattened granulosa cells (Figs. 3.5 & 3.6). Within these primordial follicle “nests”, oocytes had numerous desmosome-like junctional complexes with adjacent oocytes.

Figure 3.3 Primordial follicle – adult koala. Primordial follicle of an adult koala surrounded by the ovarian interstitium (I). The primary oocyte within the primordial follicle is surrounded by a simple follicular epithelium (F); Note large lipid droplet (L) and nucleus (N) with dispersed chromatin. Bar = 5 μ m.

Figure 3.4 Primordial follicle – western grey kangaroo. Oocyte of primordial follicle of an adult western grey kangaroo. Note part of a prominent nucleus (N), Golgi complexes (G) within the cytoplasm, and desmosome-like junctional complexes (arrow) and interdigitation of the membranes (arrowhead) between the oolemma and follicle cells (F). Bar = 2 μ m.

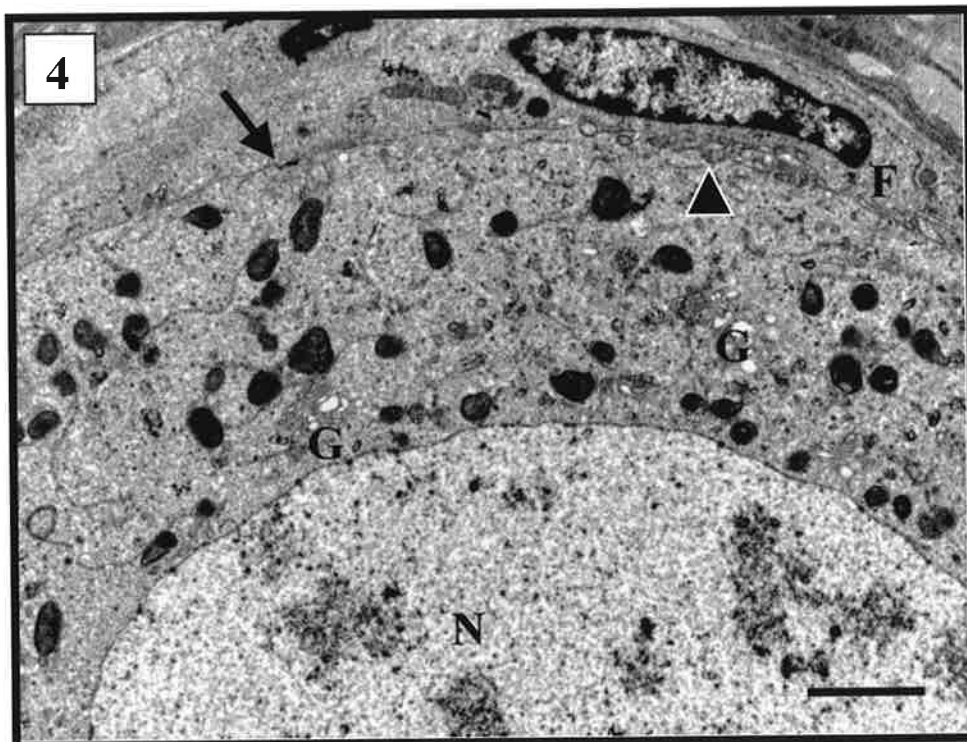
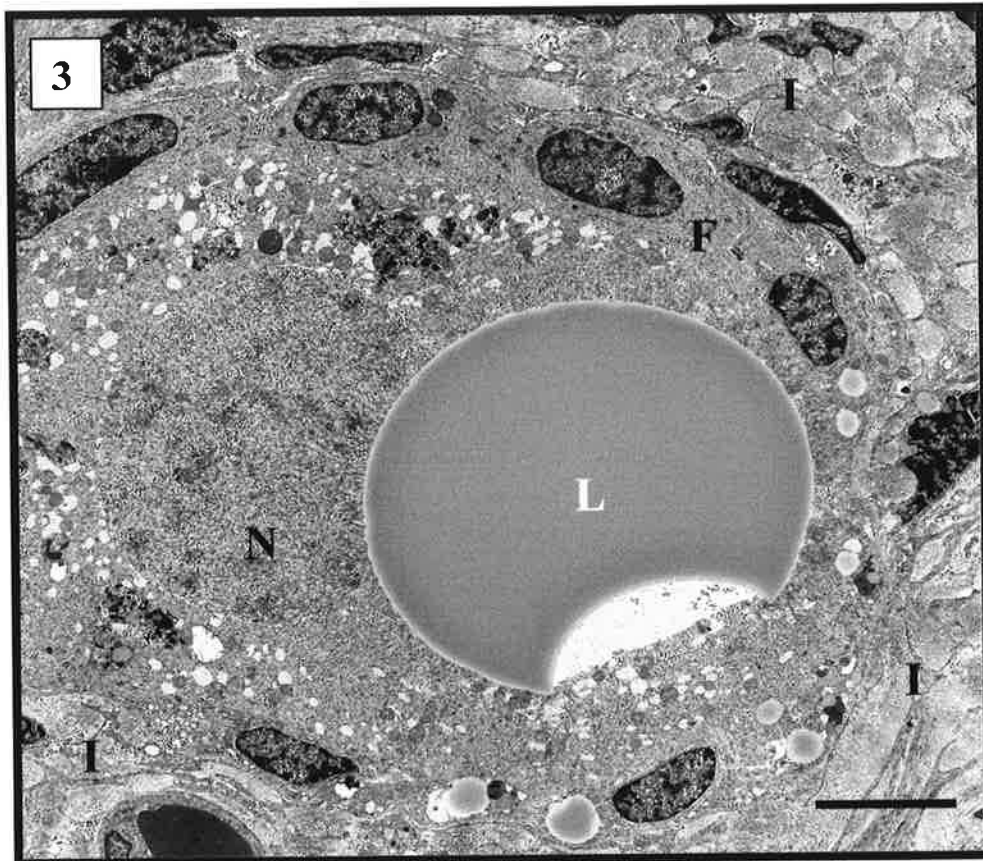
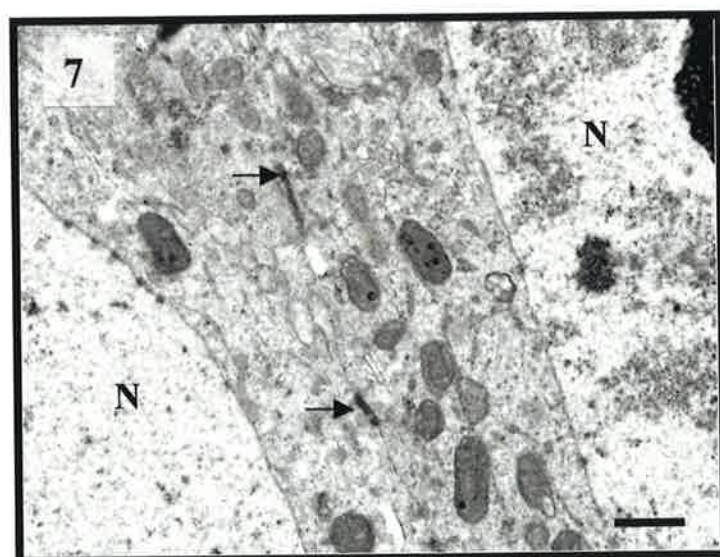
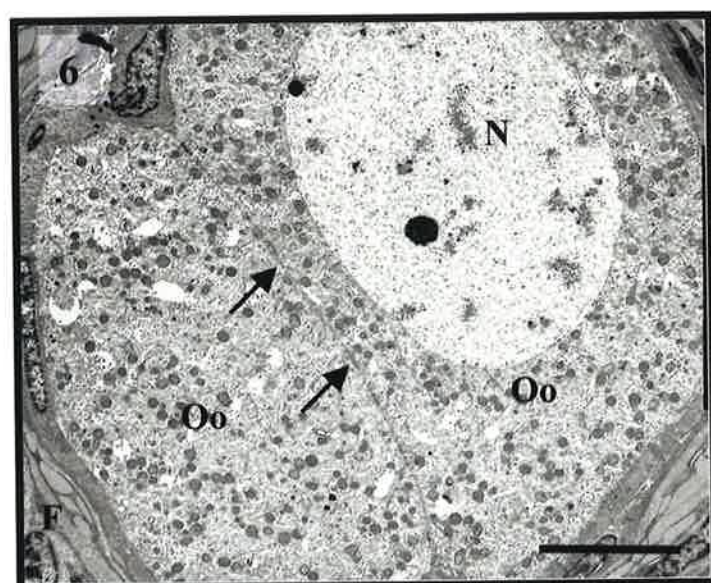
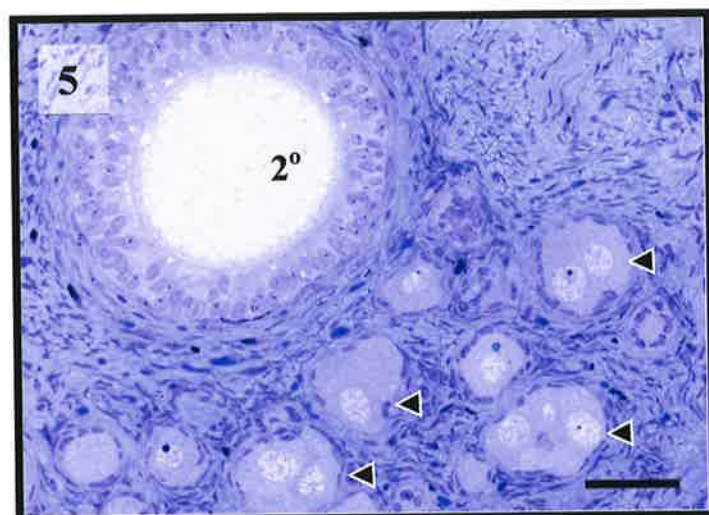


Figure 3.5 Western grey kangaroo ovary. Thick plastic section of ovary of western grey kangaroo demonstrating secondary follicle (2°) and several polyovular primordial follicles (arrowheads). Note primordial follicle ‘nests’ comprised of two or more primary oocytes surrounded by a simple squamous layer of follicle cells. Bar = 50µm.

Figure 3.6 Polyovular primordial follicle – western grey kangaroo. A polyovular primordial follicle of a western grey kangaroo with two primary oocytes (Oo) and prominent nucleus (N). Note: desmosome-like junctions (arrows) between oolemma of the adjacent oocytes. Bar = 10µm.

Figure 3.7 Polyovular primordial follicle – western grey kangaroo. Cell membranes between adjacent primary oocytes and their nuclei (N) in a polyovular primordial follicle of a western grey kangaroo. Desmosome-like junctions (arrows) were found between the adjacent oolemmas. Bar = 1µm.



3.3.4 Primary follicles

Early primary follicles were those characterised as having a single layer of follicle cells that was intermediate between the flattened squamous type and cuboidal in shape. The plasma membranes between the oocyte and follicle cells of early primary follicles in all species appeared highly folded and/or interdigitated in places (Figs. 3.8 & 3.9). No microvilli were present on the oolemma of oocytes of early primary follicles. In the southern hairy-nosed wombat, however, electron dense granular material was present within the highly folded membranes between the oocyte and follicle cells, which may indicate the early secretion of the ZP (Fig. 3.10). At higher magnification, small vesicles containing electron dense material were present within the oocyte cytoplasm of the wombat, immediately adjacent to the highly folded membranes (Fig. 3.11). No such vesicles were noted within the follicle cell cytoplasm at this stage.

In primary follicles that were slightly more advanced than the previous stage, follicle cells were clearly cuboidal in shape (Fig. 3.12). Characteristic of ZP formation at this stage in the possum, wombat and koala, discrete pockets of ZP material were found dispersed between the follicle cells and even extending as far as the basement membrane (Figs. 3.12 & 3.13). No secretory vesicles could be seen within the follicle cells surrounding these pockets of ZP matrix. The pockets of ZP matrix coincided, however, with the first appearance of microvilli on the oocyte surface, and the associated pockets of ZP matrix between the oocyte and follicle cells (Fig. 3.14). This feature of pockets of microvilli and the associated ZP matrix on the surface of the oocytes was common to all species during this stage of ZP formation. Follicle cells continued to maintain their junctional complexes with the oocyte via cytoplasmic extensions (Fig. 3.14).

Figure 3.8 Primary follicle – western grey kangaroo. An oocyte (Oo) of a western grey kangaroo within a primary follicle demonstrating follicle cells (F) that are more cuboidal than the follicle cells typical of primordial follicles. Bar = 2 μ m.

Figure 3.9 Primary follicle – western grey kangaroo. High magnification of the interdigitated membranes of the oocyte (Oo) and follicle cell (F) of the primary follicle from Fig. 3.8. Bar = 500nm.

Figure 3.10 Primary follicle – southern hairy-nosed wombat. The primary follicle of the southern hairy-nosed wombat has large cuboidal follicle cells (F) surrounding the oocyte (Oo). The membranes between the follicle cells and oocyte are highly interdigitated, while in some areas electron dense material appears between the membranes, perhaps representing early deposition of zona matrix. Microvilli are not present on the oolemma at this stage. Bar = 1 μ m.

Figure 3.11 Primary follicle – southern hairy-nosed wombat. High magnification of part of the primary follicle seen in Fig. 3.10 highlighting the high degree of interdigitation of the follicle cell (F) and oocyte (Oo) cell membranes. Electron dense material can be seen between the membranes and small electron dense vesicles (arrowheads) are found underlying the oolemma. Bar = 500nm.

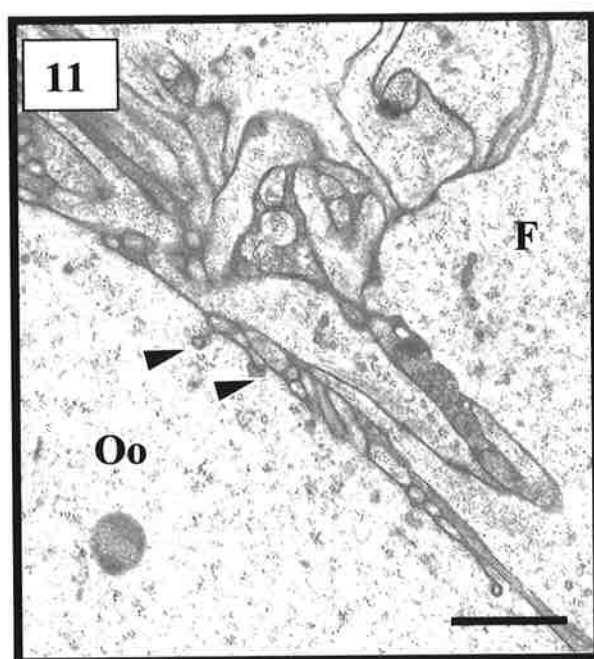
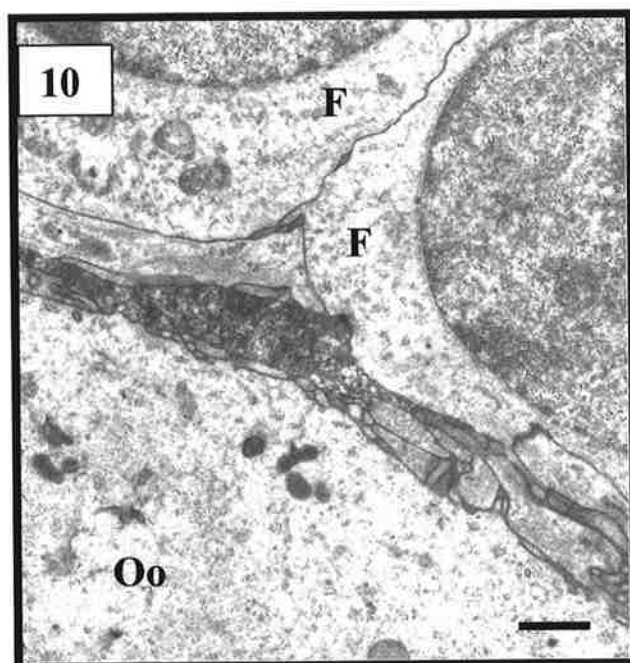
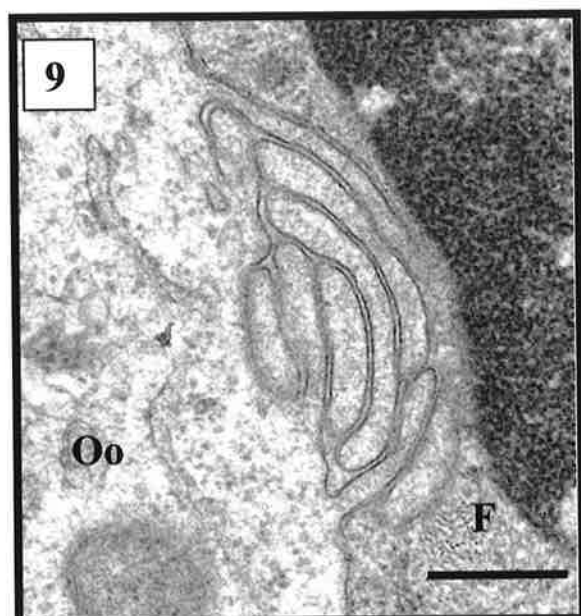
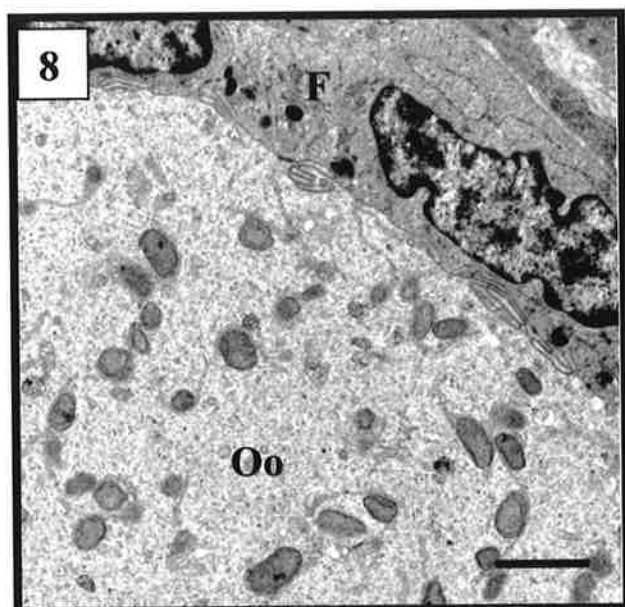
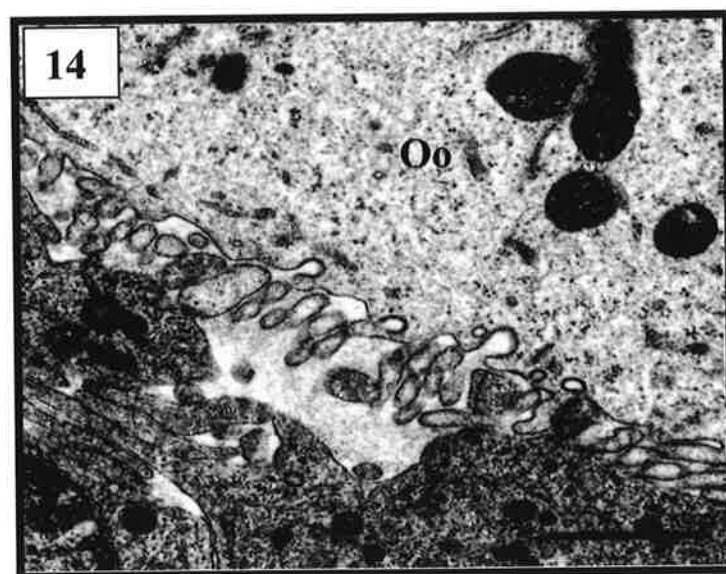
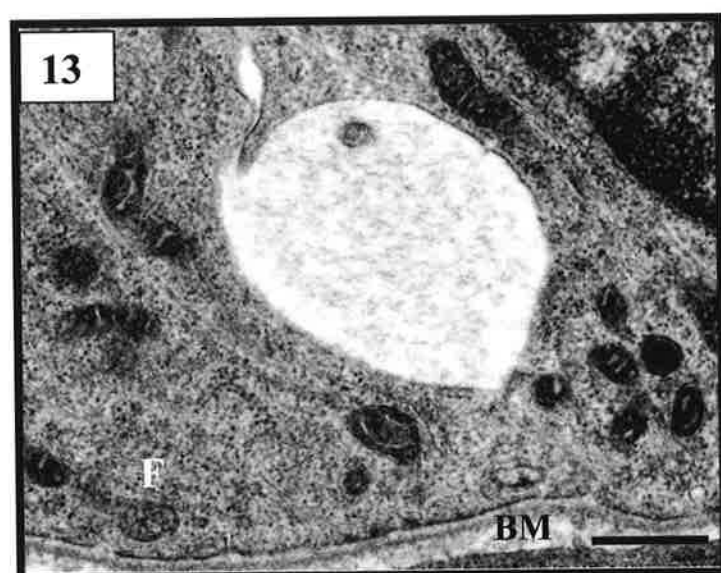
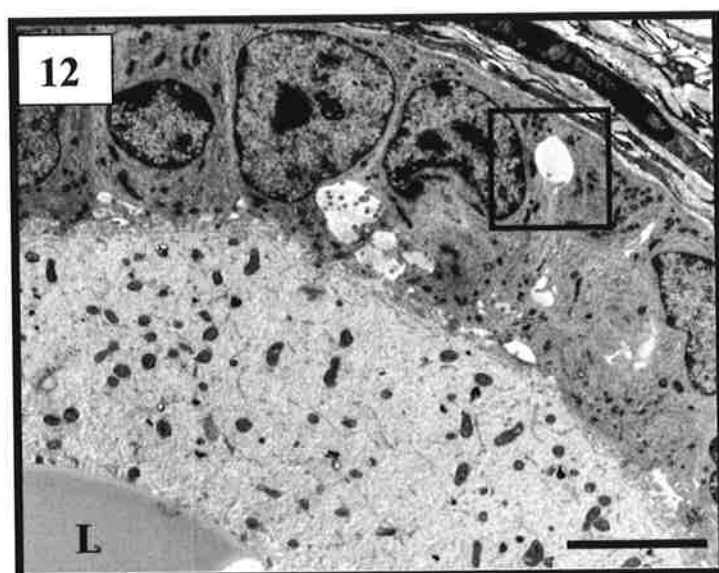


Figure 3.12 Primary follicle – southern hairy-nosed wombat. Primary follicle of the southern hairy-nosed wombat showing early formation of the zona pellucida. The oocyte contains a large lipid droplet (L) and is surrounded by a simple cuboidal follicular epithelium. Formation of the zona pellucida at this stage is represented by pockets of zona matrix appearing between the oocyte and follicle cells, or between adjacent follicle cells (boxed area). Bar = 5µm.

Figure 3.13 Primary follicle – southern hairy-nosed wombat. Higher magnification of the boxed area in Fig. 3.12 demonstrating one of the pockets of zona matrix completely surrounded by follicle cells (F) and close to the basement membrane (BM) of the follicle. Bar = 1µm.

Figure 3.14 Higher magnification of the oolemma of the oocyte (Oo) seen in Fig. 3.12 demonstrating the first appearance of microvilli. Associated with the appearance of the microvilli, pockets of zona matrix are found adjacent to the microvilli of the oolemma within the intercellular space. Bar = 1µm.



In all species, later stage primary follicles were distinguished from those of the previous stages by the finding that microvilli covered the entire surface of the oocytes. ZP matrix was found within the intercellular space, interspersed between the oocyte and follicular epithelium, with follicle cells maintaining junctional complexes with the oocyte via pedicel-shaped cytoplasmic projections (Fig. 3.15). Elongated Golgi complexes with flattened cisternae were commonly found in a subcortical region in all species, while, in some instances, small Golgi-derived electron dense vesicles could also be seen directly under the oolemma (Fig. 3.16). Prior to the formation of the second layer of follicle cells, the ZP matrix was found to have fused into a dense, uniform matrix around the oocytes of all the marsupials (Fig. 3.17).

3.3.5 Secondary follicles

In the secondary follicles of all species, the ZP appeared relatively dense, granular and homogeneous, with the outer area still somewhat interspersed between the follicle cells and irregular in appearance (Figs. 3.18 & 3.19). The inner area of the ZP was less irregular, although parts still extended between the numerous microvilli of the oocyte (Figs. 3.18 & 3.19). Numerous follicle cell cytoplasmic extensions could be seen throughout the width of the ZP in all species as they traversed and maintained their contact with the oolemma (Fig. 3.19). In the koala and wombat, large vacuoles containing granular flocculent material also first become evident within the ooplasm at this stage (Fig. 3.19), but were not noted in the other species until the tertiary follicular stage.

In pre-antral follicles, the granulosa cells appeared to be tightly arranged, as in the brushtail possum and southern hairy nosed wombat (Fig. 3.20), or appeared to be elongated as seen in the

Figure 3.15 Primary follicle – koala. Primary follicle of an adult koala with pockets of zona matrix (ZP) interspersed between the microvilli (M) of the oocyte (Oo) and the surrounding follicle cells (F). The follicle cells maintain contact with the oocyte via pedicel-shaped cytoplasmic extensions that form desmosome-like junctions with the oolemma (arrow). Golgi complexes (G) were also typically found in a sub-cortical position. Bar = 1 μ m.

Figure 3.16 Primary follicle – koala. Slightly more advanced primary follicle of an adult koala than shown in Fig. 3.15, with a broader and more uniform zona pellucida (ZP) surrounding the oocyte (Oo). Golgi complexes (arrows) are conspicuous adjacent to the oolemma Bar = 1 μ m.

Figure 3.17 Primary follicle – fat-tailed dunnart. Oocyte (Oo) of a late primary follicle of the fat-tailed dunnart is surrounded by a simple cuboidal layer of follicle cells (F) and a relatively electron dense zona pellucida (ZP). The structurally dense zona pellucida matrix appears to fill the entire intercellular space and is tightly interspersed between the microvilli of the oocyte; N (nucleus). Bar = 5 μ m.

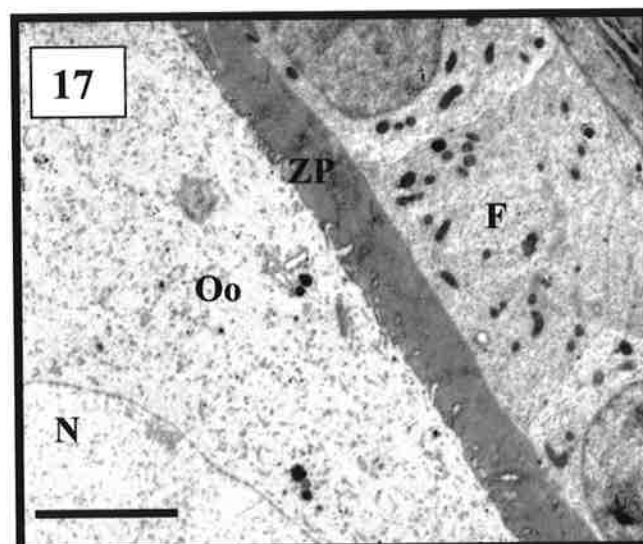
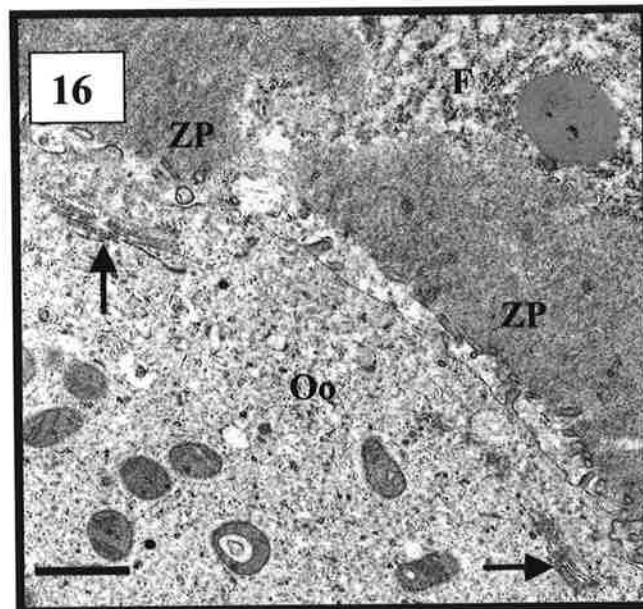
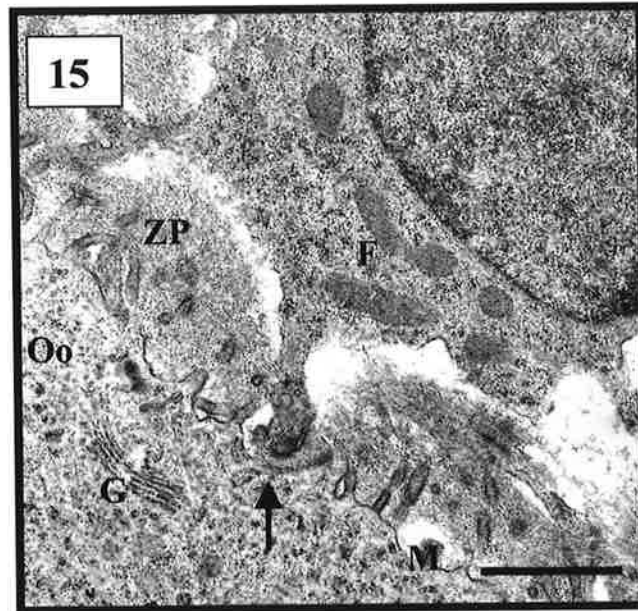
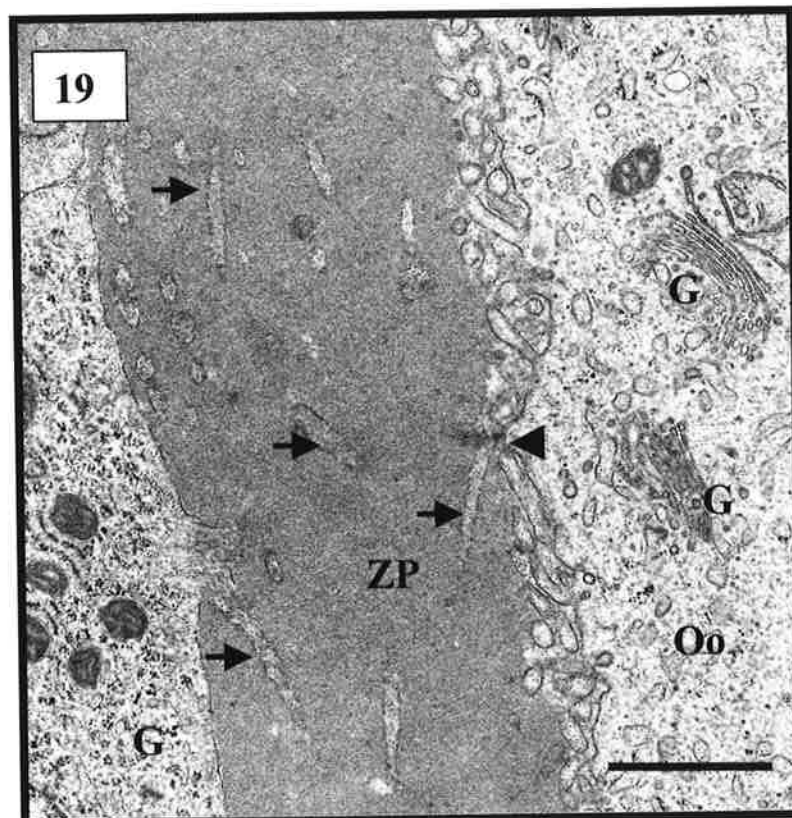
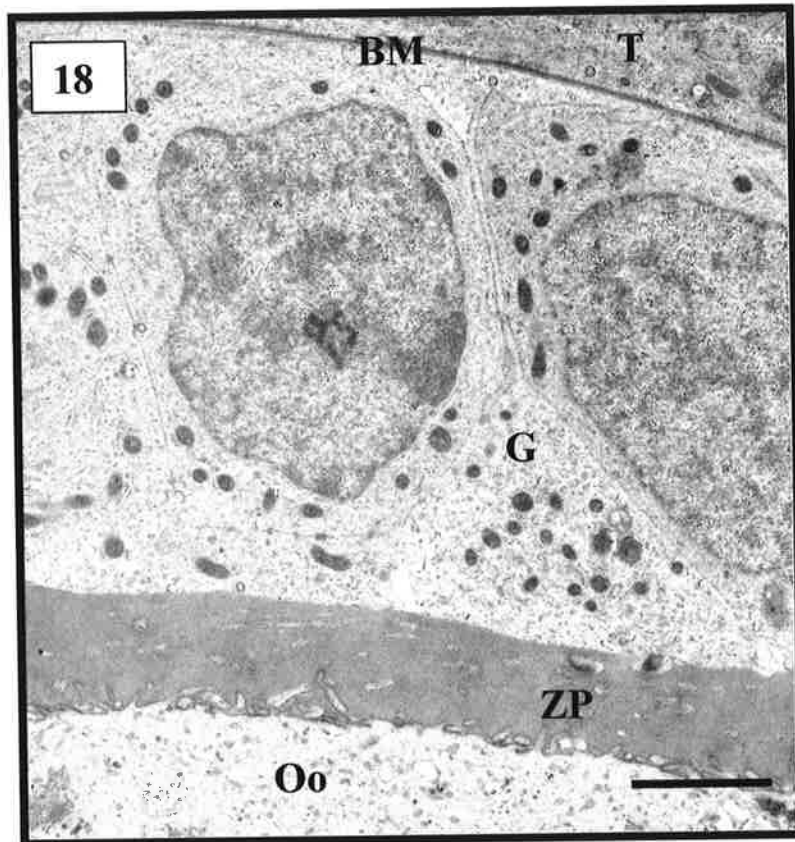


Figure 3.18 Secondary follicle – fat-tailed dunnart. Early secondary follicle of fat-tailed dunnart showing primary oocyte (Oo) surrounded by an electron dense and granular zona pellucida matrix (ZP), two layers of granulosa cells (G), the basement membrane (BM) and the theca interna cells (T). Bar = 2 μ m.

Figure 3.19 Secondary follicle – fat-tailed dunnart. Zona pellucida (ZP) surrounding oocyte (Oo) of the secondary follicle of fat-tailed dunnart is extremely dense and granular and completely fills the intercellular space between the oolemma and the granulosa cells (GC). Cytoplasmic extensions (arrows) of the granulosa cells traverse the zona pellucida and maintain desmosome-like junctions (arrowhead) with the oocyte. Within the oocyte, Golgi complexes (G) are still found in a subcortical position. Bar = 1 μ m.



fat-tailed dunnart, koala and western grey kangaroo (Fig. 3.21). The ZP of all species appeared extremely dense and compacted around the oocyte, invading the entire extracellular space between the ZP and oolemma, making contact with the oocyte surface and tightly interspersed between the microvilli (Fig. 3.22).

3.3.6 Tertiary follicles

In early tertiary follicles of all species, numerous spaces were evident between the granulosa cells, indicating the early development of a follicular antrum (Figs. 3.23 & 3.24). A number of the granulosa cells closest to the ZP appeared to have begun to develop the characteristic spherical shape of cumulus cells, while they still maintained their contact with the oocyte through the cytoplasmic extensions, which were found traversing the ZP (Fig. 3.25 & 3.26). The ZP of the fat-tailed dunnart appeared slightly irregular adjacent to the granulosa cells. A perivitelline space (PVS) was clearly evident in the oocytes of the fat-tailed dunnart at this stage (Fig. 3.25). The ZP of the koala appeared relatively thick and dense (see Table 3.1), while it did not appear as compacted within the extracellular space between the oocyte and ZP as in the previous stage (Fig. 3.26). The PVS in the brushtail possum, southern hairy-nosed wombat and western grey kangaroo, like that of the koala, was not as evident as that found in the fat-tailed dunnart at this early stage of antral development.

3.3.7 Graafian follicles

The early Graafian follicles of all species had completely formed antra while the granulosa cells closest to the ZP had fully differentiated into one or two layers of rounded cumulus cells (Figs.

Figure 3.20 Secondary follicle – southern hairy-nosed wombat. Pre-antral follicle of southern hairy-nosed wombat demonstrating well developed layer of theca interna (T) and three or more layers of cuboidal granulosa cells (G). The oocyte (Oo) is surrounded by a dense zona pellucida. Bar = 10µm.

Figure 3.21 Secondary follicle – koala. Pre-antral follicle of an adult koala with two to three layers of elongated columnar granulosa cells (G), and, like that found in other marsupials at this stage, the oocyte (Oo) is surrounded by a dense zona pellucida (ZP). Bar = 10µm.

Figure 3.22 Secondary follicle – southern hairy-nosed wombat. Higher magnification of the zona pellucida (ZP) surrounding the oocyte (Oo) of a secondary follicle of southern hairy-nosed wombat seen in Fig. 3.20. The dense and granular zona pellucida completely fills the intercellular space and is interspersed between the microvilli (M) of the oolemma. Granulosa cells (G) continue to maintain cell junctions (arrowhead) with the oocyte via cytoplasmic extensions (CE) that traverse the zona pellucida. Bar = 1µm.

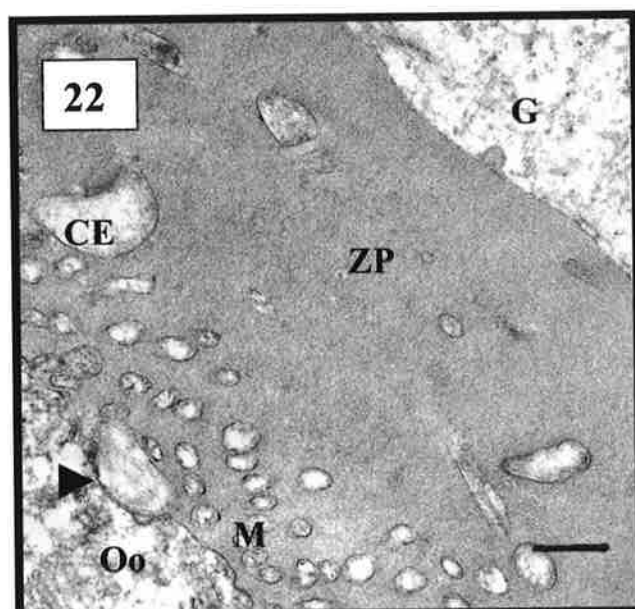
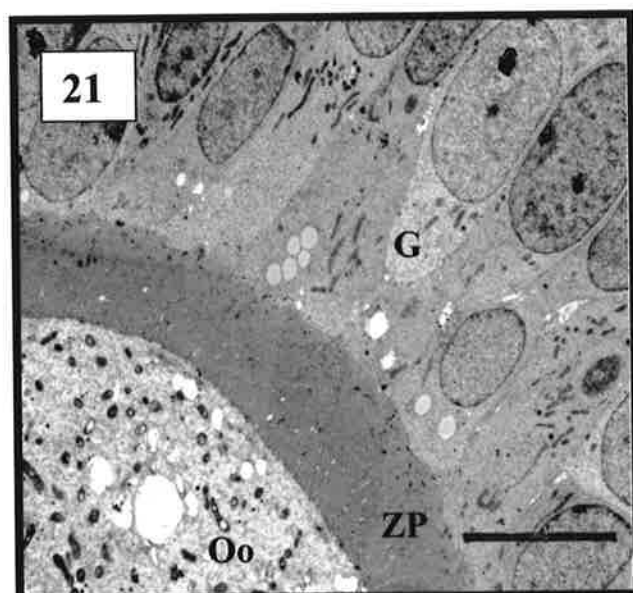
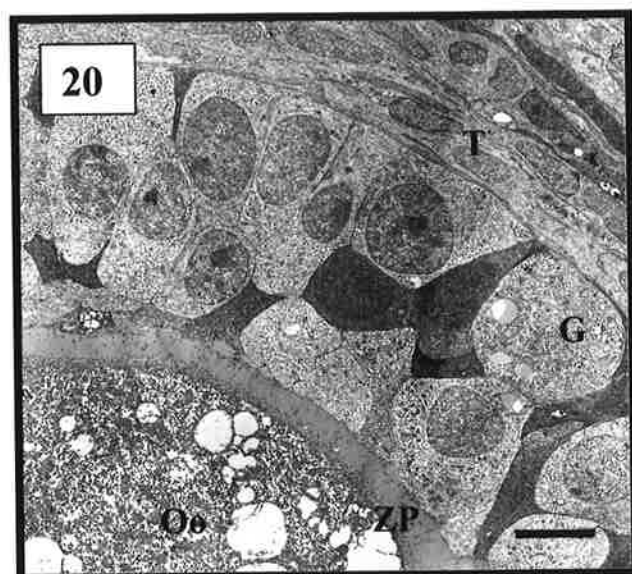
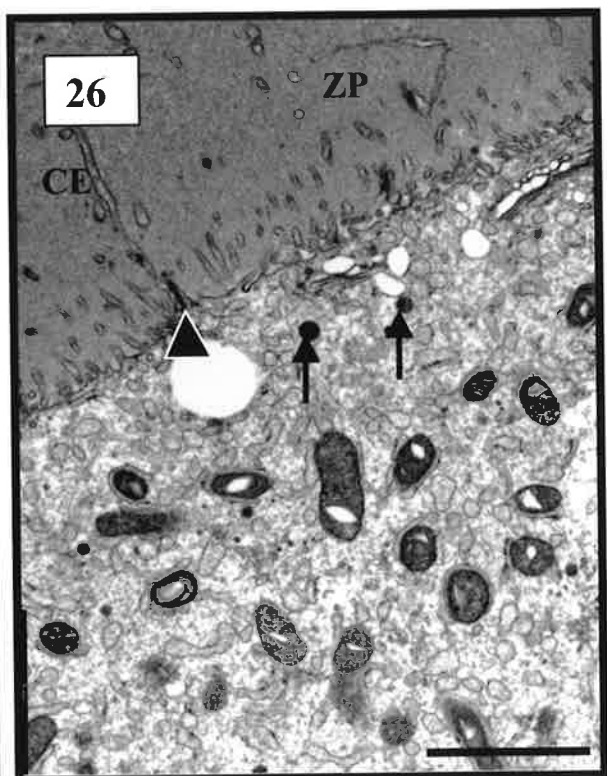
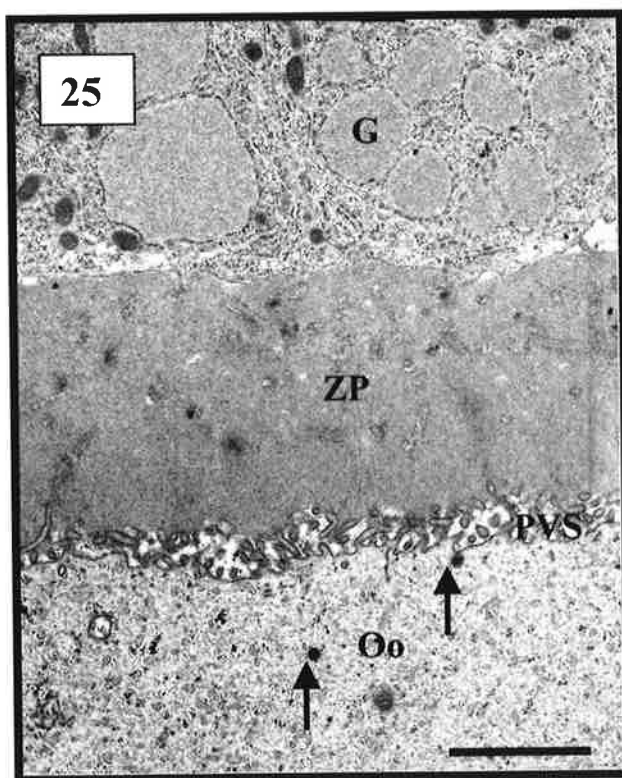
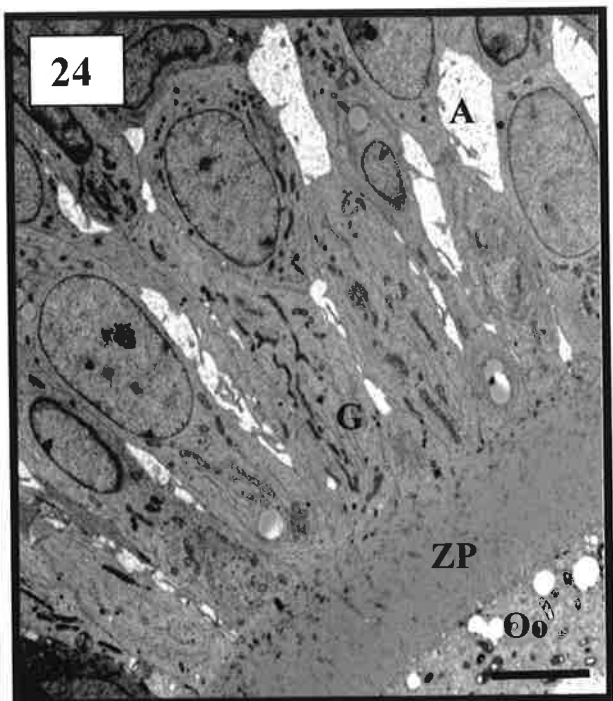
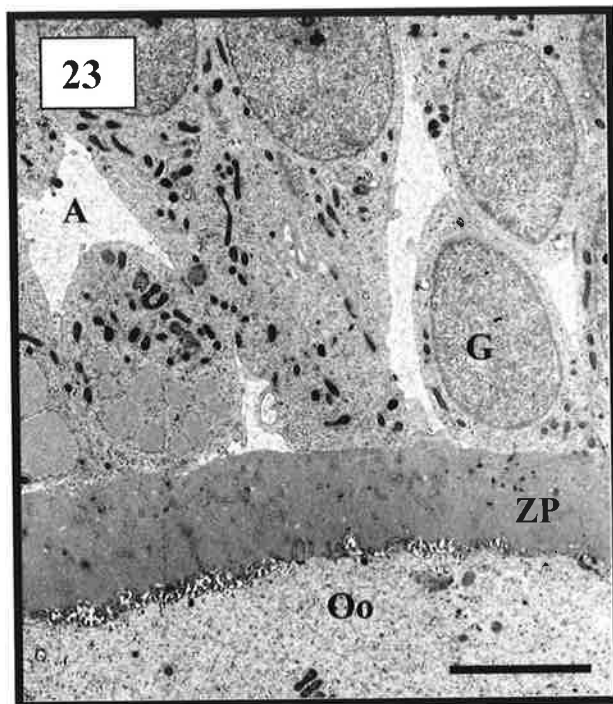


Figure 3.23 Tertiary follicle – fat-tailed dunnart. Early tertiary follicle of fat-tailed dunnart demonstrating antral spaces (A) between granulosa cells (G). Several granulosa cells closest to the zona pellucida (ZP) appear rounded in shape and may represent early cumulus cells; oocyte (Oo). Bar = 5µm.

Figure 3.24 Tertiary follicle - koala. Early tertiary follicle of an adult koala showing similar features to that of the fat-tailed dunnart seen in Fig. 3.23 with antral spaces (A) evident between granulosa cells (G). Zona pellucida (ZP) surrounding the oocyte (Oo) appears broad and very dense. Bar = 5µm.

Figure 3.25 Tertiary follicle – fat-tailed dunnart. Higher magnification of the oocyte (Oo) from the early tertiary follicle of the fat-tailed dunnart seen in Fig. 3.23. Within the oocyte, cortical granules (arrows) become evident within the cortical cytoplasm, while the perivitelline space (PVS) is also clearly evident at this stage; granulosa cells (G); zona pellucida (ZP). Bar = 2µm.

Figure 3.26 Tertiary follicle – koala. Higher magnification of the koala oocyte (Oo) seen in Fig. 3.24 showing the dense zona pellucida (ZP) being traversed by cytoplasmic extensions (CE) of the granulosa cells. Granulosa cells communicate with the oocyte via cytoplasmic extensions that maintain desmosome-like junctions with the oocyte (arrowhead). Within the ooplasm, cortical granules (arrows) are also clearly present in a sub-cortical position. Bar = 1µm.



3.27-3.29). There appeared to be less compaction of the ZP than in the previous stages and the PVS was more clearly evident in all species (Fig. 3.30). The ZP of all species appeared less dense, particularly close to the oolemma, with numerous cumulus cell cytoplasmic extensions still evident traversing the zona matrix and ending in pedicel-shaped processes that continued to maintain the desmosome junctions with the oolemma (Fig. 3.30). Large reticulated vacuoles were a dominant feature of the ooplasm in all species from this stage onwards, confining the majority of organelles to a cortical position (Figs. 3.27 & 3.28).

As compared to the earlier maturational stages, oocytes from large Graafian follicles in all species had large expanded antra. The oocytes of these stage follicles in all species displayed a well-developed PVS and were surrounded by relatively broad ZP (Fig. 3.31 - 3.33; Table 3.1). Spaces between adjacent cumulus cells were evident, though in the early tertiary follicular stage the cumulus cells still maintained contact with the oocyte through their cytoplasmic extensions (Fig. 3.33).

Pre-ovulatory follicles, noted only in the brushtail possum and koala, were characterised by the loss of cumulus cells on the surface of the ZP, with a lack of cumulus cell cytoplasmic extensions traversing the ZP in that area (Fig. 3.34). The majority of the ZP of the brushtail possum and koala was relatively dense, while the area adjacent to the oolemma and PVS was less dense and highly irregular in appearance (Fig. 3.35). Granular material was also clearly associated with the microvilli at this stage.

Figure 3.27 Graafian follicle – fat-tailed dunnart. Toluidine blue-stained thick plastic section of oocyte (Oo) from Graafian follicle of a fat-tailed dunnart. Positioned within a large follicular antrum (A), the oocyte is surrounded by two or more layers of cumulus cells (C). Bar = 100µm.

Figure 3.28 Graafian follicle – koala. Oocyte (Oo) of Graafian follicle of an adult koala is surrounded by a broad zona pellucida (ZP). Externally, the zona pellucida is surrounded by several layers of rounded cumulus cells (C); follicular antrum (A). Bar = 10µm.

Figure 3.29 Graafian follicle – fat-tailed dunnart. Higher magnification of boxed area seen in Fig. 3.27 showing the eccentrically-located nucleus (N) within the oocyte (Oo). The surrounding granular zona pellucida (ZP) and cumulus cells (C) are separated from the oocyte by a clearly defined perivitelline space (PVS). Bar = 10µm.

Figure 3.30 Graafian follicle – fat-tailed dunnart. Higher magnification of oolemma of koala oocyte (Oo) seen in Fig. 3.28 showing a less dense zona pellucida (ZP) than that found in previous stages, and the first appearance of a perivitelline space (PVS). Desmosome-like junctions (arrows) continue to be maintained between the cytoplasmic extensions of the cumulus cells and the oolemma, while Golgi complexes (G) are still a common feature of the sub-cortex. Bar = 2µm.

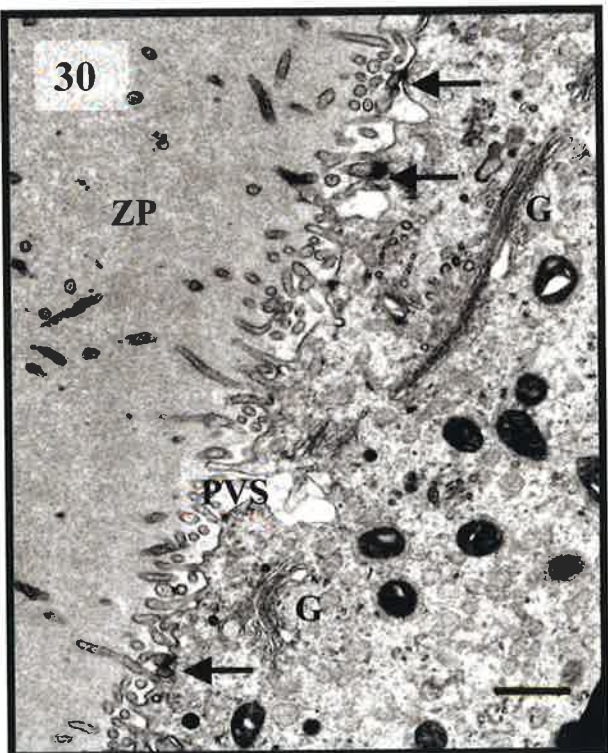
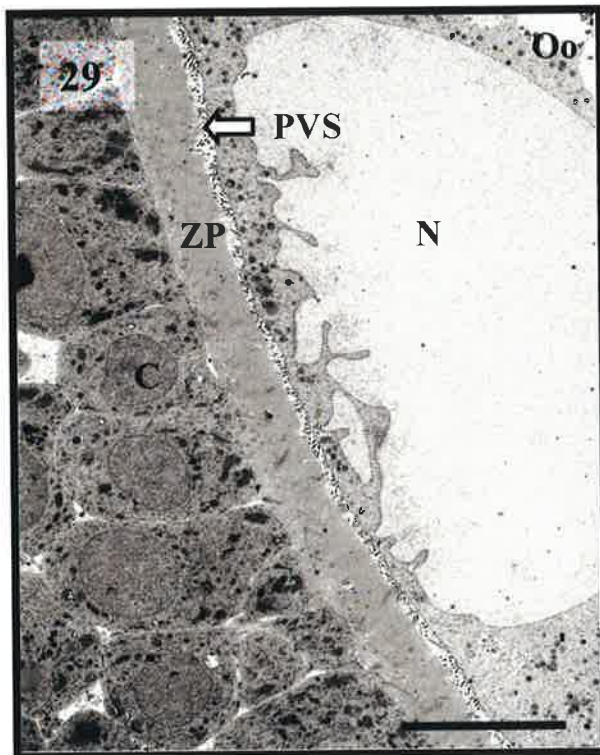
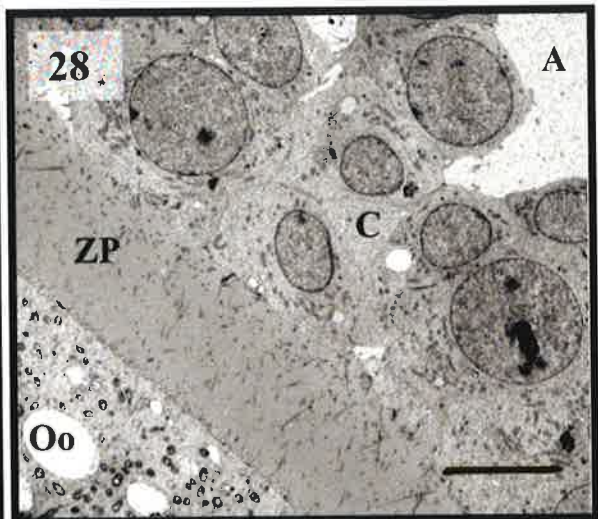
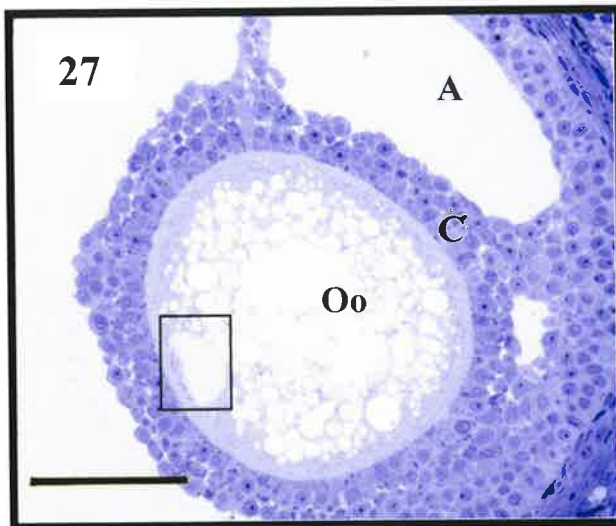


Figure 3.31 Graafian follicle – koala. Thick plastic section stained with toluidine blue of an oocyte from a large Graafian follicle of an adult koala. Within the cytoplasm of the oocyte, the nucleus (N) is surrounded by a large mass of reticulated clear vesicles (V), as well as several lipid droplets. Bar = 25µm.

Figure 3.32 Graafian follicle – koala. Transmission electron micrograph of koala oocyte (Oo) from Fig. 3.31 demonstrating that the broad zona pellucida (ZP) is less dense than that found in previous stages, while a perivitelline space (PVS) is clearly evident. Within the oocyte, granular material is found within electron lucent vesicles (V) and Golgi complexes (G) immediately adjacent to the oolemma. Bar = 5µm.

Figure 3.33 Graafian follicle – koala. Higher magnification of oolemma of koala oocyte (Oo) from Figs 3.31 and 3.32. Zona pellucida (ZP) appears more filamentous than in previous stages and continues to be traversed by cytoplasmic extensions that maintain desmosome-like junctions (arrows) with the oocyte; Golgi complexes (G); perivitelline space (PVS). Bar = 1µm.

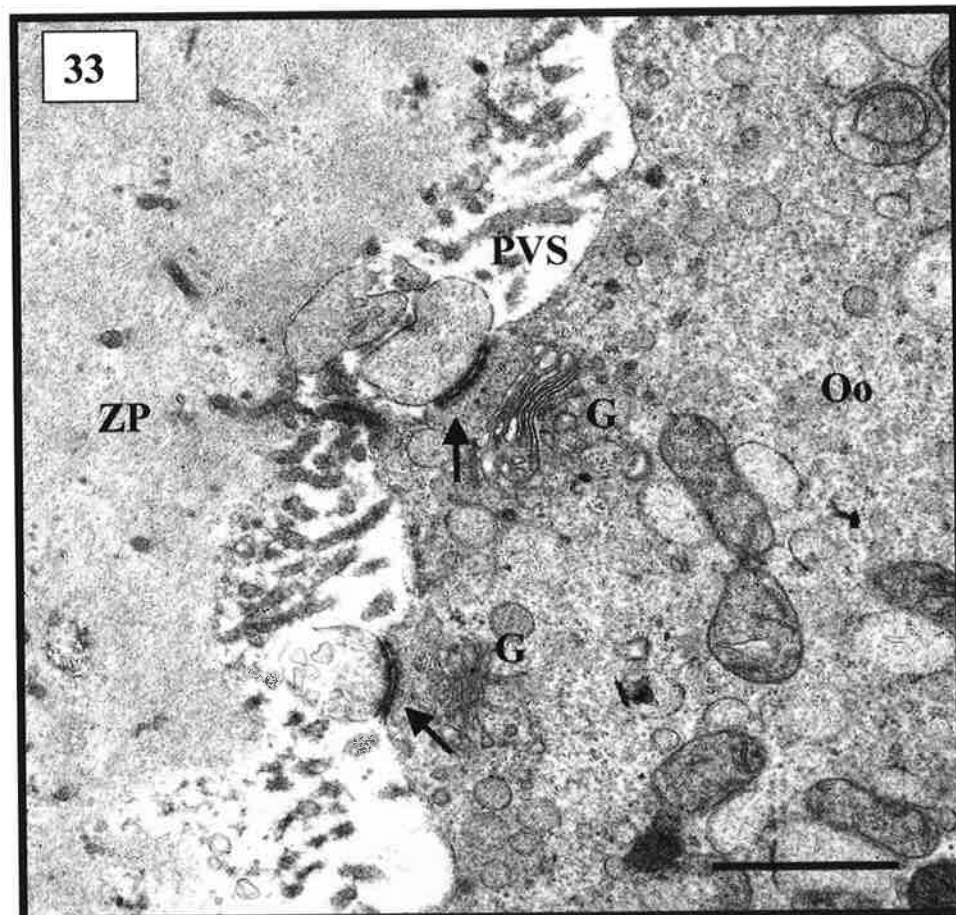
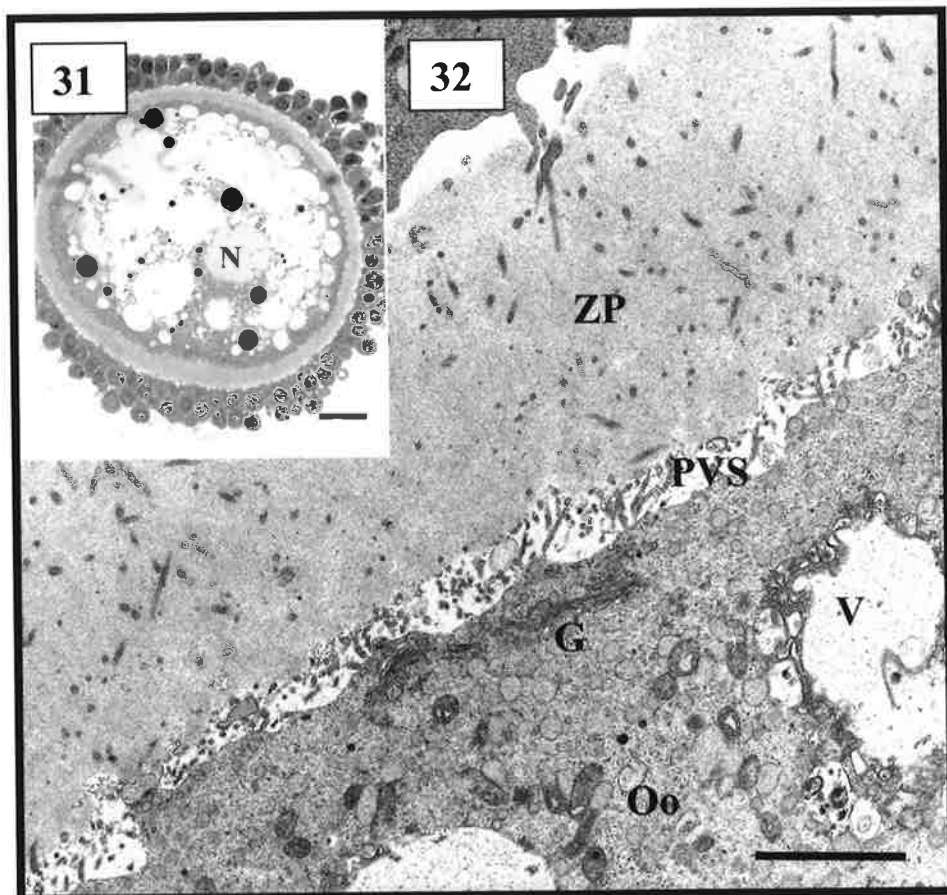
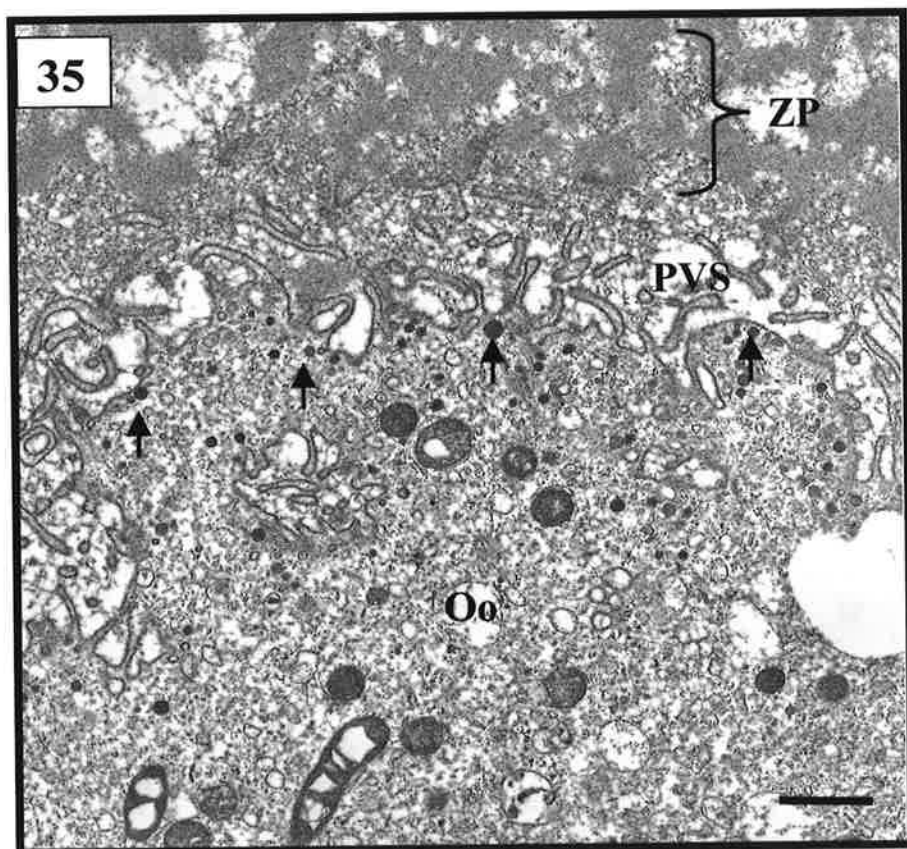
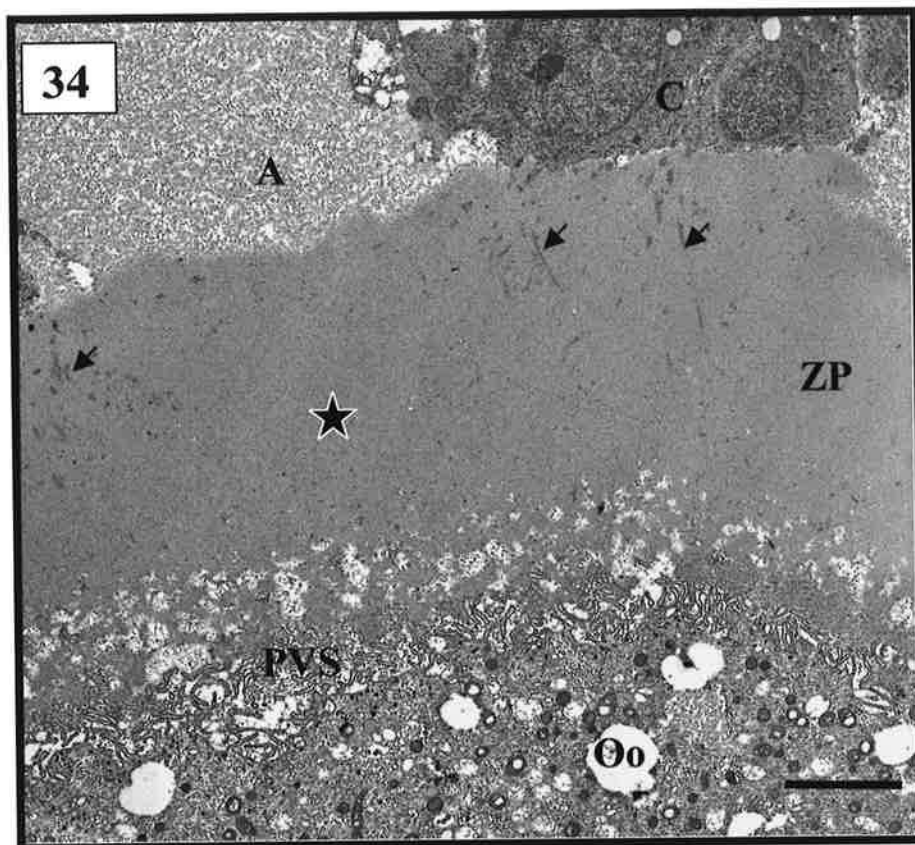


Figure 3.34 Peri-ovulatory follicle – koala. Peri-ovulatory oocyte (Oo) of adult koala surrounded by a broad zona pellucida (ZP) that appears less dense close to the perivitelline space (PVS). External to zona pellucida, some cumulus cells (C) are evident with cytoplasmic extensions (arrows) still embedded within the zona matrix. Some cumulus cells appear to have denuded from the surface of the zona pellucida, with no cytoplasmic extensions found within the zona matrix in that area (star); Antrum (A). Bar = 2 μ m.

Figure 3.35 Peri-ovulatory follicle – koala. Higher magnification of the oolemma of oocyte (Oo) seen in Fig. 3.34. The highly irregular and less dense region of the zona pellucida (ZP) immediately overlies the perivitelline space (PVS). Within the oocyte, numerous cortical granules (arrows) of varying sizes can be found adjacent to the oolemma. Bar = 1 μ m.



3.4 Discussion

In marsupials, little is known about the synthesis of the ZP, but what little is known comes from studies of oogenesis, primarily on the brushtail possum (Frankenberg *et al.*, 1996; Shackell *et al.*, 1996; Mate, 1998; Frankenberg & Selwood, 2001) and the tammar wallaby (Alcorn & Robinson, 1983; Mate, 1998). For example, light microscopical studies noted that ZP formation first occurred during the transition of primordial follicles into primary follicles in the tammar wallaby (Alcorn, 1975), while in the brushtail possum the ZP was found surrounding oocytes of early primary follicles, but not primordial follicles (Frankenberg *et al.*, 1996). More recently, a study on the timing and features of ZP formation in brushtail possum and tammar wallaby pouch young and adults was performed using basic histochemical, immunohistochemical and electron microscopical methods (Mate, 1998). Like that found previously (Alcorn, 1975; Frankenberg *et al.*, 1996), the ZP first formed around primary follicular oocytes, at least 98 days post-partum in pouch young, following the differentiation of the follicular epithelium from squamous-type to cuboidal-type follicle cells (Mate, 1998).

A number of ultrastructural studies of oogenesis and oocyte growth in marsupials also found a primary follicular stage secretion of the ZP (*M. domestica*: Falconnier & Kress, 1992; *T. vulpecula*: Frankenberg & Selwood, 2001; *S. macroura*: Kress *et al.*, 2001). The ultrastructural results of this thesis further complement these findings with the onset of secretion of the ZP of fat-tailed dunnarts, brushtail possums, koalas, wombats and western grey kangaroos beginning in early primary follicles.

One feature noted by this study of the early stages of ZP formation in the brushtail possum, wombat and koala might raise questions over the cellular site of origin of the ZP in these species. Previously noted in the rabbit (Dietl, 1989) and the brushtail possum (Mate, 1998), in the present study it was found that during the early stages of ZP formation within primary follicles, pockets of ZP matrix were present highly interspersed between the follicle cells, and in some cases quite a distance from the oolemma, some even reaching the basement membrane of the follicle. Whether this represents some contribution by the follicle cells to the formation of the ZP, or is a result of fixation artefact, is unknown. No ultrastructural evidence such as secretory vesicles or exocytosis, however, was seen within the follicle cells immediately surrounding the pockets of the ZP matrix. It is unclear whether any construction of the ZP filaments occurs prior to the secretion of the ZP glycoproteins into the extracellular space, or if filament construction occurs within the extracellular space itself (Green, 1997). It is possible that ZP glycoproteins might be secreted as monomeric, or small (e.g. several glycoproteins long) filament, units from the oocyte and that they merely infiltrate any available extracellular spaces, including between the basal and lateral membranes of the granulosa cells, before assembling into their filamentous form.

A seemingly common ultrastructural feature of ZP formation in marsupials is the finding that the ZP from late primary follicles to late secondary follicles becomes compressed and completely fills the extracellular space between the oocyte and follicle cells. Such a feature has been noted in species such as *M. domestica* (Falconnier & Kress, 1992), *Macropus eugenii* (Mate, 1998) and *S. macroura* (Kress *et al.*, 2001) and, in this thesis, in all of the marsupial species studied. As folliculogenesis proceeds from the primary to the secondary follicular stages, subsequent proliferation of the granulosa cells may reduce the extracellular spaces between the follicle cells, while also forcing the existing ZP matrix into the space immediately surrounding the oocyte.

Prior to the formation of the follicular antrum, the increase in granulosa cell numbers within the defined space created by the follicle may also increase the intra-follicular pressure so that the ZP becomes compacted around the oocyte. Evidence for this was seen in preantral oocytes of all species with dense, granular zona material observed completely filling the extracellular space between the oocyte and follicular epithelium, including between the microvilli of the oolemma.

While the compaction of the ZP around the oocyte may merely be a consequence of follicular growth, it might also serve other functional purposes. For example, Lauer and McCarthy (2002) have recently shown that in order for a robust extracellular matrix to form *in vitro*, the matrix components produced by the cultured endoderm cells must reach an appropriate saturation, termed the critical assembly concentration (CAC), before assembly will occur. Although a different system, the ZP is also an extracellular matrix that requires assembly (Green, 1997) and a similar situation may be required before assembly of the ZP filaments can occur. The compaction of the ZP into the finite area of the extracellular space between the oocyte and follicle cells may enable the ZP glycoproteins to reach their CAC and filament construction to reach a peak. Alternatively, although somewhat related, if the CAC is reached prior to the preantral stage of folliculogenesis, which might occur as some areas of the ZP matrix do appear filamentous prior to this stage, then the compaction of the ZP glycoproteins may merely increase their concentration thereby increasing the interaction of the glycoproteins and enhancing self-assembly of the filaments.

Whatever the function of the compaction of the ZP in marsupials is, following the preantral stage, the ZP appears less irregular and takes on its characteristic spherical shape suggesting that much of the core matrix construction takes place at this time. This notwithstanding, the ZP matrix was

still highly granular in appearance and final maturation of the three-dimensional structure of the ZP may take place at a later time, probably following the withdrawal of the cytoplasmic extensions of the cumulus cells. This question does, however, form the basis of another chapter of this thesis (see Chapter 6).

The origin of the ZP in marsupials has only recently been investigated, particularly following the isolation and sequencing of the possum ZP genes (Mate & McCartney, 1998; Haines *et al.*, 1999; McCartney & Mate, 1999; Voyle *et al.*, 1999), with mixed results. Non-radioactive *in situ* hybridisation using riboprobes for possum ZPA and ZPB mRNA has suggested that while the ZPA and ZPB genes were expressed in some oocytes of primordial follicles, the greatest expression was found in oocytes of primary follicles following which expression decreased (Haines *et al.*, 1999; Voyle *et al.*, 1999). No expression above background staining, however, was detected within the follicular epithelium. Using polyclonal antibodies specific for pig ZP, Mate (1998) performed immunohistochemistry and immunocytochemistry on the ovaries of possums and wallabies and found evidence for a multi-origin site of synthesis for the ZP. For example, immunohistochemistry labelled the ZP surrounding oocytes of all follicles, with the exception of primordial follicles, while labelling was also noted within the granulosa cells of primary follicles (Mate, 1998). Immunocytochemistry results from the same study, however, localised ZP glycoproteins within the membranes of the oocytes and follicle cells of primary follicles prior to their ultrastructural visualisation, but failed to detect any ZP glycoproteins in the granulosa cells (Mate, 1998).

As the ultrastructural results of this study have shown, ZP matrix may be highly dispersed within the follicular extracellular space during the early stages of ZP secretion (i.e. during the primary

follicular stage). This feature, combined with the relatively poor resolving ability of fluorescence microscopy on paraffin sections, may have resulted in the misinterpretation of ZP material to be within, and not between, the granulosa cells (Mate, 1998). Neither the immunocytochemical nor *in situ* hybridisation results suggested any contribution to the ZP by the granulosa cells, while the ultrastructural results from the present study also failed to detect any features associated with the secretion of the ZP by the follicle cells. The oocytes of primary follicles, however, displayed several features associated with secretion of the ZP, including characteristic Golgi bodies located within the sub-cortical position and small electron-dense secretory vesicles near the oolemma. Taken together, these results suggest that the ZP of the possum, at the very least, has an oocyte-specific site of origin. Supporting this, a recent study on the immunological characteristics of *E. coli*-expressed recombinant possum ZP proteins found that immunohistochemistry failed to detect any ZP proteins within the granulosa cells (Mate *et al.*, 2003). It must be said, however, that determining the site of origin of the ZP by ultrastructural methods alone, is unlikely to provide definitive results. As such, further investigations using methods such as *in situ* hybridisation and immuno-localisation for the other marsupial species (through immunohistochemistry and immunocytochemistry) are required before any answers can be provided for the question of the cellular site of synthesis of the ZP.

The final aim of this chapter was to determine if there were any unique features of the ZP around the oocytes of the wombat and koala that might account for the unique morphological features of their spermatozoa. As such, wombat and koala oocytes formed much of the basis for this ultrastructural study, predominantly due to the fact that the ultrastructural features of the oocytes of these species have been rarely studied, but also to identify any comparatively unique features of the ZP. One unique feature of the oocytes of wombats and koalas appears to be the relative

thicknesses of their ZP compared with other marsupials. While numbers of individuals and numbers of mature oocytes that had their ZP measured were relatively low, due mainly to the fact that these species were obtained opportunistically, the greater thickness of the ZP of koalas has been noted by at least one other researcher (S. Johnston, personal communication).

With ZP thicknesses on average around, or just above, that of the laboratory mouse (ZP measures around 6µm: Dunbar *et al.*, 1991, 1994), the gametes of the wombat and koala display an unusual convergence with those of laboratory rodents. For example, the spermatozoa of both wombats and koalas and laboratory rodents have falciform-shaped heads (Hughes, 1965; Harding & Aplin, 1990; Temple-Smith & Taggart, 1990), although the position of the acrosome differs significantly (Temple-Smith & Taggart, 1990; Breed *et al.*, 2001). The similar morphology is the only convergence, however, with the finding that the chromatin of the wombat and koala sperm are as easily disrupted by dilute detergents as those of other marsupials, and unlike that of rodents (Breed *et al.*, 2001). The reason for the unique structure of wombat and koala sperm remains unknown and although their oocytes appear to be surrounded by a relatively thicker ZP, the lack of stabilising features within the sperm chromatin suggests that they have a similar mode of ZP penetration as suggested for other marsupial species, namely enzymatic (Bedford, 1991, 1996, 1998).

In conclusion, this chapter has highlighted the major ultrastructural features of ZP formation during oocyte growth in five marsupial species, including the first ultrastructural characterisation of the general features of oocyte growth and ZP formation in the southern hairy-nosed wombat and koala. While there were a number of common features of ZP formation between species, there appeared to be interspecific differences in final ZP thicknesses, with those of the wombat

and koala relatively thicker than those of the other species. Whether this interspecific variation in ZP thickness relates to a species-specific method of sperm-ZP binding and/or ZP penetration is unknown. The species-specificity of the ZP, although debatable, is said to be afforded through the glycoconjugates of the ZP, and the interspecific variation of ZP glycoconjugates amongst marsupials forms the basis for the investigations of the next chapter.

Chapter 4 Glycoconjugates of the marsupial zona pellucida

4.1 Introduction

The eutherian mammalian zona pellucida (ZP) is composed of three sulphated glycoproteins: ZPA (or ZP2), ZPB (or ZP1) and ZPC (or ZP3), each of which is encoded by a gene that is highly conserved among species (Harris *et al.*, 1994). Although the protein components of the ZP glycoproteins are highly homologous among species, interspecific differences in the sugar components of the *N*- and *O*-linked oligosaccharides are evident, and these may relate to species specificity of sperm-zona pellucida binding if this occurs. The importance of the oligosaccharides for sperm binding is well established and supported by the finding that addition of various saccharides and lectins to the *in vitro* fertilization environment can prevent sperm-zona pellucida binding and fertilization (Shalgi *et al.*, 1986; Cornwall *et al.*, 1991; Mori *et al.*, 1993; Tulsiani *et al.*, 1997).

In mice, the *O*-linked oligosaccharides of ZPC facilitate primary sperm binding (Florman and Wassarman, 1985), either through a terminal α -galactose (α -Gal) (Bleil and Wassarman, 1988) or *N*-acetylglucosamine (GlcNAc) (Miller *et al.*, 1992*b*). In pigs, a *N*-linked oligosaccharide attached to Asn220 of ZPB in a ZPB–ZPC heterocomplex appears to be the primary binding site for spermatozoa (Kudo *et al.*, 1998; Yurewicz *et al.*, 1998; Nakano and Yonezawa, 2001) while, in humans, sperm–ZP binding may be mediated through a selectin-like ligand on the ZP which recognizes fucose (Fuc)-rich oligosaccharides (Patankar *et al.*, 1993; Clark *et al.*, 1996*b*; Oehninger *et al.*, 1998; Oehninger, 2001). Removal of terminal sialic acid residues (desialylation) from both the human ZP and sperm surface has also been shown to result in an increase in sperm binding (Lassalle and Testart, 1994; Banerjee and Chowdhury, 1997; Ozgur *et al.*, 1998).

Lectin histochemistry has been used to identify the saccharides and their distribution within eutherian ZP (Nicolson *et al.*, 1975; Skutelsky *et al.*, 1994). Observational studies on the ZP of eutherian species such as rats (Shalgi *et al.*, 1991), humans (Bar-Shira Maymon *et al.*, 1994) and river buffalo (Parillo *et al.*, 1998) have demonstrated interspecific variation in the intensity of lectin-binding patterns and the distribution of various saccharide components.

The ZP of marsupials appear to be thinner and more readily solubilised by proteases than those of eutherians (Bedford, 1991, 1996; Bedford and Breed, 1994). The three genes encoding the ZP proteins in brushtail possums have been cloned and the putative amino acid sequences deduced (Mate and McCartney, 1998; Haines *et al.*, 1999; McCartney and Mate, 1999; Voyle *et al.*, 1999). As yet, no study has characterized the glycoconjugate composition of marsupial ZP. In the present study differential lectin binding, together with enzyme elimination and saponification, is used to investigate the glycoconjugate components of the ZP of follicular oocytes of seven marsupial species from different families within this infraclass.

4.2 Materials and Methods

4.2.1 Animals

The following Australian species were used in this study; fat-tailed dunnarts (*Sminthopsis crassicaudata*; Family: Dasyuridae; $n = 4$); a southern brown bandicoot (*Isodon obesulus*; Family: Peramelidae; $n = 1$); brushtail possums (*Trichosurus vulpecula*; Family:

ERRATUM

Grey short-tailed opossums (*Monodelphis domestica*; family: Didelphidae; $n = 2$) originating from the colony bred at the South West Foundation for Biomedical Research, San Antonio, Texas, were also used.

Phalangeridae; $n = 4$); common ringtail possums (*Pseudocheirus peregrinus*; Family: Pseudocheiridae; $n = 2$); koalas (*Phascolarctos cinereus*; Family: Phascolarctidae; $n = 3$); and eastern grey kangaroos (*Macropus giganteus*; Family: Macropodidae; $n = 2$). Animals were obtained as previously described in section 2.1 and all were adults that had never been subjected to treatments that could affect natural follicular growth. Staining with each lectin, and each disruptive treatment, was performed at least twice on ovaries of all species.

* Note: see erratum – opposite page

4.2.2 Ovary collection

Excised ovaries from the above animals, except opossums, were immediately fixed in Rossman's fluid (90% ethanol saturated with picric acid and 10% formalin) for 12–24 h, washed in 95% alcohol for 24–48 h, routinely processed, embedded in paraffin wax and sectioned at 7 μm (see sections 2.3 and 2.4). Opossum ovaries were also fixed and then stored in Rossman's fluid for several weeks before processing as above.

4.2.3 Lectin histochemistry

Sections were deparaffinized by immersion in Histoclear (National Diagnostics, Atlanta, GA), rehydrated by passing through a series of alcohols (100–50%) and brought to Tris-buffered saline (TBS, pH 7.6). The sections were then covered with 1% BSA in TBS for 30 min, excess solution was shaken off, and the slides were blotted with tissue paper. Sections were then incubated with one of ten fluorescein isothiocyanate (FITC)-conjugated lectins (Vector Laboratories, Burlingame, CA) (Table 4.1). These lectins were used at a concentration of 20 mg ml^{-1} , except for WGA, which was used at a concentration of 10 mg ml^{-1} , for 30 min at room temperature in a humidified light-safe chamber. The sections were then rinsed three times in TBS, one drop of antifade solution was added (Slowfade

Antifade kit, Molecular Probes, USA), and then a coverslip was applied. Sections were viewed with an Olympus BH epifluorescent and phase-contrast microscope, using a 515 nm excitation filter and an IFK90 nm barrier filter with an absorption wavelength of 535 nm and an emission wavelength of 617 nm. The intensity of lectin staining was determined qualitatively using a scoring system adapted from the light microscopical lectin histochemistry investigations of ZP by Skutelsky *et al.* (1994), Legge (1995), Parillo *et al.* (1996, 1998) and Parillo and Verini-Supplizi (1999, 2001). A positive result was indicated by (+) and a negative result by (–), with the intensity of fluorescence being defined as mild (+), strong (++) or intense (+++). Only the ZP of antral follicular oocytes were compared among species, while exposure times for photography of fluorescence results were kept identical at 51.2 s and photographed on T-Max 400 ASA black and white film.

The sugar specificities and the inhibiting sugars for the lectins used in this study are listed (Table 4.1).

4.2.4 Enzymatic and saponification treatments

Sections were incubated at 37°C for 1 h with 1 U neuraminidase ml⁻¹ (from *Clostridium perfringens*, Sigma, St. Louis, MO) in 0.05 mol acetate buffer l⁻¹ (pH 5.5) before incubation with SBA, PNA, SNA, RCA-II and WGA (Riley and Elhay, 1994) to determine sugar residues masked by terminal sialic acid residues. In addition, sections were subjected to mild alkali hydrolysis (saponification) in a 1% (w/v) potassium hydroxide solution in 70% ethanol at room temperature for 1 h before incubation with the lectins (Wiebkin, 1994) to detect the presence of sugar residues masked by *O*-acetylated sialic acid groups.

Table 4.1. Lectins used and their corresponding saccharide specificities

Origin of lectin	Common name	Acronym	Sugar specificity	Sugar inhibitor
<i>Arachis hypogea</i>	Peanut	PNA	β Gal-(1-3)-GalNAc	Lactose
<i>Erythrina cristagalli</i>	Coral Tree	ECA	β Gal-(1-4)-GlcNAc	D-GlcNAc
<i>Concanavalina ensiformis</i>	Jack bean	Con A	α -D-Man, α -D-Glc	D-Man, D-Glc
<i>Pisum sativum</i>	Garden pea	PSA	α -D-Man	D-Man
<i>Ricinus communis I</i>	Castor bean	RCA-I	β -D-Gal	Lactose
<i>Triticum vulgaris</i>	Wheat germ	WGA	$[\beta$ -(1-4)-D-GlcNAc] ₂ , NeuNAc	D-GlcNAc*, NeuNAc
<i>Sambucus nigra</i>	Elderberry	SNA	α -NeuNAc-(2-6)-Gal/GalNAc	NeuNAc
<i>Glycine max</i>	Soybean	SBA	α -D-GalNAc, α -D-Gal	α -D-GalNAc
<i>Ulex europeaus I</i>	Gorse	UEA-I	α -L-Fuc	α -L-Fuc
<i>Lotus tetragonolobus</i>	Winged pea	LTA	α -L-Fuc	α -L-Fuc

* Sugar inhibitor used in this study

Fuc: fucose; Gal: galactose; GalNAc: *N*-acetylgalactosamine; Glc: glucose; GlcNAc: *N*-acetylglucosamine; Man: mannose; NeuNAc: *N*-acetylneuraminic acid (sialic acid) (from Wu *et al.*, 1988).

4.2.5 Controls

Incubation of the lectins with 0.2–0.4 mol l⁻¹ of their inhibitory sugar made up in distilled water for 30 min before the application of the lectins to the sections served as specific controls. For the desialylation treatments, control sections were incubated in acetate buffer alone while, for the saponification treatment, control sections were incubated in 70% ethanol.

4.3 Results

Ovaries from all individuals had follicles at various stages of development, with oocytes surrounded by a ZP from the early primary follicular stage onwards. No variation in lectin staining was noted among the ZP surrounding oocytes of secondary and tertiary follicles in

the same animal, and there was no observable difference in lectin binding between individuals of the same species. The intensity of fluorescence of ten lectins with the ZP around the antral follicular oocytes of fat-tailed dunnarts, the southern brown bandicoot, grey short-tailed opossums, brushtail possums, ringtail possums, koalas, and eastern grey kangaroos is summarized (Table 4.2).

4.3.1 *Arachis hypogea* (peanut) lectin (PNA)

The staining of the marsupial ZP with PNA varied from negative in the dunnarts to intense fluorescence in the ringtail possums. However, after neuraminidase treatment, there was an increase in fluorescence of the ZP in all species, except for those of the opossums and ringtail possums. Saponification also increased the fluorescence of the ZP of all the species (Fig. 4.1), except those of the ringtail possums and koalas.

4.3.2 *Erythrina cristagalli* lectin (ECA)

Before the saponification treatments, no fluorescence was detected with ECA on the ZP in fat-tailed dunnarts, brushtail possums and ringtail possums, while there was mild fluorescence in the ZP of opossums, koalas and kangaroos, and strong fluorescence in the ZP of the bandicoot. However, after removal of *O*-acetyl groups, intense fluorescence with ECA was found in the ZP of the dunnarts, bandicoot, brushtail possums and ringtail possums and there was an increase in fluorescence in the ZP of the other species.

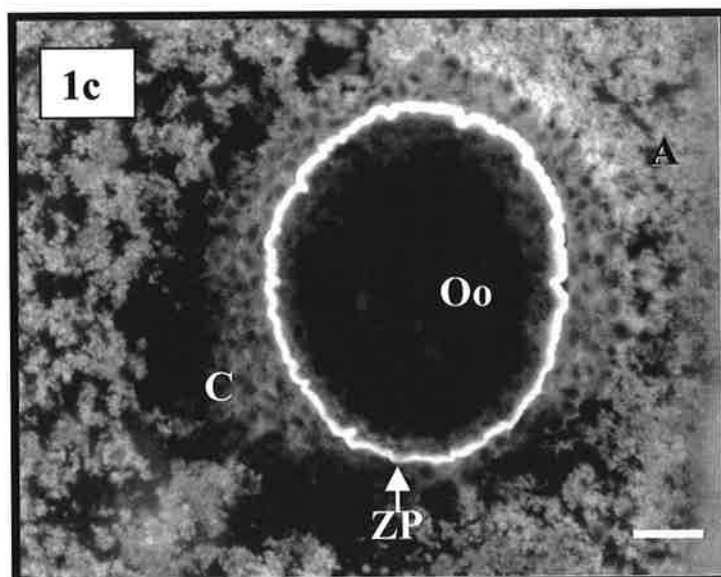
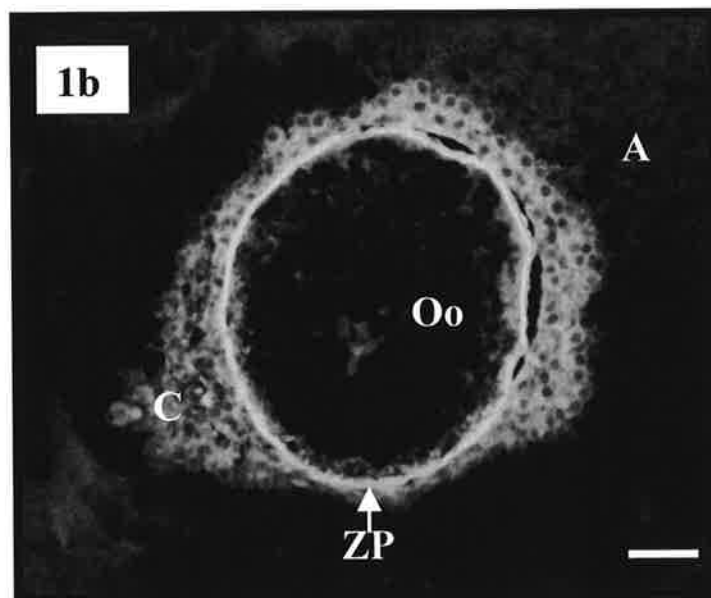
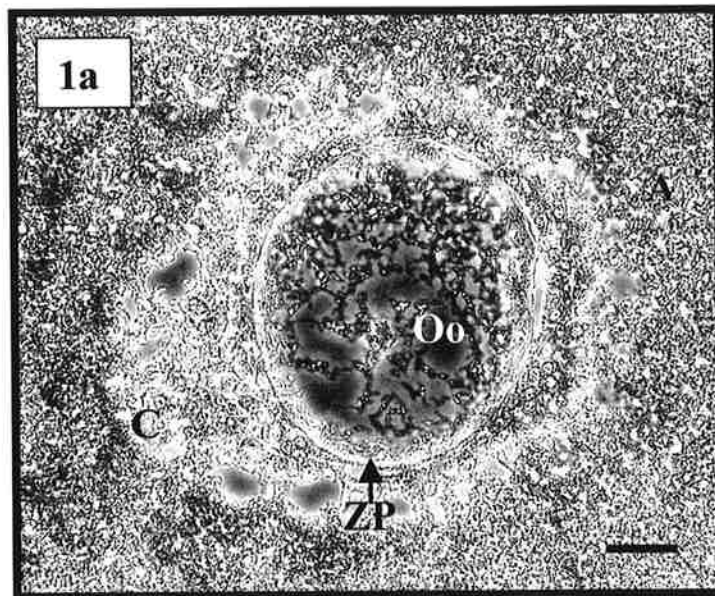
Table 4.2. Lectin binding to zonae pellucidae of marsupial follicular oocytes

Lectin and Treatment	Fat-tailed dunnart (Dasyuridae)	Southern brown bandicoot (Peramelidae)	Grey short-tailed opossum (Didelphidae)	Brush-tail possum (Phalangeridae)	Ringtail possum (Pseudocheiridae)	Koala (Phascolarctidae)	Eastern grey kangaroo (Macropodidae)
PNA	-	+	+	++	+++	++	++
NEU-PNA ^a	++	++	+	+++	+++	+++	+++
KOH-PNA ^b	+	++	++	+++	+++	++	+++
ECA	-	++	+	-	-	+	+
KOH-ECA ^b	+++	+++	++	+++	+++	++	++
Con A	-	-	-	-	-	-	-
KOH-Con A ^b	++	-	-	-	-	-	-
PSA	-	++	-	-	++	++	-
KOH-PSA ^b	+++	++	++	++	++	++	++
RCA-I	+	+	-	-	+++	+	-
NEU-RCA-I ^a	++	++	-	-	+++	+	+
KOH-RCA-I ^b	+	++	-	-	+++	+	+
WGA	++	+++	+++	+++	++	+++	+++
NEU-WGA ^a	+++	+++	+++	+++	++	+++	+++
KOH-WGA ^b	++	+++	+++	+++	+++	+++	+++
SNA	+	+	-	-	++	++	-
NEU-SNA ^a	++	++	-	+	+	++	+
KOH-SNA ^b	+	+	++	-	+	++	++
SBA	-	+	-	-	-	-	-
NEU-SBA ^a	+	++	-	+	+	++	++
KOH-SBA ^b	++	+	-	-	-	-	-
UEA-I	-	-	-	-	-	-	-
KOH-UEA-I ^b	-	-	-	-	-	-	-
LTA	-	-	-	-	-	-	-
KOH-LTA ^b	-	-	-	-	-	-	-

^aNeuraminidase treatment before binding study with respective lectin.

^bSaponification with alcoholic KOH before binding study with respective lectin.

Figure 4.1 Antral follicular oocytes (Oo) and zonae pellucidae (ZP) from the brushtail possum. Phase contrast (1a) and fluorescent (1b,c) micrographs of the zona pellucida stained with fluorescein isothiocyanate-*Arachis hypogea* (peanut) lectin (FITC-PNA) before (1b) and after (1c) saponification. Note the greater fluorescence of the zona pellucida in 1c; Antrum (A); Cumulus cells (C). PNA binding is specific for β -Galactose (1-3) *N*-acetylgalactosamine. Bar = 45 μ m.



4.3.3 *Concanavalia ensiformis* lectin (Con A)

Apart from the saponified ZP of the fat-tailed dunnarts, the ZP of other species did not stain with Con A. Specific controls showed that this fluorescence was inhibited by α -D-mannose but not α -D-glucose.

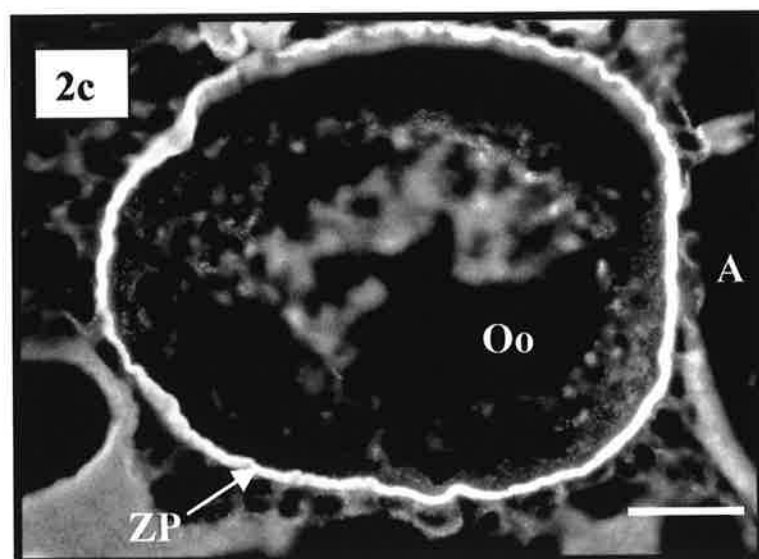
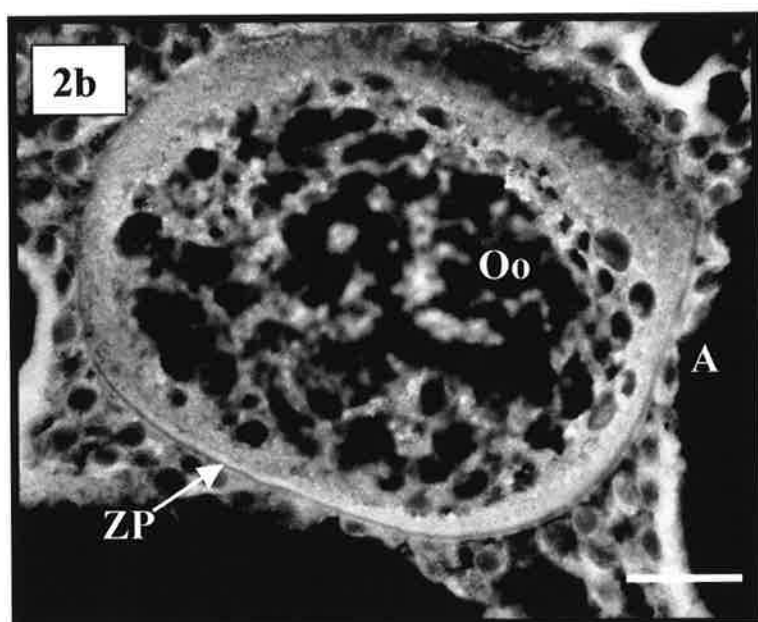
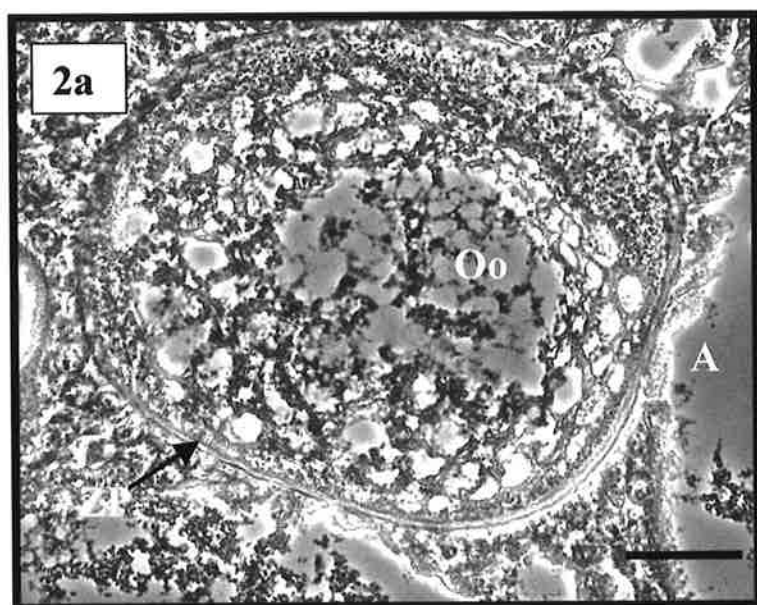
4.3.4 *Pisum sativum* lectin (PSA)

Before saponification, only the ZP of the bandicoot, ringtail possums and koalas strongly fluoresced with PSA and there was no change in intensity of fluorescence after saponification. The ZP of the other species exhibited fluorescence only after saponification, and then fluoresced strongly or intensely (Fig. 4.2).

4.3.5 *Ricinus communis* I lectin (RCA-I)

Intense fluorescence of RCA-I was found in the ZP of ringtail possums, while mild fluorescence was observed in the ZP of dunnarts, the bandicoot and koalas. The ZP of the opossums and brushtail possums did not fluoresce with RCA-I before, or after, either of the treatments, while the ZP of the kangaroos stained mildly only with RCA-I after they were desialylated or saponified. After neuraminidase treatment, the fluorescence of the ZP of the dunnarts, the bandicoot and kangaroos increased while, after saponification, only the ZP of the bandicoot and kangaroos increased in fluorescence, with the ZP of the bandicoot fluorescing intensely with this lectin.

Figure 4.2 Antral follicular oocytes (Oo) and zonae pellucidae (ZP) from the fat-tailed dunnart. Phase contrast (2a) and fluorescent (2b,c) micrographs stained with fluorescein isothiocyanate-*Pisum sativum* (garden pea) lectin (FITC-PSA) before (2b) and after (2c) saponification. Note greater fluorescence of zona pellucida after saponification in 2c; Antrum (A); Cumulus cells (C). PSA binding is specific for α -D-Mannose. Bar = 45 μ m.



4.3.6 *Triticum vulgaris* (wheat germ) lectin (WGA)

The ZP of all species exhibited strong to intense fluorescence with WGA, which was localized to the inner and outer regions of the ZP before desialylation or saponification treatments (Fig. 4.3). There was an increase in fluorescence with this lectin after neuraminidase treatment of the ZP of fat-tailed dunnarts, and an increase in fluorescence of the ZP of the ringtail possums after saponification. Although there were no changes in the intensity of fluorescence of this lectin to the ZP of the other species after either treatment, saponified ZP of all species exhibited a loss of inner and outer ZP localization and demonstrated a uniform fluorescence throughout the entire ZP.

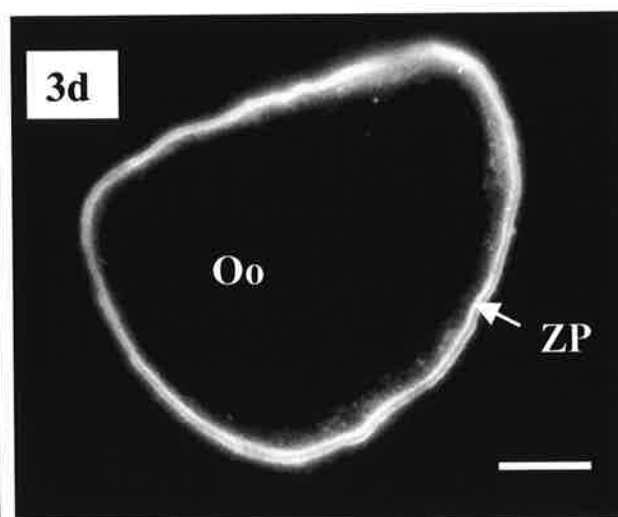
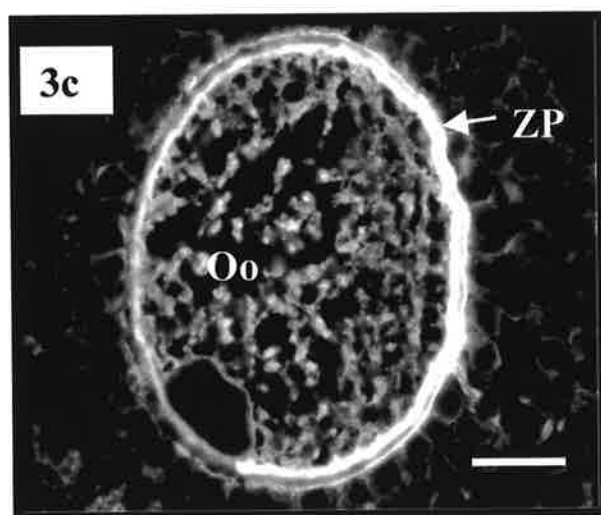
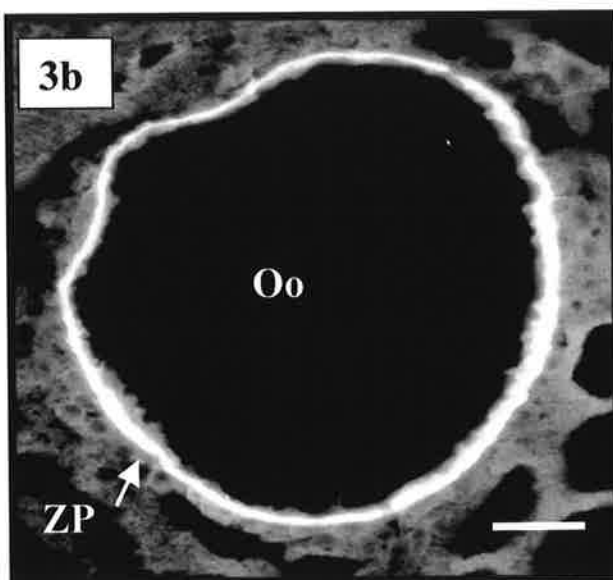
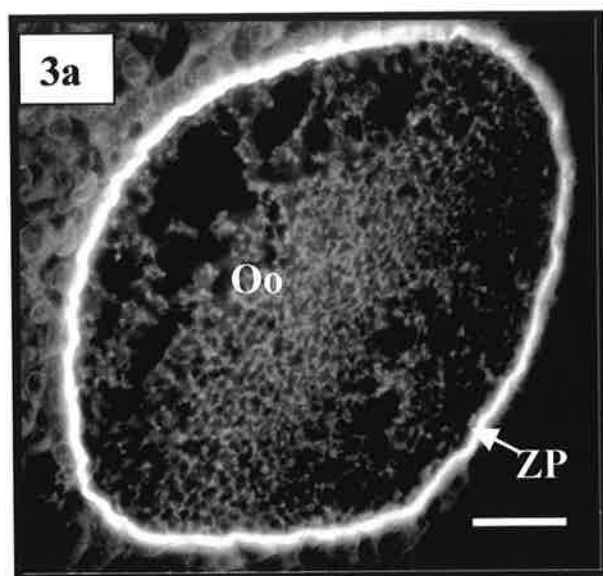
4.3.7 *Sambucus nigra* lectin (SNA)

Fluorescence with SNA was demonstrated in the ZP of the fat-tailed dunnarts, bandicoot, ringtail possums and koalas, before exposure to neuraminidase or mild alkali hydrolysis. An increase in the intensity of fluorescence was evident after pre-treatment with neuraminidase in the ZP of fat-tailed dunnarts, the bandicoot, brushtail possums and kangaroos, while a decrease in fluorescence was noted in the desialylated and saponified ZP of ringtail possums. An increase in fluorescence with this lectin was noted in the ZP of the opossums and kangaroos, from an absence of evident fluorescence before saponification to strong fluorescence after removal of O-acetyl groups.

4.3.8 *Glycine max* (soybean) lectin (SBA)

Only the ZP of the bandicoot fluoresced with SBA before desialylation or saponification treatments. An increase in fluorescence with this lectin was evident in the ZP of all species,

Figure 4.3 Antral follicular oocytes (Oo) and zonae pellucidae (ZP) from grey short-tailed opossum (3a), brushtail possum (3b), ringtail possum (3c) and eastern grey kangaroo (3d) all stained with fluorescein isothiocyanate-*Triticum vulgaris* (wheat germ) lectin (FITC-WGA). Note intense fluorescence of the inner and outer regions of the zonae pellucidae, particularly in the ringtail possum (3c) and eastern grey kangaroo (3d). WGA binding is specific for β (1-4) *N*-acetylglucosamine, *N*-acetylneuraminic acid (sialic acid). Bar = 45 μ m.



except those of the opossums, after incubation with neuraminidase, while only the ZP of the dunnarts demonstrated an increase in fluorescence with this lectin after saponification (Fig. 4.4).

4.3.9 *Ulex europeaus* I lectin (UEA-I) and *Lotus tetragonolobus* lectin (LTA)

No fluorescence with either UEA-I or LTA was evident in any of the ZP tested before, or after, any of the treatments.

4.3.10 Controls

Incubation of the lectins with their complementary sugar before incubation with the sections resulted in elimination of fluorescence in the ZP of all species (Fig. 4.5), except for that of WGA, which still demonstrated mild fluorescence after incubation with D-GlcNAc.

4.4 Discussion

Eutherian ZP are composed of highly glycosylated glycoproteins, and both *N*- and *O*-linked oligosaccharides are present (Benoff, 1997; Shalgi and Raz, 1997). Where it has been possible to obtain large numbers of ZP, detailed analysis of their oligosaccharides has been performed by high performance liquid chromatography (HPLC) and such studies have demonstrated interspecific differences in the carbohydrate components. For example, mouse ZPA and ZPC have β -GlcNAc and α -galactose at the non-reducing ends of *N*-linked chains that are absent from pig ZPC (Noguchi and Nakano, 1993), whereas bovine

Figure 4.4 Antral follicular oocytes (Oo) and zonae pellucidae (ZP) from the southern brown bandicoot. Phase contrast (4a) and fluorescent (4b,c) micrographs of the zona pellucida stained with fluorescein isothiocyanate-*Glycine max* (soybean) lectin (FITC-SBA) before (4b) and after (4c) incubation in the enzyme neuraminidase. Note fluorescence of the zona pellucida increases from mild fluorescence in 4b to strong fluorescence in 4c indicating greater fluorescence after desialylation; Antrum (A). SBA binding is specific for α -D-N-acetylgalactosamine/ α -D-galactose. Bar = 45 μ m.

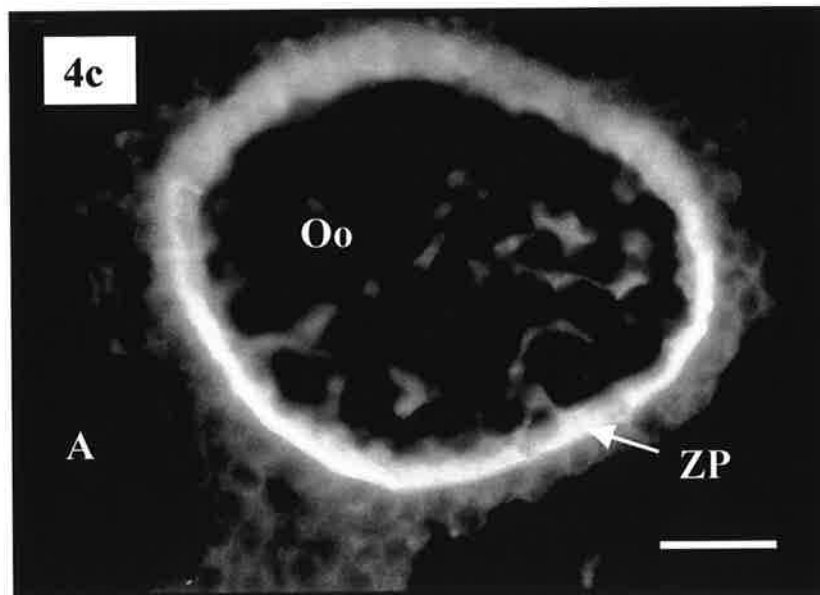
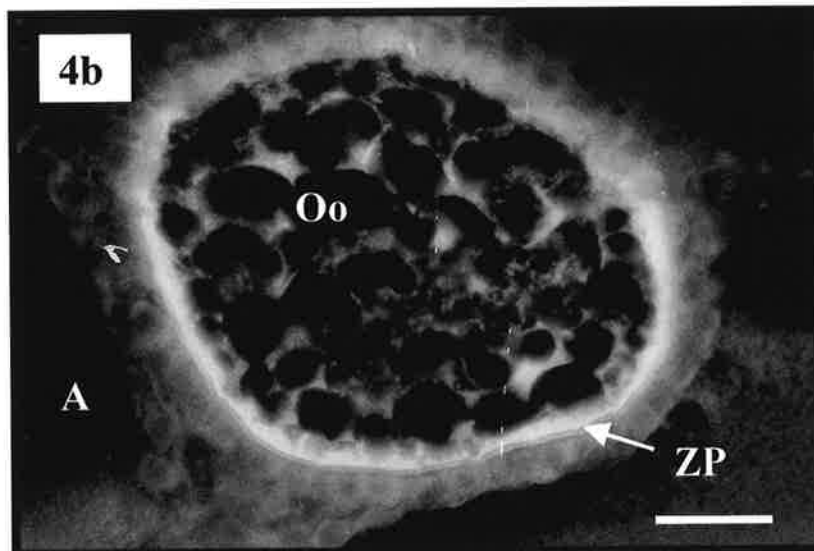
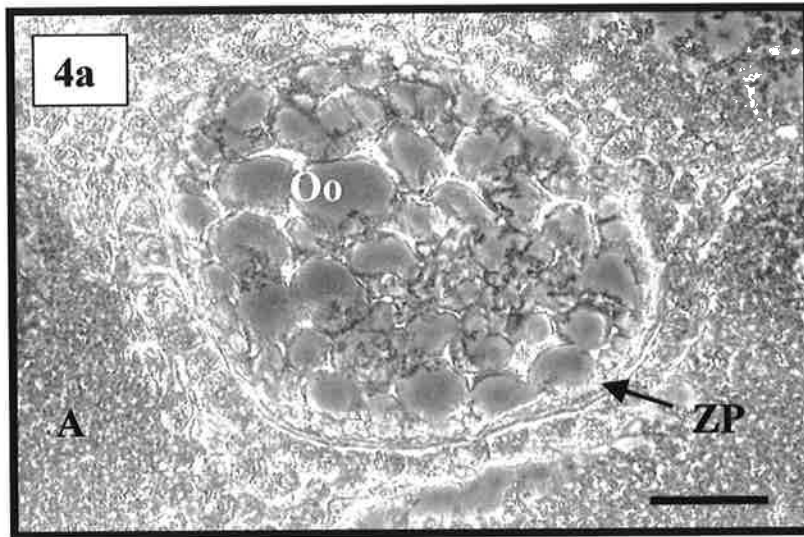
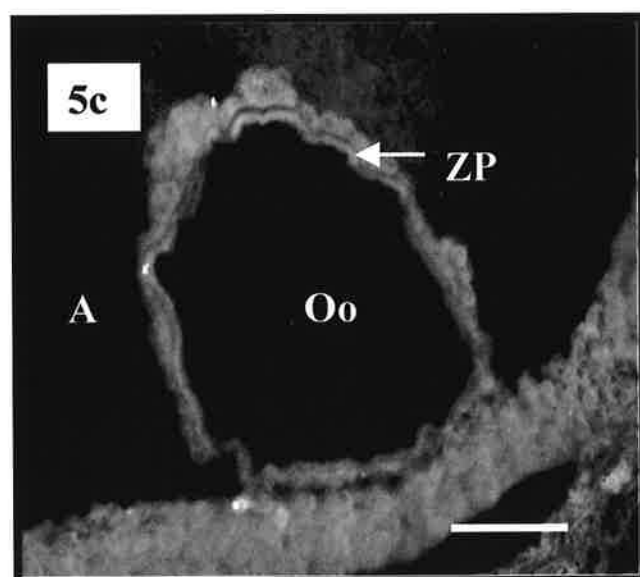
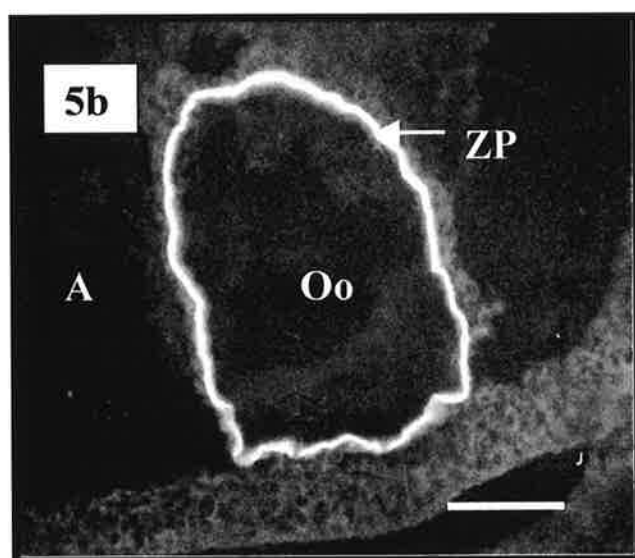
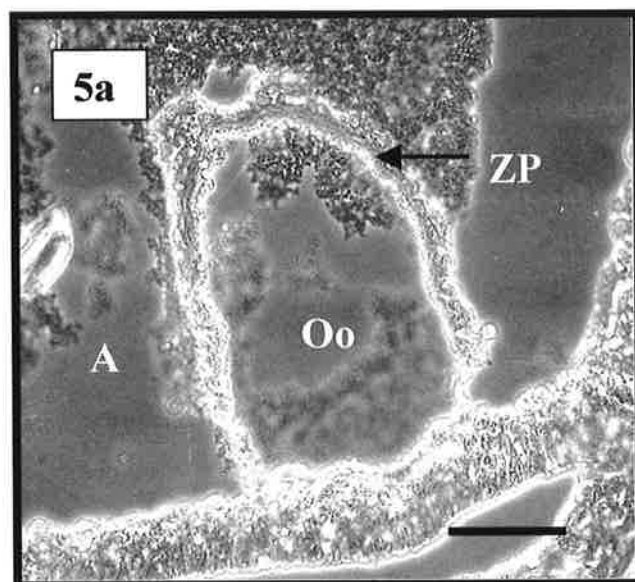


Figure 4.5 Antral follicular oocytes (Oo) and zonae pellucidae (ZP) from the brushtail possum. Phase contrast (5a) and fluorescent (5b,c) micrographs of the zona pellucida after saponification stained with fluorescein isothiocyanate-*Erythrina cristagalli* (coral tree) lectin (FITC-ECA) without (5b) and with (5c) the addition of 0.4M D-N-acetylglucosamine to the lectin 30 min prior to application to the sections. Note the loss of fluorescence of the zona pellucida following the incubation of the lectin with its inhibiting sugar. ECA binding is specific for β galactose (1-4) N-acetylglucosamine/ α -D-galactose. Bar = 45 μ m.



ZP contain high-mannose-type neutral oligosaccharides which are absent from both mouse and pig ZP (Katsumata *et al.*, 1996).

For species in which large amounts of ZP are unavailable, or where glycoconjugate localization is required, lectin histochemistry has been used widely for identification of the saccharides (Shalgi *et al.*, 1991; Bar-Shira Maymon *et al.*, 1994; Avilés *et al.*, 1999). Staining with lectins has shown interspecific variation of sugar residues within eutherian ZP (Nicolson *et al.*, 1975; Skutelsky *et al.*, 1994; Parillo *et al.*, 1996; Parillo & Supplizi, 1999, 2001). In the present study, differential lectin histochemistry has also demonstrated interspecific variation in saccharide components in the ZP of seven marsupial species. For example, in the present study interspecific differences in α -D-mannose were detected. Before saponification, α -D-mannose could not be identified with Con A but, after saponification, only the ZP of the fat-tailed dunnarts fluoresced with Con A. However, staining with PSA, which is also specific for α -D-mannose, resulted in fluorescence of the ZP of the southern brown bandicoot, ringtail possums and koalas before saponification and in the ZP of all species after saponification. These results not only highlight the need to stain with more than one lectin specific for the same saccharides but also show that the differences in staining between Con A and PSA may be due to an interspecific variation in glycoconjugates. While both lectins are specific for the tri-mannosidic core of *N*-linked oligosaccharides, PSA has a greater affinity for those that also possess α -L-fucose linked to GlcNAc at their reducing ends (Debray *et al.*, 1981; Kornfeld *et al.*, 1981; Wu *et al.*, 1988). While α -L-fucose was not positively identified in the ZP of the marsupials investigated in the present study, both of the fucose-specific lectins used recognize di- and tri-saccharides through an L-fucose- α (1-2) linkage to galactose, whereas PSA recognizes α -L-fucose through a (1-6) linkage (Wu *et al.*, 1988).

The identification of mannose suggests, for the first time, the presence of *N*-linked oligosaccharides in the ZP glycoproteins of all the marsupial species used in the present study. This finding is in agreement with studies from the cDNA and deduced amino acid sequences of the three ZP glycoproteins in brushtail possums, which indicate that the ZP polypeptides contain several potential *N*-linked glycosylation sites (Mate and McCartney, 1998; Haines *et al.*, 1999; McCartney and Mate, 1999). The number of potential *N*-linked glycosylation sites is eight in ZPA, seven in ZPB and two in ZPC (Mate and McCartney, 1998; Haines *et al.*, 1999; McCartney and Mate, 1999; Voyle *et al.*, 1999), after discounting one site in ZPA and ZPC in which proline was in the *X* position of the determining sequin N-*X*-S/T. Several authors have noted that sugar substitution at this site is extremely unlikely (Mononen and Karjalainen, 1984; Kornfeld and Kornfeld, 1985; Shakin-Eshleman *et al.*, 1996). Moreover, the potential for *N*-linked sites to be glycosylated varies according to factors that determine glycosylation efficiency. For example, sequons with Thr in the hydroxy position are more likely to be glycosylated than those with Ser (Kasturi *et al.*, 1995). The amino acid in the *X* position of N-*X*-S/T also determines glycosylation efficiency (Shakin-Eshleman *et al.*, 1996). The third, fourth and fifth potential *N*-linked glycosylation sites in both ZPA and ZPB in brushtail possums contain Thr in the hydroxy position, while the fifth site of both glycoproteins also contain Ser in the *X* position, with a core glycosylation efficiency of approximately 95% (Shakin-Eshleman *et al.*, 1996). Taken together, these factors indicate that the possum zona pellucida may contain *N*-linked oligosaccharides.

While a number of interspecific differences were observed in glycoconjugate content, major variation or similarities appeared to relate to a large extent to the sialylation of the glycoproteins. For instance, all the ZP of the marsupials tested, except for those of the opossum, appeared to contain masking residues of sialic acid since neuraminidase

treatment increased fluorescence with PNA and SBA. Sialic acid is commonly found as a capping sugar of the terminal saccharide units of oligosaccharides and may affect the ability of the internal sugar residues to interact with lectins. Removal of these sialic acid residues by neuraminidase before incubation of the ZP with the lectins has demonstrated that terminal β -Gal (1-3) *N*-acetylgalactosamine (β -Gal (1-3)GalNAc) and α -D-Gal(NAc) residues in the ZP are indeed masked by sialic acid. This also appears to be the case for ZP of eutherian mammals in which increases in binding to PNA and SBA after desialylation occur in the ZP of sheep, goats, pigs (Parillo *et al.*, 1996), rabbits and hares (Parillo and Verini-Supplizi, 2001), rats (Avilés *et al.*, 1996) and river buffalo (Parillo *et al.*, 1998).

Marsupial ZP also appear to contain masking sialic acids that relate to their *O*-acetylation, some of which are common across all species. The disaccharide β Gal(1-4)GlcNAc appears to be masked by *O*-acetylated sialic acids in all marsupial ZP, with increases to strong and intense fluorescence with ECA after saponification. In the fat-tailed dunnarts and brushtail possums, this disaccharide was not evident before saponification, indicating that the *O*-acetyl groups on sialic acids are responsible for a conformational masking of these residues. In marsupial ZP, *O*-acetylated sialic acids are also found to mask other glycoconjugates conformationally, including the aforementioned mannose in the dunnarts, opossums, brushtail possums and kangaroos, β Gal(1-3)GalNAc in all species except the ringtail possums and koalas, and various other residues consistent with interspecific variation.

Another apparent conservation of glycoconjugate content of the ZP across mammals is the relative abundance and distribution of the disaccharide $[\beta$ -(1-4)-D-GlcNAc]₂, to which WGA binds. In all of the marsupial ZP studied, there was strong-to-intense fluorescence with this lectin, localized to the inner and outer regions. Similar intensities and

localizations have been described in the ZP of almost all eutherian species studied, except for perhaps those of cats (Skutelsky *et al.*, 1994; Parillo and Verini-Supplizi, 1999), and hares that displayed a uniform distribution of GlcNAc throughout the entire ZP (Parillo and Verini-Supplizi, 2001). While WGA is also specific for sialic acid, binding intensity in the present study remained either unchanged or increased after removal of the sialic acid with neuraminidase, indicating that the staining by WGA is at least partly due to its binding to $[\beta\text{-(1-4)-D-GlcNAc}]_2$.

The localization of WGA binding to the inner and outer regions of the ZP and the loss of such localization after saponification indicates that the sialic acids of the internal glycoproteins may be *O*-acetylated, whereas the residues of the glycoproteins of the inner and outer regions appear not to be. This apparent complexity of structure supports a re-evaluation of the structural organisation of the ZP as proposed by Wassarman (1988), at least as it applies to marsupials. Such complexity of structure, in terms of regional variation of glycoconjugates within the ZP, may also occur in other eutherian species with the findings of the localization of GlcNAc residues on the inner and outer compartments (as mentioned above), and other examples such as a monoclonal antibody raised against a poly-*N*-acetyllactosaminoglycan of *N*-linked oligosaccharide being localized to concentric rings within the ZP of pigs (Dunbar *et al.*, 2001).

The *O*-acetylated sialic acids in ZP may play a major role in increasing the lifespan of a glycoprotein by preventing or retarding degradation of glycoconjugates (Schauer, 1982, 1988). An *O*-acetyl residue at the *O*-4 position of a sialic acid has been found to completely block the action of a neuraminidase which, in turn, prevents further glycosidases from acting on internal saccharide residues (Schauer, 1988). The inability to detect masking sialic acid residues in the opossum ZP may relate to this, as saponification

increased the reactivity of the ZP of this species to various lectins, indicating that *O*-acetylated sialic acids are, in fact, present. The apparent disparity of sialic acid *O*-acetylation between the inner and outer parts of the ZP (in which there is little or none), from the internal compartment (in which there is much), may relate to the function and longevity of the ZP. The high ratio of *O*-acetylated sialic acids at the core of the ZP may make this area extremely resistant to degradation and, thereby, help to maintain its structural integrity throughout the lifetime of the oocyte and early embryo. Alternatively, the lack of *O*-acetylated sialic acids on the outer surface of the ZP may allow selective glycolysis and proteolysis by the acrosomal enzymes of the spermatozoon.

The viscoelasticity and protease resistance of a glycoprotein has been related to the extent of glycosylation of the protein. Mucins with long oligosaccharide side-chains have been found to have more pronounced viscoelastic properties (Sellers *et al.*, 1988; Jentoft, 1990), whereas the more glycosylated a protein, the more resistant it may be to proteases (Semino *et al.*, 1985; Jentoft, 1990). The reported inability of marsupial ZP to retain their spherical shape after removal of the oocyte and their increased sensitivity to dilute proteases (Bedford, 1991; Bedford and Breed, 1994) may relate to their ZP glycoproteins being less glycosylated, with shorter *O*-linked oligosaccharides, than those of eutherian ZP. The observations of Bedford (1991) pertained mainly to the ZP of the American opossums, however, few biochemical investigations have been performed on the ZP of Australian marsupials. Whether the ZP of Australian marsupials display similar biochemical properties as those of the American species and/or to eutherian species, is unknown.

Chapter 5 Glycoconjugates of the oviduct of the brushtail possum at the time of ovulation

5.1 Introduction

In eutherian mammals, the oviduct was thought, until recently, to provide an optimal yet passive conduit and microenvironment for gamete interaction and early embryonic development (Hunter, 1988; Leese, 1988; Buhi *et al.*, 1997). However, recently it has been found that the oviduct secretes fluid and other factors (e.g. oviduct-specific glycoproteins or OGPs) that enhance the chances of successful gamete maturation and fertilisation *in vivo* (for review see Bleau & St.-Jacques, 1989; Malette *et al.* 1995; Buhi *et al.* 2000). Oviductal glycoprotein(s) have been shown to associate with, or induce changes to, the eggs and embryos (and their surrounding egg coats) of hamsters (Araki *et al.* 1987; L  veill   *et al.* 1987a; Yang & Yanagimachi, 1989; Brown *et al.* 1990; Kan *et al.* 1990; St.-Jacques *et al.* 1992; Boatman *et al.* 1994), mice (Kapur & Johnson, 1986, 1988; Kim & Schuetz, 1993; Kim *et al.* 1996), pigs (Hedrick *et al.* 1987; Buhi *et al.* 1993), baboons (Boice *et al.* 1990) and cows (Staros & Killian, 1998).

In eutherians, many of these associating oviductal glycoproteins have been found to be oviduct-specific and are commonly referred to as oviductins (Bleau & St.-Jacques, 1989). There is a high degree of conservation of cDNA and putative amino acid sequences of oviductins between eutherian species (Donnelly *et al.*, 1991; Arias *et al.*, 1994; Sendai *et al.*, 1994, 1995; Suzuki *et al.*, 1995; Buhi *et al.*, 1996; Verhage *et al.*, 1997b). Even with such a high degree of homology, OGPs may enhance the species-specificity of sperm-zona pellucida

interactions (Schmidt *et al.*, 1997) perhaps due to their high degree of glycosylation. El-Mestrah and Kan (1999*a,b*), using high-resolution lectin-gold cytochemistry, demonstrated that the secretory granules of the ampullary epithelium of the hamster contain a number of glycoconjugates, however, some glycoconjugates such as *N*-acetyl-glucosamine (GlcNAc), were found to occur only around the time of ovulation.

In marsupials, the oviduct secretes the tertiary egg envelopes, the mucoid coat and perhaps the shell membrane (for reviews see Hughes, 1977, 1984; Selwood, 2000). The mucoid coat of marsupials has been shown to be composed of PAS+ mucoprotein that may be sulphated in some species (Hughes, 1977; Roberts *et al.*, 1994; Selwood, 2000). Ultrastructural data on the mucoid coat and ampullary and isthmic epithelium in *Sminthopsis crassicaudata* has suggested that some variation may exist between the mucoid secretions of the different regions of the oviduct (Roberts & Breed, 1996*b*), although this has not been shown biochemically. Recently, in the brushtail possum, the secretions of the oviduct have also been shown to associate with spermatozoa and induce capacitation-like changes in their morphology (Sidhu *et al.*, 1998, 1999), a pre-requisite for achieving sperm-ZP binding and penetration in vitro (Mate *et al.*, 2000). This chapter, therefore, used the brushtail possum to investigate the following questions: 1) which glycoconjugates are present within the oviduct epithelium around the time of ovulation? And 2) is there any variation in the glycoconjugates secreted by the epithelium of the ampulla and isthmus of the oviduct?

5.2 Materials and Methods

5.2.1 Animals

Female brushtail possums (*Trichosurus vulpecula*) were obtained and housed as previously described in Section 2.1.1.

5.2.2 Superovulation and Collection of Oviducts

Female possums were primed to superovulate as previously described in section 2.2.

For the determination of the glycoconjugate content of the oviductal epithelium, oviducts were collected from superovulated possums as described in section 2.3. Briefly, reproductive tracts were removed and placed into pre-warmed sterile Tyrode solution in culture dishes. Under a dissecting microscope, excess peritoneal tissue containing blood vessels were removed and the oviduct straightened so that the infundibulum, the ovarian segment (ampulla), and the isthmus could be clearly delineated (Figure 5.1). Following retrograde flushing with Tyrode solution to recover superovulated eggs, two to four sections, measuring ~1mm in length, were taken of the ampulla (closest to the infundibulum) and isthmus from each reproductive tract and immediately placed either into Rossman's fluid for lectin histochemistry or 2% glutaraldehyde made up in 0.1M cacodylate buffer pH 7.4 for high resolution lectin-gold immunocytochemistry and processed as described below.

5.2.3 Determination of the Glycoconjugate Composition of the Oviductal Epithelium by Lectin Histochemistry and High Resolution Lectin-gold Immunocytochemistry

5.2.3.1 Lectin Histochemistry of the Oviductal Epithelium

Lectin histochemistry was used to determine the glycoconjugate composition of the secretory granules and glycocalyx of the oviductal epithelium. Rossman's fluid-fixed pieces of ampulla and isthmus were processed, embedded into paraffin wax (see section 2.3), sections were cut

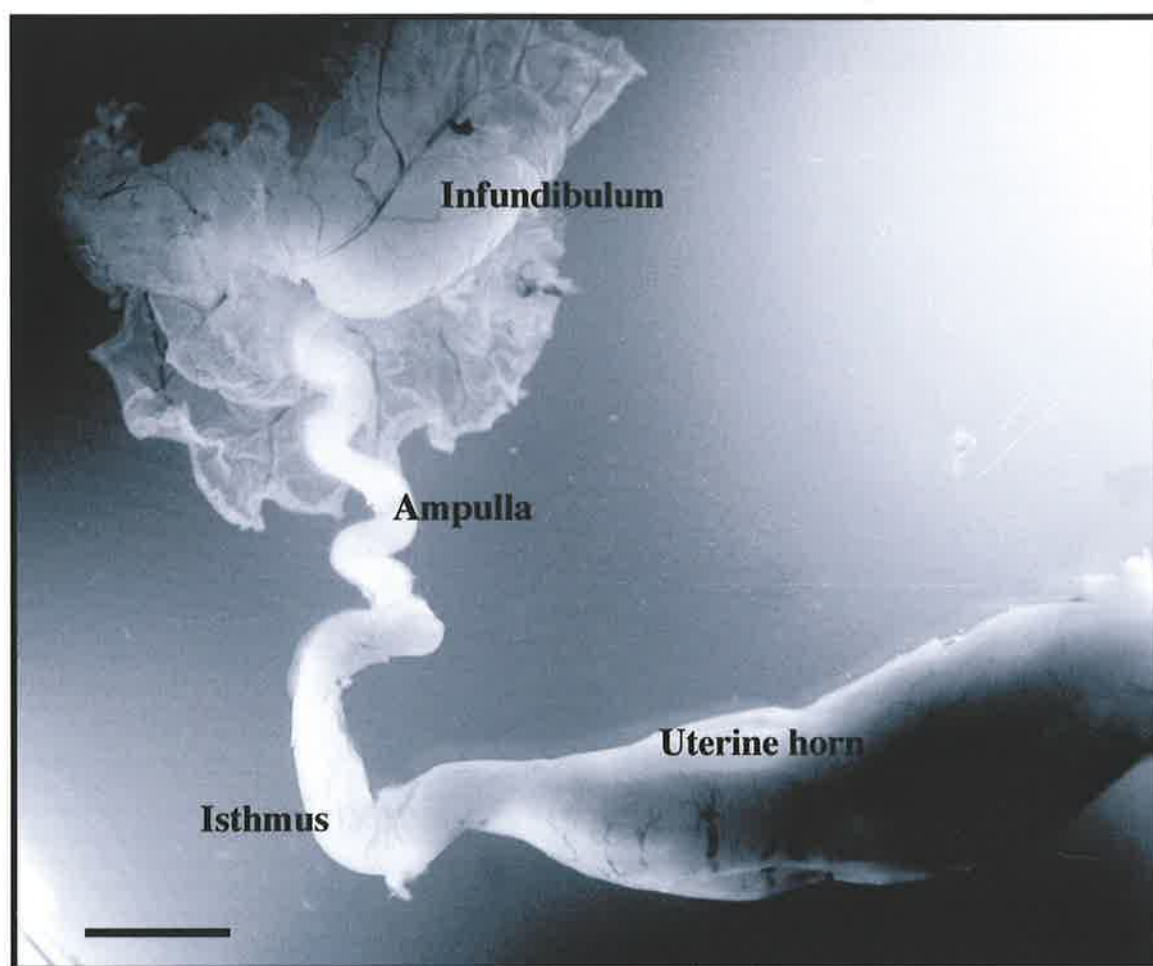


Figure 5.1 Gross anatomy of the upper reproductive tract of the brushtail possum *Trichosurus vulpecula* following the removal of the surrounding peritoneum, highlighting the major divisions of the oviduct, the infundibulum, the ampulla and the isthmus. Bar = 500mm.

and placed onto slides as previously described (see section 2.4). The sections were de-waxed in HistoClear, rehydrated through a decreasing series of alcohols (3 x 100%, 70% and 50%) and brought to 0.05M Tris buffered saline (TBS), pH 7.6. They were then covered in 1% BSA made up in TBS for 30 min at room temperature and incubated in one of nine fluorescein isothiocyanate (FITC-)-conjugated lectins (Vector Laboratories, Burlingame, CA). For lectins and their sugar specificity, see Table 5.1. As mentioned in Chapter 4, the intensity of lectin staining was determined qualitatively using a scoring system adapted from light microscopical observations, with a positive result indicated by (+) and a negative result by (-), and the intensity of fluorescence being defined as mild (+), strong (++) or intense (+++). Exposure times for photography of fluorescence results were kept identical at 51.2 s and photographed on T-Max 400 ASA black and white film. Lectin histochemistry using each lectin was performed on the oviducts at least 3 times, on three individuals.

5.2.3.2 Lectin Immunocytochemistry of Oviductal Epithelium

In order to determine, localise and quantify the glycoconjugates of the oviductal epithelium at high-resolution, lectin-gold immunocytochemistry was undertaken. Samples of ampullary and isthmus oviductal epithelium were fixed in 2% glutaraldehyde made up in 0.1M cacodylate buffer, pH 7.4 and processed into LR Gold. Semi-thick (~1µm) survey sections were cut on a Reichert-Jung Ultracut ultramicrotome with a glass knife and stained with 0.025% Toluidine Blue made up in 0.5% sodium tetraborate (Borax). Once appropriate sections were found, excess resin was trimmed away, thin (70-90nm) sections were cut, placed onto nickel grids coated with a supporting film of 2% Collodion in Amyl Acetate (Electron Microscopy Sciences, Fort Washington, PA) and allowed to air dry for 24h.

Table 5.1. Lectins used and their corresponding saccharide specificities

Origin of lectin	Common name	Acronym	Sugar specificity	Sugar inhibitor
<i>Arachis hypogea</i>	Peanut	PNA	β Gal-(1-3)-GalNAc	Lactose
<i>Erythrina cristagalli</i>	Coral Tree	ECA	β Gal-(1-4)-GlcNAc	D-GlcNAc
<i>Concanavalina ensiformis</i>	Jack bean	Con A	α -D-Man, α -D-Glc	D-Man, D-Glc
<i>Lens culinaris</i>	Lentil	LCA	α -D-Man, α -D-Glc	D-Man, D-Glc
<i>Ricinus communis I</i>	Castor bean	RCA-I	β -D-Gal	Lactose
<i>Triticum vulgaris</i>	Wheat germ	WGA	$[\beta$ -(1-4)-D-GlcNAc] ₂ , NeuNAc	D-GlcNAc*, NeuNAc
<i>Sambucus nigra</i>	Elderberry	SNA	α -NeuNAc-(2-6)-Gal/GalNAc	NeuNAc
<i>Glycine max</i>	Soybean	SBA	α -D-GalNAc, α -D-Gal	α -D-GalNAc
<i>Lotus tetragonolobus</i>	Winged pea	LTA	α -L-Fuc	α -L-Fuc

* Sugar inhibitor used in this study

Fuc: fucose; Gal: galactose; GalNAc: *N*-acetylgalactosamine; Glc: glucose; GlcNAc: *N*-acetylglucosamine; Man: mannose; NeuNAc: *N*-acetylneuraminic acid (sialic acid) (from Wu *et al.*, 1988).

Lectin-gold immunocytochemistry was then performed following the method described in Chapman *et al.* (1994). Briefly, the sections had non-specific labelling blocked with 1% BSA, 1% gelatin and 0.2M glycine before being washed in 0.5M TBS containing 1mM CaCl and 0.5% Tween 20 (washing buffer, WB) and incubated in one of four biotinylated lectins (Vector Laboratories, Burlingame, CA). Following incubation in the lectin, sections were washed in WB and placed onto a drop of 10nm colloidal gold-conjugated goat anti-biotin antibody (diluted 1:30 in 0.5M TBS) for 1 hour. Grids were then washed in WB again, counter-stained in 70% alcoholic uranyl acetate and viewed on a Philips CM100 TEM with digital images captured on a Mega-View II digital image capture system (Soft Imaging System, GmbH, Germany). Gold particles were counted and quantitated using the analySIS 2.1-image analysis software (Soft Imaging System, GmbH, Germany).

The biotinylated lectins used in this part of the study were: (1) Garden Pea lectin (*Pisum sativum*: PSA) for α -D-Mannose, (2) Castor bean lectin (*Ricinus communis* I: RCA-I) for β -D-galactose, 3) Wheat germ agglutinin (*Triticum vulgaris*: WGA) for $(\beta$ -(1-4)-D-*N*-acetylglucosamine)₂, and *N*-acetylneuraminic acid (sialic acid), and 4) Soybean lectin (*Glycine max*: SBA) for α -D-*N*-acetylgalactosamine/ α -D-galactose. The lectins were used at a concentration of 20 μ g/ml, except for WGA that was used at a concentration of 10 μ g/ml. For specificity control, the biotinylated lectins were incubated with 0.4-1.0M/L of their specific sugar made up in distilled water for 30 min prior to the application of the lectins to the grids. Lectin immunocytochemistry was performed at least twice for each lectin, on oviducts of three individuals.

Statistical analyses, specifically the student's t-test, were performed on gold particle counts of the same lectin between the two regions of the oviduct (i.e. ampulla versus isthmus) using Systat statistical analysis software (Systat Software Inc., California) and differences were considered statistically significant where $p < 0.05$.

5.3 Results

5.3.1 Glycoconjugates of the Oviductal Epithelium at Ovulation

5.3.1.1 Lectin histochemistry

The ampullary and isthmus regions of the oviduct of peri-ovulatory possums were investigated for glycoconjugate content using fluorescence lectin histochemistry. The intensity of

fluorescence of nine lectins within the secretory and ciliated cells of the epithelium of the ampulla and isthmus of brushtail possums is summarised in Table 5.2.

Arachis hypogea (peanut) lectin (PNA)

The fluorescence of the ampullary and isthmic regions of the possum oviduct were similar in intensity. In both the ampulla and isthmus, the secretory cells were stained strongly with PNA, with fluorescence localised to granular material within the cells. The ciliated cells in the ampulla and isthmus demonstrated an intense binding, particularly to the external surface of the epithelium (Fig. 5.2).

Erythrina cristagalli lectin (ECA)

Mild fluorescence with ECA was found on the secretory cells of the ampulla and isthmus, while strong fluorescence was localised on the ciliated cells of both regions, with some isthmic ciliated cells staining throughout the entire cytoplasm (Fig. 5.3).

Concanavalia ensiformis lectin (Con A) and Lens culinaris lectin (LCA)

Mild fluorescence with Con A and LCA were noted on both the secretory and ciliated cells, in both the ampulla and isthmus.

Ricinus communis I lectin (RCA-I)

Intense fluorescence with RCA-I was found on the secretory cells of the ampulla and isthmus, with much of the staining localised to the apical, protruding portion of the cells. Strong fluorescence was also localised to the external surface of the ciliated cells of both the ampullary and isthmic regions.

Figure 5.2 Ampullary region of oviduct of a peri-ovulatory brushtail possum. Phase contrast (2a) and fluorescent (2b) micrographs of the ampulla stained with fluorescein isothiocyanate-*Arachis hypogea* (peanut) lectin (FITC-PNA). Note intense fluorescence on the ciliated cells of the ampullary epithelium (E) and strong staining of the non-ciliated secretory cells; lumen (L); lamina propria (LP). Bar = 45µm.

Figure 5.3 Isthmic region of oviduct of a peri-ovulatory brushtail possum. Phase contrast (3a) and fluorescent (3b) micrographs of the isthmus stained with fluorescein isothiocyanate-*Erythrina cristagalli* (coral tree) lectin (FITC-ECA). Note strong labelling of the ciliated cells of the epithelium (E) of the isthmus, with some ciliated cells labelled throughout their entire cytoplasm (arrowheads); lumen (L); lamina propria (LP). PNA binding is specific for β -Galactose (1-3) *N*-acetylgalactosamine. Bar = 45µm.

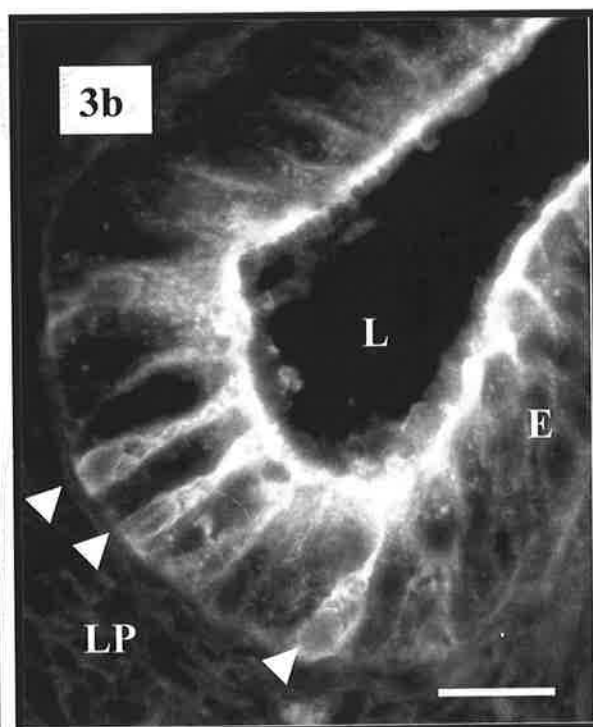
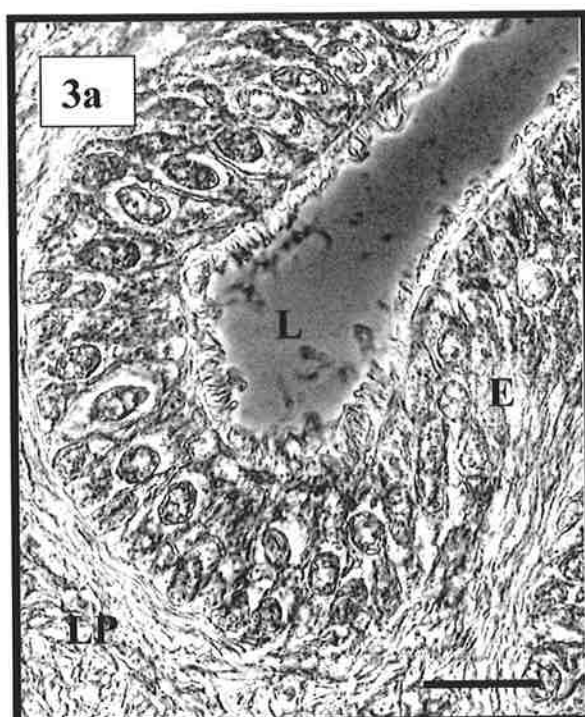
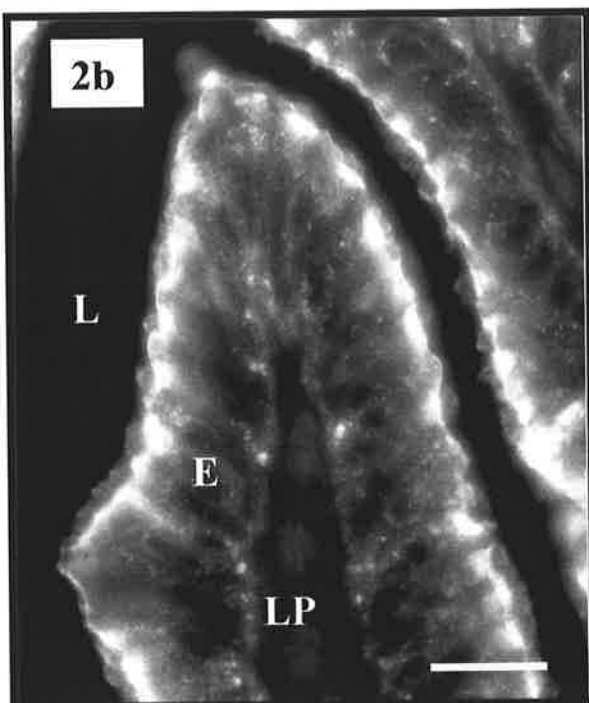
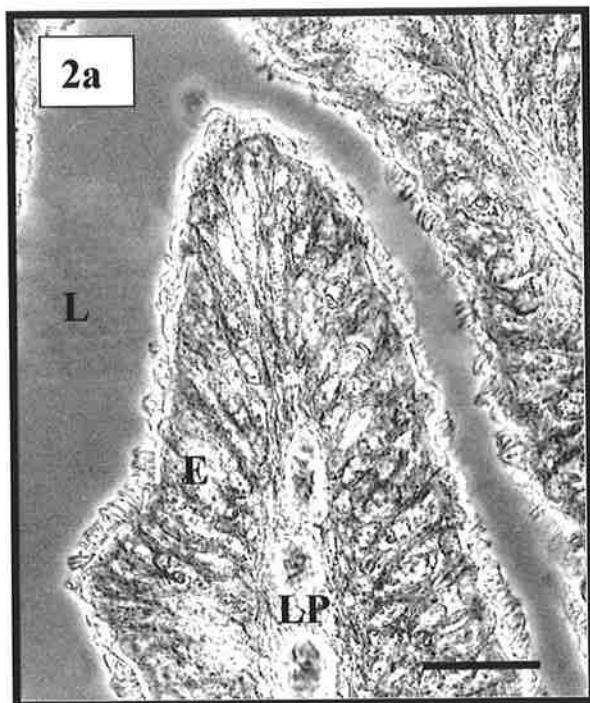


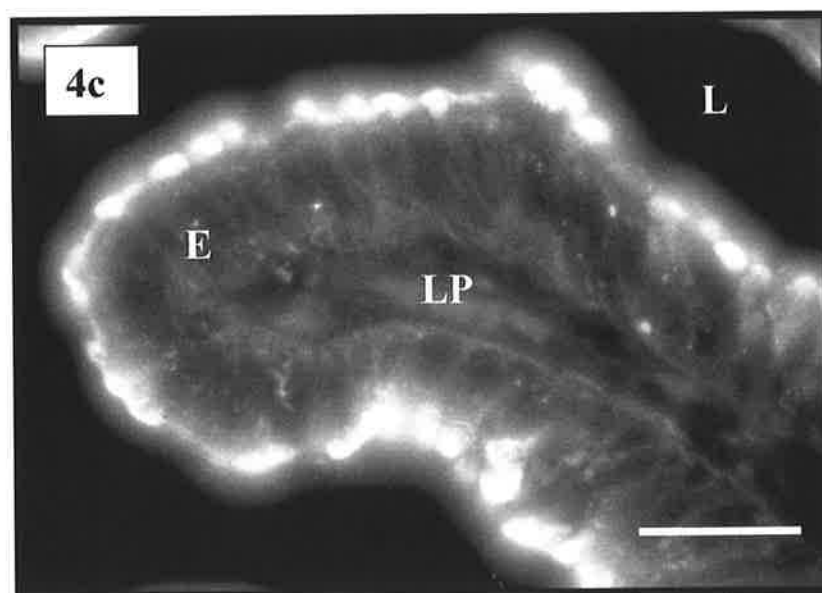
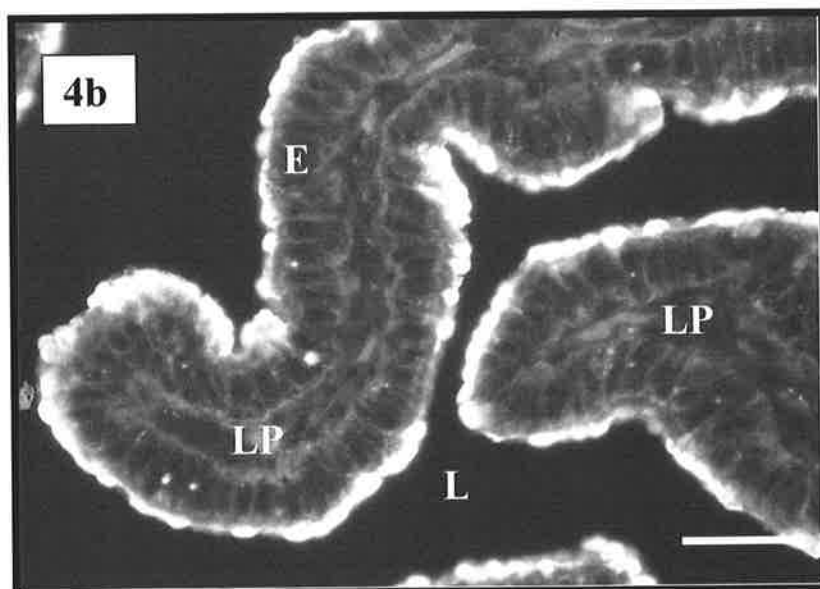
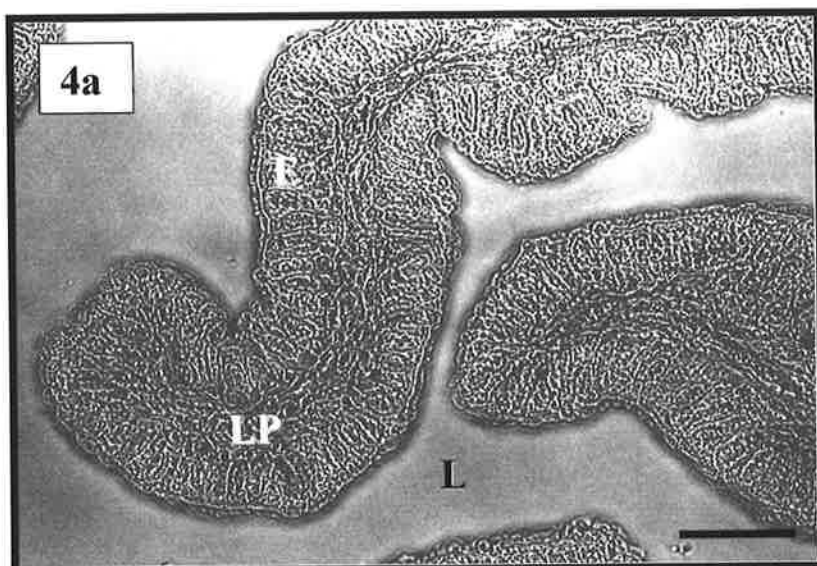
Table 5.2. Summary results of the intensity of lectin binding to the oviductal epithelial cells of the peri-ovulatory brushtail possum. Fluorescence was qualitatively scored as - negative; + mild; ++ strong; +++ intense. (*PNA* peanut agglutinin; *ECA* *Erythrina cristagalli* agglutinin; *Con A* *Concanavalia ensiformis* agglutinin; *LCA* *Lens culinaris* agglutinin; *RCA-I* *Ricinus communis* -I agglutinin; *WGA* wheat germ agglutinin; *SNA* *Sambucus nigra* agglutinin; *SBA* soybean agglutinin; *LTA* *Lotus tetragonolobus* agglutinin).

Lectin	Ampulla		Isthmus	
	Secretory cell	Ciliated cell	Secretory cell	Ciliated cell
PNA	++	+++	++	+++
ECA	+	++	+	++
Con A	+	+	+	+
LCA	+	+	+	+
RCA-I	+++	++	+++	++
WGA	+++	+	+++	+
SNA	++	-	++	-
SBA	++	-	-	-
LTA	-	++	-	++

Triticum vulgaris (wheat germ) lectin (WGA)

Staining with WGA produced intense fluorescence to the secretory cells of both the ampulla and isthmus. While staining was particularly localised to the apical, protruding portion of the secretory cells, fluorescence was also found throughout most of the cytoplasm (Fig. 5.4). Mild fluorescence with WGA was found on the ciliated cells that were localised to the surface in both the ampulla and isthmus.

Figure 5.4 Ampullary epithelium of a peri-ovulatory oviduct of a brushtail possum. Phase contrast (4a) and fluorescent (4b,c) micrographs of the ampulla stained with fluorescein isothiocyanate-*Triticum vulgaris* (wheatgerm) lectin (FITC-WGA). Note the non-ciliated secretory cells of the ampullary epithelium (E) are stained intensely with FITC-WGA, while the ciliated cells are stained only mildly. At higher magnification (4c), intense labelling with FITC-WGA is evident on the protruding apical portion of the non-ciliated secretory cells; lumen (L); lamina propria (LP). WGA binding is specific for β (1-4) *N*-acetylglucosamine, *N*-acetylneuraminic acid (sialic acid). Bar = 100 μ m (4a,b) and 50 μ m (4c).



Sambucus nigra lectin (SNA)

Strong staining with SNA was found on the secretory cells of both the ampullary and isthmic epithelia. Again, much of this fluorescence was localised to the apical surface of the secretory cells, but was also evident near the basement membrane. Little to no fluorescence was found on the ciliated cells of either the ampulla or isthmus using this lectin.

Glycine max (soybean) lectin (SBA)

Strong fluorescence with SBA was only found on the secretory cells of the ampulla, and only the very apical portions of the secretory cells were labelled (Fig. 5.5). Neither the secretory cells of the isthmus nor the ciliated cells of either the ampulla or isthmus were stained using SBA (Fig. 5.6).

Lotus tetragonolobus lectin (LTA)

Staining with LTA was only found on the ciliated cells at strong levels in the ampulla and isthmus (Fig. 5.7). No staining was found on any secretory cells in either the ampullary or isthmic epithelia.

Controls

Incubation of the lectins with their inhibitory sugar before incubation with the sections resulted in elimination of fluorescence in the oviductal epithelium, except for the lectin WGA, which still demonstrated mild fluorescence after incubation with D-GlcNAc, likely due to the secondary specificity of WGA for *N*-acetylneuraminic acid (sialic acid).

Figure 5.5 Ampulla of the peri-ovulatory oviduct of a brushtail possum. Fluorescent micrograph of the ampulla stained with fluorescein isothiocyanate-*Glycine max* (soybean) lectin (FITC-SBA). Note the apical regions of some of the non-ciliated secretory cells (arrowheads) of the ampullary epithelium (E) are stained strongly with FITC-SBA; lumen (L); lamina propria (LP). SBA binding is specific for α -D-*N*-acetylgalactosamine/ α -D-galactose. Bar = 75 μ m.

Figure 5.6 Isthmus of the peri-ovulatory oviduct of a brushtail possum. Fluorescent micrograph of the isthmus stained with fluorescein isothiocyanate-*Glycine max* (soybean) lectin (FITC-SBA). Note lack of staining with FITC-SBA of the epithelium (E), with only autofluorescence noted on the red blood cells within the lamina propria (LP); lumen (L). SBA binding is specific for α -D-*N*-acetylgalactosamine/ α -D-galactose. Bar = 75 μ m.

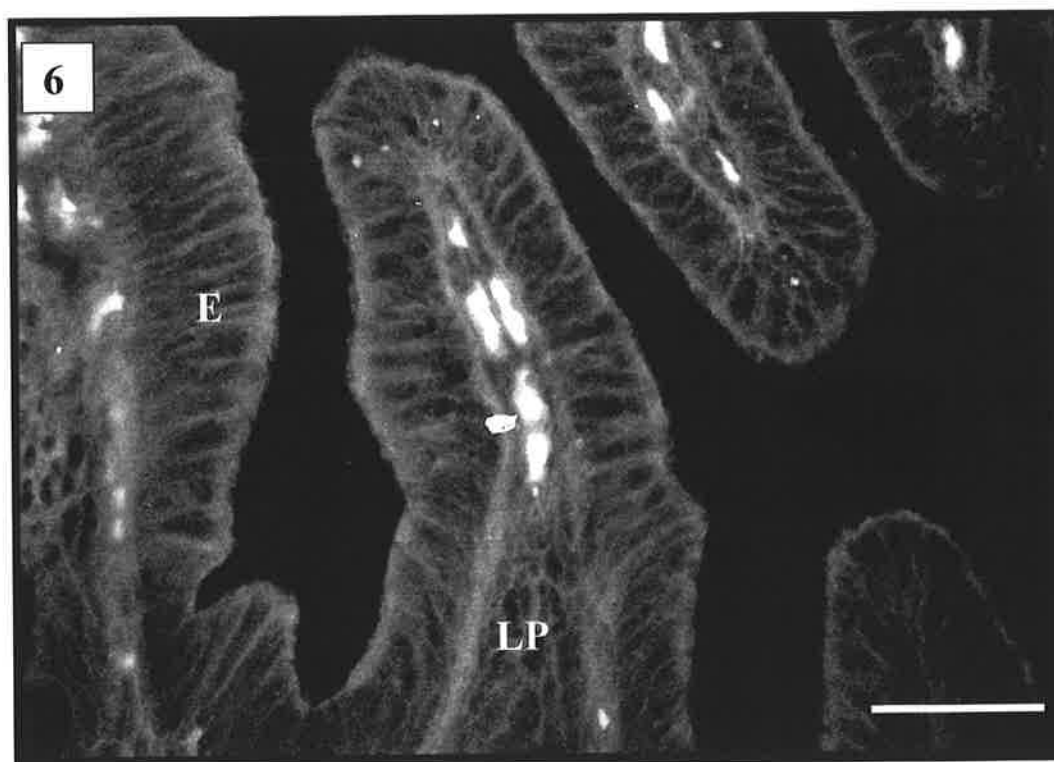
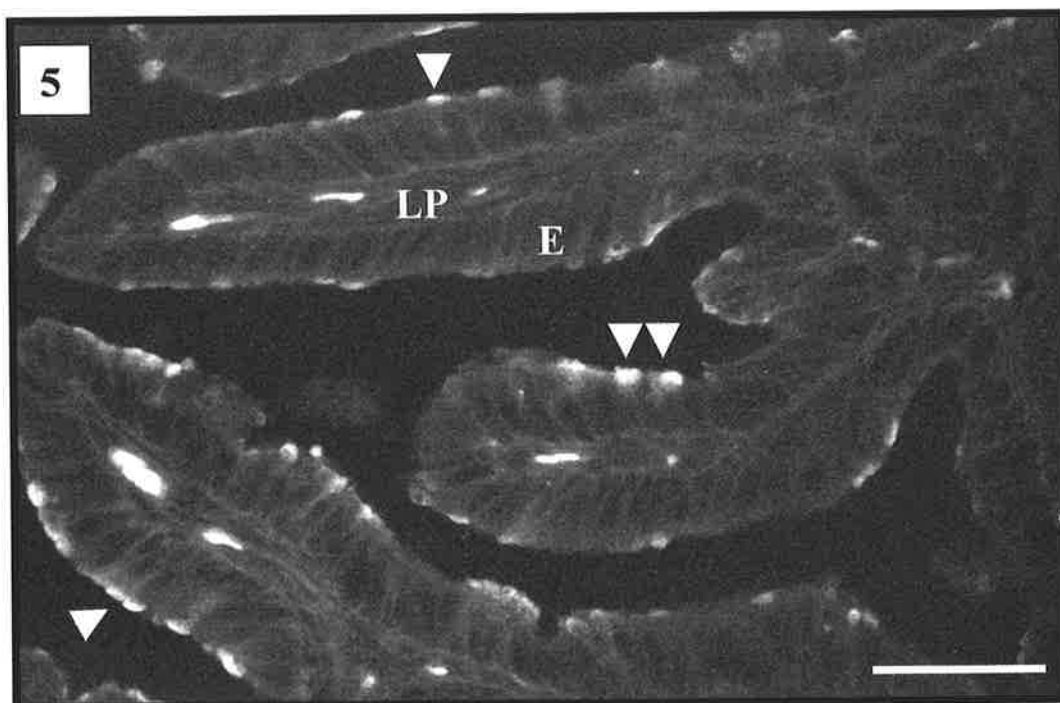
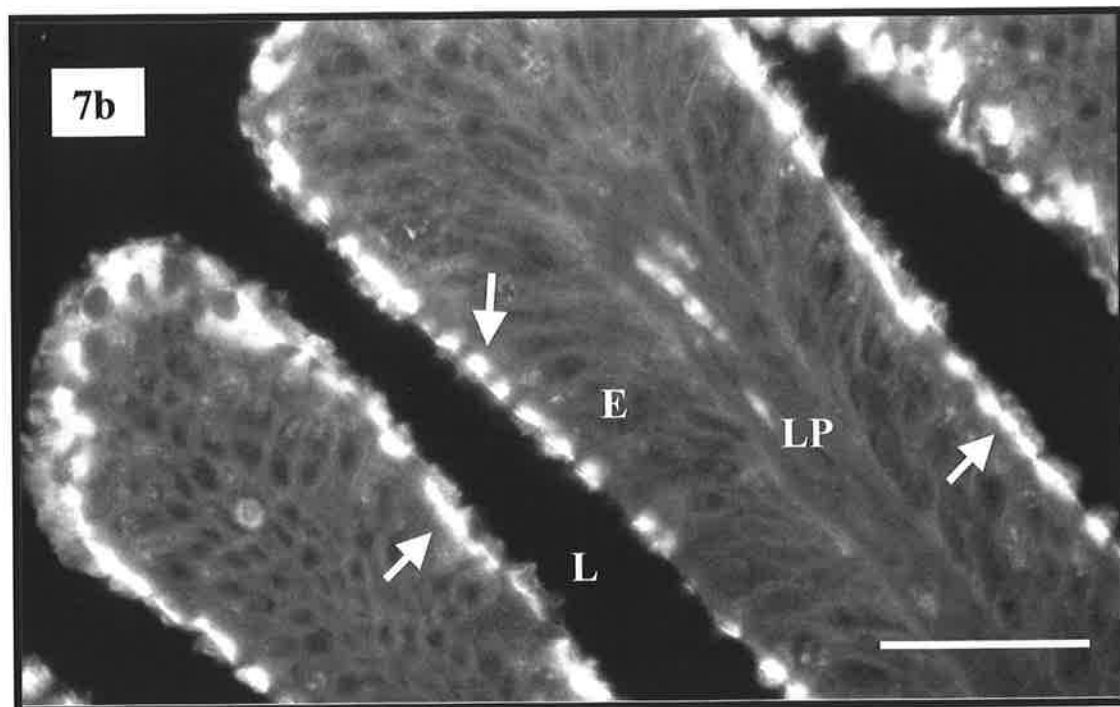
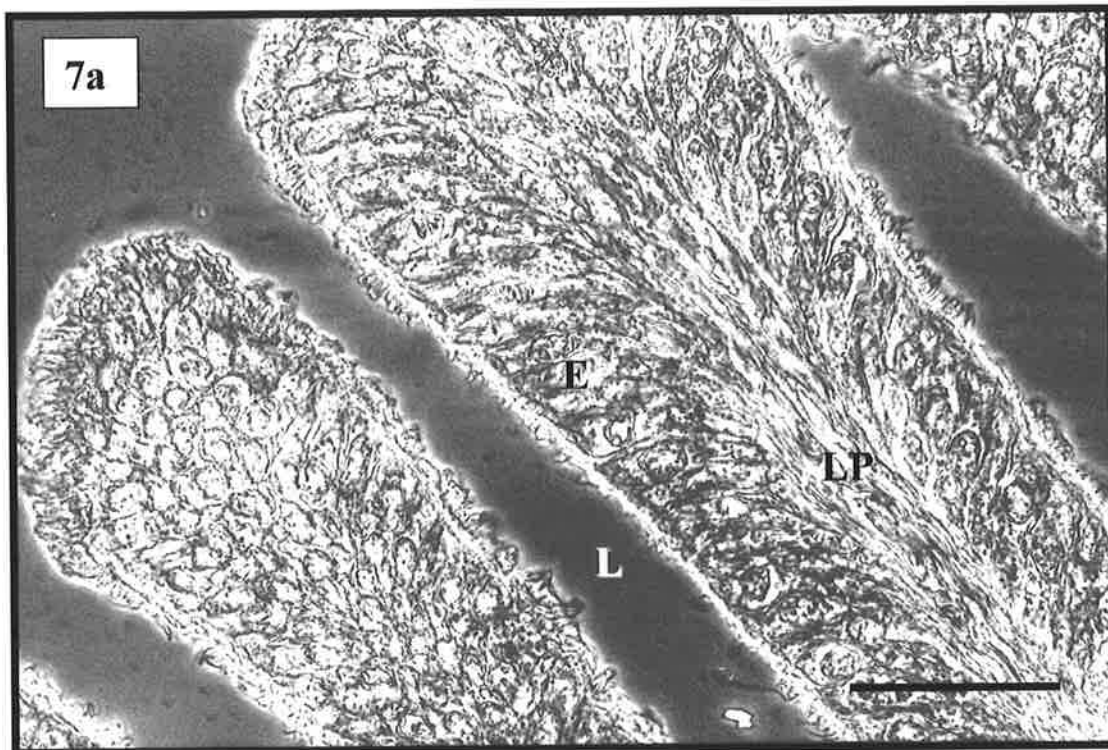


Figure 5.7 Isthmus of the oviduct of a peri-ovulatory brushtail possum. Phase contrast (7a) and fluorescent (7b) micrographs of the isthmus stained with fluorescein isothiocyanate-*Lotus tetragonolobus* (winged pea) lectin (FITC-LTA). Note the strong staining of the ciliated cells of the isthmus epithelium (E), which is particularly localised to the region of the basal bodies (arrows) at the apex of the cells; lumen (L); lamina propria (LP). LTA binding is specific for α -L-Fucose. Bar = 50 μ m.



5.3.2 Regional Variation in Secretory Glycoconjugates within the Oviduct

5.3.2.1 Lectin immunocytochemistry

Lectin immunocytochemistry was performed on the oviductal epithelium of peri-ovulatory possums in order to determine and localize which glycoconjugates were present in secretions of the ampulla and isthmus around the time of ovulation. The densities of gold labelling of secretory granules of the ampullary and isthmic secretory cells were determined for each of the four lectins and are summarised in Table 5.3.

Pisum sativum lectin (PSA)

Ultrastructurally, the secretory cells of the ampullary epithelium were comprised of columnar cells that had numerous microvilli on their luminal surface and a number of electron dense secretory granules within the cytoplasm (Fig. 5.8a). Lectin-gold labelling, best seen with less contrast (Fig. 5.8b), was localised primarily to the secretory granules and the surface microvilli. The epithelium of the isthmus appeared ultrastructurally similar to the ampulla, although lectin gold labelling with PSA was of a significantly lower density ($p < 0.001$) (Fig. 5.9a,b; Table 5.3).

Ricinus communis I lectin (RCA-I)

Lectin-gold labelling intensities for β -D-Galactose, as demonstrated with RCA-I agglutinin, were similar for the secretory cells of the epithelium of the ampulla and isthmus. In these sections, however, there appeared to be variation in some of the secretory granules with a population of electron-light and electron-dense granules within the same cell. Qualitatively,

Figure 5.8 Lectin immunocytochemistry of ampulla of oviduct of a peri-ovulatory brushtail possum. Electron micrographs of ampullary sections incubated with biotinylated-*Pisum sativum* (garden pea) lectin (biotin-PSA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Gold labelling (best seen with less contrast, 8b) was localised to the secretory granules (SG) and microvilli (M) of the non-ciliated secretory cells of the ampullary epithelium; cell junction (CJ). PSA binding is specific for α -D-Mannose. Bar = 1 μ m.

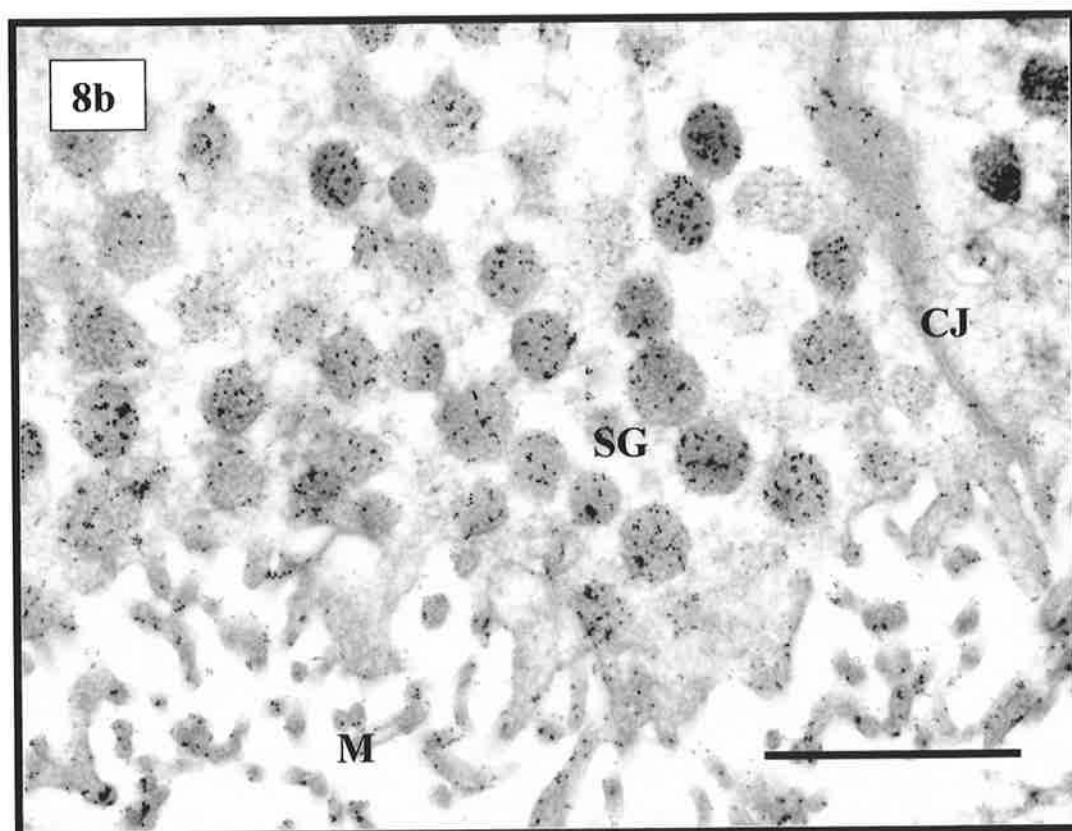
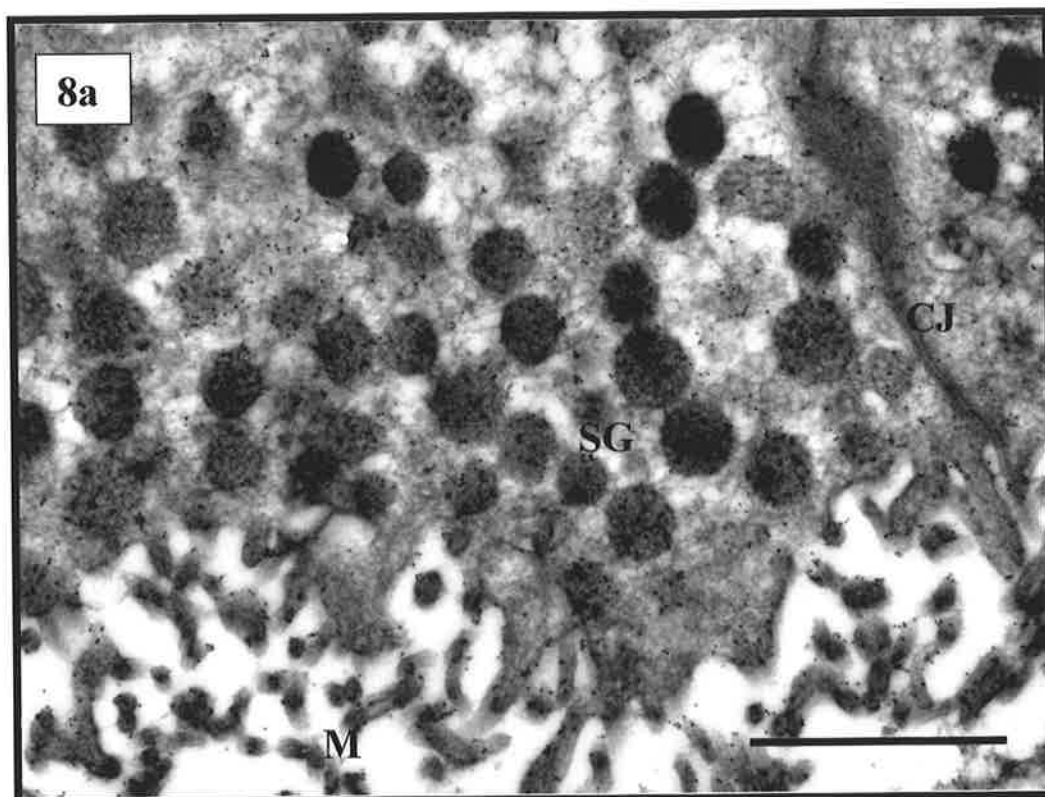


Figure 5.9 Lectin immunocytochemistry of isthmus of oviduct of a peri-ovulatory brushtail possum. Electron micrographs of isthmic sections incubated with biotinylated-*Pisum sativum* (garden pea) lectin (biotin-PSA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Compared with Fig. 5.8, gold labelling (best seen with less contrast, 9b) was found in lower density on the secretory granules (SG) of the non-ciliated secretory cells of the isthmic epithelium than that found in the ampulla; lumen (L); microvilli (M). PSA binding is specific for α -D-Mannose. Bar = 1 μ m.

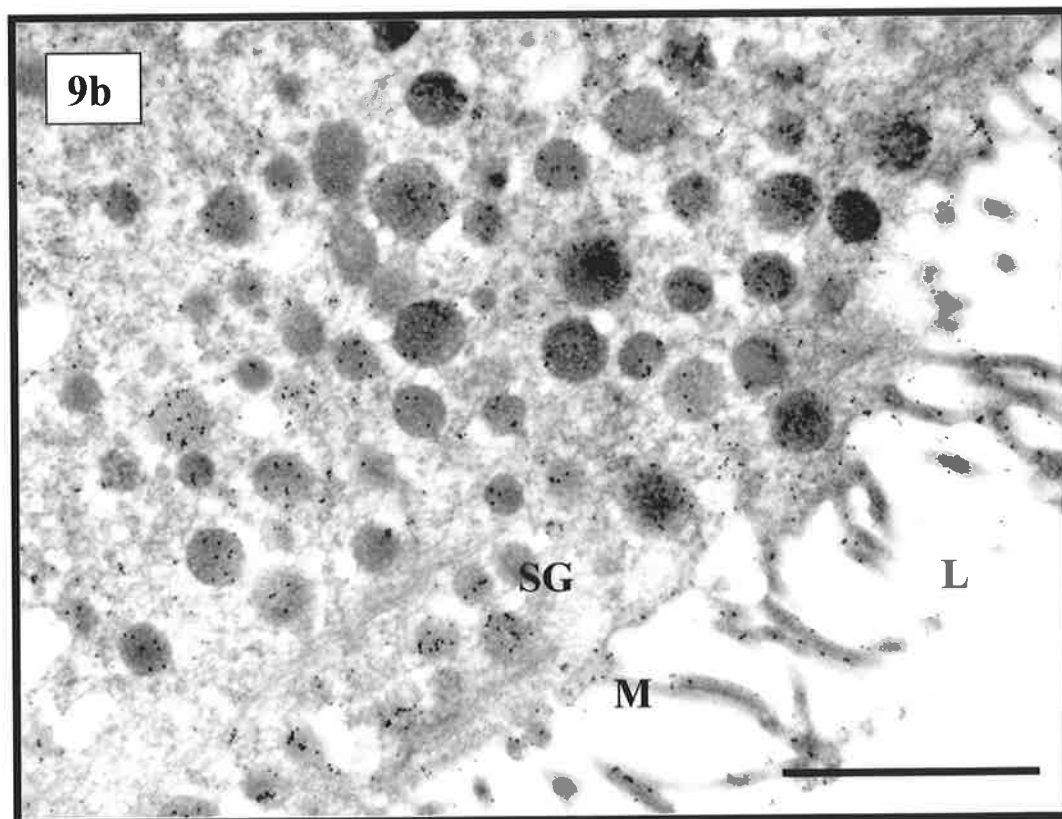
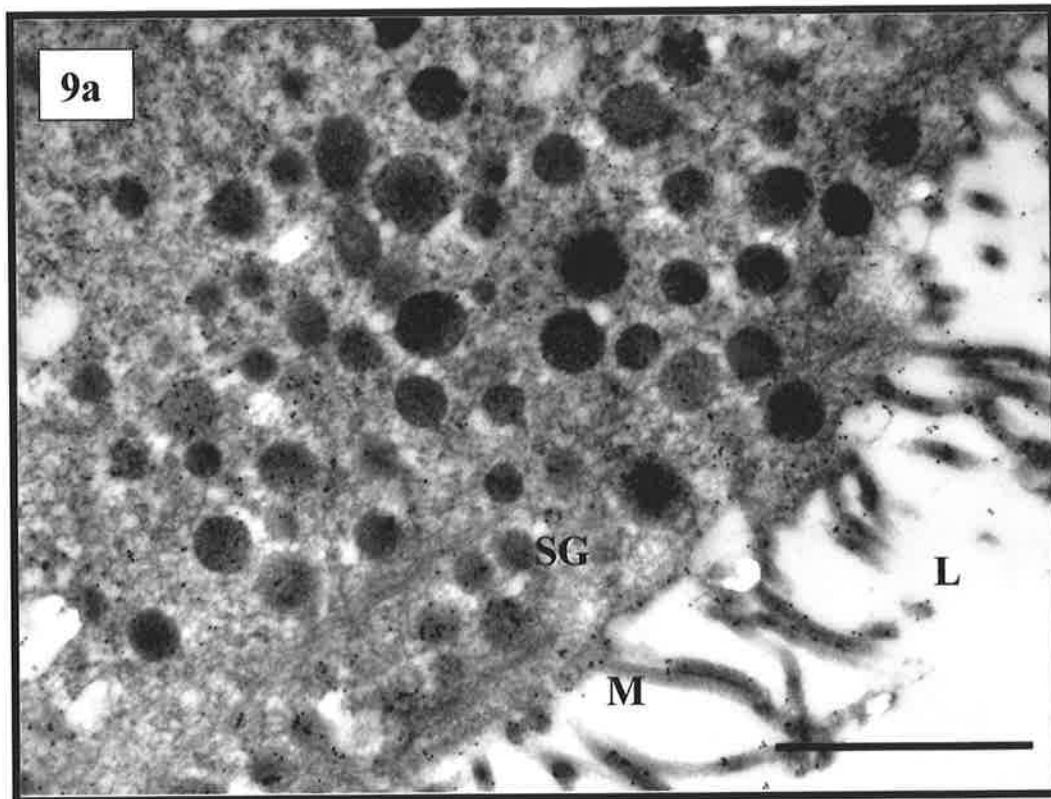


Table 5.3. Density of gold labelling of secretory granules of the ampullary and isthmus regions of the peri-ovulatory oviduct of the brushtail possum following incubation with four lectins. The labelling intensities are shown as the number of gold particles per secretory granule; mean values $\pm$ SEM. (*PSA* *Pisum sativum* agglutinin; *RCA-I* *Ricinus communis-I* agglutinin; *WGA* wheat germ agglutinin; *SBA* soybean agglutinin).

Lectin	Ampulla	Isthmus
PSA	21.79 $\pm$ 1.11	9.92 $\pm$ 0.59 ^a
RCA-I	51.70 $\pm$ 3.12	55.02 $\pm$ 3.93
WGA	23.43 $\pm$ 2.69	39.58 $\pm$ 3.47 ^b
SBA	95.28 $\pm$ 8.03	0.7 $\pm$ 0.22 ^a

^aThe labelling densities of secretory granules using these lectins was highly significantly less ($p < 0.001$) in the isthmus non-ciliated secretory cells than in the ampullary non-ciliated secretory cells

^bThe labelling densities of secretory granules using this lectin was highly significantly less ($p < 0.001$) in the ampullary non-ciliated secretory cells than in the isthmus non-ciliated secretory cells.

lectin-gold labelling of the varying populations of secretory granules appeared to show a higher density of labelling of the electron-dense granules.

Triticum vulgaris (wheat germ) lectin (*WGA*)

Significantly lower levels of gold-labelling were found on the secretory granules of ampullary secretory cells than on those of the isthmus ($p < 0.001$). *WGA* labelling was especially localised to the secretory granules of the isthmus and the microvilli of the epithelium (Fig. 5.10a,b).

Glycine max (soybean) lectin (*SBA*)

High densities of lectin-gold labelling were present on the secretory granules and microvilli of the ampullary epithelium (Fig. 5.11a,b). Virtually no labelling was detected on ciliated cells of

Figure 5.10 Lectin immunocytochemistry of isthmus of oviduct of a peri-ovulatory brushtail possum. Electron micrographs of isthmic sections incubated with biotinylated-*Triticum vulgaris* (wheatgerm) lectin (biotin-WGA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Gold labelling (best seen with less contrast, 10b) was localised to the secretory granules (SG) of the non-ciliated secretory cells of the isthmus in greater density than that found in the ampulla; lumen (L); microvilli (M). WGA binding is specific for β (1-4) *N*-acetylglucosamine, *N*-acetylneuraminic acid (sialic acid). Bar = 1 μ m.

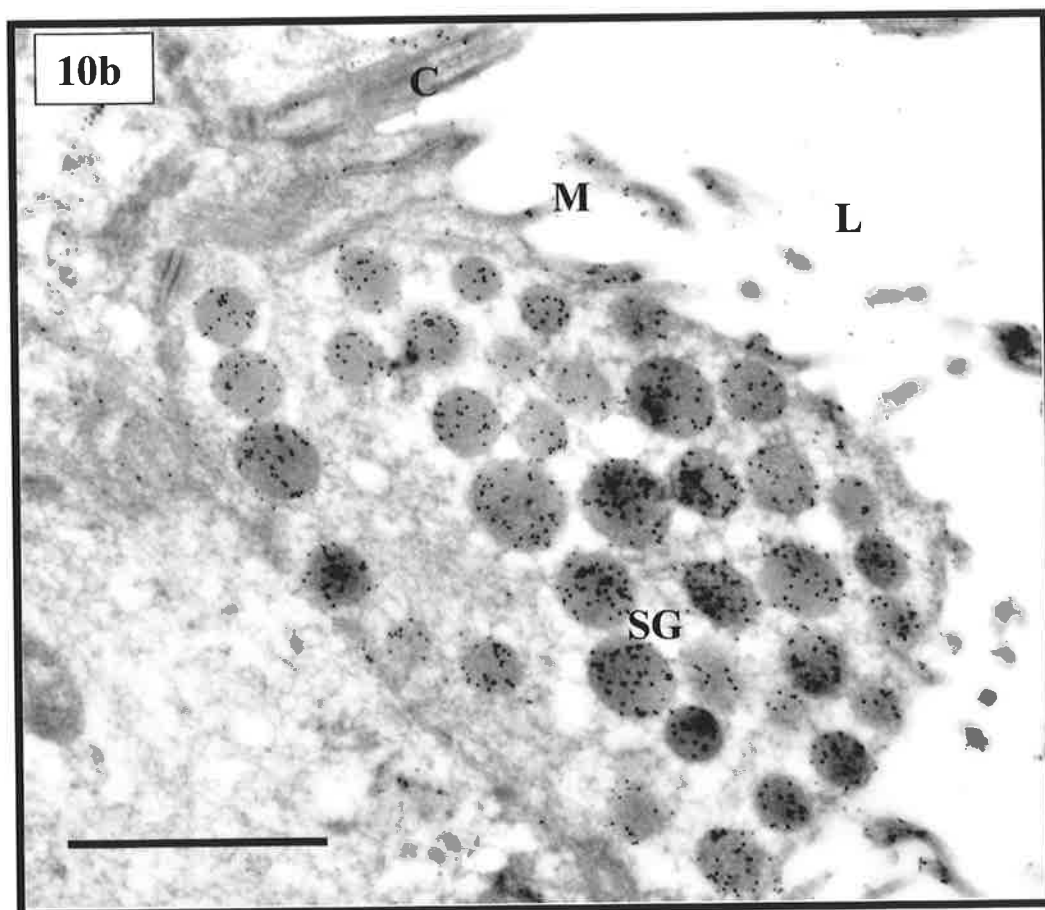
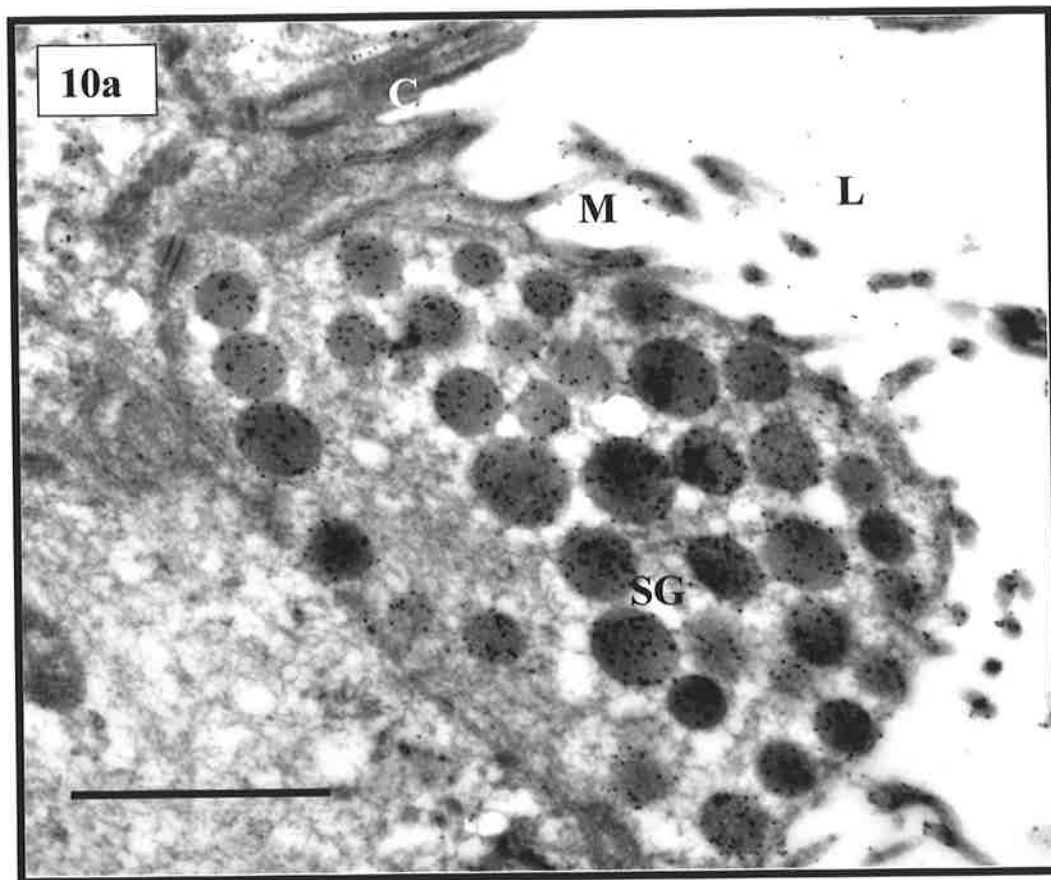
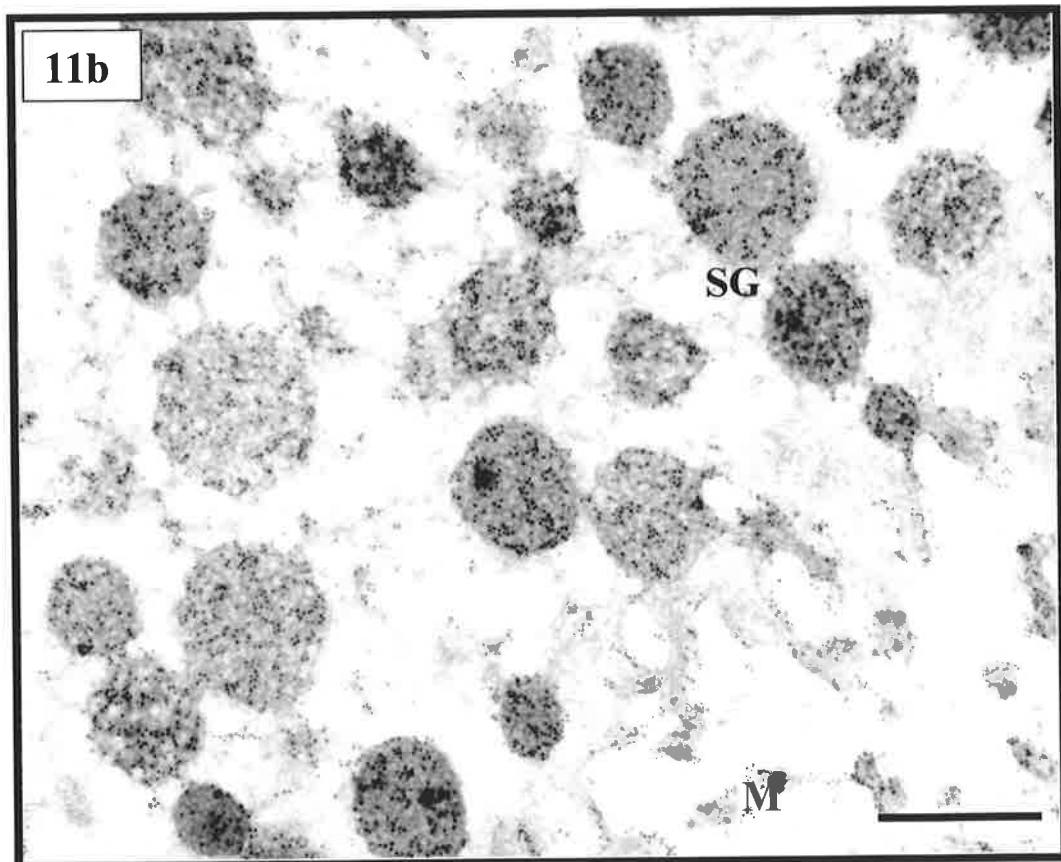
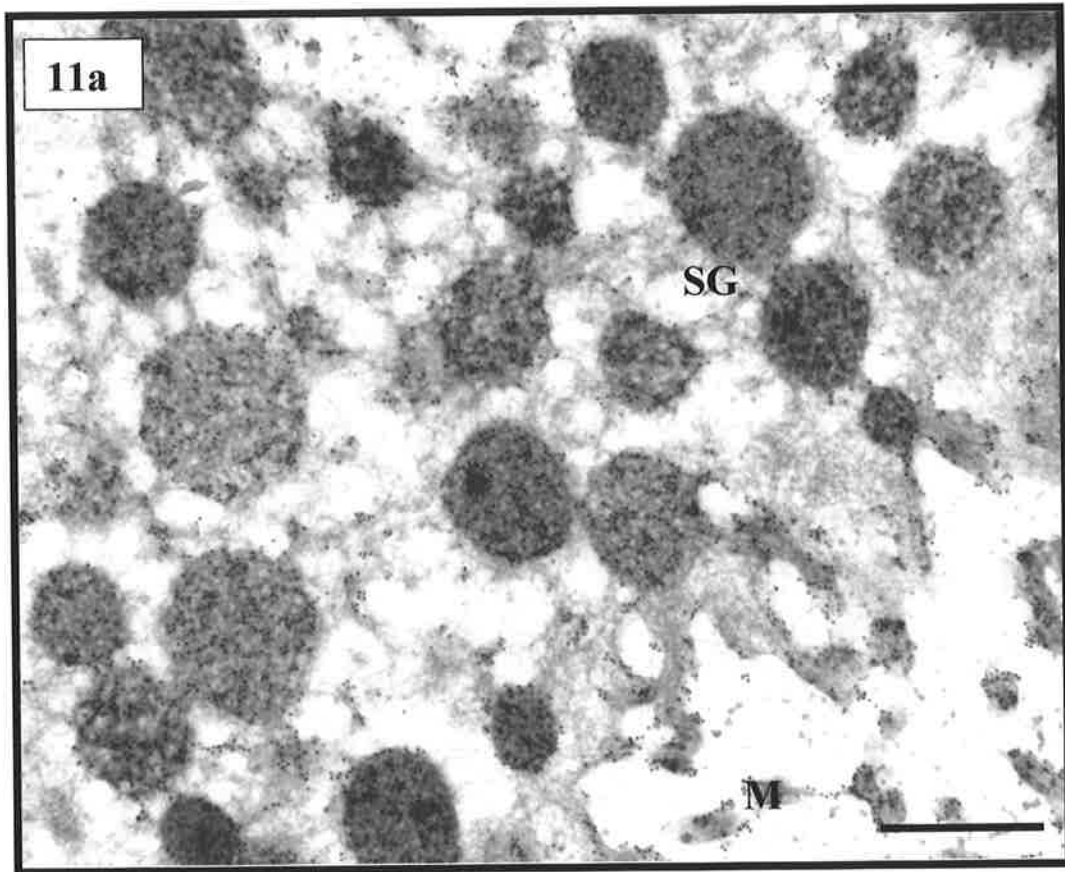


Figure 5.11 Lectin immunocytochemistry of ampulla of oviduct of a peri-ovulatory brushtail possum. Electron micrographs of ampullary sections incubated with biotinylated-*Glycine max* (soybean) lectin (biotin-SBA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Gold labelling (best seen with less contrast, 11b) was of relatively high density on the secretory granules (SG) and microvilli (M) of the non-ciliated secretory cells of the ampulla; lumen (L). SBA binding is specific for α -D-N-acetylgalactosamine/ α -D-galactose. Bar = 500nm.



the ampulla and isthmus with the exception of material on the external cilia themselves, while practically no labelling was present on the secretory cells of the isthmus, significantly less than in the ampulla ($p < 0.001$) (Fig. 5.12a,b).

Controls

Gold labelling was reduced to practically nil following incubation of the biotinylated lectins with their appropriate inhibiting sugars prior to their application to the sections (data not shown).

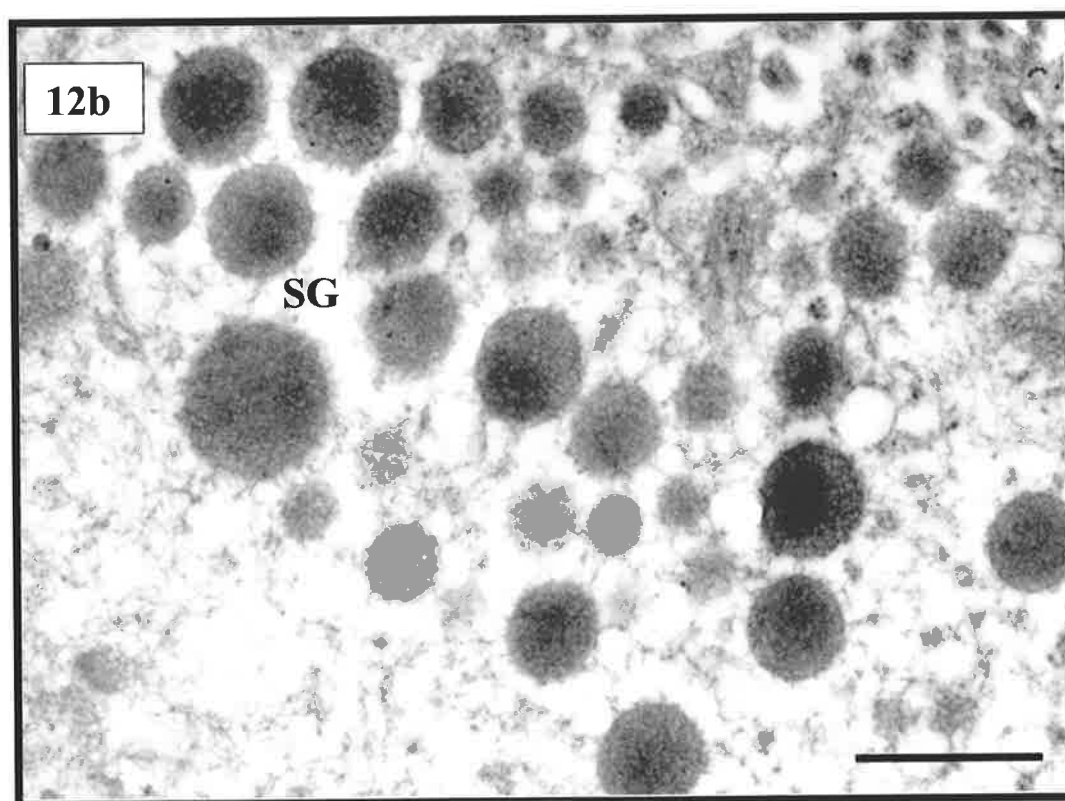
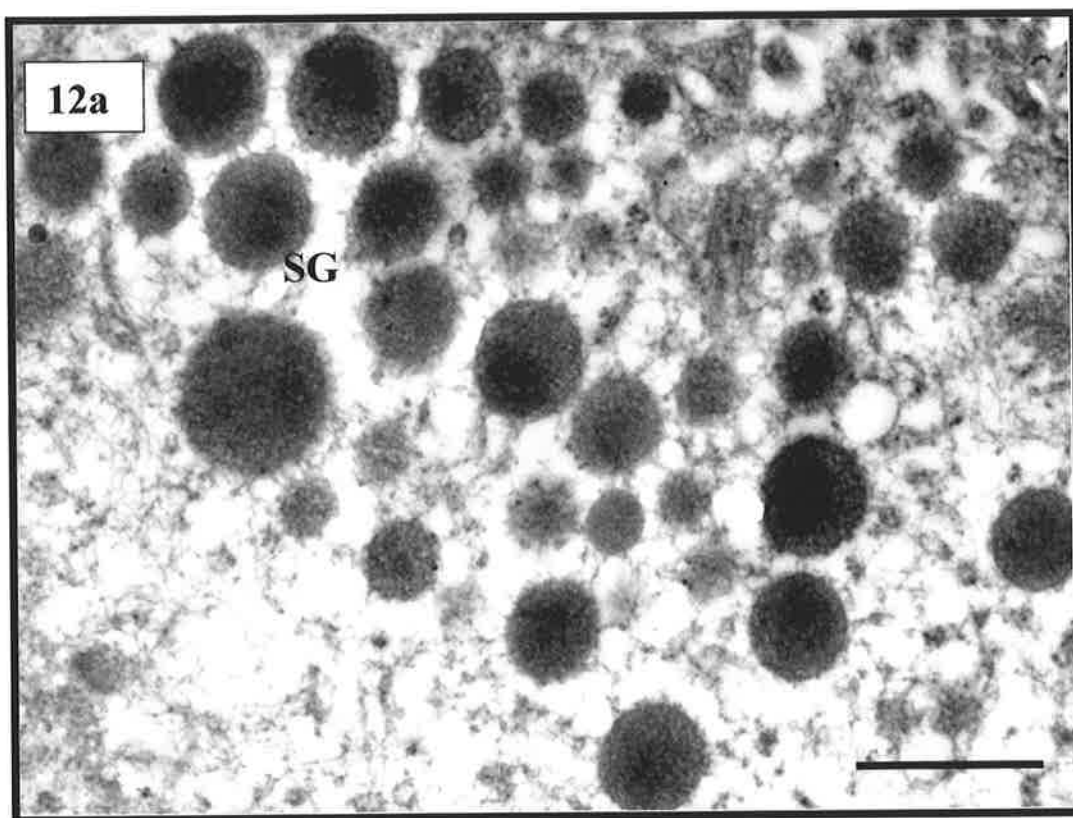
5.4 Discussion

5.4.1 Overview

In eutherians, carbohydrates play a vital role in the events leading up to, and including, fertilisation (for reviews see Benoff, 1997; Shalgi & Raz, 1997; Tulsiani *et al.*, 1997; Dell *et al.*, 1999; Topfer-Petersen, 1999). Initial binding of sperm to the zona pellucida (ZP) takes place through the glycoconjugates of the ZP glycoproteins, while it has been argued that with the large degree of homology of the ZP genes and proteins between species, it is the oligosaccharide components of the glycoproteins that are responsible for the species-specificity of this interaction (Florman & Wassarman, 1985; Wassarman, 1988, 1990, 1995; Bleil & Wassarman, 1988; Thaler & Cardullo, 1996; Ozgur *et al.*, 1998; Takasaki *et al.*, 1999).

Carbohydrates have also been found to play an important role in events within the oviduct. For example, in eutherians, carbohydrates have been found to be responsible for the binding of

Figure 5.12 Lectin immunocytochemistry of isthmus of oviduct of a peri-ovulatory brushtail possum. Electron micrographs of isthmic sections incubated with biotinylated-*Glycine max* (soybean) lectin (biotin-SBA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Compared with Fig. 5.11, only very little gold labelling (best seen with less contrast, 11b) was evident over the secretory granules (SG) and rest of the non-ciliated secretory cells of the isthmus. SBA binding is specific for α -D-*N*-acetylgalactosamine/ α -D-galactose. Bar = 500nm.



spermatozoa to the isthmic epithelium resulting in the formation of a sperm reservoir within the oviduct (Demott *et al.*, 1995; Lefebvre *et al.*, 1997; Suarez, 1998, 2001, 2002; Suarez *et al.*, 1998; Green *et al.*, 2001; Talevi & Gualtieri, 2001; Wagner *et al.*, 2002). The binding appears to be important for a number of reasons including: formation of a selective barrier to physiologically 'abnormal' sperm (e. g. those sperm that have undergone premature capacitation and acrosome reaction) (Fazeli *et al.*, 1999); decrease of incidence of polyspermy by the selective release of limited numbers of sperm at the time of ovulation (Hunter, 1973); and, maintenance of sperm viability (Pollard *et al.*, 1991; Smith & Nothnick, 1997).

The oviduct is also responsible for the production of oviduct-specific glycoproteins (OGPs), which play an important role in sperm-egg interactions. For example, oviductal exposure is commonly associated with an increase in penetrability and fertilisability of oocytes (Boatman *et al.*, 1994; King *et al.*, 1994; Kito & Bavister, 1996), while OGPs have been found in intimate association with oocytes, eggs, embryos and their vestments in a number of species (for reviews see Bleau & St.-Jacques, 1989; Malette *et al.* 1995; Buhi *et al.* 2000). OGPs may even be responsible for the species-specificity of sperm-ZP binding (Schmidt *et al.*, 1997), which again highlights the importance of carbohydrates due to the fact that, like the situation with the ZP, there is a high degree of homology of the genes and glycoproteins of OGPs (oviductins) between species and, therefore, specificity is likely to be afforded through the oligosaccharides.

In marsupials, the oviduct has only recently been investigated in facilitating gamete maturation and interaction. It was previously known that the oviduct was responsible for the production of one or both of the tertiary egg coats, but particularly the mucoid layer, which is akin to that found around other mammalian (e.g. rabbit) and non-mammalian (e.g. birds,

reptiles and amphibians) oocytes (for reviews see Hughes, 1977; Breed, 1996; Selwood, 2000). The function of the mucoid layer in marsupials was generally thought to play little role in sperm-egg interaction, although it has recently been speculated that the early deposition of mucoid might enhance sperm-ZP binding (Selwood, 2000). In fact, the mucoid layer has also been suggested to prevent sperm-egg interaction in marsupials, at least in terms of the prevention of polyspermy, due to its relatively rapid acquisition and the observation of sperm trapped within its concentric layers (Rodger & Bedford, 1982; Selwood, 1982, 1992).

More recently, however, the enhancing characteristics of oviductal glycoproteins for sperm-egg interaction have been investigated in the brushtail possum with the finding that proteins from the oviduct bind to sperm, maintain their viability and increase their ability to undergo capacitation, binding and penetration of the ZP (Sidhu *et al.*, 1999; Mate *et al.*, 2000). The possibility of oviductal contribution to the post-ovulatory oocyte and ZP had not been investigated, so this chapter attempted to qualify and quantify the glycoconjugates present within the epithelium of the ampulla and isthmus around the time of ovulation.

5.4.2 Glycoconjugates of the Peri-Ovulatory Oviductal Epithelium of the Brushtail Possum

Using nine fluorescently conjugated lectins, lectin histochemistry was performed on the ampullary and isthmic regions of the oviducts of peri-ovulatory stage possums. It was found that there was remarkable similarity in the binding of lectins between the ampullary and isthmic epithelial cells. The secretory cells of both the ampulla and isthmus bound to every lectin used, with the exception of SBA in the isthmus and LTA in both regions, while the ciliated cells bound all lectins except SNA and SBA. The greatest intensity of binding in the

secretory cells was found with the lectins RCA-I and WGA, indicating that β -Galactose (β -Gal) and *N*-acetylglucosamine (GlcNAc) and/or sialic acid were major constituents of the oviductal secretions. The ciliated cells, however, displayed the greatest affinity for the disaccharide β -Galactose-(1-3)-*N*-acetylgalactosamine (β Gal-(1-3)-GalNAc) with intense labelling with the lectin PNA, while ECA, RCA-I and LTA binding were also found at strong levels.

Similar results have been found in some eutherian species including the pig, with intense staining of both the isthmus and ampulla with RCA-I (Raychoudhury *et al.*, 1993; Walter & Bavdek, 1997), and in the hamster, where GalNAc, galactose and sialic acid are present in the secretory granules of the ampulla throughout the oestrous cycle, while labelling for GlcNAc was found to peak at ovulation (Kan *et al.*, 1990; El-Mestrah & Kan, 1999*a,b*). Fucose, as demonstrated through labelling with LTA and/or *Ulex europeaus* I (UEA-I), appeared to be a common glycoconjugate within the ciliated cells of possums, rabbits and pigs, but not hamsters (Menghi *et al.*, 1985, 1989, 1995; Raychoudhury *et al.*, 1993; Walter & Bavdek, 1997; El-Mestrah & Kan, 1999*a*).

These results appear to be quite different, however, from those obtained using lectin histochemistry on the oviduct of the rabbit. Like that of marsupials, the rabbit oviduct produces a mucoid coat that comes to surround the oocyte and/or embryo prior to them reaching the uterus. Unlike the situation in marsupials whereby eggs/embryos traverse the oviduct in around 7-24 hours (see Selwood, 2000 for references), rabbit oocytes spend around 3 days in the oviduct, during which time there is an increase in thickness of the mucoid coat that is required before passage of the egg/embryo into the uterus can occur (Gould, 1974; Menghi *et al.*, 1995).

The oviduct of the rabbit appears to show great regional variability with the finding that the ampulla produces more mucin than the isthmus (Greenwald, 1957), however, ultrastructurally, the secretory cells of the isthmus have been suggested as appearing to be more responsible for mucin secretion than the ampulla (Jansen & Bajpai, 1982). The ampullary epithelium of the rabbit oviduct appears to be more reactive to lectin histochemistry than the isthmus, however, with localisation of β Gal-(1-3)-GalNAc only found on the secretory cells of the ampulla (Menghi *et al.*, 1985). Hormonal administration to the rabbits combined with lectin histochemistry following neuraminidase treatments found that while the ampulla was more reactive to lectins than the isthmus, removal of sialic acid displayed that both regions of the oviduct contributed to the mucoid coat under different hormonal conditions (Menghi *et al.*, 1995). The length of time that the rabbit egg spent within the oviduct meant that a fairly precise stratification of the mucin glycoconjugates could take place (Menghi *et al.*, 1995), a situation postulated to occur in *Sminthopsis crassicaudata* (Roberts & Breed, 1996b). Such regional variability of reactivity to the lectins was not noted to such a degree in the possum oviduct using fluorescein-conjugated lectin histochemistry.

5.4.3 Regional Variation in the Glycoconjugate Secretions of the Peri-Ovulatory Oviduct of the Brushtail Possum

The largest regional difference, however, was noted for the localisation of α -D-GalNAc/ α -D-Gal, as demonstrated by binding of SBA, which was localised to the secretory cells of the ampulla but nowhere else. This difference in binding was supported and quantified using lectin immunocytochemistry with significantly more gold labelling with SBA on the secretory granules of the ampulla than in the isthmus. In fact, lectin immunocytochemistry proved to be

much more sensitive in terms of quantification of regional differences in glycoconjugate content of the secretory granules of the non-ciliated secretory cells, with three out of the four lectins displaying significantly greater labelling in one region. Greater intensities of labelling of the secretory granules within the ampulla were also found for α -D-Mannose as indicated by PSA, while significantly greater labelling of GlcNAc and/or sialic acid with WGA was noted in the secretory granules of the isthmus. Only β -Gal, with the second highest labelling intensity in the secretory granules of the ampulla, appeared to be equally distributed between the ampulla and isthmus.

In eutherians, regional variation in secretions of the oviduct have been demonstrated to occur in a number of species (for review see Abe, 1996), and probably reflects the different roles of the regions. For example, the glycoconjugates of the isthmus have been investigated into their role in the binding of sperm to the epithelium and the subsequent formation of the sperm reservoir in species such as the pig, cow and hamster (for reviews see Topfer-Petersen, 1999; Suarez, 2001). Alternatively, the glycoconjugates of the ampulla have been investigated for their role in sperm-egg interactions (Kan *et al.*, 1990; El-Mestrah & Kan, 1999*a,b*).

In marsupials, sperm have been shown to be stored within the oviduct for varying lengths of time from several hours to up to two weeks (for reviews see Rodger, 1994; Taggart, 1994; Taggart *et al.*, 1998). In the brushtail possum, sperm were found to adhere to the cells of the epithelial folds of the isthmus following artificial insemination (Jungnickel *et al.*, 2000), while sperm from the tammar wallaby were found to adhere to oviduct epithelial cell monolayers *in vitro* (Sidhu *et al.*, 1998). Whether glycoconjugates are responsible for the binding of sperm to the oviduct in marsupials is unknown. However, with the development of the oviduct epithelial cell monolayer co-culture system (Sidhu *et al.*, 1998), this question can now be

considered in more detail. The high levels of β -Gal and GlcNAc within the non-ciliated secretory cells of the isthmus of the brushtail possum, as indicated by labelling with RCA-I and WGA, respectively, in this study, suggest that the role of these glycoconjugates in sperm storage should be investigated.

The finding of higher levels of α -D-GalNAc and α -D-Man in the ampulla in this study, as indicated by labelling with SBA and PSA respectively, could mean that the oviductal secretions containing these glycoconjugates may play a role in the pre-fertilisation maturation of sperm and eggs, and/or have a role in sperm-egg interactions. Alternatively, as the mucoid coat begins to be laid down within the ampulla, these glycoconjugates could be part of the oviductal secretions that form the PAS+ mucoid coat. Whatever their role, the difference in the secretory glycoconjugates found within the ampulla and isthmus of the possum oviduct highlight that these two regions likely have quite different roles in gamete maturation and sperm-egg interaction. Determination of the role of the oviduct in the pre-fertilisation maturation of the ZP and the glycoconjugate content of pre- and post-ovulatory ZP, and its mucoid coat, form the major aims of the next chapter.

Chapter 6 Ultrastructural and glycoconjugate changes to the zona pellucida of the brushtail possum following ovulation

6.1 Introduction

As mentioned in Chapter 5, oviductal glycoprotein(s) have been shown to associate with, or induce changes to, the eggs and embryos (and their surrounding egg coats) of hamsters (Araki *et al.* 1987; L  veill   *et al.* 1987a; Yang & Yanagimachi, 1989; Brown *et al.* 1990; Kan *et al.* 1990; St.-Jacques *et al.* 1992; Boatman *et al.* 1994), mice (Kapur & Johnson, 1986, 1988; Kim & Schuetz, 1993; Kim *et al.* 1996), pigs (Hedrick *et al.* 1987; Buhi *et al.* 1993), baboons (Boice *et al.* 1990) and cows (Staros & Killian, 1998).

Within the hamster zona pellucida (ZP), lectin-gold cytochemistry showed that glycoconjugates changed in distribution from a generally uniform labelling throughout the ZP prior to ovulation to a more heterogeneous distribution post-ovulation (El-Mestrah & Kan, 2001). This supports the role of the oviduct as a contributor to the composition of the post-ovulatory ZP and suggests a possible functional significance in the process of fertilisation.

In marsupials, the oviduct secretes the tertiary egg envelopes, and in particular, the mucoid coat (for reviews see Hughes, 1977; Selwood, 2000). However, it is not known if there is any contribution by the oviduct to the post-ovulatory ZP, although the ZP of the brushtail possum has been found to undergo a post-ovulatory morphological change from 'broad and diffuse' to 'thin and compact' (Rodger & Mate, 1993). Whether this occurs as a result of some oviductal contribution to the ZP is unknown.

The importance of the oviduct to successful gamete interaction in marsupials has recently been suggested by the finding that sperm from the brushtail possum will only bind to, and penetrate, the ZP *in vitro* after the sperm have been pre-incubated in oviduct-conditioned media (Mate *et al.*, 2000). Sperm-oolemma binding and fertilisation did not occur, however, which suggests that some component from the *in vivo* environment was missing from the *in vitro* culture system. As the eggs used by Mate *et al.* (2000) were *in vitro* matured ovarian oocytes, the missing component might be some contribution by the oviduct to the egg and its surrounding coats.

With the finding of regional variation in glycoconjugate secretion of the oviduct in the brushtail possum in Chapter 5 of this thesis, this chapter, therefore, again used the brushtail possum as a model to investigate the following questions: 1) are there morphological changes that occur to the zona pellucida following ovulation? 2) are there changes in glycoconjugate content of the zona pellucida following ovulation?, and 3) which glycoconjugates are present within the mucoid coat? In order to answer the first question, a number of electron microscopical techniques were utilised such as ultra-rapid freezing and cryo-electron microscopy in an attempt to preserve the ZP as close to the natural state as possible. The latter questions were investigated using high resolution lectin-gold immunocytochemistry as described in the previous chapter.

6.2 Materials and Methods

6.2.1 Animals

Female brushtail possums (*Trichosurus vulpecula*) were obtained and housed as previously described in Section 2.2.1.

6.2.2 Superovulation and Collection of Eggs

Female possums were primed as detailed in section 2.2 and their ovulated eggs collected. To obtain ovarian oocytes for the determination of the ultrastructure and glycoconjugate content of the ZP, a similar regime to that described in section 2.2 was used except that the female possums, following removal of pouch young (if present), received the initial 15i.u. injection of PMSG but no injection of GnRH. Instead, animals were euthanased 72-96h following the injection of PMSG. Ovaries were removed, placed into pre-warmed physiological saline, and viewed under a dissecting microscope. Two 1ml syringes with 30G needles attached were then used to puncture the large follicles to release the oocytes into the culture media.

6.2.3 Post-Ovulatory Changes to the Zona Pellucida

In order to determine if there is a morphological change to the ZP following ovulation, ovarian and ovulated oocytes were subjected to several methods of cytochemical preparation for ultrastructural investigations.

The goal of specimen preparation for electron microscopy is to preserve the tissue as close to a 'natural state' as is possible. Typically, ultrastructural investigations of biological tissues involve some kind of chemical fixation prior to dehydration and, in the case of transmission electron microscopy (TEM), embedding in resins at high temperatures. Unfortunately, these processes can lead to changes to the native state of the tissue. In order to overcome this, several methods of specimen preparation were used to preserve the natural states of the ZP. The first of these involved treating ovarian and ovulated oocytes with saponin to remove the amorphous material in between ZP filaments. They were then processed in the presence of ruthenium red (RR) following the methods of Familiari *et al.*

(1989, 1992) (for details, see Appendix 1). RR stabilises structural components of glycoproteins while saponin allows the true structure of the ZP glycoprotein filaments to be viewed unencumbered from amorphous material. The oocytes and their coats were then washed in cacodylate buffer and processed as normal for routine scanning electron microscopy (SEM) as described in section 2.3. As a control, ovarian and ovulated eggs were also processed routinely for SEM as described in section 2.3.

As the marsupial ZP is highly glycosylated (see Chapter 4, this thesis) it is also likely to be highly hydrated, which means that processing for routine electron microscopy may result in shrinkage and other artefacts of the ZP. Thus, in order to minimize artefacts, several methods of low temperature fixation and processing for electron microscopy were employed. These were:

- 1) Cryo-scanning electron microscopy. This involved pipetting ovarian or ovulated oocytes, that were cultured in Tyrode solution at 35-37°C while the cryo-transfer system of the Philips XL30 Field Emission Gun SEM (FE-SEM) was sufficiently cooled with liquid nitrogen (approximately 20 min), onto SEM stubs covered with a carbon sticky tab. Excess culture media was removed using a small piece of filter paper since excess frozen water can impede visualisation as well as possibly cause mechanical damage through ice crystal formation during freezing. The SEM stub with the oocytes was then transferred into a cryo-holder and plunge frozen into liquid nitrogen slush for approximately 30 sec. The stub was then transferred to the liquid nitrogen pre-cooled cryo-transfer system of the FE-SEM and sealed at a temperature of around -120°C. Samples then had surface ice sublimed for 180 sec at -90°C before being sputter-coated with 2-3nm Au/Pd and viewed in the FE-SEM, operated at 1-5kV.

2) Ultra-rapid cooling (plunge freezing) and freeze substitution. This technique involves cooling a tissue (usually at $>10^4$ °C s⁻¹) that immobilises water within cells without the formation of ice crystals (i.e. a vitrified state). It results in cryo-immobilisation of the tissue and its components below -80°C (Sitte *et al.*, 1987). As the vitrified state of water within the cells is akin to liquid water, components within cells become immobilised in a similar state to their 'native' form (Bachmann & Mayer, 1987; Wilson *et al.*, 1998). Following this procedure, freeze substitution may be performed, whereby fixation and dehydration of tissue is carried out at a temperature below -80°C, which results in less disruption of the cellular components than when processing is performed at room temperature (Kellenberger, 1987; Wilson *et al.*, 1998). This method was used on ultra-rapidly cooled and freeze substituted ovarian and ovulated oocytes before routine embedding in TAAB resin at room temperature and polymerisation at 60°C. The method described by Yudin *et al.* (1988) and Cherr *et al.* (1990, 1992) for rapid freezing and freeze substitution of hamster eggs and sea urchin embryos was employed and can be found in Appendix 2. Following freeze substitution, the eggs were then processed and embedded into TAAB resin as described in section 2.3. Sections of the ovarian and ovulated eggs were then cut and prepared for TEM as described in section 2.4.

6.2.4 Glycoconjugate Composition of the Zonae Pellucidae and Mucoïd Coats of Ovarian and Ovulated Oocytes

In order to determine, localise and quantify the glycoconjugates of the ZP of ovarian oocytes and the ZP and mucoïd coats of ovulated oocytes at high-resolution, lectin-gold immunocytochemistry was undertaken. Ovarian and ovulated oocytes were fixed in 2% glutaraldehyde made up in 0.1M cacodylate buffer, pH 7.4, washed in cacodylate buffer and dehydrated by passing through a series of alcohols. Oocytes were then infiltrated in LR Gold (3:1, 1:1 and 1:3 ethanol: LR Gold for 60 min each) at -20°C. Following infiltration

in 100% LR Gold without initiator (0.1% Benzil) overnight at -20°C, oocytes were infiltrated in 100% LR Gold with initiator added for 60 min prior to embedding in BEEM capsules and polymerisation under UV light conditions at -20°C for 24 h. Semi-thick (~1µm) survey sections were cut on a Reichert-Jung Ultracut ultramicrotome with a glass knife and stained with 0.025% Toluidine Blue made up in 0.5% sodium tetraborate (Borax). Once appropriate sections were found, excess resin was trimmed away, thin (~90nm) sections were cut, and placed onto nickel grids coated with a supporting film of 2% Collodoin in Amyl Acetate (Electron Microscopy Sciences, Fort Washington, PA) and allowed to air dry for 24h.

Lectin-gold immunocytochemistry was then performed following the method described in Chapman *et al.* (1994) and section 5.2.3.2 of this thesis. Briefly, the sections had non-specific labelling blocked with 1% BSA, 1% gelatin and 0.2M glycine before being washed in 0.5M TBS containing 1mM CaCl and 0.5% Tween 20 (washing buffer, WB) and incubated in one of four biotinylated lectins (Vector Laboratories, Burlingame, CA). Following incubation in the lectin, sections were washed and placed onto a drop of 10nm gold-conjugated goat anti-biotin antibody (diluted 1:30 in 0.5M TBS) for 1 hour. Grids were then again washed, counter-stained in 70% alcoholic uranyl acetate and viewed on a Philips CM100 TEM with digital images captured on a Mega-View II digital image capture system (Soft Imaging System, GmbH, Germany). Gold particles were counted and quantitation carried out using the analySIS 2.1-image analysis software (Soft Imaging System, GmbH, Germany).

The biotinylated lectins used in this part of the study, like those used in Chapter 5, were: (1) Garden Pea lectin (*Pisum sativum*: PSA) for α-D-Mannose, (2) Castor bean lectin (*Ricinus communis* I: RCA-I) for β-D-galactose, 3) Wheat germ agglutinin (*Triticum vulgaris*: WGA) for (β-(1-4)-D-*N*-acetylglucosamine)₂, and *N*-acetylneuraminic acid (sialic

acid), and 4) Soybean lectin (*Glycine max*: SBA) for α -D-N-acetylgalactosamine/ α -D-galactose. The lectins were used at a concentration of 20 μ g/ml, except for WGA that was used at a concentration of 10 μ g/ml. For specificity controls, the biotinylated lectins were incubated with 0.4-1.0M/L of their specific sugar made up in distilled water for 30 min prior to the application of the lectins to the grids.

Statistical analyses, specifically the student's t-test, were performed on gold particle counts of the same lectin between the three developmental stages of the zona pellucida (i.e. ovarian, ovulated and artificially activated) using Systat statistical analysis software (Systat Software Inc., California) and differences were considered statistically significant where $p < 0.05$.

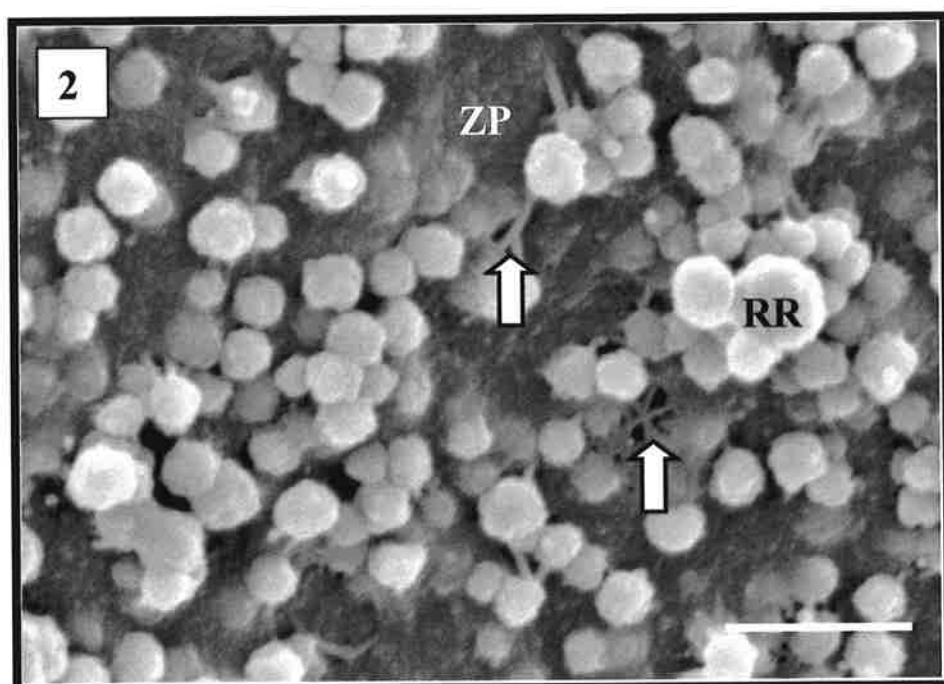
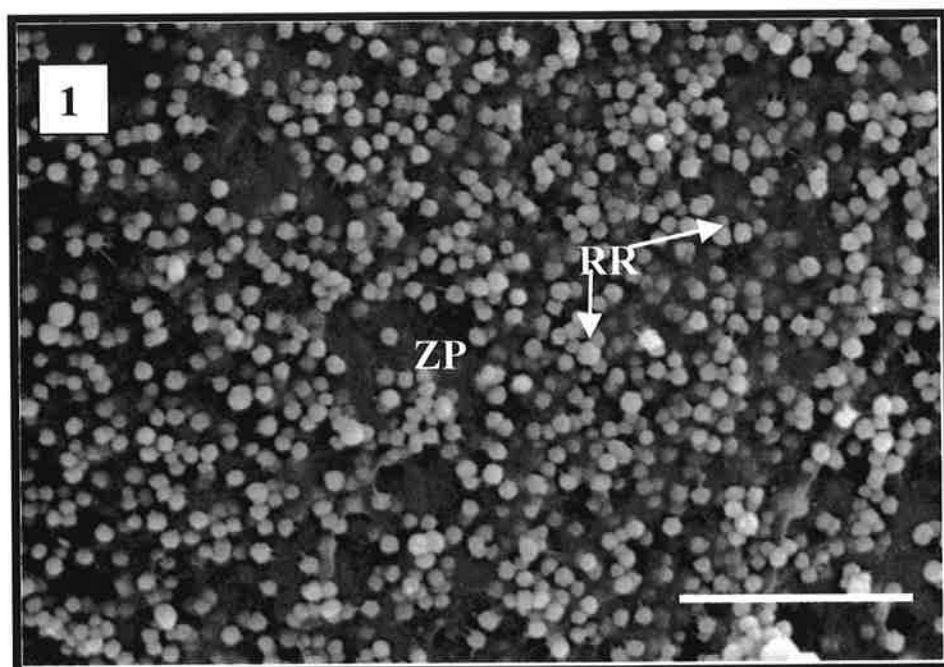
6.3 Results

6.3.1 Ultrastructural Changes to the Zona Pellucida following Ovulation

Fixation and processing of ovarian and ovulated eggs in the presence of ruthenium red (RR) and saponin was performed in the hope that many of the artefacts derived from routine fixation and preparation for scanning electron microscopy (SEM) would be eliminated. Unfortunately, when compared to routine SEM, the ultrastructure of the ZP of ovarian and ovulated eggs was partially obscured by the presence of small (~200-400nm) globular particles, as can be seen on the ZP of an ovarian egg (Fig. 6.1). Despite this, however, it can still be seen that the ZP of the ovarian egg was very dense and granular in structure with very few filaments evident (Fig. 6.1). At higher magnification, some evidence of a filamentous structure is present, although most of the ZP appears dense and uniform in structure (Fig. 6.2). This dense structure of ZP surrounding ovarian oocytes was also seen in those oocyte prepared using routine SEM.

Figure 6.1 Scanning electron micrograph of an ovarian oocyte from a brushtail possum pretreated with ruthenium red and saponin. Much of the electron- and structurally-dense zona pellucida (ZP) is obscured by small globular particles, presumably composed of, or artefactually induced by, ruthenium red (RR). Bar = 10 μ m.

Figure 6.2 Scanning electron micrograph of higher magnification of the brushtail possum ovarian oocyte seen in Fig. 6.1 showing the dense structure of the zona pellucida (ZP) and the ruthenium red-associated particles (RR). While mainly compact in structure, some areas of the zona pellucida of the ovarian oocyte were filamentous (arrows). Bar = 1 μ m.



The ZP surrounding ovulated eggs prepared in this manner, however, were much more filamentous in structure (Fig. 6.3). The thin zona filaments are more clearly seen at higher magnification and traverse numerous fenestrae (Fig. 6.4). Routine SEM also demonstrated that the zonae of ovulated eggs were highly filamentous in structure with numerous fenestrae externally (Fig. 6.5). At higher magnification, the ZP appeared web-like in structure with thicker filaments externally, which joined to branching points for the filaments with fenestrae interspersed between the branches (Fig. 6.6). One ovulated oocyte had the outer layer of the ZP disrupted at some stage of processing (Fig. 6.7) that displayed the underlying zona structure, which appeared less filamentous, with fewer fenestrae and more compact in structure (Fig. 6.8).

Cryo-SEM of the freshly frozen, hydrated ovarian and ovulated eggs was very poor due to the resolving ability of the microscope, while there was also difficulty in determining a sufficient sublimation of ice without causing specimen damage. As such, few features of the ZP of ovarian and ovulated eggs were resolved using this technique. Cryo-SEM of the ovarian ZP demonstrated a relatively featureless surface appearance with no discernable filaments (Fig. 6.9). The ZP around the ovulated oocytes, however, were slightly more filamentous in appearance, although there also appeared to be much amorphous material on the surface interspersed between the filaments giving the zona a relatively uniform structure (Fig. 6.10).

The third method used to more naturally preserve the ZP was ultra-rapid freezing and freeze substitution for transmission electron microscopy (TEM). Rapid frozen and freeze substituted ovarian oocytes had 1 to 2 layers of cumulus cells external to the ZP (Fig. 6.11). The ZP appeared variable in thickness and numerous cumulus cell cytoplasmic extensions were seen traversing them. High magnification of the ZP surrounding the ovarian oocytes demonstrated that they were well preserved (Fig. 6.12). The ZP appeared

Figure 6.3 Scanning electron micrograph of an ovulated oocyte from a brushtail possum pre-treated with ruthenium red and saponin. Like that in Figs 6.1 and 6.2, much of the underlying zona pellucida (ZP) is obscured by ruthenium red-associated particles (RR) but the zona pellucida matrix can be seen to be highly filamentous. Bar = 10 μ m.

Figure 6.4 Scanning electron micrograph of higher magnification of the brushtail possum ovulated oocyte seen in Fig. 6.3 demonstrating the highly filamentous (arrows) structure of the zona pellucida (ZP). The ruthenium red-associated particles (RR) appear to be less dense than that seen on the zona pellucida of ovarian oocytes. Bar = 1 μ m.

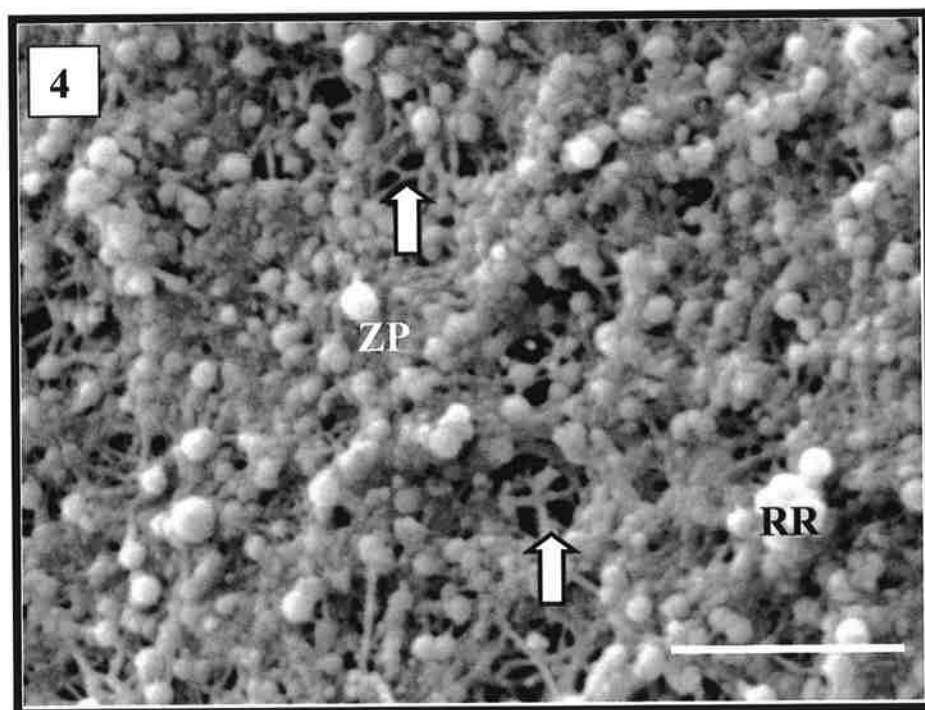
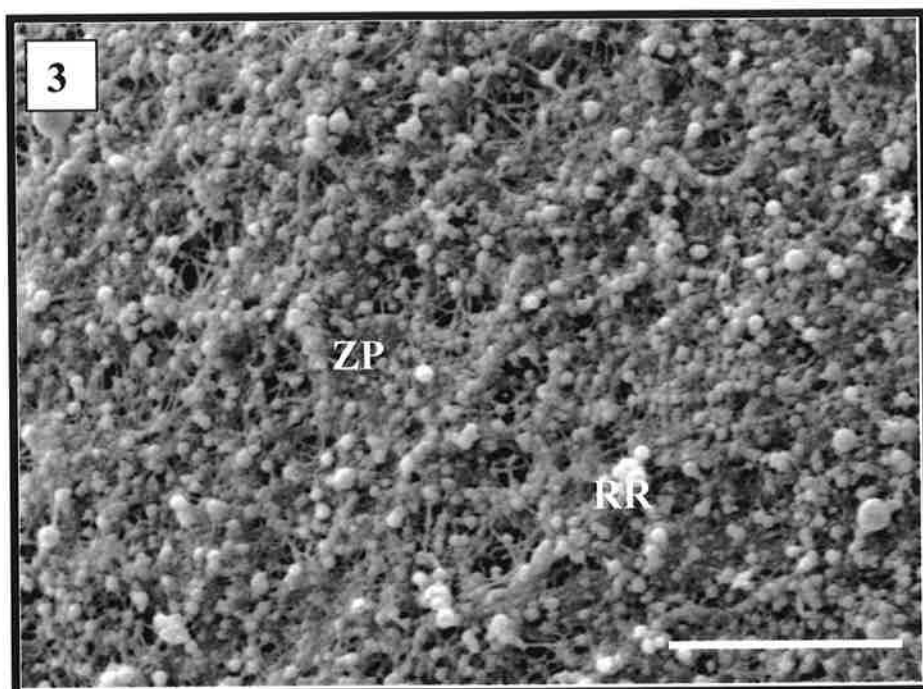


Figure 6.5 Scanning electron micrograph of the zona pellucida around an ovulated oocyte from a brushtail possum processed routinely for scanning electron microscopy. Zona pellucida (ZP) is highly filamentous with numerous fenestrae giving the surface a mesh-like appearance. Bar = 10 μ m.

Figure 6.6 Scanning electron micrograph of the zona pellucida from brushtail possum ovulated oocyte seen in Fig. 6.5. Note the highly filamentous zona pellucida (ZP) with the external surface composed of large, branching (large arrows) filaments that link with other large filaments to produce the fenestrae. The fenestrae are traversed by smaller filaments (small arrows). Bar = 1 μ m.

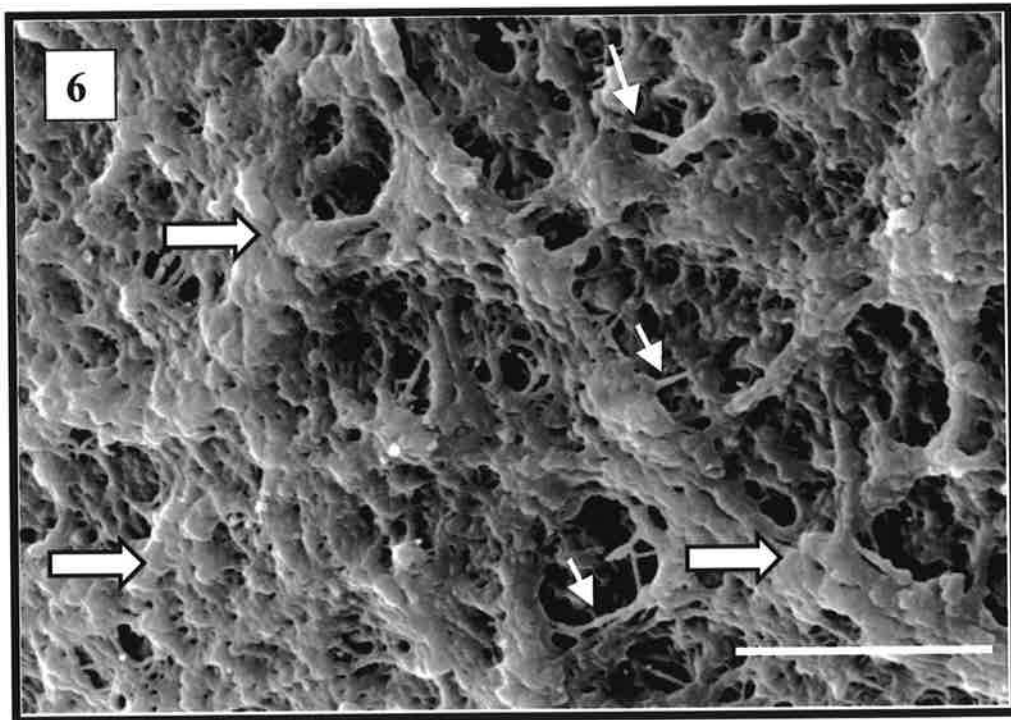
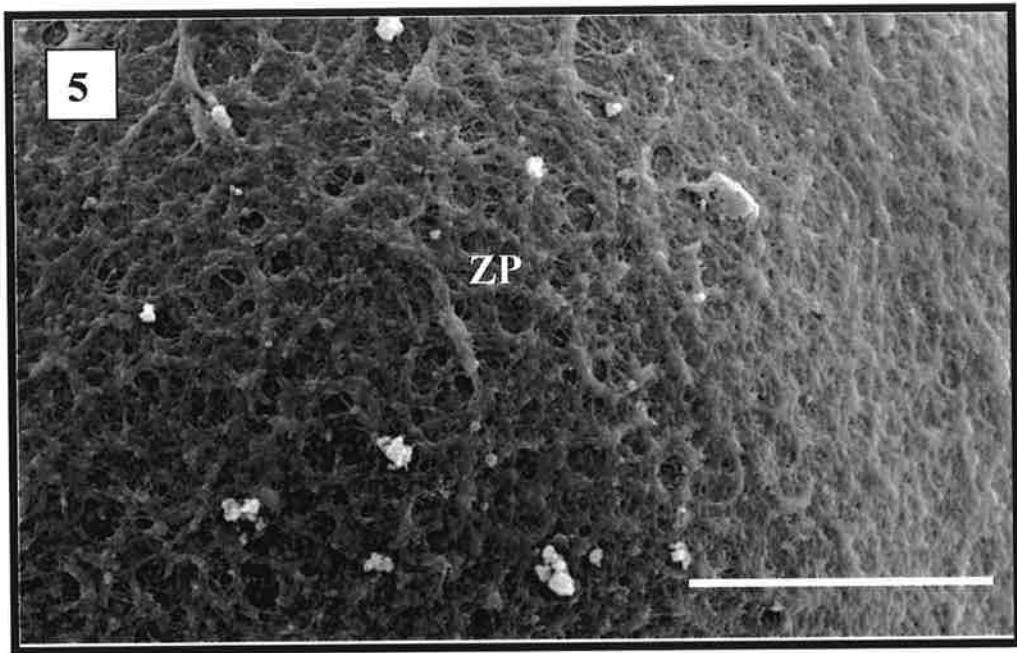


Figure 6.7 Scanning electron micrograph of the zona pellucida of two ovulated oocytes from a brushtail possum. The zona pellucida (ZP) has been disrupted through the preparative process revealing the relatively dense underlying zona pellucida matrix (arrow). Bar = 5 μ m.

Figure 6.8 Scanning electron micrograph of higher magnification of the brushtail possum ovulated oocyte seen in Fig. 6.7 highlighting the highly filamentous structure of the external zona pellucida (ZP), and the internal surface of the zona matrix (arrow). The inner surface of the zona pellucida appears much denser than that of the outer surface. Bar = 5 μ m.

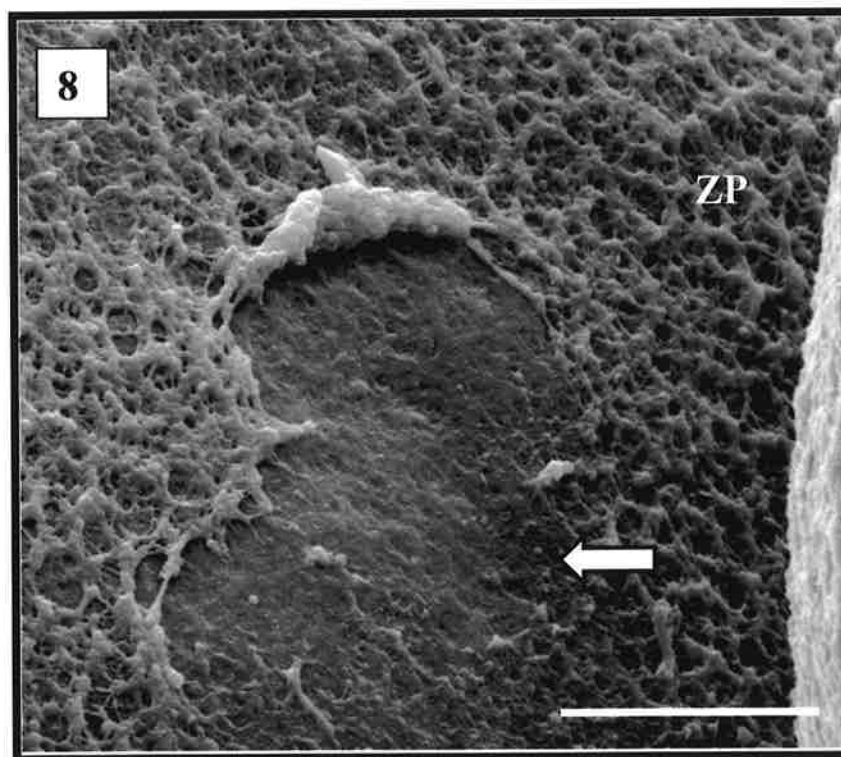
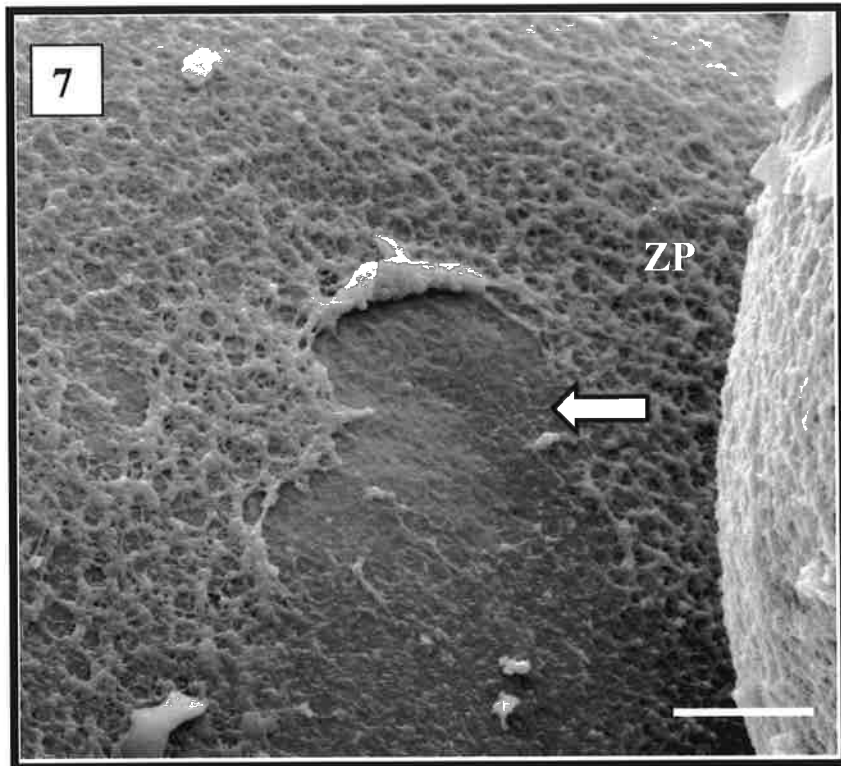


Figure 6.9 Cryo-scanning electron micrograph of the zona pellucida around a frozen hydrated ovarian oocyte from a brushtail possum that was frozen in liquid nitrogen slush, sublimed, and sputter coated prior to being viewed under low voltage in the scanning electron microscope. The zona pellucida (ZP) is relatively featureless and dense in structure with few filaments evident. Bar = 2 μ m.

Figure 6.10 Cryo-scanning electron micrograph of the zona pellucida (ZP) around a frozen hydrated ovulated oocyte from a brushtail possum that was frozen in liquid nitrogen slush, sublimed, and sputter coated prior to being viewed under low voltage in the scanning electron microscope. It appeared filamentous (F) in structure, although relatively dense with no fenestrae evident. Bar = 1 μ m.

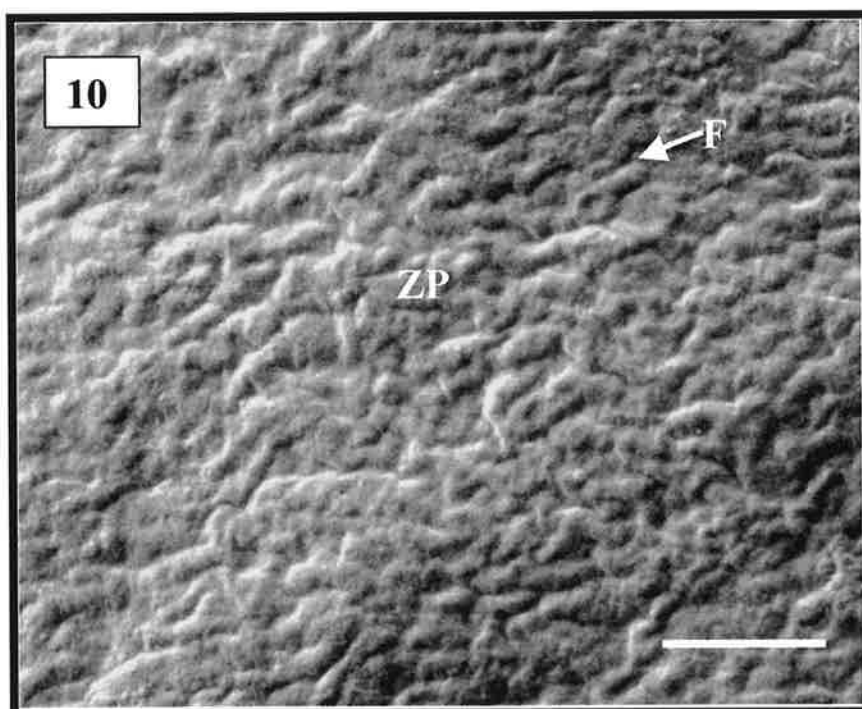
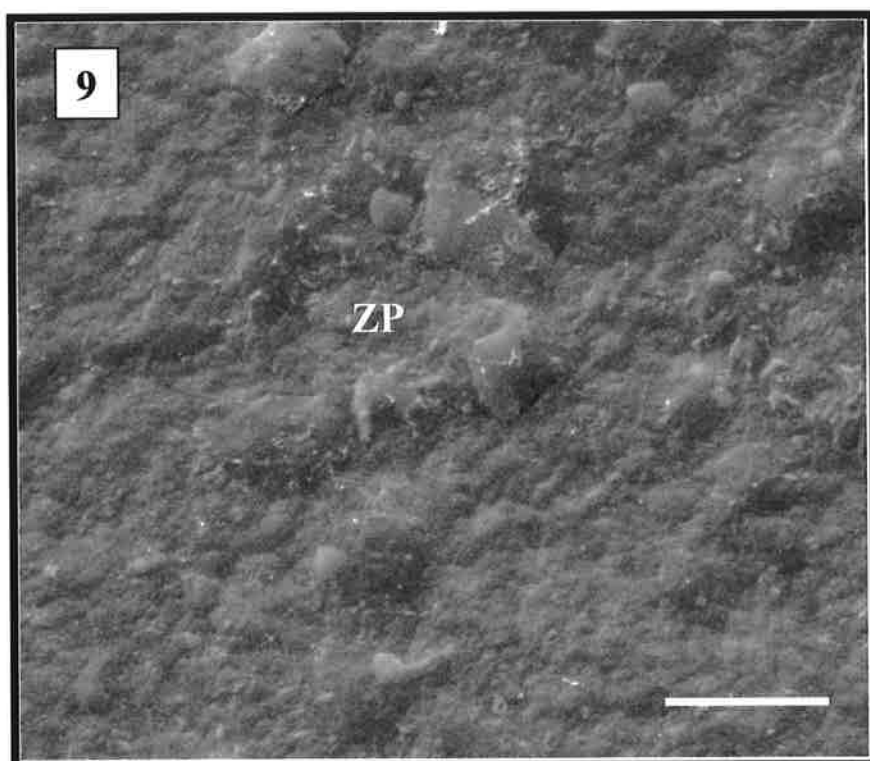
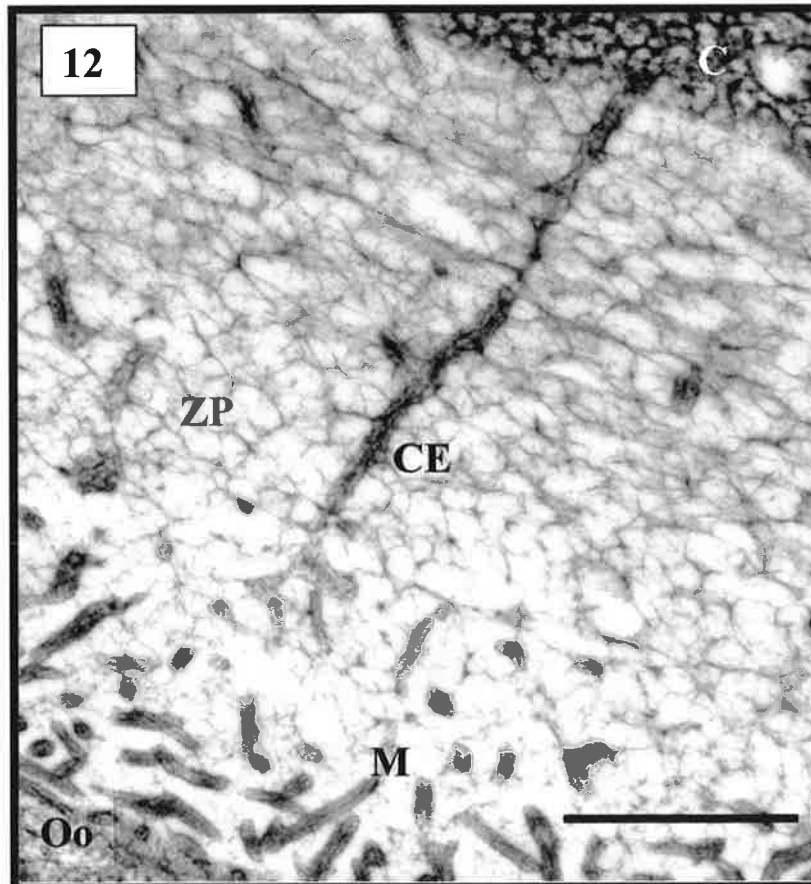
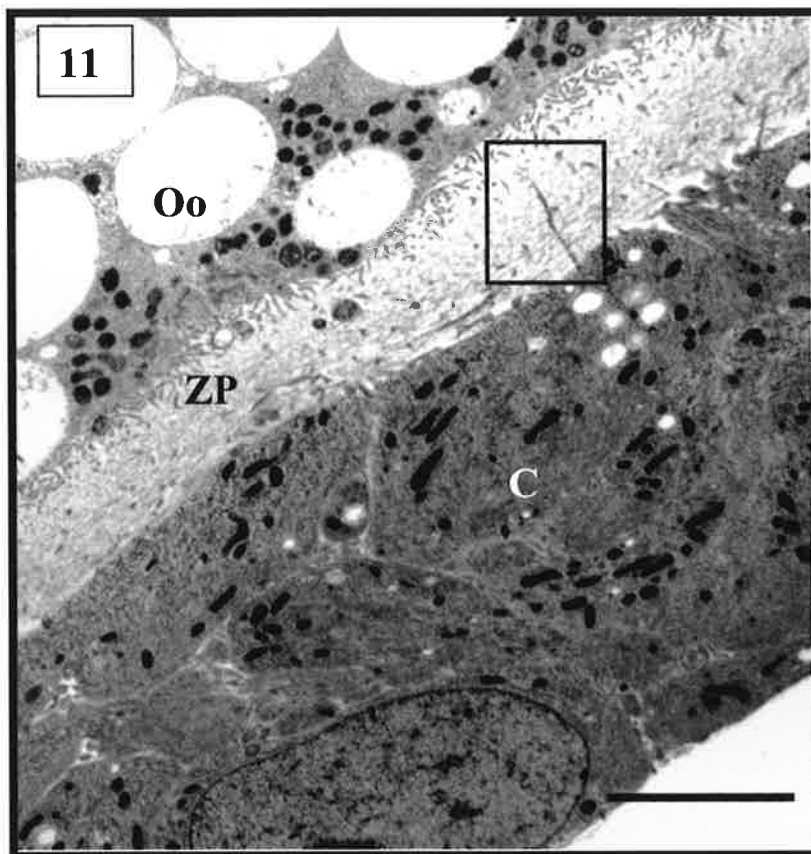


Figure 6.11 Transmission electron micrograph of a rapidly-frozen, freeze substituted pre-ovulatory ovarian oocyte and surrounding zona pellucida from a brushtail possum. The ovarian oocyte (Oo) is surrounded by a relatively broad zona pellucida (ZP) and one to two layers of cumulus cells. Bar = 10 μ m.

Figure 6.12 Transmission electron micrograph of higher magnification of the boxed area of the rapidly-frozen, freeze substituted zona pellucida surrounding the ovarian oocyte (Oo) from Fig. 6.11. Note its filamentous structure and that it is traversed by cytoplasmic extensions (CE) of the cumulus cells (C), while the filaments appear more linearly aligned close to the cumulus cell than those closest to the microvilli (M) of the oocyte. Bar = 1 μ m.



filamentous although disorganised, while the area of the zonae adjacent to the cumulus cells appeared denser with relatively compact filaments, in comparison to the area closest to the oocyte.

The ultra-rapid frozen and freeze substituted ovulated oocytes were commonly encased within a mucoid coat (Fig. 6.13). The ZP had compacted into the perivitelline space and was found interspersed between the microvilli of the oocyte. Associated with this was some minimal tearing in the cortical cytoplasm, although numerous cortical granules were still present. At higher magnification, the ZP of the ovulated oocyte appeared highly filamentous with a regular pattern of branching with numerous fenestrae (Fig. 6.14). This was particularly highlighted at even higher magnification, with the ZP displaying a web-like structure with numerous branching points for the filaments that appeared to be slightly thicker than the branching filaments (Fig. 6.15). The structure of the ovulated ZP appeared highly organised with the electron dense filaments interspersed by electron lucent fenestrae. In close association with the electron dense protein filaments was a more diffuse electron density, more than likely smaller filaments traversing the zona fenestrae (Fig. 6.15).

6.3.2 Glycoconjugates of the Zonae Pellucidae Before and After Ovulation

Lectin immunocytochemistry was performed on two stages of development (ovarian and ovulated) of the ZP of the brushtail possum in order to determine what changes, if any, occur to the glycoconjugate composition of the ZP following ovulation. The glycoconjugates present within the mucoid coat were also determined. Gold labelling densities of the ZP surrounding ovarian and ovulated oocytes and the mucoid coats following incubation in one of four lectins are summarised in Table 6.1 and Figure 6.16.

Figure 6.13 Transmission electron micrograph of a rapidly-frozen, freeze substituted ovulated oocyte and surrounding zona pellucida (ZP) from a brushtail possum. Note also the mucoid coat (MC) external to the zona. Slight artefactual disruption of the oocyte is evident immediately underlying the microvilli (M) of the oolemma, although this was not evident in all of the oocytes. Bar = 2 μ m.

Figure 6.14 Transmission electron micrograph of higher magnification of a rapidly-frozen, freeze substituted zona pellucida and part of the ovulated oocyte (Oo). Note the highly filamentous structure of the zona pellucida (ZP). Immediately underlying the microvilli (M) of the oocyte, several cortical granules can be seen (arrows). Bar = 1 μ m.

Figure 6.15 Transmission electron micrograph of the zona pellucida (ZP) after rapidly-freezing and freeze substitution demonstrating the highly ordered filamentous structure. The electron dense zona filaments appear to arise from, and join other, branching points (arrows) giving the zona pellucida a 'honeycomb'-like appearance. Material of more diffuse electron density surrounds the electron dense zona filaments, which may represent smaller zona filaments or the oligosaccharides of the zona pellucida glycoproteins. Bar = 500nm.

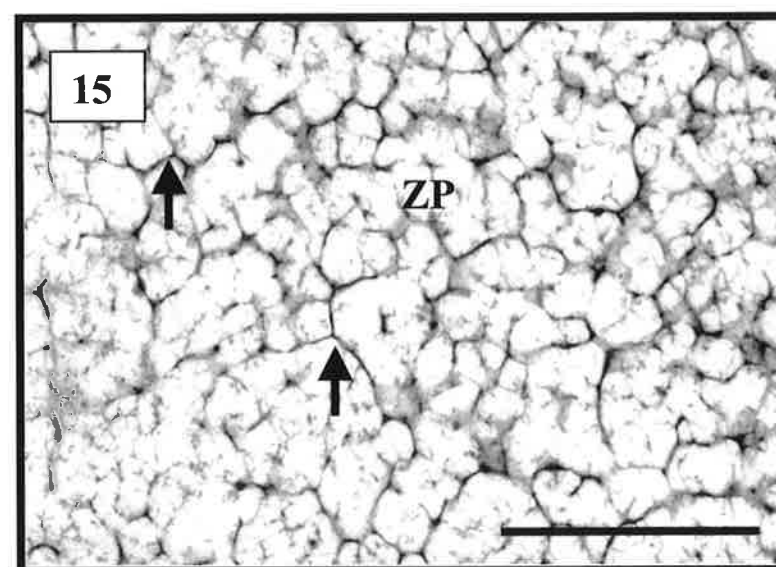
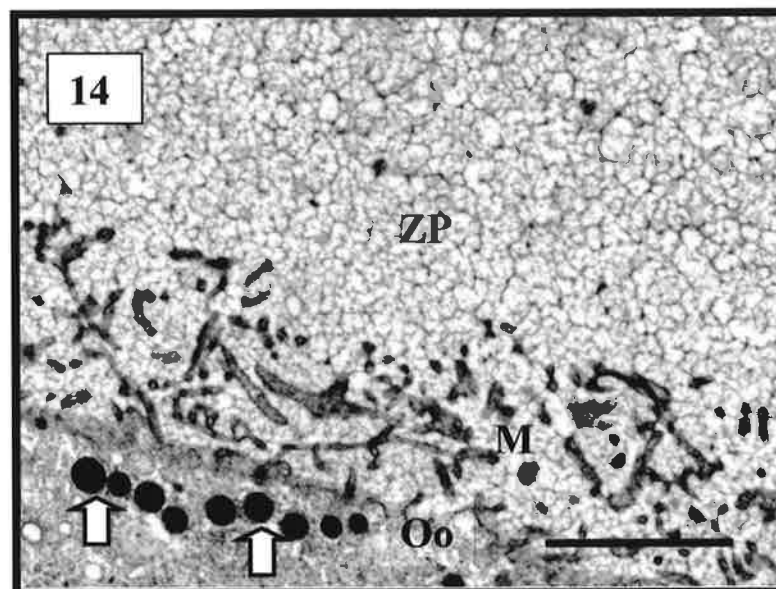
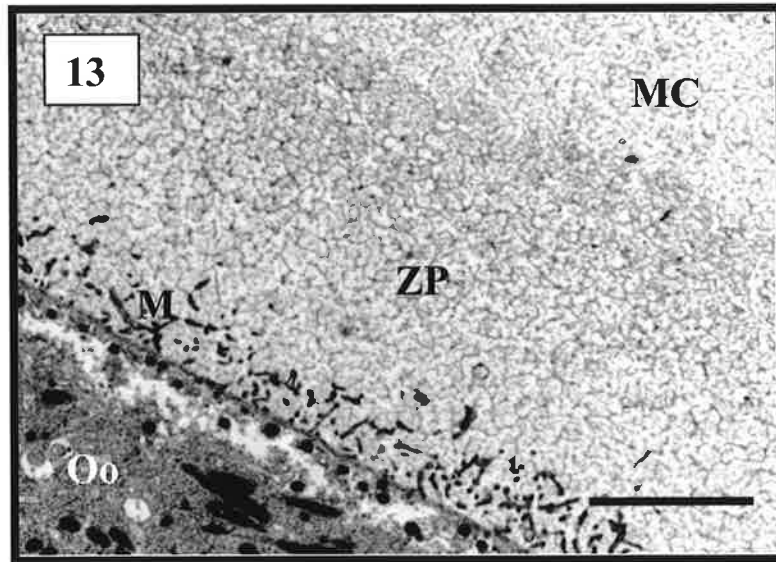


Table 6.1 Density of gold labelling of the zona pellucida of ovarian and ovulated (tubal) oocytes and the mucoid coat of the brushtail possum following incubation with four lectins. The labelling intensities are shown as the number of gold particles μm^2 ; mean values $\pm$ SEM. (*PSA* *Pisum sativum* agglutinin; *RCA-I* *Ricinus communis-I* agglutinin; *WGA* wheat germ agglutinin; *SBA* soybean agglutinin).

Lectin	Ovarian ZP	Tubal ZP	Mucoid Coat
PSA	14.72 $\pm$ 5.31	162.82 $\pm$ 19.61 ^a	42.69 $\pm$ 10.23
RCA-I	48.52 $\pm$ 11.76	187.03 $\pm$ 28.68 ^a	115.48 $\pm$ 23.05
WGA	91.11 $\pm$ 8.55	146.31 $\pm$ 21.84	178.22 $\pm$ 48.22
SBA	10.45 $\pm$ 1.85	90.55 $\pm$ 4.80 ^a	73.42 $\pm$ 1.75

^a The labelling densities for these lectins was significantly higher ($p < 0.05$) on the ZP of ovulated eggs than the ZP of ovarian oocytes;

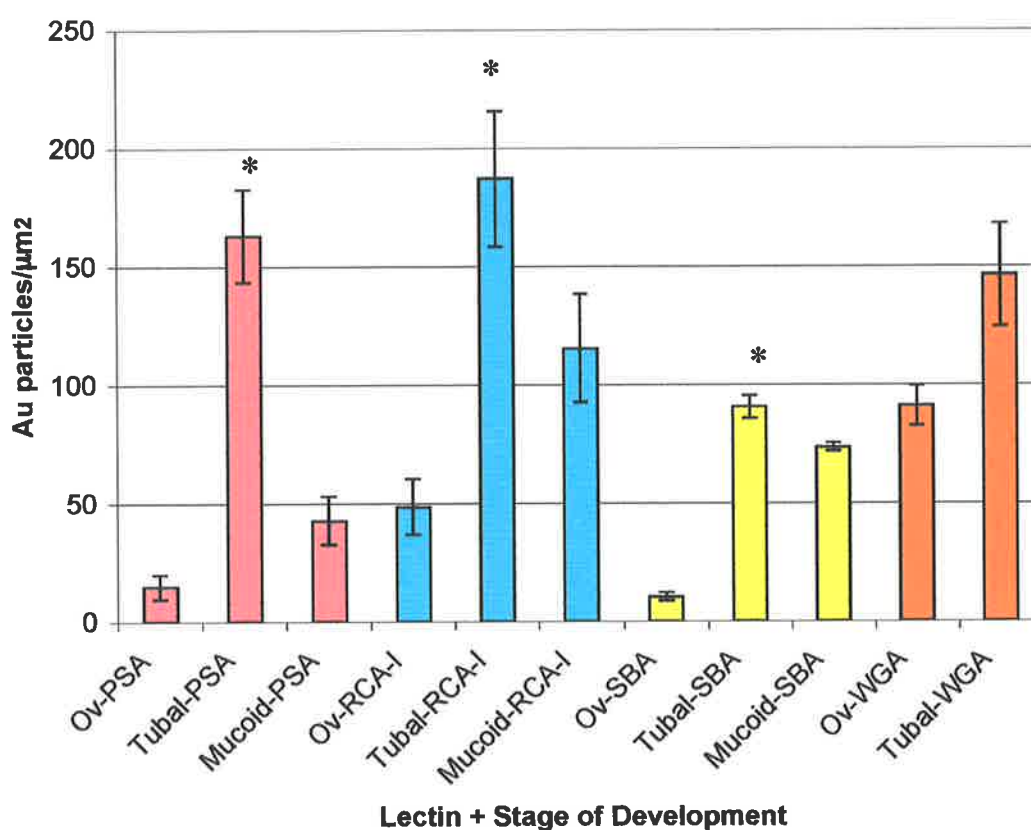


Figure 6.16. Density of gold labelling of the zona pellucida of the ovarian (Ov) and ovulated (Tubal) oocytes of the brushtail possum following incubation in four different lectins. The labelling intensities are shown as the number of gold particles μm^2 ; mean values $\pm$ SEM. Stars (*) represent significant differences in gold labeling of the same lectin from the previous stage of development. (*PSA* *Pisum sativum* agglutinin; *RCA-I* *Ricinus communis-I* agglutinin; *WGA* wheat germ agglutinin; *SBA* soybean agglutinin).

Pisum sativum lectin (PSA)

Follicular oocytes, surrounded by granulosa cells, had relatively broad ZP with many microvilli of the oocyte projecting into it (Fig. 6.17). Relatively little gold labelling was present on the ZP of ovarian oocytes using PSA, however, the labelling increased significantly following ovulation ($p=0.001$) (Fig. 6.18). While there only appeared to be minimal gold labelling of the mucoid coat, labelling of the ZP and microvilli within the perivitelline space was considerable (Fig. 6.18). At higher magnification, the labelling of the ZP appeared to closely adhere to the filaments, with evidence of a number of specific lines of gold particles overlying the filaments beneath (Fig. 6.19).

Ricinus communis I lectin (RCA-I)

Moderate densities of gold labelling were found on the ZP of ovarian oocytes using RCA-I (Fig. 6.20). Labelling increased significantly following ovulation ($p=0.008$), with high densities of labelling over the mucoid coat as well as the ZP and the microvilli of the oocyte (Fig. 6.21). Labelling of β -Gal residues was also noted on the cortical granules within the cortical cytoplasm of the oocyte while there appeared no specific labelling over the surrounding mitochondria (Fig. 6.22).

Triticum vulgaris (wheat germ) lectin (WGA)

Gold labelling with WGA demonstrated the greatest density of particles for ovarian ZP. Following ovulation, labelling increased non-significantly ($p=0.08$), again with relatively high densities of labelling over the mucoid coat, ZP and oocyte's microvilli within the perivitelline space. Also, cortical granules were again labeled within the cortical cytoplasm.

Figure 6.17 Zona pellucida of the peri-ovulatory ovarian oocyte of the brushtail possum incubated with biotinylated-*Pisum sativum* (garden pea) lectin (biotin-PSA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Note very little gold labelling of the zona pellucida (ZP); cumulus cells (C); microvilli (M); oocyte (Oo). PSA binding is specific for α -D-Mannose. Bar = 1 μ m.

Figure 6.18 Zona pellucida around an ovulated oocyte of the brushtail possum incubated with biotinylated-*Pisum sativum* (garden pea) lectin (biotin-PSA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Note abundant gold labelling of the zona pellucida (ZP) and microvilli (M) of the ovulated oocyte (Oo). Much less gold labelling was noted on the mucoid coat (MC) external to the zona pellucida; perivitelline space (PVS). PSA binding is specific for α -D-Mannose. Bar = 1 μ m.

Figure 6.19 Higher magnification of the zona pellucida of an ovulated oocyte of a brushtail possum incubated with biotinylated-*Pisum sativum* (garden pea) lectin (biotin-PSA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Gold labelling of zona pellucida (ZP) appeared specific to underlying filaments with rows of gold particles evident throughout the zona (arrows); microvilli (M); mucoid coat (MC); perivitelline space (PVS). PSA binding is specific for α -D-Mannose. Bar = 1 μ m.

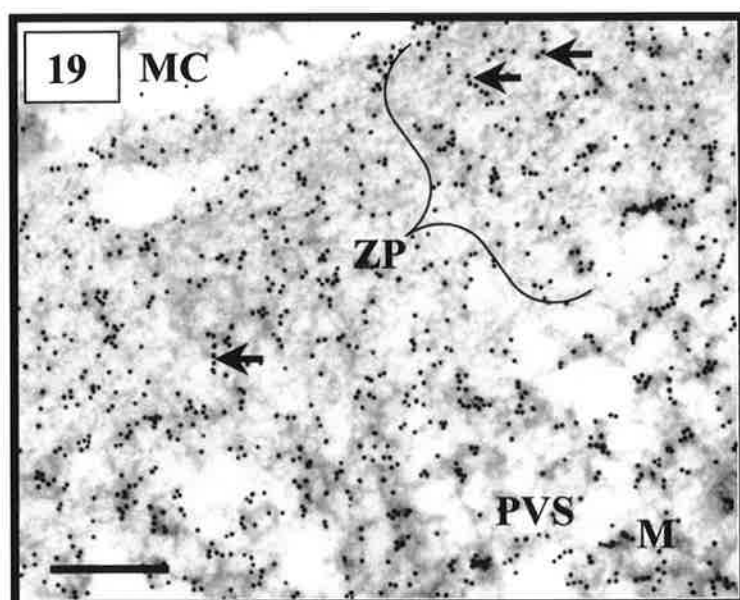
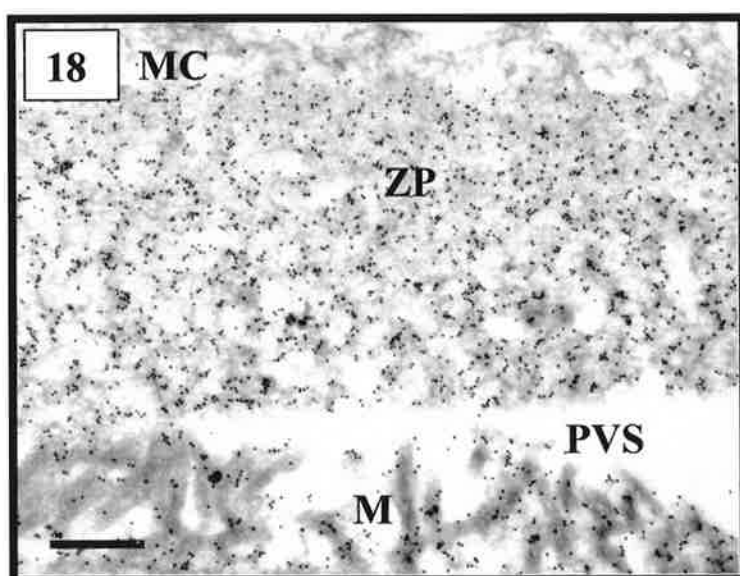
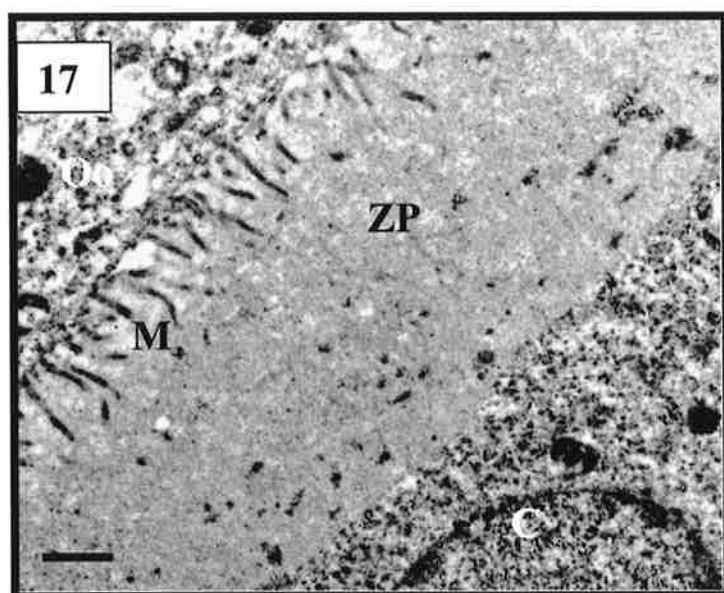
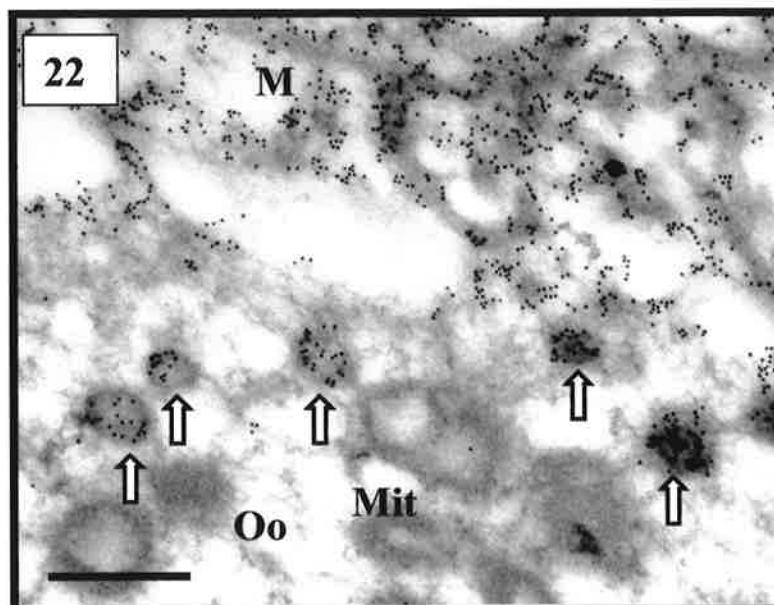
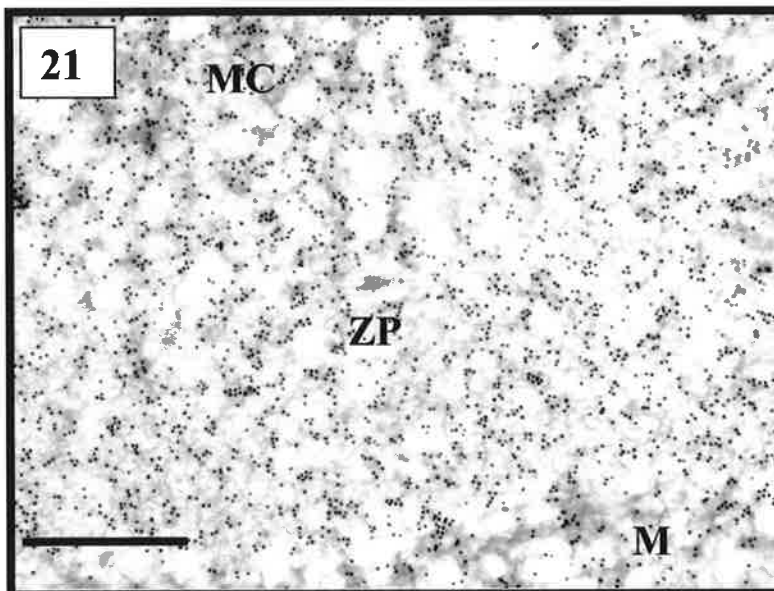
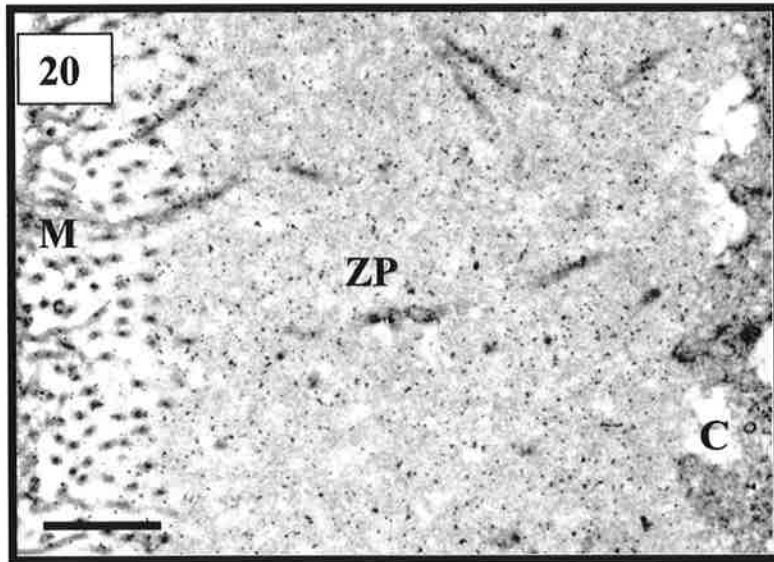


Figure 6.20 Zona pellucida of the peri-ovulatory ovarian oocyte of the brushtail possum incubated with biotinylated-*Ricinus communis I* (castor bean) lectin (biotin-RCA-I) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Gold labelling is evident throughout the zona pellucida (ZP) of the ovarian oocyte (Oo); cumulus cells (C); microvilli (M). RCA-I binding is specific for β -D-Galactose. Bar = 1 μ m.

Figure 6.21 Zona pellucida of an ovulated oocyte of the brushtail possum incubated with biotinylated-*Ricinus communis I* (castor bean) lectin (biotin-RCA-I) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Note the relatively abundant labelling of the zona pellucida (ZP), microvilli (M) and mucoid coat (MC) of the ovulated oocyte (Oo). Like that found with PSA, gold labelling of the zona pellucida appeared highly specific with a number of the gold particles evident in rows over the underlying filaments. RCA-I binding is specific for β -D-Galactose. Bar = 1 μ m.

Figure 6.22 Higher magnification of the oolemma and cortical cytoplasm of an ovulated oocyte of a brushtail possum incubated with biotinylated-*Ricinus communis I* (castor bean) lectin (biotin-RCA-I) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Note high densities of gold labelling on the microvilli (M) of the oocyte (Oo). Within the cortical region of the cytoplasm, cortical granules (arrows) were also labelled while the surrounding mitochondria (Mit) remained unlabelled. RCA-I binding is specific for β -D-Galactose. Bar = 1 μ m.



Glycine max (*soybean*) lectin (SBA)

Very little gold labelling was noted on ovarian ZP using SBA. A statistical significant increase in gold labelling was found on the ZP following ovulation ($p < 0.001$) (Fig. 6.23), with a similar labelling intensity of the mucoid layer, the ZP and the microvilli within the perivitelline space (Fig. 6.24). Labelling within the ZP of ovulated oocytes appeared to be localised to distinct underlying filaments of higher electron density.

Controls

Virtually no gold labelling was noted on those ZP that were incubated with biotinylated lectins which were pre-incubated with their corresponding inhibitory sugar (data not shown).

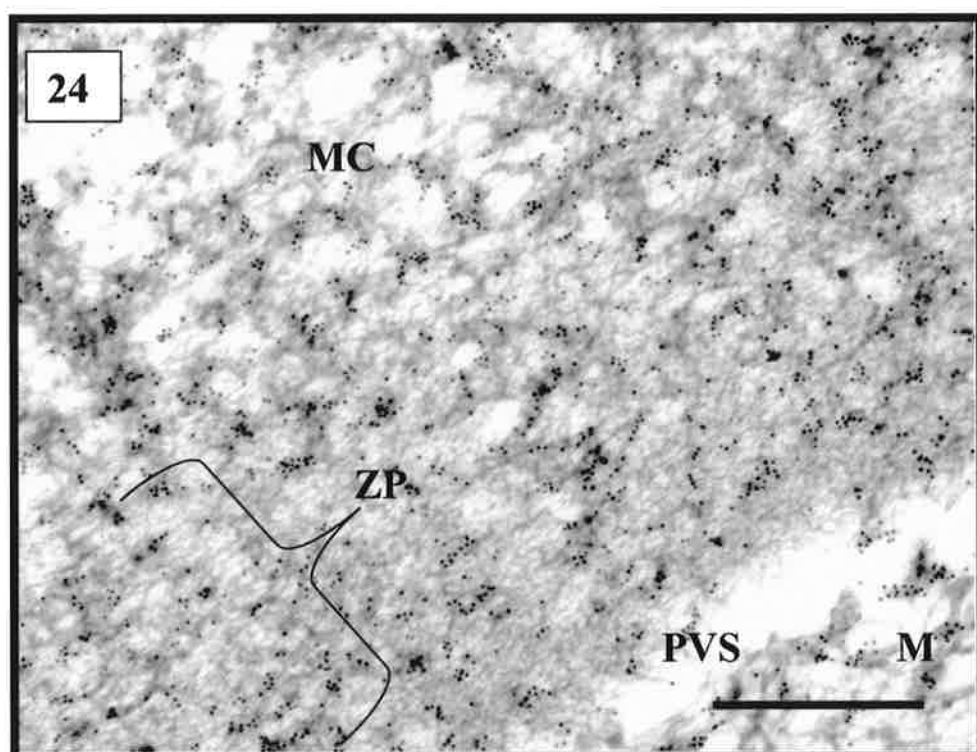
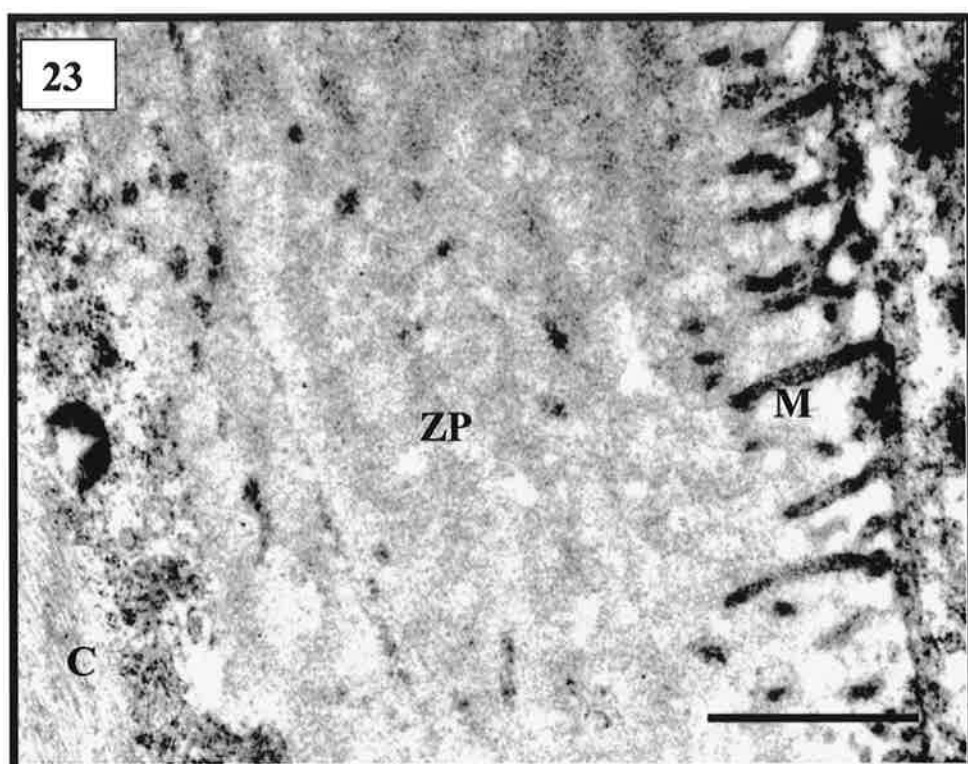
6.4 Discussion

6.4.1 Overview

The later stages of maturation of the marsupial gametes are not clearly understood. In the male, post-testicular maturation is an integral component of the development of male fertility and occurs both within the male and female reproductive tract (for reviews see Bedford, 1991, 1996; Rodger, 1991, 1994; Breed, 1994, 1996; Cooper *et al.*, 1998). Maturation of spermatozoa involves both morphological and biochemical changes. The morphological changes during epididymal transit include a realignment of the head of the spermatozoon on the tail from a T-shape to a more streamlined profile with the head lying parallel to the long axis of the tail (Harding *et al.*, 1976; Temple-Smith & Bedford, 1976, 1980; Taggart *et al.*, 1995), a remodeling of the acrosome into a distinct organelle either on the dorsal surface of the sperm head, as in possums, wallabies and bandicoots (Harding *et al.*, 1976; Temple-Smith & Bedford, 1976; Taggart *et al.*, 1995; Lin & Rodger, 1999), or

Figure 6.23 Zona pellucida of a peri-ovulatory ovarian oocyte of the brushtail possum incubated with biotinylated-*Glycine max* (soybean) lectin (biotin-SBA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Note little gold labelling throughout the zona pellucida (ZP) of the ovarian oocyte (Oo); cumulus cells (C); microvilli (M). SBA binding is specific for α -D-N-acetylgalactosamine/ α -D-galactose. Bar = 1 μ m.

Figure 6.24 Zona pellucida of an ovulated oocyte of the brushtail possum incubated with biotinylated-*Glycine max* (soybean) lectin (biotin-SBA) and labelled with 10nm gold-conjugated goat anti-biotin antibody. Note the moderate levels of labelling of the zona pellucida (ZP), microvilli (M) and mucoid coat (MC) of the ovulated oocyte (Oo). Gold labelling of the zona pellucida appeared to be specific to the regions of higher electron density. SBA binding is specific for α -D-N-acetylgalactosamine/ α -D-galactose. Bar = 1 μ m.



on the ventral surface, as in koalas and wombats (Harding *et al.*, 1987; Temple-Smith & Taggart, 1990; Breed *et al.*, 2001), and uniquely, in the American opossums, sperm-head pairing, whereby two sperm become joined at the acrosomal region of the head (Temple-Smith & Bedford, 1980; Moore & Taggart, 1993; Taggart *et al.*, 1993; Moore, 1996).

Biochemical modifications also occur to marsupial sperm as they traverse the epididymis with evidence of regionally specific secretion of glycoproteins from the epididymal epithelium (Lamont *et al.*, 1998), as well as a rearrangement of proteins on the surface of the sperm head as demonstrated by freeze-fracture studies (Cooper *et al.*, 1998). Modifications of the glycoconjugates on the sperm surface during epididymal transit (Cooper *et al.*, 2001) also occur that are consistent with variations in glycosidase activity within epididymal fluid (Knowling *et al.*, 1999).

Pre-fertilisation maturational changes of marsupial spermatozoa within the female reproductive tract are less clearly understood. Depending on the species, sperm may reside within the oviduct for varying lengths of time. In dasyurid and American didelphid marsupials, specialised infolding or crypts of the oviductal epithelium in the isthmic region occur within which sperm reside, primarily immotile, until ovulation (Rodger & Bedford, 1982; Selwood & McCallum, 1987; Bedford & Breed, 1994; Taggart *et al.*, 1998). At ovulation, sperm display a change in morphology: in the dasyurid *Sminthopsis crassicaudata*, sperm change from a streamlined configuration to once again become T-shaped (Bedford & Breed, 1994); in the didelphid *Didelphis virginiana*, paired sperm joined at their heads separate (Rodger & Bedford, 1982). This change to a T-shaped configuration and separation of the sperm pairs have thereby been suggested to represent the marsupial equivalent of capacitation that is commonly represented in eutherian sperm as a hyperactive state.

Following "capacitation" marsupial sperm then leave the isthmic crypts and make their way to the site of fertilisation (Rodger & Bedford, 1982; Bedford & Breed, 1994). To date, while such specialised isthmic crypts have not been described in other marsupial species, sperm appear to adhere to oviductal epithelium, albeit temporarily, both *in vivo* where sperm from the brushtail possum were found localised to epithelial folds within the isthmus (Jungnickel *et al.*, 2000), and *in vitro* with tammar wallaby sperm adhering to oviduct epithelial cell monolayers (Sidhu *et al.*, 1998). Following adherence, a greater number of both tammar wallaby and possum sperm appeared to have the "capacitated" T-shape configuration as seen in the *in vivo* dasyurid studies (Sidhu *et al.*, 1998; Jungnickel *et al.*, 2000).

In Australian marsupials, modifications of spermatozoa within the oviducts appear to be necessary for successful sperm-egg interaction. Sidhu *et al.* (1999) found that oviductal proteins bind to the surface of sperm and significantly increase their survival rate *in vitro*, while also increasing the number of sperm in the T-shape configuration. Even with the "capacitated" T-shape configuration, however, sperm do not necessarily bind to and/or penetrate the ZP (Bedford & Breed, 1994). It is only after pre-incubation in oviduct-conditioned media that possum spermatozoa have been found to bind and penetrate the ZP *in vitro*, although sperm-oolemma binding and fertilisation still did not occur (Mate *et al.*, 2000). The importance, therefore, of the oviduct in the pre-fertilisation maturation of spermatozoa is therefore indicated, although the inability of sperm to bind to the oolemma, even after incubation in oviduct-conditioned media, suggests that some factor was still missing from the *in vitro* environment in these experiments. The fact that the oocytes used in the above experiments were *in vitro* matured ovarian oocytes that had had no contact with the oviduct environment suggests that the possible pre-fertilisation maturation of oocytes within the oviduct may have been overlooked.

While some of the pre-fertilisation maturation events that occur to marsupial oocytes immediately prior to ovulation have been described, less is known about the events that occur following ovulation. Almost all of the events described, however, for either pre- or post-ovulatory maturation, have been purely morphological observations. For example, it has been suggested that formation of cortical granules only occurs immediately prior to ovulation (Mate *et al.*, 1992; Mate, 1996), although evidence suggests that they are formed at an earlier stage with the majority only moving to a sub-cortical position during the pre-ovulatory period (Breed, 1996; Ullmann & Butcher, 1996; Frankenberg & Selwood, 2001; Kress *et al.* 2001).

Marsupial oocytes also appear to increase in size dramatically during the pre-ovulatory period, suggested to occur either due to an accumulation of fluid or due to an increase in lipid and/or electron lucent vesicles (Rodger *et al.*, 1992; Mate, 1996), and while there was a reported decrease in vitellus diameter of the brushtail possum oocyte following ovulation, there was even a reported increase in oocyte diameter following ovulation in the bettong (Rodger *et al.*, 1992). This finding differs from that described for that of eutherians, where oocytes complete their growth phase during follicular antrum formation (Lintern-Moore *et al.*, 1976; Wassarman & Albertini, 1994). The only subsequent increase in diameter of eutherian oocytes appears to be in the volume of the perivitelline space following ovulation and exposure to the oviductal environment (Yang & Yanagimachi, 1989; Boatman *et al.*, 1994).

Apart from the changes indicated above, there is also a claim that the ZP of the brushtail possum changes from being "thick and diffuse" before ovulation to "thin and compact" after (Rodger & Mate, 1993). This observation was not supported by observations on the pre- and post-ovulatory structure of the ZP of *Sminthopsis crassicaudata* (Breed, 1996). Despite the fact that there have been several studies into the biochemical changes that

occur to spermatozoa during pre-fertilisation maturation, there have been no attempts to investigate if any biochemical changes occur to the oocyte during pre-fertilisation maturation, especially following ovulation. This chapter, therefore, attempted to qualify and quantify the morphological and biochemical changes that occur to the oocyte, particularly the ZP, following ovulation. Morphologically, the ultrastructural changes of the ZP following ovulation were investigated using a variety of ultrastructural techniques, whilst biochemically, the glycoconjugate composition of the ZP before and after ovulation were quantified using high resolution lectin-gold cytochemistry.

6.4.2 Ultrastructure of the Pre- and Post-Ovulatory Zona Pellucida

6.4.2.1 Overview of the Ultrastructure of the Eutherian Zona Pellucida

A number of studies have used ultrastructural methods to investigate the development of the ZP, from those surrounding immature oocytes to those surrounding fertilised zygotes and embryos, with many of these studies particularly using scanning electron microscopy (SEM) as their investigative tool. Early ultrastructural studies using routine SEM demonstrated the characteristic "Swiss-cheese" appearance of the ZP of mature ovulated and fertilised eggs of hamsters and mice (Jackowski & Dumont, 1979; Phillips & Shalgi, 1980*a,b*). There was some controversy, however, over whether fertilisation resulted in a change in morphology of the ZP (Jackowski & Dumont, 1979; Phillips & Shalgi, 1980*a*).

The ultrastructural characteristics of developing ZP from juvenile mice were also investigated using routine SEM (von Weymarn *et al.*, 1979). This study found that the ZP gradually changed from having a smooth surface to one that showed a smooth fibrous network-like structure with some small pores that eventually became more pronounced resulting in a fine fibrous network. These characteristic ultrastructural features of ZP

development have been reported in other eutherian species such as the human (Familiari *et al.*, 1988), cow (Suzuki *et al.*, 1994) and pig (Funahashi *et al.*, 2000, 2001), in which the mesh-like arrangement of the ZP only appeared following maturation. More detailed investigations of the ultrastructure of the mouse ZP (Familiari *et al.*, 1989) and human (Familiari *et al.*, 1992) were performed using oocytes pre-treated with the detergent saponin, to remove masking amorphous material; these were contrasted, and stabilized, with the cationic dye ruthenium red (RR). Using this method, the ZP of immature and atretic follicular oocytes were found to be smooth and compact, whilst those around mature oocytes at the time of fertilisation had the typical "spongy" appearance (Familiari *et al.*, 1992). While some mature oocytes also appeared to have smooth and compact ZP, they were found less frequently, and were less likely to be fertilised (Familiari *et al.*, 1992).

6.4.2.2 SEM of Routine and Ruthenium Red and Saponin-treated Oocytes and Zona Pellucida of the Brushtail Possum

In the present study, ovarian and ovulated oocytes of brushtail possums were processed both routinely and using saponin and RR for SEM. Routine SEM demonstrated that the ZP of the brushtail possum appeared to exhibit a similar pattern of maturation to that described for the eutherian species, with ovarian oocytes having a smooth and highly compacted appearance, while the ZP around ovulated oocytes were highly filamentous. The stage at which this morphological change takes place was not determined as all of the ovarian oocytes used in this study were from large follicles. Such a late maturational change to the structure of the ZP, during the peri-ovulatory period may differ from that of most eutherian zonae which appear to become filamentous in structure well before ovulation. The exception, as mentioned above, is the human in which some of the mature oocytes demonstrated ZP with a smooth and compact appearance (Familiari *et al.*, 1992). As these mature oocytes with "immature" zonae were much less likely to be fertilised, however, the

late maturation of the possum ZP may be at least one factor that reduces the chances of successful *in vitro* fertilisation in this species and, perhaps, other Australian marsupials.

The maturational change in morphology of the ZP was also found when oocytes were processed in the presence of saponin and RR, although this was not as easily observed due to artefactual masking by globular material. Ruthenium red was used in this study in an attempt to act as a stabilising and contrasting agent with the cationic dye preserving the anionic components of the ZP glycoproteins (Familiari *et al.*, 1989, 1992). Unfortunately, this was not always successful since the addition of RR to the fixative sometimes resulted in the bovine ZP having globular units that covered the underlying matrix and thus obscured its detail (Tengowski & Schatten, 1997). Only following the present study was it found that, in some cases, the addition of cationic dyes such as RR could cause a breakdown of polyanionic chains resulting in them appearing as condensed granules (Hunziker & Schenk, 1984). This does not always appear to occur, however, with the ZP of the mouse and human superbly visualised using RR (Familiari *et al.*, 1989, 1992). For some reason, the bovine ZP and, now, it would seem the possum ZP have glycoproteins that do not preserve well with RR for visualisation by SEM. As RR has been used on the oocytes of possums previously in this thesis with great success as a visualising tool (see Chapter 7), it should still be considered as having some value in investigations into the egg coats of marsupials.

6.4.2.3 Cryo-EM of Ovarian and Ovulated Oocytes and Zona Pellucida

The second technique used was that of Cryo-electron microscopy (cryo-EM). Cryo-EM is based on the concept that if tissue is frozen very rapidly then, at an appropriately cold temperature (usually below -80°C), intracellular water will not form ice crystals and will be immobilised, basically frozen in time, thereby preserving the native state of the tissue

(Bachmann & Mayer, 1987; Sitte *et al.*, 1987; Wilson *et al.*, 1998). This technique has the advantage of overcoming many of the artefacts introduced through chemical fixation and dehydration that are required for routine EM. It potentially has many applications including providing a more accurate visualisation of the molecular and ultrastructure of intra- and extra-cellular components (e.g. ribosomes: for review see Frank, 2001; mitochondria: Nicastro *et al.*, 2000; general biological components: Erk *et al.*, 1998), and immuno-localisation (Tokuyasu, 1997; Monaghan *et al.*, 1998). Cryo-EM has only rarely been used to study the ultrastructure of egg coats with rapid freezing and freeze substitution (FS) for transmission EM (TEM) used to visualise the extracellular matrices of hamster (Yudin *et al.*, 1988; Cherr *et al.*, 1990) and sea urchin eggs (Cherr *et al.*, 1992), while high-pressure freezing and FS has been used for visualisation of the bovine ZP using SEM (Tengowski & Schatten, 1997).

In the present study, two methods of cryo-EM were investigated on the ovarian and ovulated oocytes of possums, cryo-SEM and rapid freezing and FS for TEM. The first method, cryo-SEM, involved freezing oocytes in liquid nitrogen slush before sublimation of excess ice, sputter-coating and visualisation. This method proved quite difficult to master as the sublimation step required excess ice to be removed by increasing the temperature of the cryo-transfer chamber from around -120°C to -90°C for a few minutes, during which time visualisation of this process was virtually impossible. As a result, excess sublimation resulted in structural damage to some parts of the external surface of the ZP. Once specimens were moved into the main vacuum chamber of the microscope, it was also found that the resolving power of the microscope of the frozen specimens was very poor and provided little ability to visualise the fine detail of the surface characteristics of the ZP. This may have been due to the following reasons.

Firstly, in order to view the frozen specimens, despite being sputter-coated, only a low-voltage electron beam could be used, and even that for only short amounts of time, as higher voltage and lengthier investigations were found to result in severe structural damage to the surface features. Higher voltage equates with better resolving ability but also increases the capacity of the electron beam to cause damage, even with fixed, metal-coated specimens. The electron beam, in the case here, was found to increase the temperature of the frozen samples fairly rapidly (in less than 120 sec), thereby destroying the ZP structural integrity, the very thing the method of cryo-SEM was trying to preserve. As a consequence of this, only a very low-voltage (~1kV) electron beam could be used, which, of course, resulted in a trade-off of poor resolving power.

Secondly, the fine structure of the ZP appeared to be obscured, with only the general features of underlying filaments being resolved. It is unclear whether this was due to structural damage caused by excess sublimation, the true representation of a high proportion of amorphous material interspersed between the ZP filaments, or a result of the sputter coating.

Familiari *et al.* (1989, 1992) used the detergent saponin during fixation of mouse and human oocytes in order to remove any amorphous material from the ZP that may have obscured the fine structural detail of the filaments. Saponin was used in the fixation steps for SEM of the ZP of ovarian and ovulated oocytes, as described above, and, if anything, the filaments of the ZP of routinely processed oocytes (without the addition of saponin) appeared to be more easily resolved than those processed in the presence of saponin. Whether the decrease in resolution of the ZP filaments in the saponin-treated oocytes was due to the artefact caused by RR (as discussed above), or perhaps a stabilisation of the amorphous material by RR, is not clear. The finding that RR bound to material within the perivitelline space of ovulated oocytes, as well as the mucoid layer outside of the ZP (see

Chapter 7), however, might suggest that the RR may have preserved some oviductal glycoproteins on the surface of the ZP, thereby partially obscuring the underlying ZP filaments. Due to the great variability in the timing of ovulation of super-ovulated possums, ovulated oocytes have differing times of exposure to the oviductal environment before collection and fixation. Although ovulated oocytes without any noticeable mucoid layer were selected for study, it is possible that even small amounts of oviductal glycoproteins may have adhered, and therefore even obscured, the ZP following fixation, both in the presence of RR, and in oocytes frozen for cryo-SEM.

The second possible obstruction to observation of the fine filamentous detail of the possum ZP using cryo-SEM is the fine-metal sputter coating used to stabilise the surface and provide sufficient contrast. Tengowski and Schatten (1997) compared several methods of fixation, processing and preparation of bovine oocytes in order to produce the best detail of ZP structure under low voltage SEM. Conventional chemical fixation with no sputter-coating and high-pressure freezing and FS were found to be the best protocols, while conventional sputter-coating and fixation in the presence of RR hindered the ability to resolve the fine detail of ZP filaments (Tengowski & Schatten, 1997). Conventional sputter coating uses metals of up to 30nm (e.g. gold) which, if the measurements of 9.5nm for the ZPA:ZPC heterodimer "beads on the string" are to be used as an example (Greve and Wassarman, 1985), would clearly interfere with an accurate visualisation of the filaments. The likelihood that the sputter coating of the cryo-SEM oocytes obscured the filaments of the ZP is relatively unlikely, however, due to the fact that 2-3nm gold/palladium was used. Whatever the reason for the poor resolution of the possum ZP using cryo-SEM, it was not as informative of the ZP structure as conventional SEM, or even saponin- and RR-treated oocytes despite the artefacts. While this cryo-EM technique did not yield good results, the method of high-pressure freezing and FS for SEM provided Tengowski and Schatten (1997) with some of excellent results for visualization of the bovine ZP.

Rapid freezing and FS were used here to visualise the ultrastructure of the ZP of possum ovarian and ovulated oocytes using TEM, based on the work by Yudin *et al.* (1988) and Cherr *et al.* (1990, 1992). Using this method, the ZP of the possum ovarian and ovulated oocytes appeared to differ significantly from each other in morphology. The ZP of the ovarian oocytes were filamentous in nature but with little order to the arrangement and orientation of the filaments. The ZP of the ovulated oocytes, however, were also highly filamentous in nature but with a highly ordered structure, such that it appeared honeycomb-like in regularity. The filamentous nature of the ZP of the possum ovarian oocytes was unusual in that it was rarely seen as such in routine TEM and SEM, the ZP typically appearing more granular than filamentous. Whether this was due to some form of compaction of the ovarian ZP during the dehydration steps used in routine processing that resulted in the collapse of the filaments so that the ZP appeared dense and granular is unknown. The outer cumulus cells may also exert an external compressive force, particularly following dehydration, which could result in further compaction of the ovarian zonae.

Supporting its appearance using SEM, however, the outer region of the matrix of the ovarian ZP, close to the cumulus cells, appeared denser than the inner region of the matrix that was closest to the oocyte. No such morphological variation was noted of the ZP of ovulated oocytes, with its entire width appearing uniformly structured; this was similar to the observations of the ZP matrix for the golden hamster (Yudin *et al.*, 1988). This homogeneous structure is contrary to the multilaminar structure found for hamster ZP by polarised light microscopy, where it is described as being divided into inner and outer layers separated by an intermediate layer of low retardance, with the inner layer composed of filaments arranged radially and the outer layer composed of filaments arranged tangentially (Keefe *et al.*, 1997). Eutherian ZP have often been described as consisting of

two layers: an inner, densely packed layer that is closest to the oolemma; and an outer, less dense layer close to the cumulus cells (for references, see Yanagimachi, 1994). This appears to be quite different for the structure of the mature possum ovarian ZP, with the outer region of the ZP appearing much denser than the area immediately adjacent to the oolemma. Such a feature has also been noted in the mature ovarian oocytes of the koala (see Chapter 3) and noted previously in the brushtail possum (Hughes, 1984). Such a heterogeneous structure of marsupial ZP is not seen under light microscopy on living cells (see Fig. 8.1, Chapter 8, for Nomarski differential optics of possum ZP of unfixed ovarian oocyte), however, the relative thinness of marsupial ZP may make visualisation of any heterogeneity difficult.

The ZP filamentous structure, as determined by rapid freezing and FS, of the zonae of ovulated oocytes of possums seems to be unique in terms of its complexity and regularity. These filaments give the matrix a highly distinctive trabeculae-like appearance with electron-dense filament backbones surrounded by diffuse material that branches into the fenestrae, which at higher magnification give the filaments a "snowflake" resemblance. Each filament appears to originate or terminate at a point with two other filaments giving the matrix a hexagonal or honeycomb appearance, which is only hinted at under SEM. When comparisons are made between the results obtained by routine SEM and rapid freezing and FS for TEM of ovulated oocyte ZP, the electron-dense backbones seen with TEM may represent the larger structural branching filaments seen with SEM, while the more diffuse branching material may be the smaller filaments found underneath, and within, the fenestrae that are created by the larger filaments. This architecture and sub-architecture of the ZP has been found in several eutherian species, including those from the human (Familiari *et al.*, 1992) and cow (Tengowski & Schatten, 1997). In these species, the ZP are comprised of large structural filaments that make up the majority of the gross ZP matrix, while the larger filaments themselves appear to be composed of a fine

micromeshwork of fibrils (Tengowski & Schatten, 1997), suggesting that there is a high degree of conservation of its basic morphology between eutherian and marsupials mammals; a conclusion that is perhaps not entirely surprising due to the high degree of homology between the ZP genes and putative polypeptide sequences (Mate & McCartney, 1998; Haines *et al.*, 1999; McCartney & Mate, 1999; Voyle *et al.*, 1999).

While there may be a conservation of basic morphology of the ZP, there may be differences functionally that relate to the structural differences. In eutherians, it has been argued that spermatozoa preferably reach the oocyte by passing through the fenestrae of the ZP (Tsuiki *et al.*, 1986), and that the filaments on the surface of the ZP may be loosened by some factor (suggested to be secreted by the cumulus cells) to increase their penetrability (Tesarik *et al.*, 1988). Suzuki *et al.* (1994) has argued that the morphological change of the ZP from a smooth matrix to one with a mesh-like arrangement is evolutionarily significant in that the appearance of the fenestrae is important for orientating the spermatozoon for fertilisation. In marsupials, such a functional relationship to the structure of the ZP seems unlikely. The fenestrae of the possum ZP appear to range between 0.1-1µm in diameter, which is much smaller than the size of the sperm head of the possum, suggesting that the possum sperm could not use the fenestrae as means of penetration. This is supported by studies of sperm-egg interaction of *Monodelphis domestica* and *Sminthopsis crassicaudata* in which sperm penetrating the ZP appear much larger than the fenestrae of the surrounding matrix (see Fig. 16d, p.627 and Fig. 18, p. 628 of Breed, 1996). The fact that the cumulus cells denude from the surface of the zona pellucida before ovulation also suggests that the secretion of a 'softening' factor for the zona is unlikely in marsupials.

So does the ZP of the brushtail possum undergo a morphological change from 'broad and diffuse' prior to ovulation, to 'thin and compact' after ovulation? Using a variety of

ultrastructural methods in this study, there does appear to be a late maturation of the ZP of the brushtail possum, which is represented by a fairly distinctive morphological change from a compact, dense matrix to a highly filamentous, ordered structure. The descriptions of the ZP are less than accurate, however, as the ZP of ovarian oocytes did not appear to be diffuse. In fact under SEM it was quite the opposite, except perhaps, for the region immediately adjacent to the oolemma, while the ZP of ovulated oocytes did not appear to be compact with numerous fenestrae separating the highly filamentous matrix. The question of whether ZP change from being broad prior to ovulation to thin after ovulation was not investigated. While some differences in thickness have been noted between ZP from ovarian and ovulated oocytes using routine TEM, with the ovarian ZP appearing to be somewhat thicker than that of ovulated ZP (Chapman, unpublished observations), it is not clear if this difference is due to processing, angle of section or a true reflection of morphological variation. If any change in thickness of ZP occurs, it might take place immediately prior to ovulation when the cumulus cells break their junctions with the oolemma, retract their cytoplasmic extensions, and separate from the ZP surface. The withdrawal of the cytoplasmic extensions would more than likely cause a reduction in the volume of the ZP and, therefore, thickness, as the matrix readjusts as a result. What the functional significance is of such a change in structure is unknown. In some eutherian species, only the oocytes that have ZP that have undergone the maturational change, from a smooth surface to a mesh-like arrangement, are likely to bind sperm and become fertilised (Familiari *et al.*, 1988). In Australian marsupials, however, even those oocytes that have 'mature' ZP (i.e. ovulated oocytes) have been unable to bind sperm *in vitro*, unless the sperm have been pre-exposed to oviductal factors, while sperm-egg binding and fertilisation still remain allusive (Mate *et al.*, 2000). While the morphological changes that occur to the ZP following ovulation are a bit clearer, it seems that morphological change alone does not increase its receptivity. As such, the potential biochemical changes that might occur to the ZP within the oviduct were investigated.

6.4.3 Glycoconjugates of the Zonae Pellucidae of Ovarian Oocytes and Their Modifications Following Ovulation

As mentioned in Chapter 5, in eutherians carbohydrates have been found to play an important role in fertilisation (for reviews see Benoff, 1997; Shalgi & Raz, 1997; Tulsiani *et al.*, 1997; Dell *et al.*, 1999; Topfer-Petersen, 1999). Primary binding of sperm takes place through the glycoconjugates of the oligosaccharides of the ZP glycoproteins (for example, Huang Jr *et al.*, 1982; Bleil & Wassarman, 1988; Litscher *et al.*, 1995; Chapman & Barratt, 1996; Maegawa *et al.*, 2002). Investigations into the glycoconjugates of the ZP surrounding ovarian oocytes of a number of eutherian species found that there appeared to be variation in glycosylation patterns that have been suggested to be important in the species-specificity of the sperm-ZP binding (Nicolson *et al.*, 1975; Skutelsky *et al.*, 1994; Parillo *et al.*, 1996; Parillo & Verini-Supplizi, 1999, 2001). The glycosylation of the ZP surrounding ovarian oocytes, however, appears to be different from that that meets the spermatozoon within the oviduct, with oviductal exposure resulting in modification of ZP glycoconjugates, presumably due to the addition of oviduct-specific glycoproteins (OGPs) (for reviews see Bleau & St-Jacques, 1989; Hunter, 1994; Malette *et al.*, 1995; Boatman, 1997; Buhi *et al.*, 1997, 2000). The role of the oviduct in the post-ovulatory structure and composition of the ZP have not been investigated in marsupials. The finding that oviductal proteins bind to sperm of the brushtail possum (Sidhu *et al.*, 1999), while, in the previous chapter of this thesis, regional variation in secretory glycoconjugates of the possum oviduct was demonstrated suggest that the ampulla and isthmus may play different roles in the structure and interaction of the gametes. As such, the glycoconjugate composition of the ZP surrounding oocytes before and after ovulation, and of the mucoid coats of ovulated oocytes, were investigated to determine if there was any variation in glycoconjugates that result from oviductal exposure.

Lectin gold labelling of the possum ZP of ovarian oocytes was similar to the lectin histochemical results obtained for the possum in chapter 4 of this thesis, with low levels of labelling with PSA and SBA and much greater labelling with WGA. Gold labelling with RCA-I, however, was greater than for PSA and SBA, even though no labelling was detected using lectin histochemistry. Generally, though, lectin reactivity of the ovarian ZP was much lower than that for the ovulated oocytes, with significant increases in lectin labelling following ovulation. All of the glycoconjugates that were tested for were found to have significantly increased in concentration within the ZP following ovulation, with the exception of GlcNAc and/or sialic acid (where a similar, but not statistically significant increase was found to occur). The mucoid coats surrounding the ovulated oocytes were found to have the highest density of labelling for GlcNAc and β -Gal, and less so of α -D-GalNAc/ α -D-Gal and α -D-Mannose.

The major increase in labelling of glycoconjugates within the ZP following ovulation suggests that one of two things might be occurring. Firstly, it might suggest that coinciding with the morphological change that occurs to the ZP following ovulation, there might be some kind of biochemical change that takes place, with perhaps a rearrangement of glycoconjugates. This rearrangement might be brought about either through some kind of oocyte/cumulus-specific modification that adds to, or exposes, previously masked glycoconjugates, or brought about by some oviductal factor that modifies the existing oligosaccharides to expose the glycoconjugates that were previously inaccessible to the lectins. As shown in Chapter 4 of this thesis, reactivity to lectins was increased in ovarian ZP following removal of masking agents such as sialic acid with the use of the enzyme neuraminidase, and O-acetyl groups on sialic acids, through saponification with KOH. Neuraminidase has been found to be present, and play a role in fertility, within the oviducts of non-mammalian species such as toads (De Martinez & Olavarria, 1973; De Martinez *et al.*, 1975; Vitaioli *et al.*, 1990). It has also been suggested that in the human, the cumulus-

coronal cells may secrete a factor that increases the penetrability of the ZP (Tesarik *et al.*, 1988), while removal of sialic acid from the surface of sperm and ZP has been shown to increase sperm-ZP binding (Lassalle & Testart, 1994; Banjeree & Chowdhury, 1997; Ozgur *et al.*, 1998). Whether a similar modification is occurring to the post-ovulatory zona pellucida of the brushtail possum is unknown.

The second possibility is for an increase in glycoconjugate content of the possum ZP following ovulation as a result of an incorporation of oviductal-specific glycoproteins (OGPs). In eutherians, as mentioned above, OGPs have been found to bind to the ZP in various species, including the hamster (Kan *et al.*, 1988, 1990), pig (Buhi *et al.*, 1993), sheep (Gandolfi *et al.*, 1991), cow (Staros & Killian, 1998) and baboon (Boice *et al.*, 1990). Particularly studied in the hamster, early investigations found that the oviduct contributed a glycoprotein, termed hamster oviductin 1 (Hm OV-1), to the post-ovulatory zona pellucida (Kan *et al.*, 1988, 1989), and that its contribution resulted in the addition of GalNAc residues, not previously identified in the ovarian ZP but localised to the secretory granules of the ampulla (Kan *et al.*, 1990). More recently, galactose glycoconjugates, in the form of α - and β -Gal, and α - and β -GalNAc, have been found to be the major transfers to the ZP following ovulation, while GlcNAc residues also increased in the outer portion of the ZP only (El-Mestrah & Kan, 2001).

Similarly in the possum, α -D-GalNAc/ α -D-Gal residues, which were labelled at very low levels in the ZP surrounding ovarian oocytes, were found to significantly increase in labelling following ovulation. These glycoconjugates, like in the hamster, were localised to the secretory granules of the ampulla, but not the isthmus, and only appeared to contribute relatively moderately to the mucoid coat. These findings suggest that α -D-GalNAc/ α -D-Gal residues, originating from the ampullary region of the oviduct and transferred to the ZP after ovulation, may play a role in sperm-zona pellucida interactions.

Alternatively, labelling with PSA for α -D-Mannose, also showed similar modifications. Demonstrating low levels of labelling within the ZP of ovarian oocytes, α -D-Mannose residues showed a significant increase in labelling following ovulation. It also had the lowest density of binding within the mucoid coats, and was significantly localised more in the secretory granules of the ampulla than in the isthmus. This suggests that the ampulla may be responsible for the addition of the α -D-Mannose residues within the post-ovulatory ZP. The other two glycoconjugates, β -Gal and GlcNAc and/or sialic acid, although major contributors to the glycoconjugate composition of the post-ovulatory ZP, they also appear to be major components of the mucoid coat with highest densities of labelling. β -Galactose was found to be relatively equally distributed between the secretory granules of the ampulla and isthmus, while GlcNAc and/or sialic acid residues were labelled significantly more in the secretory granules of the isthmus than the ampulla. Although not investigated here, it would be interesting to see if there was variation in the distribution of the various glycoconjugates throughout the complete mucoid layer and whether glycoconjugates such as β -Gal would be found throughout the entire mucoid layer, while GlcNAc and/or sialic acid would be found particularly localised to the outer layers, as suggested by the lectin immunocytochemical results.

The localisation of lectin labelling to the perivitelline space and microvilli of the oolemma, in conjunction with the findings from Chapter 7 of this thesis, further support the contention of the incorporation of oviductal glycoproteins into these areas. The exact role of these incorporated glycoproteins is unknown, however, the inability to achieve sperm-oolemma binding *in vitro* following pre-incubation of sperm, but not follicular oocytes, with oviduct-conditioned media suggests that they may play a role in sperm-egg fusion (Mate *et al.*, 2000).

Notwithstanding this, the present study has further supported the vital role of the oviduct in gamete maturation and interaction in an Australian marsupial. The secretions of the oviduct not only appear to be necessary for successful capacitation of spermatozoa and formation of the mucoid coat, but also for the composition of the post-ovulatory ZP and perivitelline space. The regional variation of glycosylation of the oviductal secretions noted in the previous chapter supports a more complex interaction between the oviduct and gametes than previously thought. While it has been previously postulated that some variation may exist for the secretion of the mucoid layer between the ampulla and isthmus (Roberts & Breed, 1996*b*), such intricate variation in terms of the specific glycosylation patterns of the oviductal glycoproteins and their possible contribution to the oocyte and the post-ovulatory ZP are entirely unique.

Chapter 7 The egg coats of the brushtail possum (*Trichosurus vulpecula*) following ovulation and artificial activation with particular reference to the origin of the Perivitelline Space Matrix

7.1 Introduction

In eutherian mammals, cortical granules (CG) are small (0.1-1.0 μm) membrane-bound organelles that are synthesised in the Golgi apparatus and migrate to within 1-2 μm of the plasma membrane of maturing oocytes (or oolemma) (for reviews see Ducibella, 1991, 1996; Hoodbhoy & Talbot, 1994). At fertilisation, egg activation occurs and a wave of calcium ions are released from internal stores within the agranular endoplasmic reticulum of the oocyte that rapidly spreads out from the point of fusion (Abbott & Ducibella, 2001). This release of intracellular calcium initiates several events including cortical granule exocytosis (Abbott & Ducibella, 2001). This exocytosis plays an important role in blocking further spermatozoa entry into the egg and the occurrence of polyspermy by inducing changes to the zona pellucida (ZP). The CG exudate has been found to contain several enzymes such as ovoperoxidase and β -N-acetylglucosaminidase (Hoodbhoy & Talbot, 1994). These CG enzymes may harden the ZP and/or partly cleave O-linked glycoconjugates from the sperm ligands of the ZP, thereby preventing subsequent sperm binding and penetration (Schmell & Gulyas, 1980b; Miller *et al.*, 1993; Iwamoto *et al.*, 1999). Cortical granule exocytosis, both natural and artificial, has also been found to modify the glycoconjugates of the ZP by both adding and removing glycoconjugates in the process (El-Mestrah & Kan, 2002).

In marsupials, little detail on CG exocytosis and function is known, although, as in eutherians, egg activation appears to involve a release of calcium ions (Witton *et al.*, 1999) and thus presumably CG exocytosis. Although cortical granule exocytosis occurs around the time of sperm penetration of the egg (Breed & Leigh, 1990; Selwood, 1992; Breed, 1996), the role of CG exocytosis in the prevention of polyspermy in marsupials is controversial as a mucoid layer comes to surround the oocyte shortly after it enters the oviduct which may thus form more of the barrier to polyspermic fertilisation (Rodger & Bedford, 1982; Selwood, 1982, 1992, 2000). It is unknown, therefore, if CG exocytosis has any effect on the structure and/or composition of the ZP in marsupials.

In mammals, material accumulates within the perivitelline space (PVS) around the time of ovulation, termed the perivitelline space matrix (PVM) (Denker & Gerdes, 1979; Denker, 2000). In marsupials, this material was first described in the American opossum *Didelphis virginiana* where it was shown to be composed of filaments and granules (Talbot & DiCarlantonio, 1984) and was subsequently found to occur in *Sminthopsis crassicaudata* (Breed & Leigh, 1988). Dandekar and Talbot (1992) and Dandekar *et al.* (1992, 1995) suggested that this matrix arose from CG exocytosis. They thus termed it the 'cortical granule envelope' (CGE) even though similar material had been found within the PVS of both ovarian and unfertilised ovulated oocytes (see Talbot & DiCarlantonio, 1984; Breed & Leigh, 1988; Breed, 1996). Thus, at the present time the origin of the matrix that occurs within the perivitelline space of marsupials is not clear.

Here the following questions were investigated: 1) can cortical granule exocytosis be artificially induced in the oocytes of the brushtail possum? 2) what changes, if any, occur to the egg coats that surround the ovulated oocyte following cortical granule exocytosis and do these changes result in the formation of the PVM? 3) what glycoconjugates are present within the cortical granules? and 4) what changes in glycoconjugate content, if any, occur to the ZP that surround the ovulated oocyte following cortical granule exocytosis

7.2 Materials and Methods

7.2.1 Animals

Female brushtail possums (*Trichosurus vulpecula*) were obtained and housed as previously described in Section 2.2.1.

7.2.2 Superovulation and Collection of Oocytes

Female brushtail possums were primed and their ovulated oocytes collected as previously described in section 2.3.

In order to determine if the CG envelope forms following CG exocytosis or from the incorporation of oviductal secretions, periovulatory ovarian oocytes were artificially activated

as described in section 6.2.2. They were then collected and cultured, as described above, until needed for experimentation.

The numbers of oocytes obtained and their subsequent experimental treatments can be found in Table 7.1.

7.2.3 Oocyte Culture and Artificial Activation

In order to determine the changes in egg coat appearance following CG exocytosis at activation, oocytes were artificially activated by a similar method to that used by Ducibella *et al.*, (1988) and Green (1989). For this, after washing the oocytes several times in divalent cation-free modified Whitten's medium (Hoppe & Pitts, 1973), they were placed in one of four treatment groups and incubated as follows: (i) artificial activation media (AAM), which was composed of Whitten's medium to which 10 µl/ml of calcium ionophore A23187 (Sigma Chemical Company, St. Louis, MO) (2 mg/ml dimethyl sulphoxide) had been added, for 5 min at 37°C, (ii) AAM for 15 min at 37°C, (iii) AAM for 30 min at 37°C, (iv) Whitten's medium with no ionophore for 30 min at 37°C. The presence of CGs in the oocytes was subsequently determined by transmission electron microscopy and lectin histochemistry (see below).

7.2.4 Determination of the Artificial Induction of CG Exocytosis

To determine the length of the incubation time required for CG exocytosis, oocytes were placed into 3% paraformaldehyde/3% glutaraldehyde made up in 0.2 M-phosphate buffer, pH 7.4 overnight at 4°C and then processed as previously described in section 2.4.

Table 7.1 Experimental treatment of superovulated animals, method of artificial activation and number of oocytes obtained. The experimental outcomes of the artificially activated (A) and ovulated (O) oocytes are also highlighted.

Possum No.	No. of superovulated oocytes	No. of Oocytes Artificially Activated by Treatment ^a				Treatment of Activated (A) and Ovulated (O) Oocytes ^b		
		7% ethanol	5min Ionophore	15min Ionophore	30min Ionophore	Routine TEM ^c	RRfix TEM ^d	Lectin-gold ^e
P38-3-00	10	-	-	10	-	10 (O)	-	-
P43-3-00	15	2	2	2	2	8 (A) 7 (O)	-	-
P44-3-00	10	2	2	2	2	8 (A) 2 (O)	-	-
P45-3-00	7	-	2	3	-	5 (A) 2 (O)	-	-
P2-4-01	8	-	-	4	-	-	-	4 (A) 4 (O)
P5-4-01	10	-	-	2	-	-	4 (O)	2 (A) 4 (O)
P7-4-01	6	-	-	3	-	-	3 (A) 3 (O)	-
P10-4-01	15	-	-	7	-	-	-	7 (A) 8 (O)

^aFor experimental methods used see section 7.2 Materials and Methods;

^bFor experimental methods used see section 7.2 Materials and Methods;

^c Routine TEM: oocytes fixed and processed for routine transmission electron microscopy;

^dRRfix TEM: oocytes fixed and processed in the presence of ruthenium red for transmission electron microscopy;

^eLectin-gold: oocytes fixed and processed for lectin immunocytochemistry

7.2.5 Determination of the Presence of a Matrix within the Perivitelline Space

In order to determine whether material is deposited in the perivitelline space following CG exocytosis, ovarian and ovulated oocytes (artificially activated or not) were processed as described above, except that there were immediately fixed in 3% glutaraldehyde containing 0.5% ruthenium red (RR) (Sigma Chemical Company) in 0.1M cacodylate buffer, pH 7.4, for either 1-3 h at room temperature or overnight at 4°C. They were then washed several times in cacodylate buffer containing 0.5% RR, post-fixed with 1% OsO₄ in cacodylate buffer containing 0.5% RR for 1-2 h, washed in cacodylate buffer, dehydrated, and processed as described in section 2.4 (see Dandekar *et al.*, 1995).

Resin blocks containing the oocytes were then sectioned, stained with uranyl acetate and lead citrate and viewed under the transmission electron microscope as described in section 2.5 for determination of the presence of RR-positive material within the perivitelline space.

7.2.6 Determination of CG and ZP Glycoconjugate Composition

In order to gain some insight into the glycoconjugate composition of the CGs and any changes in glycoconjugate content of the ZP following activation, ovarian and ovulated oocytes were either fixed immediately after recovery, or following artificial activation as described above, prior to being processed for lectin histochemistry and/or lectin immuncytochemistry.

Ovulated and artificially activated oocytes were immediately immersed in 2% glutaraldehyde made up in 0.1% cacodylate buffer, pH 7.4, and fixed for 1-2 h at room temperature or overnight at 4°C. They were then washed in cacodylate buffer and dehydrated by passing them through a graded series of alcohols before being processed in LR Gold as described in section 6.2.4.

For lectin histochemistry, 1µm thick sections were then cut, placed onto a drop of distilled water on aminopropyltriethoxysilane (APES) -coated glass slides and transferred to a hot plate until dry. Sections were then covered in 1% BSA in 0.05M Tris buffered saline (TBS), pH 7.6 for 30 min at room temperature and then incubated in one of three fluorescein isothiocyanate (FITC) -conjugated lectins (Vector Laboratories, Burlingame, CA), each of which is specific for a particular sugar(s). These were: (i) wheat germ agglutinin (*Triticum vulgaris*: WGA) for (β-(1-4)-D-*N*-acetylglucosamine)₂, (=GlcNac)₂ and *N*-acetyl-neuraminic acid (sialic acid), (ii) Jack bean lectin (*Concanavalia ensiformis*: Con A) for α-D-Mannose and α-D-Glucose, and (iii) lentil lectin (*Lens culinaris*: LCA) for α-D-Mannose. The lectins were used at a concentration of 20 µg/ml for Con A and LCA and 10 µg/ml for WGA. Incubations were carried out for 30 min at room temperature in a humidified light-safe chamber. The sections were then rinsed three times in TBS, a drop of antifade solution added (Slowfade Antifade Kit, Molecular Probes, USA), and a coverslip applied. Sections were viewed with an Olympus BH epifluorescent and phase-contrast microscope, using a 515 nm excitation filter and an IFK barrier filter with an absorption wavelength of 535 nm and an emission wavelength of 617nm.

For specificity controls, lectins were incubated with 0.4-1.0 M/L of their specific sugar made up in distilled water for 30 min.

For lectin immunocytochemistry, once appropriate sections were found, excess resin was trimmed away and thin (~90nm) sections were cut and placed onto nickel grids coated with a supporting film of 2% Collodion in Amyl Acetate (Electron Microscopy Sciences, Fort Washington, PA) and allowed to air dry for 24h.

Lectin-gold immunocytochemistry was then performed following the method described previously in section 5.2.3.2. Briefly, the sections had non-specific labelling blocked with 1% BSA, 1% gelatin and 0.2M glycine prior to incubation in one of four biotinylated lectins (Vector Laboratories, Burlingame, CA). Following incubation in the lectin, sections were then washed before being placed onto a drop of 10nm colloidal gold-conjugated goat anti-biotin antibody (diluted 1:30 in 0.5M TBS) for 1 hour. Grids were then washed, counter-stained in 70% alcoholic uranyl acetate and viewed on a Philips CM100 TEM with digital images captured on a Mega-View II digital image capture system (Soft Imaging System, GmbH, Germany). Gold particles were counted and underwent quantitation using the analySIS 2.1-image analysis software (Soft Imaging System, GmbH, Germany).

The biotinylated lectins used in this part of the study, the same as those used in Chapter 6, were: (1) Garden Pea lectin (*Pisum sativum*: PSA) for α -D-Mannose, (2) Castor bean lectin (*Ricinus communis* I: RCA-I) for β -D-galactose, 3) Wheat germ agglutinin (*Triticum vulgaris*: WGA) for $(\beta$ -(1-4)-D-*N*-acetylglucosamine)₂, and *N*-acetylneuraminic acid (sialic acid), and 4)

Soybean lectin (*Glycine max*: SBA) for α -D-N-acetylgalactosamine/ α -D-galactose. Lectins were used at a concentration of 20 μ g/ml, except for WGA that was used at a concentration of 10 μ g/ml. For specificity control, the biotinylated lectins were incubated with 0.4-1.0M/L of their specific sugar made up in distilled water for 30 min prior to the application of the lectins to the grids.

Statistical analyses, specifically the student's t-test, were performed on gold particle counts of the same lectin between the ZP of artificially activated oocytes, as well as the ZP of ovarian and ovulated oocytes determined in the previous chapter using Systat statistical analysis software (Systat Software Inc., California) and differences were considered significant where $p < 0.05$.

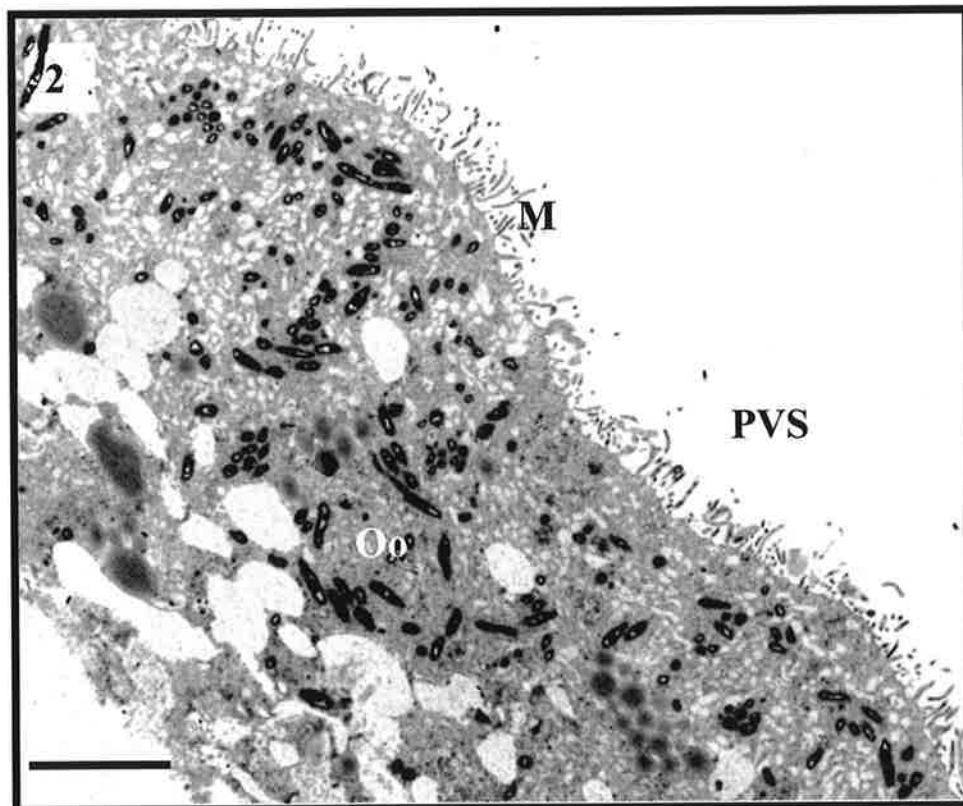
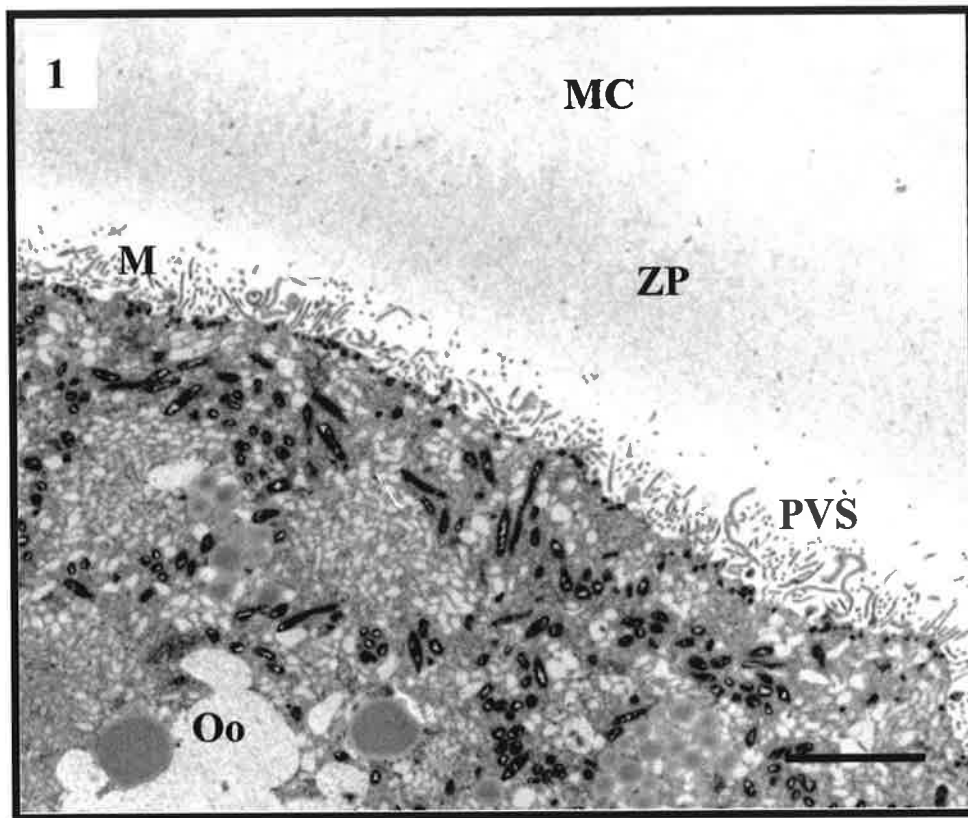
7.3 Results

7.3.1 Artificial Induction of CG Exocytosis

Control ovulated oocytes, which were incubated for 30 min in the modified Whitten's medium, displayed numerous cortical granules as well as a prominent perivitelline space (Fig. 7.1). However, ovulated eggs incubated in the medium containing the calcium ionophore, A23187, had fewer cortical granules, even after 5 min exposure, whereas by 15 min no cortical granules could be found (Fig. 7.2). As a result of this observation, subsequent studies for determination

Figure 7.1 Transmission electron micrograph of a control ovulated oocyte of a brushtail possum that was incubated for 30 min in modified Whitten's medium alone. Numerous electron dense cortical granules can be seen directly underlying the microvilli (M) of the control oocyte (Oo); muroid coat (MC); perivitelline space (PVS); zona pellucida (ZP). Bar = 2 μ m.

Figure 7.2 Transmission electron micrograph of an ovulated oocyte of a brushtail possum that was artificially activated for 15 min in calcium ionophore, A23187. Very few electron dense cortical granules are present underlying the microvilli (M) of the artificially activated oocyte (Oo); perivitelline space (PVS). Bar = 2 μ m.



of the effects of CG exudate on egg coat structure involved incubation in the ionophore for 15 min.

7.3.2 Ultrastructural Origin of the Perivitelline Space Matrix

Artificially activated ovulated oocytes that had been processed in the presence of ruthenium red (RR) had a prominent mucoid layer peripheral to the ZP (Fig. 7.3). Within the oocytes however, no CGs could be seen in the cortical region suggesting that their exocytosis had taken place. Within the PVS, a highly conspicuous, 1.5-2 μ m wide band of RR-stained material, could be seen on the inner surface of the zona pellucida as well as to a lesser extent in association with the microvilli of the oocyte. RR-stained material was also found within the ZP adjacent to the PVS and, less so, towards the outer part of the matrix (Fig. 7.4).

Non-activated ovulated eggs processed in the presence of RR also demonstrated material within the PVS. This material was found to be particularly prominent in oocytes surrounded by the most extensive mucoid layer (Fig. 7.5). In oocytes that had the thinnest mucoid layer, numerous cortical granules were evident underlying the oolemma with a small amount of ruthenium red-stained material in the PVS (Fig. 7.6). In those oocytes with more extensive mucoid coats, numerous cortical granules were still evident within the cortical ooplasm while a highly prominent RR-stained matrix was present within the PVS (Fig. 7.5). Egg microvilli also stained with RR and appeared to be more prominent in the oocytes with the thicker mucoid layers (Fig. 7.7).

Figure 7.3 Transmission electron micrograph of an ovulated oocyte of a brushtail possum that was artificially activated for 15 min in calcium ionophore, A23187 and processed in the presence of ruthenium red. The oocyte is surrounded by a prominent mucoid coat (MC) stained intensely with the ruthenium red, peripheral to the zona pellucida (ZP). Note also ruthenium red-stained material within the perivitelline space (PVS); electron lucent vesicles (V) are evident in the oocyte cytoplasm; microvilli (M). Bar = 2 μ m.

Figure 7.4 Transmission electron micrograph of higher magnification of the zona pellucida (ZP) of an ovulated oocyte of a brushtail possum that was artificially activated for 15 min in calcium ionophore, A23187 and processed in the presence of ruthenium red. The zona pellucida appears denser close to the mucoid coat (MC) than to the perivitelline space (PVS). Ruthenium red staining is also noted within the zona pellucida, particularly close to the perivitelline space, but also in bands throughout the zona matrix (arrows) leading from the external surface to the perivitelline space. Bar = 1 μ m.

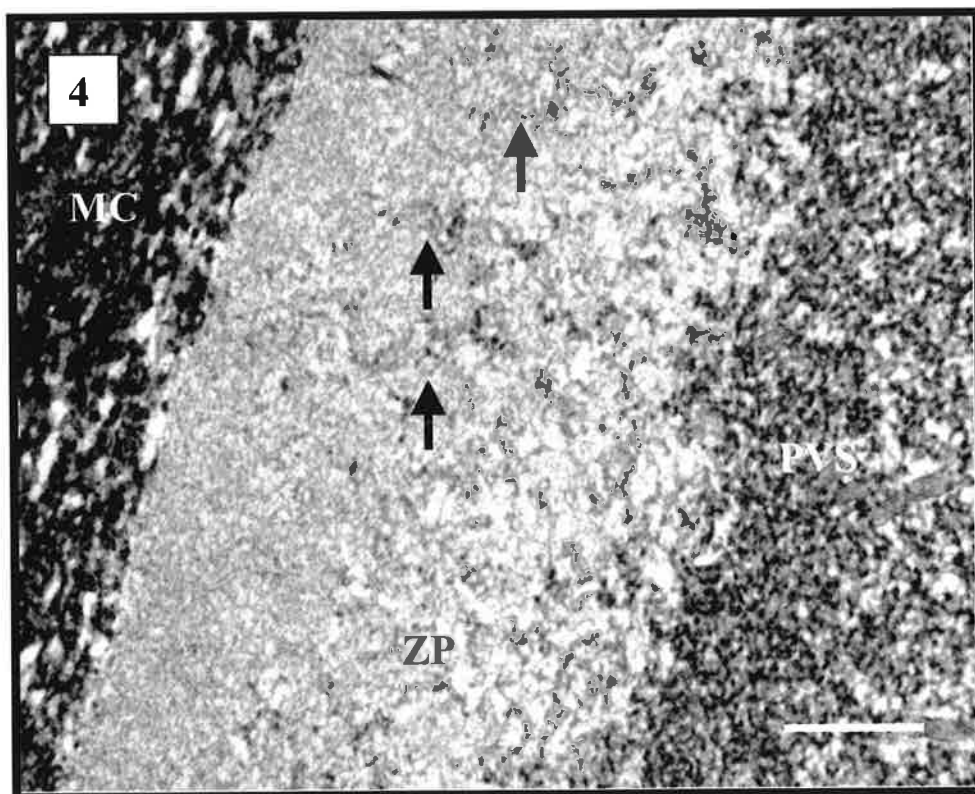
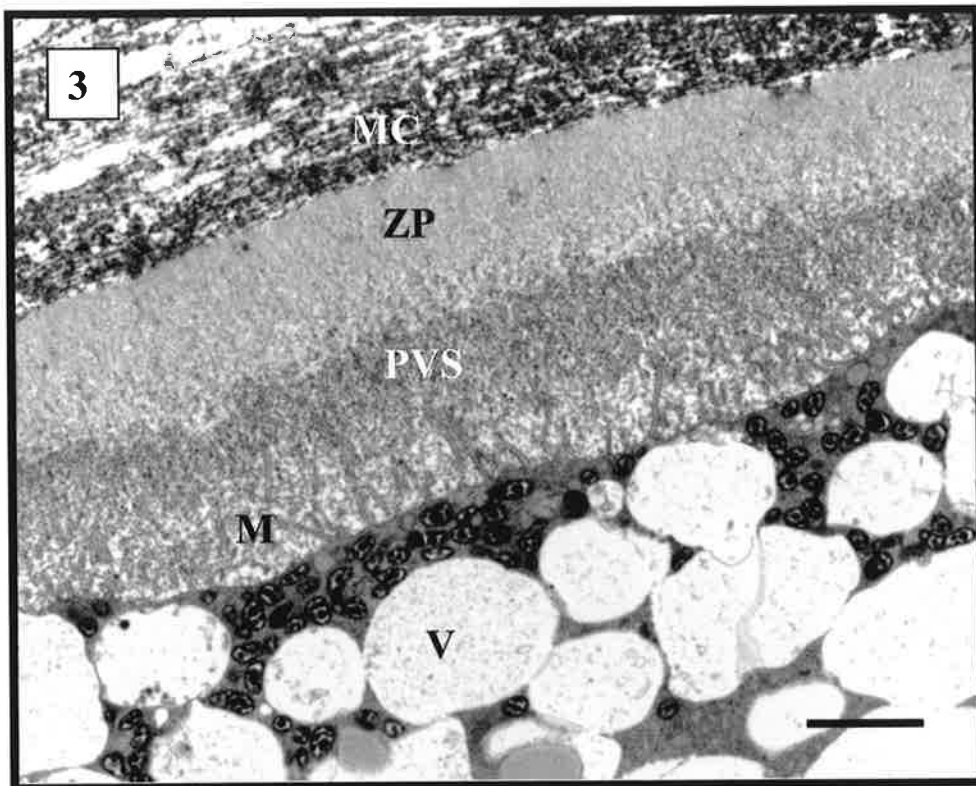
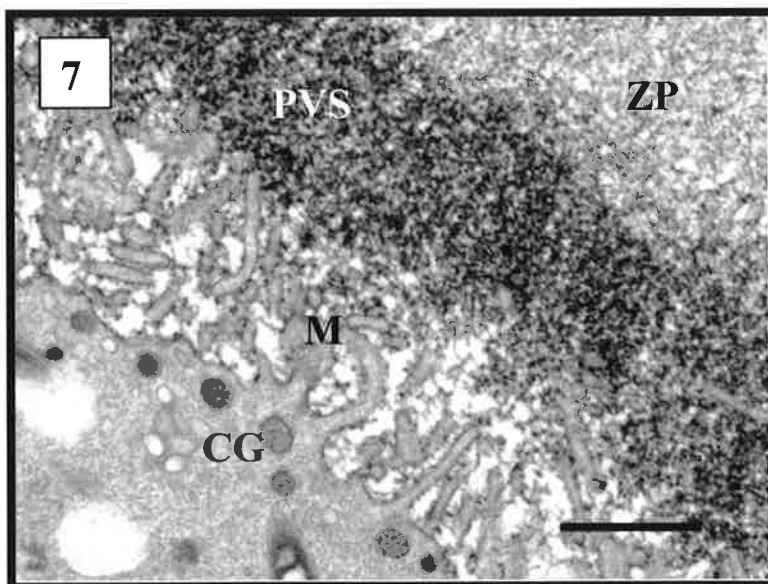
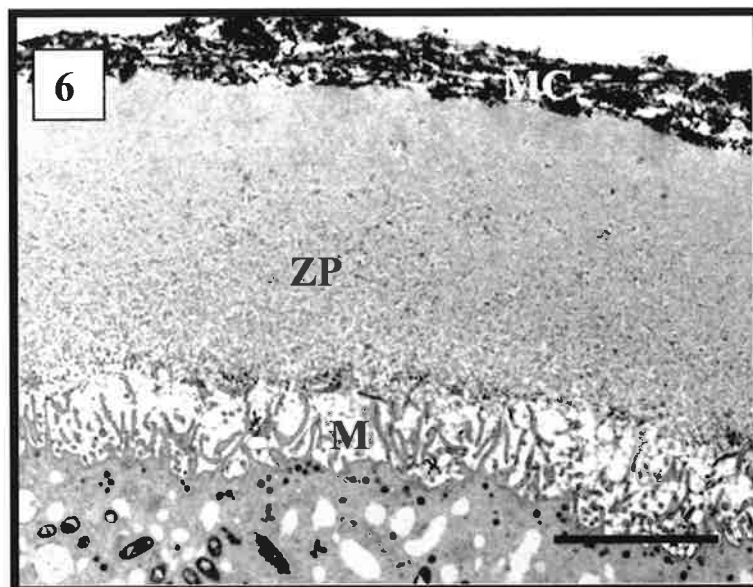
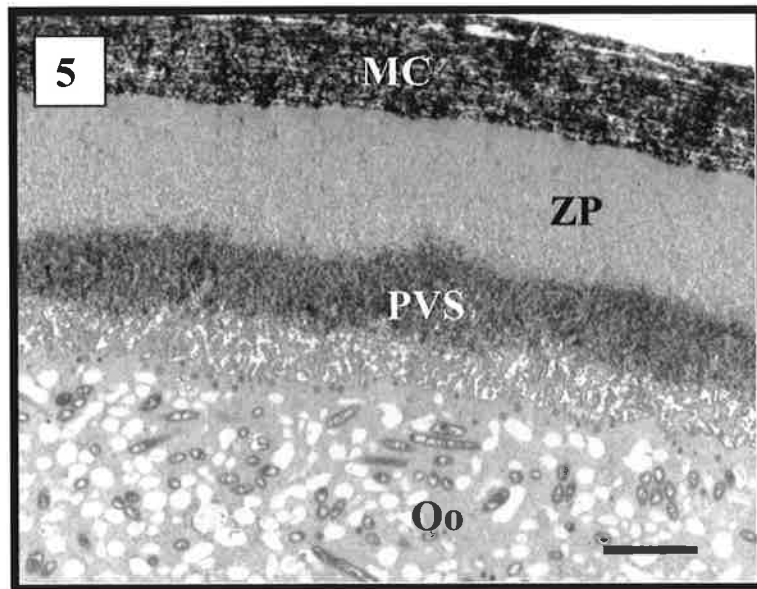


Figure 7.5 Transmission electron micrograph of a control ovulated oocyte of a brushtail possum that was incubated for 15 min in modified Whitten's medium alone and processed in the presence of ruthenium red. Like that noted in Fig. 7.3, the control oocyte (Oo) has a prominent mucoid coat (MC) peripheral to the zona pellucida (ZP) that is stained intensely with the ruthenium red. A prominent band of ruthenium red-stained material can be seen within the perivitelline space (PVS). Note numerous cortical granules within the cortical cytoplasm. Bar = 2 μ m.

Figure 7.6 Transmission electron micrograph of a control ovulated oocyte of a brushtail possum that was incubated for 15 min in modified Whitten's medium alone and processed in the presence of ruthenium red. Unlike that noted in Fig. 7.5, this control oocyte (Oo) has a much less prominent mucoid coat (MC) peripheral to the zona pellucida (ZP), while only a small amount of ruthenium red-stained material occurs within the perivitelline space (PVS). Again, note numerous cortical granules within the cortical cytoplasm. Bar = 2 μ m.

Figure 7.7 Transmission electron micrograph of higher magnification of the oolemma and perivitelline space (PVS) of the control ovulated oocyte seen in Fig. 7.5 demonstrating the prominent ruthenium red stained material within the perivitelline space. The microvilli (M) of the oocyte are also stained with ruthenium red. Cortical granules (CG) can be clearly seen within the cortical cytoplasm. Bar = 2 μ m.



One to two layers of cumulus cells, external to the ZP, surrounded artificially activated peri-ovulatory ovarian oocytes (Fig. 7.8). In these preparations the ZP appeared more compact adjacent to the cumulus cells. In the oocyte cortex, no cortical granules were found underlying the oolemma; a finding that supports the view that oocyte activation had taken place. Within the PVS, little staining with RR was observed with most of the stained material occurring immediately beneath the ZP (Fig. 7.9).

7.3.3 Glycoconjugate Composition of CGs and ZP of Artificially Activated Oocytes

In ovulated oocytes, FITC-WGA stained both the mucoid layer and, intensely, small granules within the cortex of the oocyte as well as material scattered throughout the cytoplasm (Fig. 7.10). In artificially activated ovulated oocytes, although FITC-WGA still stained the mucoid layer and material in the oocyte cortex, no stained cortical granules could be seen (Fig. 7.11), indicating their exocytosis is likely to have taken place due to incubation in the activation medium. The cortical granules did not, however, stain with either FITC-Con A or FITC-LCA in either the ovulated or artificially activated eggs, although there was some staining of material within the large, centrally-located vacuoles of the cytoplasm (Fig. 7.12).

Lectin immunocytochemistry of the ZP of artificially activated ovulated oocytes demonstrated that the glycoconjugates of the ZP decreased following CG exocytosis (a comparison of the lectin immunocytochemical results of the ZP of artificially activated oocytes and those of the previous chapter on the ZP of ovarian and ovulated oocytes can be found in Table 7.2 and Figure 7.13). Following artificial activation, while α -D-Mannose, β -Galactose and GlcNAc

Figure 7.8 Transmission electron micrograph of a peri-ovulatory ovarian oocyte of a brushtail possum artificially activated for 15 min in calcium ionophore, A23187 and processed in the presence of ruthenium red. The ovarian oocyte is surrounded by several layers of cumulus cells (C) peripheral to the zona pellucida (ZP). No cortical granules can be seen within the cortex of the oocyte cytoplasm. Bar = 5µm.

Figure 7.9 Transmission electron micrograph of higher magnification of the zona pellucida (ZP) of a peri-ovulatory ovarian oocyte (Oo) of a brushtail possum artificially activated in calcium ionophore, A23187 for 15 min and processed in the presence of ruthenium red. Filaments of zona pellucida are clearly evident, while very little material appears to be stained by ruthenium red within the perivitelline space (PVS). No cortical granules are evident immediately underlying the microvilli (M) of the oolemma. Bar = 1µm.

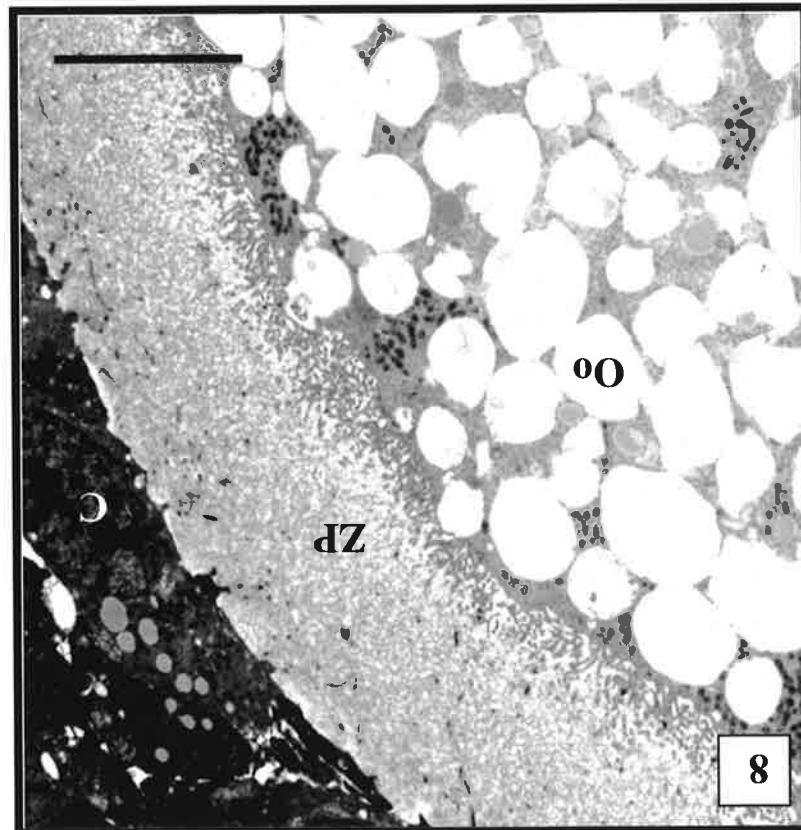
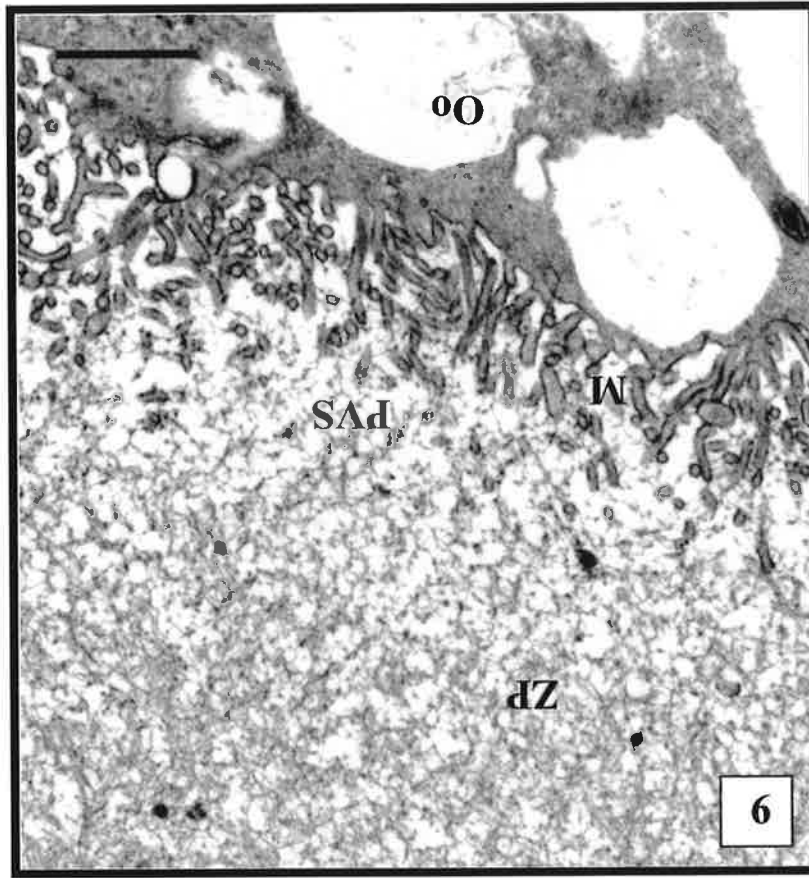
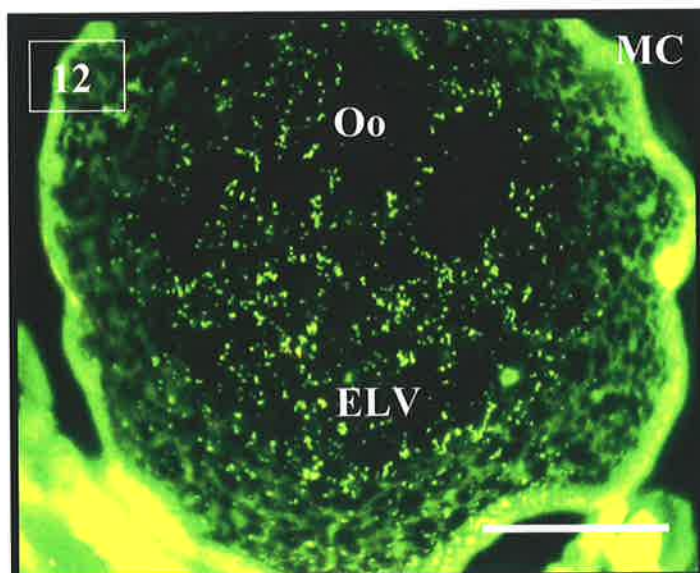
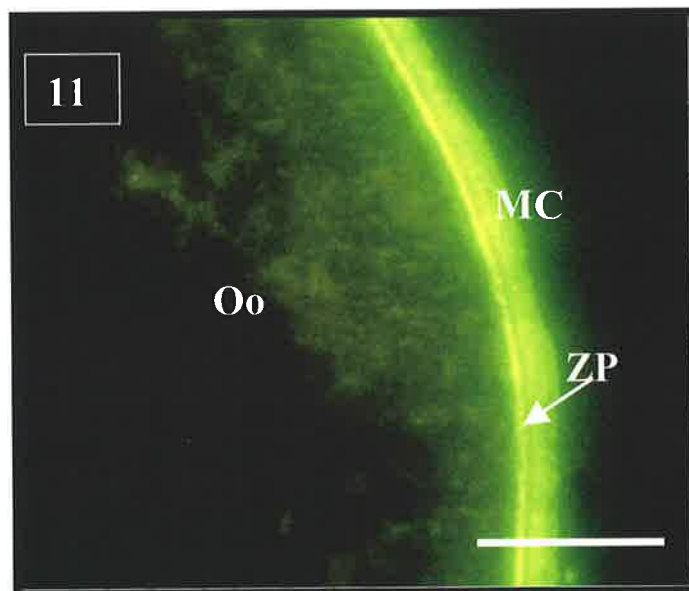
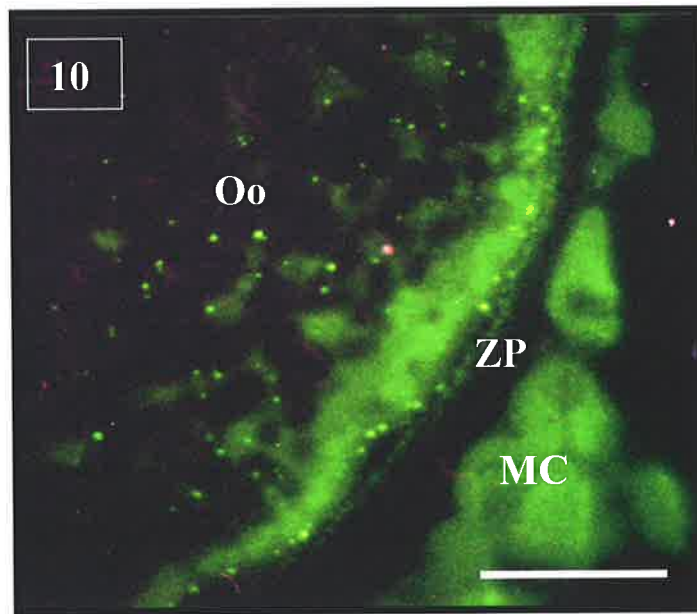


Figure 7.10 Lectin histochemistry of a control ovulated oocyte of a brushtail possum incubated for 30 min in Whitten's medium. Fluorescent micrograph of the control ovulated oocyte (Oo) stained with fluorescein isothiocyanate-*Triticum vulgaris* (wheat germ) lectin (FITC-WGA) shows fluorescence of cortical cytoplasm and surrounding mucoid coat (MC). Small granules also show strong labelling throughout the cytoplasm of the oocyte, which are particularly localised to the cortical cytoplasm, adjacent to the zona pellucida (ZP). WGA binding is specific for β (1-4) *N*-acetylglucosamine, *N*-acetylneuraminic acid (sialic acid). Bar = 25 μ m.

Figure 7.11 Lectin histochemistry of ovulated oocyte of brushtail possum artificially activated in calcium ionophore, A23187 for 15 min. Fluorescent micrograph of artificially activated ovulated oocyte (Oo) stained with fluorescein isothiocyanate-*Triticum vulgaris* (wheat germ) lectin (FITC-WGA), like that seen in Fig. 7.10, shows fluorescence of cortical cytoplasm and surrounding mucoid coat (MC). No FITC-WGA positive granules can be seen within the cytoplasm of the oocyte. WGA binding is specific for β (1-4) *N*-acetylglucosamine, *N*-acetylneuraminic acid (sialic acid). Bar = 25 μ m.

Figure 7.12 Lectin histochemistry of control ovulated oocyte of brushtail possum incubated for 30 min in Whitten's medium alone. Fluorescent micrograph of control ovulated oocyte (Oo) stained with fluorescein isothiocyanate-*Concanavalina ensiformis* (jack bean) lectin (FITC-Con A) shows fluorescence of material within electron lucent vesicles (ELV) within central region of the cytoplasm, as well as cortical region; mucoid coat (MC). Con A binding is specific for α -D-Mannose and/or α -D-Glucose. Bar = 50 μ m.



residues demonstrated a small decrease within the ZP from that of the ZP of non-activated ovulated oocytes, the glycoconjugate α -D-GalNAc and/or α -D-Galactose were found to have decreased significantly ($p=0.01$) (Table 7.2 & Fig. 7.13).

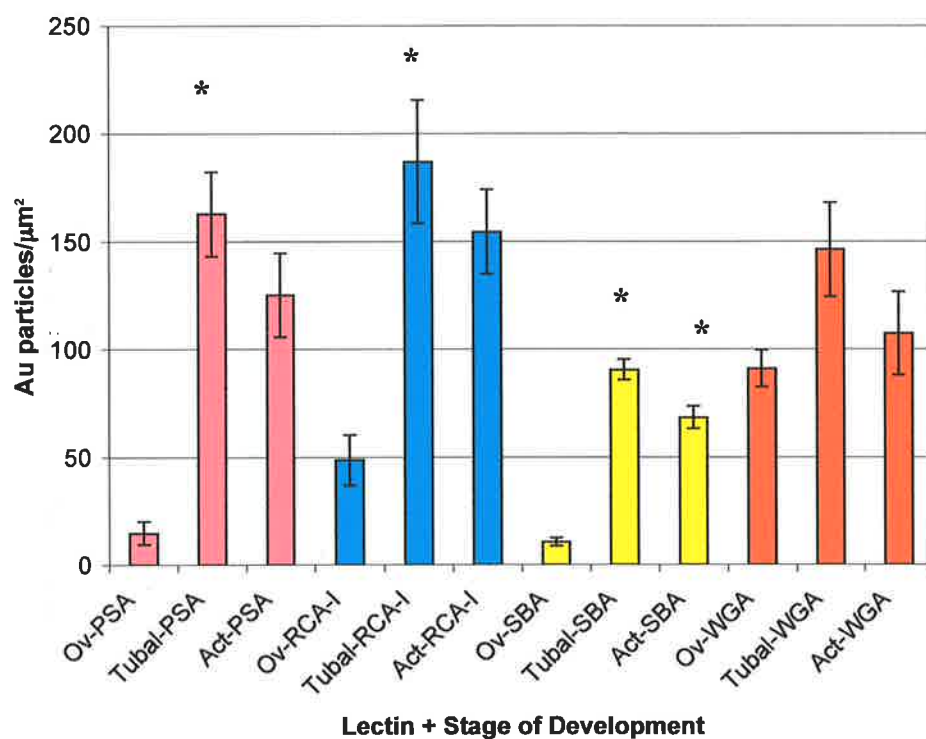


Figure 7.13. Graphic representation of gold labelling density of the zona pellucida of the oocytes of the brushtail possum following incubation in one of four lectins. The labelling intensities are shown as the number of gold particles μm^2 ; mean values $\pm$ SEM. Stars (*) represent significant differences in gold labeling of the same lectin from the previous stage of development. (PSA *Pisum sativum* agglutinin; RCA-I *Ricinus communis*-I agglutinin; WGA wheat germ agglutinin; SBA soybean agglutinin)

Table 7.2 Density of gold labelling of the zona pellucida of ovarian, ovulated (tubal) and artificially activated oocytes of the brushtail possum following incubation with four lectins. The labelling intensities are shown as the number of gold particles μm^2 ; mean values $\pm$ SEM. Gold labeling of the zona pellucida of activated oocytes is shaded to highlight that it is new data from this chapter. (*PSA Pisum sativum* agglutinin; *RCA-I Ricinus communis-I* agglutinin; *WGA* wheat germ agglutinin; *SBA* soybean agglutinin).

Lectin	Ovarian ZP	Tubal ZP	Activated ZP
PSA	14.72 $\pm$ 5.31	162.82 $\pm$ 19.61 ^a	125.13 $\pm$ 19.49
RCA-I	48.52 $\pm$ 11.76	187.03 $\pm$ 28.68 ^a	154.56 $\pm$ 19.68
WGA	91.11 $\pm$ 8.55	146.31 $\pm$ 21.84	107.38 $\pm$ 19.40
SBA	10.45 $\pm$ 1.85	90.55 $\pm$ 4.80 ^a	68.41 $\pm$ 5.25 ^b

^a The labelling densities for these lectins was significantly higher ($p < 0.05$) on the ZP of ovulated eggs than the ZP of ovarian oocytes;

^b The labelling density for this lectin was significantly lower ($p < 0.05$) on the ZP of artificially activated eggs than on the ZP of ovulated eggs.

7.4 Discussion

7.4.1 Overview

In marsupials, the events that occur at the time of fertilisation are poorly understood and, as yet, repeatable *in vitro* fertilisation only appears to have been achieved in a non-Australian marsupial (Taggart *et al.*, 1993). Consequently, the dynamic processes that take place at fertilisation can only be inferred from studies of *in vivo* fertilisation (for reviews see Selwood, 1982; Breed, 1994, 1996) and from extrapolation of investigations with eutherian species. One

of the least understood processes at this time is the origin and functions of the marsupial ZP and perivitelline space matrix (PVM) material.

In marsupials, during early embryonic development, the ZP plays an important role in maintaining polarity of the blastomeres by facilitating cell-zona attachment (Selwood, 1992, 2000), but other post-fertilisation functions of the ZP may be less critical than they are in eutherians due to the acquisition of the mucoid coat and shell membrane (Hughes, 1977; Selwood, 2000). Whilst the presence of these coats is likely to prevent ectopic pregnancy as well as maintaining the integrity of the developing embryo, it is unclear whether the occurrence of polyspermy is prevented by the acquisition of the mucoid layer or as a consequence of the changes that take place to the ZP as a result of CG exocytosis, or both. Around the time of fertilisation CG exocytosis takes place in marsupials (Selwood, 1992; Breed, 1996 for reviews) and in *Monodelphis*, material found to be present in the perivitelline space at this time has been termed the 'cortical granule envelope' suggesting that it arises as a result of CG exocytosis (Dandekar *et al.*, 1995). Here, for the first time in marsupials, a method for the artificial activation of brushtail possum oocytes using calcium ionophore was developed from methods used on eutherian oocytes. Following this, the contribution of the CG exudate to the PVM that surrounds marsupial oocytes at the time of fertilisation was investigated, while the glycoconjugates of the CGs were partially characterised. Lastly, the effect of CG exocytosis on the glycoconjugate content of the ZP of ovulated oocytes was investigated in order to determine if CG exocytosis induced any physiological change to the ZP in marsupials.

7.4.2 Ultrastructure of artificial activation

While CG exocytosis is assumed to play a similar role in inducing the block to polyspermy in marsupial eggs, as in eutherians, there is evidence of polyspermy occurring not infrequently in dasyurids (e.g. *Antechinus stuartii*: Selwood, 1982; *S. crassicaudata*: Breed & Leigh, 1990; Breed, 1996). This, combined with the finding of supernumerary sperm trapped within the mucoid layers, has led to the suggestion that, in marsupial eggs, it is the laying down of the mucoid rather than the cortical reaction that plays an important role in the block to polyspermy (Rodger & Bedford, 1982; Selwood, 1982, 2000).

Despite this possible difference in function of CGs in eutherians and marsupials, in this study it was demonstrated that CG exocytosis can be artificially induced in the oocytes of brushtail possums in a similar manner as used for eutherian oocytes. After as little as 5 min incubation in calcium ionophore A23187, the majority of CGs had undergone exocytosis in the possum oocytes, with an apparent complete exocytosis after 15 min. Although five min incubation with 7% ethanol is sufficient to result in activation in porcine oocytes (with CG exocytosis and ~22% pronuclear development: Didion *et al.* 1990; Ruddock *et al.* 2001), it did not appear to induce a widespread cortical reaction in the oocytes of the brushtail possum. The larger size of the possum oocyte (at around 220µm diameter) compared to those of eutherians (mouse ~ 80µm diameter) might account for the slow response to the 7% ethanol, and a longer incubation might be required to induce total CG exocytosis in oocytes of marsupials.

Witton *et al.* (1999) recently demonstrated that, when injected with solubilised sperm extract, the eggs of *M. domestica* experienced a series of calcium oscillations similar to that expected at fertilisation. These calcium oscillations travelled as waves around the peripheral cytoplasm of the egg, with a slower rate of propagation within the internal cytoplasm probably as a result of the presence of lipid vesicles. The internal cytoplasm of the oocytes of the brushtail possum as well as many other marsupials, are predominantly filled with large electron lucent vacuoles and these, therefore, may serve to insulate the core of the large oocyte, allowing the wave of calcium to propagate rapidly around the peripheral cytoplasm within which reside the CGs and the nucleus. This would then allow for a rapid exocytosis of the CGs creating a rapid block to polyspermy, either at the level of the ZP, or oolemma. The ability to induce CG exocytosis *in vitro*, therefore, supports the contention that despite proposed differences in the importance of the cortical reaction in the block to polyspermy, marsupial eggs are activated in a manner similar to that in eutherians (Witton *et al.* 1999).

7.4.3 Origin of the Perivitelline Space Matrix

In eutherians, following CG exocytosis, material accumulates within the PVS that has been termed the 'cortical granule envelope' (CGE) (Dandekar & Talbot, 1992; Dandekar *et al.* 1995). Ultrastructural localisation of material within the PVS using RR, however, did not support the contention that the so-called CGE was primarily CG in origin, at least in the brushtail possum. While a thick RR-stained band, corresponding to the CGE as reported by Dandekar *et al.* (1995) in *M. domestica*, appeared underneath the ZP within the PVS of artificially activated ovulated eggs in possums, it was also observed in non-activated ovulated

eggs and absent from artificially activated peri-ovulatory ovarian oocytes. Furthermore, the RR-stained material within the PVS of possum ovulated eggs appeared to be most prominent in those eggs that had accumulated the thickest mucoid coats.

Previous studies with eutherians have demonstrated that secretions of the oviduct play a major role in the final maturation of the egg (including the ZP) and sperm-egg interactions (for review see Bleau & St.-Jacques, 1989; Malette *et al.* 1995; Buhi *et al.* 2000). Recently, the importance of oviductal secretions on sperm-egg interactions in the brushtail possum have been demonstrated with sperm requiring a prior incubation in oviductal-conditioned media before they will bind to, and penetrate, the ZP *in vitro* (Mate *et al.* 2000). While the incorporation of oviductal secretions into the ZP and/or the PVS of ovulated eggs in the brushtail possum has not been reported, this thesis has demonstrated that there is an increase in glycoconjugate content of the ZP following ovulation that is closely associated with secretions from the oviduct (see Chapters 5 and 6, this thesis). Although there is a possibility that the material in the PVS of non-activated ovulated oocytes may be due to a premature release of CGs, there were still numerous CGs within the cortical cytoplasm of these oocytes, and the likelihood that the broad band of RR-stained material could result from such a minor release of CGs is remote. It seems likely, therefore, that the majority of the PVM results from the incorporation of oviductal protein(s) into the PVS, while only a minor component is likely to result from CG exocytosis, at least in the brushtail possum.

7.4.4 Glycoconjugate Composition of Cortical Granules and the Zona Pellucida following Artificial Activation

Studies with the laboratory mouse have identified four CG proteins including β -*N*-acetylglucosaminidase and ovoperoxidase that target the ZP and facilitate structural and functional changes that prevent polyspermy (Gulyas & Schmell, 1980; Miller *et al.* 1993; Hoodbhoy & Talbot, 1994). In eutherian mammals, and particularly in rodents, proteins of CGs appear to be glycosylated and stain with the lectins LCA and Con A that are specific for α -D-mannose (Cherr *et al.* 1988; Hoodbhoy & Talbot, 1994). Recently, several of the glycosylated proteins that bound LCA and Con A in hamster CGs were also found to contribute to the PVM (Hoodbhoy & Talbot, 2001). Furthermore, two proteins, p62 and p56, that were found to be present in the CGs of mice, rats, hamsters, cows and pigs also were found to contribute to the PVM and played a role in pre-implantation development in hamsters, but not to the ZP block to polyspermy (Hoodbhoy *et al.* 2001).

Although lectin histochemistry has been commonly used to determine CG distribution and exocytosis in eutherian species (e.g. Cherr *et al.* 1988; Connors *et al.* 1998; Tatemoto & Terada, 1999), this is the first demonstration of CGs using this method in a marsupial. Small granules were clearly evident within the cortical cytoplasm using FITC-WGA on control ovulated eggs, and clearly absent in artificially activated eggs. This evidence, in conjunction with the ultrastructural data, supports the contention that the granules labelled by FITC-WGA in the control eggs were indeed CGs.

Unlike in some eutherians, however, the CGs of the brushtail possum did not appear to contain α -D-mannose as indicated by the lack of binding with the lectins Con A and LCA. The lack of binding with these lectins, however, is more likely to reflect an inaccessibility of the mannosylated glycoconjugates to the lectins through steric hindrance than an absence of mannose altogether. In support of this, staining with WGA indicated that the CGs of the brushtail possum contain $(\beta$ -(1-4)-D-*N*-acetylglucosamine)₂ (abbreviated here as GlcNAc), and *N*-acetylneuraminic acid (sialic acid), similar to that reported for eutherian species (Hoodbhoy & Talbot, 2001). In mammalian glycoproteins, GlcNAc is typically found in *N*-linked oligosaccharides, particularly as the glycoconjugate forming the glycosidic linkage to the asparagine residue of the polypeptide backbone, and, as *N*-linked oligosaccharides contain a tri-mannosylated core (Kornfeld & Kornfeld, 1980; Kobata, 1992), evidence suggests that the CGs of brushtail possums would, in fact, contain mannose.

The modification of the ZP following CG exocytosis is usually cited as involving two major processes. The first is suggested to involve the removal of the functional part of the sperm receptor, either removal of specific glycoconjugates (Miller *et al.*, 1993) and/or the specific proteolysis of ZPA (Bleil *et al.*, 1981, 1988; Iwamoto *et al.*, 1999). The second process is commonly cited to involve structural hardening of the ZP, recently suggested to occur as a result of increased disulphide bonding within ZPA and between ZPB and the other ZP glycoproteins in the pig (Iwamoto *et al.*, 1999), but may also be due to cross-linking of tyrosine residues as a result of the release of CG ovoperoxidase (Gulyas & Schmell, 1980; Schmell *et al.*, 1983). Recently, however, investigations into the glycoconjugates of the ZP

following CG exocytosis have found that while some glycoconjugates are removed, others may be added (Aviles *et al.*, 1996; El-Mestrah & Kan, 2002).

This study found that there was no increase in ZP glycoconjugates following CG exocytosis, only decreases, with only GalNAc and/or α -D-Gal residues decreasing significantly. In the previous two chapters GalNAc and/or α -D-Gal residues were specifically localised to the ampullary epithelium, the site for fertilisation, and these significantly increased within the ZP following ovulation. This, combined with the finding of this chapter that only this glycoconjugate decreased significantly within the ZP following CG exocytosis, strongly supports the contention that this glycoconjugate may play an important role in sperm binding. Some caution must be taken when interpreting the results of glycoconjugate changes to the ZP following artificially-induced CG exocytosis, however, as differences in glycoconjugate modification of the ZP have been noted to result from natural and artificial activation (El-Mestrah & Kan, 2002).

7.5 Conclusions

This study has shown that despite proposed differences in the importance of the cortical reaction in the block to polyspermy in marsupials, the oocytes of brushtail possums can be artificially induced to undergo CG exocytosis with the calcium ionophore A23187 as in eutherians. The previously described 'cortical granule envelope' that results from CG exocytosis in both eutherians and marsupials, may result predominantly from the incorporation of oviductal glycoproteins into the PVS rather than CG exudate and, as such, the

term 'perivitelline space matrix' (PVM) should be used until the exact origin of this matrix can be determined. While it still remains unclear what role, if any, the exocytosis of CGs in marsupials plays in the block to polyspermy, some modification to the glycoconjugates of the ZP does appear to occur. The recent development of *in vitro* sperm-ZP binding and penetration in the brushtail possum (Mate *et al.*, 2000), in conjunction with the development of a method for artificial activation of possum oocytes by this study, can allow a more detailed investigation into the role of the cortical reaction in marsupial oocytes.

Chapter 8 Final discussion and conclusions

8.1 Overview

When the zona pellucida (ZP) surrounding a living mammalian oocyte is viewed under a light microscope (see Fig. 8.1), it appears to be a relatively simple structure: a translucent ring surrounding the oocyte. Belying its simple appearance, however, is a complexity the details of which have still not been resolved.

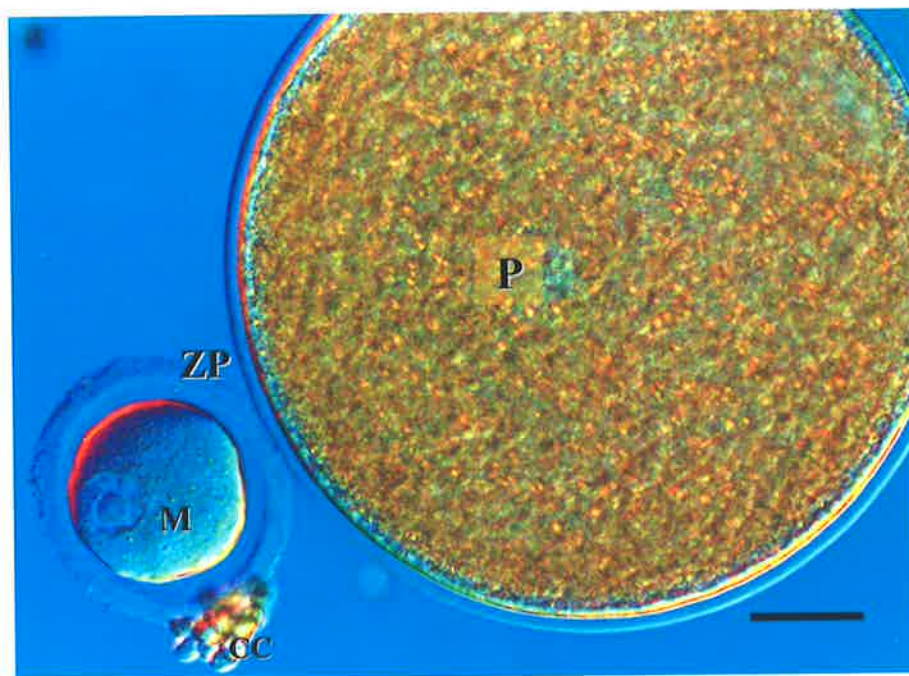


Figure 8.1. Nomarski differential interference light micrograph of (M) laboratory mouse and (P) brushtail possum oocytes obtained from large ovarian follicles. Note the much greater size of the possum oocyte surrounded by a much thinner (ZP) zona pellucida compared with the mouse oocyte. Some (CC) cumulus cells are present peripheral to the mouse ZP (following 10 min incubation in 3% hyaluronidase), whereas that is not the case around the possum oocyte (following mechanical removal). Bar equals 50 μ m.

Most research on the characterisation of the mammalian ZP has been performed on eutherian mammals and, in particular, that of the laboratory mouse. Primarily led by Paul Wassarman, Jurrien Dean and colleagues, many aspects of the ZP of the lab mouse have

been investigated including its development, secretion, composition and function. The laboratory mouse has served as a useful model for these investigations because its oocytes surrounded by ZP can be obtained in large numbers thus enabling detailed biochemical and functional studies on the ZP and sperm-ZP interaction to be investigated *in vitro*. Recent manipulation of its genome has also enabled specific investigation into the biosynthetic pathways of the ZP glycoproteins to be carried out (for reviews see Wassarman & Litscher, 1995, 2001; Wassarman *et al.*, 1996, 1999).

By contrast to that of the laboratory mouse, research into the structure and function of the ZP of Australian marsupials is relatively superficial. Other than some isolated early investigations of the egg membranes of the brushtail possum and other Australian marsupials (Hughes, 1974, 1977; Hughes & Shorey, 1973), most of the research into the marsupial ZP has been performed within the last decade or so. One major difficulty faced by researchers attempting to characterise the ZP of Australian marsupials is the fact that there are very few laboratory species from which to obtain many oocytes, and, of those that there are (for example, *Sminthopsis crassicaudata*, Bennett *et al.*, 1982, 1990; Hope *et al.*, 1986; Hope & Godfrey, 1988; *S. macroura*, Woolley, 1990*a,b*; Frigo & Woolley, 1997), breeding is not as prolific as with laboratory mice.

Another difficulty faced by researchers of the ZP of Australian marsupials is the inability to produce reliable and repeatable successful sperm-ZP and sperm-egg interactions *in vitro*. This major hurdle has hampered many investigations into the function of the ZP in marsupials, including detailed analyses of the role of the ZP glycoproteins and their components (e.g. glycoconjugates, polypeptides) in sperm-ZP binding, induction of the acrosome reaction and its possible involvement in the prevention of polyspermy following cortical granule exocytosis. Consequently, many of the structural and functional features of

the marsupial ZP have been inferred, either correctly or incorrectly, from ultrastructural studies of in vivo fertilization in marsupials and/or from eutherian models.

This thesis has investigated several aspects of the structural organization of the marsupial ZP that have been observed from oocytes at various stages of its life cycle. These investigations have included studies of the development and secretion of the ZP during oocyte maturation, its structure and composition immediately prior to, and following, ovulation and, lastly, its composition following egg activation and cortical granule exocytosis. From these findings, therefore, suggestions are made on the structure of the marsupial ZP, including its development and the organization of the glycoconjugates.

8.2 Structure of the Marsupial ZP

8.2.1 Development and Formation

The marsupial ZP first appears around the oocyte fairly soon after the differentiation of the primordial follicle into the primary follicle (Alcorn, 1975; Hughes, 1977; Frankenberg & Selwood, 1996, 2001; Mate, 1998; Kress *et al.*, 2001). In the present study, it was found that the ZP matrix first appears within the extracellular space between the oocyte and follicle cells and thus coincides with the formation of the microvilli on the oolemma such that the ZP forms isolated pockets of matrix. These eventually fuse to form a continuous matrix around the oocyte, a feature that has also been noted in a number of eutherian species (e.g. rat, Kang, 1974; mouse, Wassarman & Josefowicz, 1978; rabbit, Dietl, 1989).

One characteristic feature of ZP formation noted during this early stage of ZP formation by the present study was that it was often found to be highly dispersed between the lateral

membranes of the follicle cells. While the intercellular spaces between the follicle cells, within which the ZP matrix resided, were likely to be exaggerated as a result of shrinkage artefact through routine tissue processing, the findings of such features in all five marsupial species suggest that it may truly reflect ZP formation. As discussed in Chapter 3, this feature of ZP formation has probably led to a misinterpretation of the contribution by the follicle cells to ZP synthesis in the brushtail possum (Mate, 1998). It is possible, however, that ZP glycoproteins are secreted by the oocyte as monomeric units or, at the very most, filaments composed of only several heterodimers, with their small size allowing them to diffuse into any extracellular space. The granulosa cells of the developing follicle appear to have few specialised junctions, such as desmosomes, between them and no occluding junctions have been seen between adjacent follicle cells and, as such, diffusion of ZP glycoproteins between follicle cells could occur at least to some degree.

As folliculogenesis proceeds, subsequent mitotic divisions of follicle cells within the confined space created by the basement membrane and surrounding thecal cells may force the ZP matrix into the extracellular space immediately surrounding the oocyte (the future perivitelline space). It is postulated here that the increase in intrafollicular pressure created by the increasing number of follicle cells within the confined space of the follicle, particularly just prior to the antrum formation, may facilitate the aggregation of the ZP filaments into a compact confined structure. The possible compression of the ZP around the oocyte may serve several functions. Firstly, it may confine the ZP glycoproteins to the space between the basal membranes of the inner follicle cells and the oolemma, thereby increasing the concentration of the ZP glycoproteins in this area. This could maximise interaction between the 3 glycoproteins, allowing for a greater opportunity for self-assembly of the ZP filaments, as well as enabling cross-linking of adjacent filaments to

occur and construction of the core architecture and sub-architecture of the ZP, as described in Chapter 6 of this thesis.

Secondly, as Green (1997) pointed out, the carbohydrates of the ZP glycoproteins may result in the ZP having the physicochemical properties of an elastic gel such that the ZP retains “memory” of its spherical shape if it is mechanically disrupted. It has been postulated that the oocyte forms the mould from which the ZP generates its spherical shape (Green, 1997). In eutherians, the mature ZP has been reported to be denser close to its inner surface (Dietl, 1989; see also Yanagimachi, 1994 for references), which has been suggested as resulting from the secretion of ZP glycoproteins into the mechanically compressed space generated by the growing oocyte (Green, 1997). The compression of the ZP, therefore, may also facilitate polymerisation of the ZP into its characteristic shape.

The finding in this study of the ZP around the Graafian oocytes of the possum and koala appearing denser on its external surface and less dense on its inner surface (see also Hughes, 1977) is the reverse to that found for mature eutherian ZP. This may be a result of the compressive forces arising from the follicular epithelium rather than the oocyte as in eutherians. Folliculogenesis in eutherians has been described as biphasic, with both the oocyte and follicle growing together and then, following formation of the follicular antrum, the follicle expands but the oocyte does not (Brambell, 1956; Lintern-Moore *et al.*, 1976). In marsupials, however, despite originally being described as having a biphasic pattern of follicular growth (Lintern-Moore *et al.*, 1976; Lintern-Moore & Moore, 1977), recent evidence suggests that oocyte growth in at least some species of marsupials continues well after the formation of the follicular antrum (Selwood, 1982; Lyne & Hollis, 1983; Falconnier & Kress, 1992; Rodger *et al.*, 1992; Moritz *et al.*, 1998). This is perhaps a result of the accumulation of the electron-lucent vesicles within the oocyte cytoplasm (Mate,

1996). Whatever the means, such a difference in growth pattern may account, at least in part, for the difference in structure of the ZP of the mature ovarian oocytes between marsupials and eutherians.

Unlike in eutherians, initial compression of the ZP prior to the development of the follicular antrum may arise from the follicle cells rather than the oocyte, while compression from the growing oocyte may actually occur late in follicular development, perhaps even after ovulation (Rodger *et al.*, 1992; Mate, 1996). While the ultrastructural changes to the ZP following ovulation in the brushtail possum were investigated, measurements of changes to ZP thickness were not considered. Rodger & Mate (1993), as part of their description of the morphological changes to the ZP following ovulation, noted that the ZP changed from being “broad” prior to ovulation to “thin” following ovulation. This change in thickness was suggested to be due to compression of the ZP by the growing oocyte (Mate, 1996). While not referred to in this thesis, in the course of the present study such a change in thickness was also noted to take place, with the ZP of ovulated oocytes of the possum appearing to be thinner than that found around follicular oocytes. Such a decrease in ZP thickness has also been reported to occur in monotremes due to the accumulation of yolk, with the oocytes of the platypus having a ZP thickness of around $11\mu\text{m}$ prior to vitellogenesis and less than $0.5\mu\text{m}$ immediately prior to ovulation (Hughes, 1977). The subsequent expansion of the electron-lucent vesicles in the central region of the marsupial oocyte, sometimes referred to as yolk vesicles despite the fact that they do not appear to arise as a result of vitellogenesis (Falconnier & Kress, 1992; Kress, 1996), may in fact produce a similar outcome to the accumulation of yolk in monotremes, that of expanding the oocyte diameter, stretching the ZP, and reducing its thickness in the process.

Some loss in thickness of the ZP around the time of ovulation may also be attributed to the reduction in volume of the ZP following retraction of the cumulus cell processes and their loss from the surface of the oocyte immediately prior to ovulation. Such a loss of volume would require some reorganisation of the ZP matrix to compensate for the spaces created by the removal of the cumulus extensions, which would therefore result in some reduction of thickness. Whether such a reduction in ZP thickness occurs in marsupials other than in the brushtail possum is unknown, but it has not been noted in any other marsupial, particularly the opossums and dasyurids that have probably had the most investigations of their mature oocytes performed (for reviews see Selwood, 1982; Breed, 1994, 1996; Kress, 1996).

8.2.2 Relative Thickness and Morphology

Marsupial oocytes, in contrast with those of eutherians, are typically cited as being larger, while also having a much thinner ZP. Bedford (1991) referred to the ZP of marsupials as 'trivial' when compared to that of eutherians. The present study too, supported other findings that the oocytes of some marsupials are surrounded by a relatively thinner ZP than that which typically occurs in eutherians. The ZP of fat-tailed dunnart, possum and kangaroo Graafian follicular oocytes all measure, on average, less than that found in most eutherians (with the lab mouse ZP measuring on average around 6µm, commonly cited as the thinnest of eutherian ZP: see Dunbar *et al.*, 1991, 1994). Amongst marsupials the exceptions occur in the wombat and koala, where the ZP is relatively thicker, compared to most marsupials, and is similar in thickness to that of the laboratory mouse.

The finding here of the similarity in thickness of the ZP of the wombat and koala to that of laboratory rodents lends further support to the 'remarkable' convergence in gamete

morphology between these two groups, the vombatiforms and murid rodents (Harding & Aplin, 1990), with the morphology of wombat and koala sperm also resembling that of laboratory mice and rats (Hughes, 1965; Harding & Aplin, 1990; Temple-Smith & Taggart, 1990). The general morphology of the falciform-shaped sperm head is where the convergence seems to end, however, with the finding that the chromatin of wombat and koala sperm are much more readily dispersed by detergents than those of the laboratory rodents, similar to that of other marsupials (Breed *et al.*, 2001). It has been argued that in eutherians, as a result of the evolution of a significantly thicker ZP, spermatozoa have co-evolved stabilising features in the sperm head such as increased disulphide bonding of protamines to facilitate penetration by forward thrust of the spermatozoa (Bedford, 1991, 1996, 1998). Marsupial sperm, on the other hand, are said to have retained a predominantly enzymatic mode of penetration of the significantly thinner ZP and, as such, spermatozoa have co-evolved stabilising features in their acrosomes but not their chromatin (Bedford & Calvin, 1974; Cummins, 1980; Bedford, 1991, 1996, 1998; Lin *et al.*, 1995). The lack of stabilising features in the sperm chromatin of wombats and koalas suggests that although the sperm from these species are morphologically similar to those of some eutherians, the mode of penetration is likely to be similar to that of other marsupials.

The reason for the convergence in morphology between the sperm of wombats and koalas and laboratory rodents still remains unexplained then. Scanning and transmission electron micrographs have demonstrated that the shape of the sperm head in both wombats and koalas are highly pleomorphic, ranging from highly curved to almost fusiform in shape (Temple-Smith & Taggart, 1990; Breed *et al.*, 2001). The flexibility of the curved nucleus has not been established, however, all of the sperm heads appear to taper at the end. If the head of the sperm of the wombat and koala are relatively flexible, then the curvature may flatten somewhat upon contact with the zona providing the acrosomal face of the sperm

head (the ventral region) the greatest surface area of contact with the ZP matrix. The sperm tail is attached to the dorsal surface of the sperm head presumably so that the forward thrust that it generates would push the sperm head downwards in a diagonal direction. Most transmission electron micrographs show much of the acrosome of the wombat and koala sperm situated in the superior portion of the ventral surface of the sperm head (Harding & Aplin, 1990; Temple-Smith & Taggart, 1990; Breed *et al.*, 2001). The hydrolytic enzymes released at the time of the acrosome reaction, therefore, would be near the tapered end facilitating the diagonal penetration of the ZP. Although difficult to achieve from a practical point of view, future studies on sperm-ZP and sperm-egg interaction are required to determine the processes by which the wombat and koala sperm bind to, and penetrate, the ZP of these species.

8.2.3 Glycoconjugates of the Ovarian Zona Pellucida

In eutherians, the binding of sperm to the ZP is facilitated through the glycoconjugates of the oligosaccharides of the ZP glycoproteins (Florman & Wassarman, 1985; Bleil & Wassarman, 1988). It has been argued that sperm-ZP binding is species-specific and, therefore, variation in the glycoconjugates of the ZP glycoproteins may be responsible for this specificity (Wassarman *et al.*, 1996, 1997, 1999). Although the degree of the species-specificity of the ZP is highly questionable (Maddock & Dawson, 1974; Liu *et al.*, 1991; Wakayama *et al.*, 1996; Sinowatz *et al.*, 2003), there does appear to be variation in ZP glycoconjugates between species of eutherian mammals, as indicated by lectin histochemistry (Nicolson *et al.*, 1975; Skutelsky *et al.*, 1994; Parillo *et al.*, 1996; Parillo & Verini-Supplizi, 1999, 2001; Aviles *et al.*, 2000) and high performance liquid chromatography (Hokke *et al.*, 1993; Nagdas *et al.*, 1994; Katsumata *et al.*, 1996; Nakano *et al.*, 1996; Takasaki *et al.*, 1999).

In marsupials, little was known about the glycoconjugates of the ZP other than the fact that the ZP was composed of sulphated glycoproteins as indicated by staining with Periodic Acid Schiff's reagent (PAS) and alcian blue pH 0.2 and pH 2.6 (Hughes, 1974; Frankenberg *et al.*, 1996; Mate, 1998). In this thesis, however, a detailed analysis of the specific glycoconjugates present in the ZP and oocytes of Graafian follicles of seven marsupial species, each representing a separate family, was investigated using differential lectin histochemistry. Also, in order to investigate the role of sialic acids and their *O*-acetyl substitutions in the structure of the ZP oligosaccharides, the ZP were pre-treated with neuraminidase and mild alkaline hydrolysis respectively, prior to carrying out lectin histochemistry.

The ZP of marsupials was found to contain a wide array of glycoconjugates, of both *N*-linked and *O*-linked oligosaccharides. Evidence of *N*-linked oligosaccharides was found within the ZP of all the species studied and was identified here by localisation of two main glycoconjugates. Firstly, *N*-acetylglucosamine (GlcNAc), the glycoconjugate responsible for the glycosidic linkage of *N*-linked glycans to asparagine residues of the polypeptide backbone, was demonstrated by labelling with the lectins WGA and ECA. Secondly, α -D-Mannose (α -D-Man), present as the tri-mannosidic core of *N*-glycans, was labelled with the lectins PSA and/or Con A. *O*-linked oligosaccharides, indicated by *N*-acetylgalactosamine (GalNAc), the glycoconjugate responsible for the glycosidic linkage of *O*-linked oligosaccharides to serine and/or threonine residues of the polypeptide backbone, was demonstrated by labelling with PNA and SBA, and was also present in the ZP of all marsupial species investigated.

The glycoconjugates found within the oligosaccharide chains of the marsupial ZP glycoproteins could generally be divided into two types: structural and functional. The structural glycoconjugates, like those described above, are those that are commonly found in all mammalian glycoproteins such as GlcNAc and GalNAc (Kornfeld & Kornfeld, 1980). Similarly, N-acetyl neuraminic acid (sialic acid) could also be described as a structural glycoconjugate in that most sialic acids are found in the terminal positions of oligosaccharides (with fucose another common terminal glycoconjugate), while mannose forms the structural core of all *N*-linked oligosaccharides (Kornfeld & Kornfeld, 1980). Functional glycoconjugates, therefore, could be described as those that have roles other than being structural components. For example, some glycoconjugates may provide a glycoprotein with cell-, tissue-, individual-, or even species-specificity, while others may function as ligands for receptors. Of course, structural glycoconjugates may also have important functions and functional glycoconjugates may be important in the structure of the oligosaccharide.

One structural glycoconjugate that may also have a number of important functions, and appears to be responsible for much of the interspecific variation in ZP glycoproteins of marsupials, is sialic acid. With the exception of the opossums, sialic acid was found to be the terminal sugar in the oligosaccharides of the marsupial ZP and was responsible for the masking of galactose, and GalNAc- and galactose-containing saccharides. The *O*-acetylation of sialic acids also appeared to play a major role in the structure of the ZP oligosaccharides such that de-*O*-acetylation of the sialic acids resulted in the unmasking of predominantly GalNAc-containing glycoconjugates in all species and α -D-Man residues in the ZP of the fat-tailed dunnarts, grey short-tailed opossums, brushtail possums and eastern grey kangaroos. The present study also found a change in binding pattern with WGA from being localised to the inner and outer compartments on the native ZP to having uniform

binding throughout the de-*O*-acetylated ZP in all marsupial species studied. This localisation is postulated to be due to a higher proportion of *O*-acetylated sialic acids within the central region of the ZP and, while based on relatively crude methods, is the first time that a biochemical explanation has been offered for the relatively long half-life of the ZP.

O-acetyl substitutions on sialic acids have been shown to play very important roles in the structure, function and lifespan of various glycoproteins (for reviews see Schauer, 1982, 1988; Kelm & Schauer, 1997). For example, an *O*-acetyl substitution at the *O*-4 position of a sialic acid has been found to completely block the action of a neuraminidase, which thereby prevents any further action by glycosidases and proteases in degrading the glycoprotein (Schauer, 1988). Even the presence of sialic acid itself has been shown to inhibit infection by certain strains of bacteria (Barthelson *et al.*, 1998). The greater concentration of *O*-acetylated sialylated glycoconjugates in the internal region of the ZP in marsupials may play an important role in increasing the lifespan of the ZP, particularly following ovulation when the ZP appears to play an important role during early embryonic development (Selwood, 1992, 1994; Frankenberg & Selwood, 1998). The lack of *O*-acetylated sialylated glycoconjugates on the outer and inner regions of the ZP, however, may facilitate selective glycolysis and enzymatic disruption by acrosomal enzymes following sperm-ZP binding and cortical granule exocytosis after sperm-egg binding.

8.2.4 Glycoconjugates of the Oviduct

The functional glycoconjugates of the marsupial ZP responsible for the binding of sperm are unknown. Despite the ultrastructural investigations of *in vivo* fertilisation (for reviews see Selwood, 1982; Rodger, 1991; Breed, 1994, 1996), the inability to achieve sperm-ZP

binding and fertilisation *in vitro* has prevented detailed investigations into many of the biochemical features of sperm-ZP and sperm-egg interactions in marsupials. Whilst the present study was in progress, sperm-ZP binding and ZP penetration was achieved *in vitro* in the brushtail possum (Mate *et al.*, 2000). In order for this to occur, sperm first had to be pre-incubated in oviduct-conditioned media prior to their incubation with *in vitro* matured ovarian oocytes, while sperm-egg binding still remained unachieved (Mate *et al.*, 2000). The previous inability to get successful sperm-ZP and sperm-egg interactions *in vitro*, and the finding that it was only achieved following the incubation of sperm in oviduct-conditioned media, highlights the importance of the oviductal environment for fertilisation. The failure of sperm-egg binding *in vitro* and the use of *in vitro*-matured ovarian oocytes by Mate *et al.* (2000) also suggests that the oviductal influence on the egg and egg coats may have been overlooked.

The functions of the secretory products of the marsupial oviduct are commonly only associated with the tertiary egg coats that it produces, namely, the mucoid coat and the shell membrane (although the shell membrane may arise from the uterotubal junction and from secretions of some of the glands of the uterus [Roberts & Breed, 1996*a,b*; Casey *et al.*, 2002]). It is only recently that any other functions of the oviductal secretions, other than the production of these tertiary membranes and the importance of these secretions in the maintenance of sperm in isthmic crypts of didelphids and dasyurids (Bedford, 1996), have been suggested (Sidhu *et al.*, 1998, 1999; Mate *et al.*, 2000). Oviductal proteins from the brushtail possum have been found to bind to spermatozoa and increase their survival *in vitro* while also inducing capacitation-associated morphological changes to the sperm in which they transform from a streamlined shape to a T-shaped form (Sidhu *et al.*, 1999). It was these T-shaped 'capacitated' possum sperm that were then found to bind to the ZP, and penetrate it, *in vitro*.

As sperm-ZP binding in marsupials is likely to be facilitated through the glycoconjugates of the ZP, and the obvious importance of the oviductal glycoproteins in the pre-fertilisation maturation of the spermatozoa, the glycoconjugates present in the oviductal epithelium of the brushtail possum at the time of ovulation were investigated in the present study using lectin histochemistry and lectin immuno-gold cytochemistry. Following on from this, lectin immuno-gold cytochemistry was also performed on the ovarian and ovulated oocytes of the brushtail possum in order to determine if there were any glycoconjugate changes in the ZP following ovulation, as well as determining the glycoconjugates present within the mucoid coat. Lastly, in order to consider which glycoconjugates might play a role in sperm-ZP binding, and which therefore may be removed from the ZP following egg activation to prevent polyspermy, lectin immuno-gold cytochemistry was also performed on the artificially activated oocytes of the brushtail possum.

Lectin histochemistry demonstrated that a number of glycoconjugates were present within the possum oviduct at the time of ovulation. Most prevalent were β -galactose (β -Gal) and GlcNAc residues in the secretory cells of both the ampulla and isthmus. Although lectin histochemistry only recognised one major regional difference in secretory glycoconjugates between the ampulla and isthmus, namely the presence of α -D-GalNAc and/or α -Gal residues in the secretory cells of the ampulla but not the isthmus, lectin immuno-gold cytochemistry highlighted much greater regional variation. For example, α -D-GalNAc and/or α -Gal residues were only found within the secretory granules of the ampulla, while significantly more α -D-Man residues were found in the secretory granules of the ampulla than in the isthmus. On the other hand, GlcNAc residues were found to be significantly greater in the secretory granules of the isthmus than in the ampulla, while β -Gal residues were found in relatively equal distribution between the ampulla and isthmus. While some

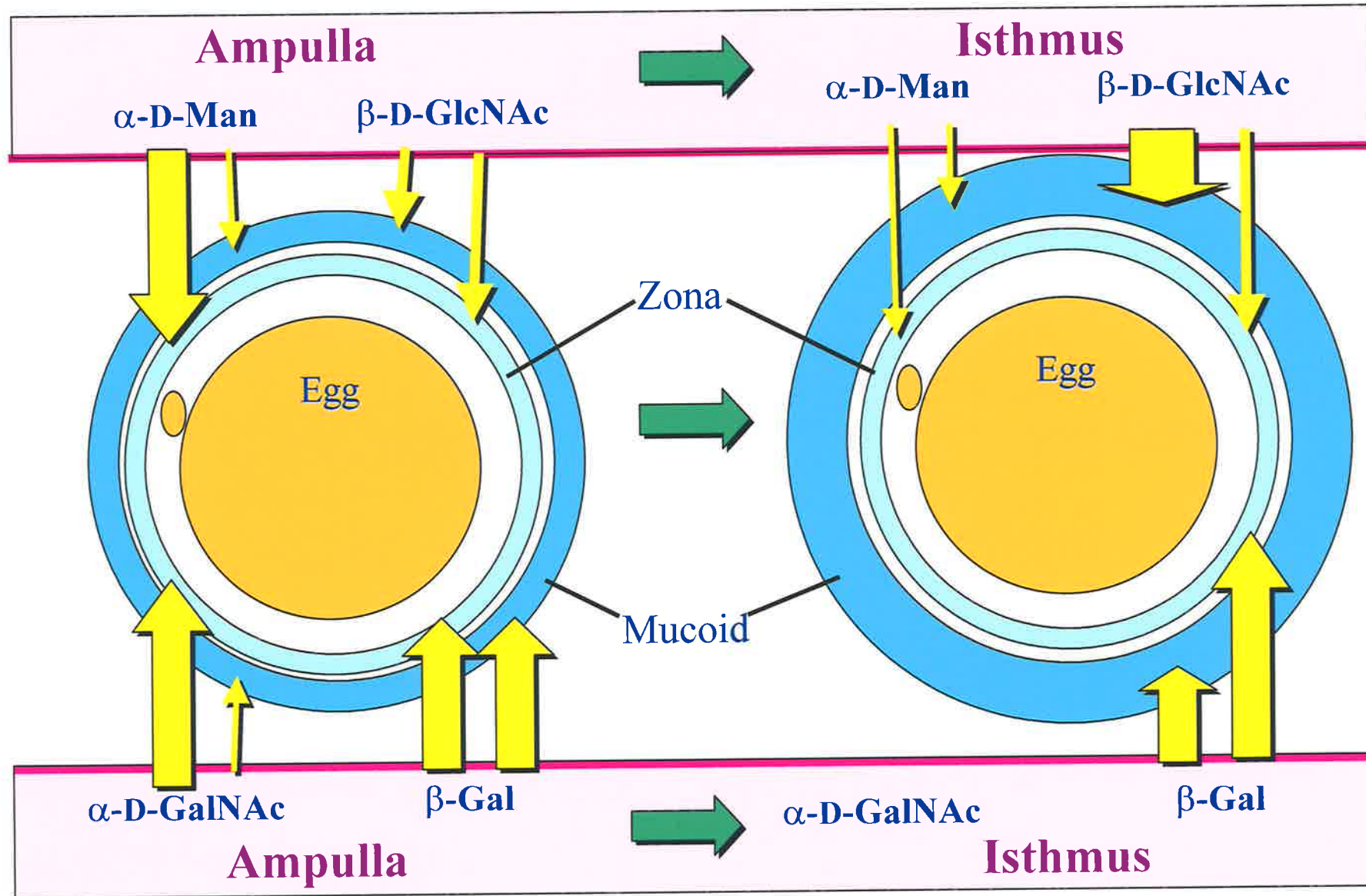
regional variation has been postulated for the secretion of the mucoid coat in *S. crassicaudata* (Roberts & Breed, 1996b; Selwood, 2000), this is the first biochemical evidence that regional variation exists in the secretions of the ampullary and isthmic epithelium of the brushtail possum.

8.2.5 Glycoconjugates of the Post-Ovulatory Zona Pellucida

Lectin immuno-gold cytochemistry also highlighted the influence of the oviductal secretions on the ZP of the brushtail possum, with a significant increase in α -D-Man, β -Gal, and α -D-GalNAc and/or α -Gal and a small increase in GlcNAc residues, in the ZP following ovulation. The mucoid coat, however, appeared to be primarily composed of GlcNAc and β -Gal residues, and less so by the other two glycoconjugates. Taking the lectin immuno-gold cytochemistry results of the oviduct, ZP and mucoid coat together, a hypothesised summary of the regional influence of the oviductal glycoconjugates on the ovulated oocytes is presented (Fig. 8.2).

The newly ovulated oocyte first enters the oviduct via the infundibulum and reaches the ampulla. In marsupials, as fertilisation takes place within the ampulla (Selwood, 1982; Bedford & Breed, 1994; Jungnickel *et al.*, 2000) and this is also the site for the first accumulation of the mucoid coat, any influence that the oviduct is going to have on sperm-ZP interaction, either positive or negative, is going to occur within this region. Within the ampulla, therefore, α -D-GalNAc (and/or α -D-Gal) and α -D-Man appear to have the greatest effect on the post-ovulatory ZP (Fig. 8.2). For example, α -D-GalNAc was the most highly labelled glycoconjugate within the secretory granules of the ampulla and it appeared to be almost entirely localised to this region, while α -D-Man was significantly greater in the ampulla than in the isthmus. Also, both α -D-GalNAc and α -D-Man residues were found

Figure 8.2. Diagrammatic representation of the possible glycoconjugate contribution by the ampullary and isthmic epithelium of the oviduct to the post-ovulatory oocyte of *T. vulpecula*. Note: The larger the arrows, the greater the contribution.



to have significantly increased within the ZP following ovulation, while they both only made minor contributions to the mucoid coat. The finding that α -D-GalNAc (and/or α -D-Gal) residues also significantly decreased within the ZP following cortical granule exocytosis suggests that this glycoconjugate may have an important role in sperm-ZP binding.

This is not to say that β -Gal does not play a functional role in the ZP of the ovulated oocyte, as was found to be the second most highly labelled glycoconjugate within the ampulla and also significantly increases within the ZP following ovulation (Fig. 8.2). However, the equal distribution of β -Gal along the oviduct and the fact that it was the second highest labelled glycoconjugate within the mucoid coat, suggests that it may be more likely to play a role in the formation of the mucoid coat. The finding that GlcNAc residues were found in higher density within the isthmus, increased only non-significantly within the ZP following ovulation and were the major components of the mucoid coat also suggests that this glycoconjugate may have more of a role in the formation of the mucoid coat.

There is an alternative hypothesis for the influence of the oviduct on the ZP. If the oviduct was not contributing glycoconjugates to the ZP, then why is there an increase in glycoconjugates following ovulation? One possible reason (as was utilised by this study to investigate the masked glycoconjugates of marsupial ovarian ZP) is that the oviduct may produce glycosidases such as neuraminidase that modify the existing glycoconjugates of the ZP. These oviductal glycosidases would then function by exposing masked glycoconjugates within the ZP, allowing them to bind to sperm, as well as to lectins.

Neuraminidase activity has been previously noted in the oviducts of non-mammalian vertebrates such as amphibians (De Martinez & Olavarria, 1973; De Martinez *et al.*, 1975; Vitaoli *et al.*, 1990). While it has not been noted directly in mammalian oviducts, the removal of sialic acid from the surface of human sperm has been shown to significantly increase sperm-ZP binding (Lasalle & Testart, 1994). One possible hypothesis for the prerequisite of incubation of possum sperm in oviduct-conditioned media before successful sperm-ZP binding *in vitro* occurs, therefore, could follow a similar theme, that is, that an oviductal neuraminidase is required to remove sialic acid from the sperm surface before sperm-ZP binding can occur. As no neuraminidase, or any other glycosidase activity, has been demonstrated to occur within the marsupial oviduct (with the exception of acrosomal and cortical granule glycosidases during sperm-egg interactions), more investigations are required to test these hypotheses.

8.2.6 Role of Cortical Granule Exocytosis on the Structure of the Zona Pellucida

The oviduct obviously plays an important role in sperm-egg interaction in marsupials. Secretions of the oviduct, namely those that produce the mucoid coat, have been suggested to both enhance and hinder fertilisation. For example, early deposits of the mucoid layer on the ZP have been suggested to enhance sperm adherence to the ZP (Breed, 1994; Selwood, 2000), while excess mucoid has been suggested to act as a barrier to polyspermy (Rodger & Bedford, 1982; Selwood, 1982, 2000). The mucoid coat in marsupials, therefore, could play a number of the roles analogous to the ZP of eutherians, including possibly being responsible for the block to polyspermy, maintenance of the structural integrity of the developing embryo and the prevention of the premature attachment of the embryo to the oviductal epithelium.

Whether there is any block to polyspermy at the level of the ZP in marsupials is unknown, however, this study showed that there is at least some biochemical reaction on the possum ZP following cortical granule exocytosis. ZP from artificially activated possum oocytes had decreased levels of glycoconjugates compared with non-activated oocytes, although only α -D-GalNAc (and/or α -D-Gal) was significantly reduced. Glycosidases have been shown to be released from cortical granules following egg activation (for review see Hoodbhoy & Talbot, 1994), while the glycoconjugate content of hamster ZP has been shown to change following natural and artificial egg activation (although some differences are evident between the two methods) (El-Mestrah & Kan, 2002). More work is needed to establish if a block to polyspermy is established at the ZP in marsupials.

Despite the previous finding in *M. domestica* of a 'cortical granule envelope' within the perivitelline space (PVS) following egg activation (Dandekar *et al.*, 1995), this study found that the perivitelline space matrix (PVM) in both non-activated and activated possum oocytes appeared to result primarily from the incorporation of oviductal secretions and less so from cortical granule exocytosis. The incorporation of oviductal glycoproteins into the PVS has been found to occur in mouse oocytes (Kapur & Johnson, 1986, 1988; Kim *et al.*, 1996), while Roberts and Breed (1996a) demonstrated that polyclonal antibodies raised against the mucoid and shell coats of *S. crassicaudata* immunolocalised within the PVS of unfertilised mouse oocytes. The cross-reactivity of the oviductal glycoproteins within the PVS of the mouse oocytes and the mucoid/shell membranes of the fat-tailed dunnart highlights the high degree of conservation of oviduct-specific glycoproteins across mammals. The failure to achieve sperm-oolemma binding *in vitro* (Mate *et al.*, 2000), combined with the finding in this thesis of oviductal glycoproteins within the PVS of unfertilised tubal oocytes of the possum, suggests that the oviduct may also play a role in sperm-egg binding. This, of course, requires further investigation.

8.3 Future studies

The justification for the research into the egg coats of marsupials of late has fallen into two main areas: potential immunocontraceptive applications and potential benefits for endangered marsupials via assisted reproduction techniques (for review see Mate *et al.*, 1998). Both rely somewhat on further scientific investigations into the structure and function of marsupial egg coats and their role in fertilisation. The discussion above of the major findings of this thesis has highlighted a number of areas that would benefit from further research.

One obvious area for further research is to continue investigations into the role of the oviduct in pre-fertilisation maturation of the gametes, sperm-ZP and sperm-egg interaction and in facilitating early embryonic development. The cross-reactivity of the antiserum generated against the mucoid and shell membrane of the fat-tailed dunnart with the PVM of the laboratory mouse demonstrates a high degree of conservation of the oviduct-specific glycoproteins (OSG), even across mammalian infraclasses (Roberts *et al.*, 1997). Initial steps, then, could be to biochemically characterise the OSG in marsupials. As genes have been sequenced for the OSG of a number of eutherian species (for review see Malette *et al.*, 1995), primers could be designed to the highly conserved regions of the OSG and the gene for the marsupial OSG could be cloned and sequenced.

Sequencing of the OSG gene in marsupials could then be used for determining homologous regions of this gene across the infraclasses, which would provide an insight into this gene's evolution. Sequencing would also allow for an analysis of the biochemical structure of the OSG gene and glycoprotein providing insight into its transcription, post-translational modifications such as sulphation, glycosylation and phosphorylation, as well

as its method of secretion. Specific primers could then be designed to enable studies such as Northern blot analyses and *in situ* hybridisation to investigate the expression of the OSG gene including the location and timing of its expression during the oestrous cycle, as well as determining if there is any regional variation (e.g. between the ampulla and isthmus) of its expression.

The development of the oviduct cell culture system in marsupials (Sidhu *et al.*, 1998, 1999) has enabled a great many investigations to proceed, including the development of sperm-oviduct cell monolayer co-culture and the first successful method for achieving *in vitro* sperm-ZP binding and penetration in an Australian marsupial (Sidhu *et al.*, 1998, 1999; Mate *et al.*, 2000). Oviduct cell culture would enable sufficient quantities of OSG to be produced so that a number of biochemical studies could be performed, as well as enabling the production of antibodies generated against the marsupial OSG. This method of generating antibodies, that is on the native glycosylated form of OSG, reduces much time and effort required to generate enough protein from some form of expression system (e.g. bacterial, mammalian cells such as chinese hamster ovary cells), as well as ensuring that the glycoprotein is naturally glycosylated.

Immunohistochemistry and immunocytochemistry could then be performed allowing for the immuno-localisation of the OGP within the oviductal epithelium. Immuno-localisation could also be used on ovulated oocytes to determine if the increase in glycosylation within the ZP, as demonstrated in this thesis, was a result of the incorporation of OGP or from some other factor, while also allowing an exact determination of whether the PVM in marsupials results from cortical granule exocytosis or is oviductal in origin. Immunocytochemistry could also be used to determine if OGP or some other oviductal factors bind to spermatozoa (binding of oviduct proteins to possum sperm has been

previously demonstrated by Sidhu *et al.*, 1999). If OGP did bind to sperm, immunocytochemistry could be used to determine if binding of OGP to spermatozoa was localised to a specific region of the sperm that might, therefore, provide clues into its role in enabling sperm-ZP binding *in vitro*.

Further attempts at achieving *in vitro* fertilisation in Australian marsupials should also be continued, with a focus on developing an oocyte-oviduct cell monolayer co-culture in conjunction with the sperm-oviduct cell monolayer co-culture. *In vitro*-matured ovarian oocytes have been used by Mate *et al.* (2000) for *in vitro* fertilisation studies, presumably because of problems of obtaining precisely timed ovulated oocytes which, therefore, results in the retrieval of mucoid-encased oocytes from the oviducts. Whether a mucoid coat would be formed during oocyte-oviduct cell monolayer co-culture is unknown. The concentric nature of the mucoid coats found around oocytes of a number of marsupials, however, suggests that ovulated oocytes accumulate their mucoid coat as they roll down the confined space of the oviduct (Selwood, 1982, 2000; Breed & Leigh, 1990), which is obviously very different from what would happen in co-culture. Alternatively, oocytes may be incubated in oviduct-conditioned media to prevent any physical contact of the oocyte with the oviduct.

Lastly, the glycoconjugates responsible for sperm-ZP binding can now be investigated. Although the postulated incorporation of OGP into the ZP complicates the issue, competitive binding studies of sperm using a variety of glycoconjugates should be attempted following their pre-required incubation in oviduct-conditioned media. Inhibition of sperm-ZP binding *in vitro* would then enable the glycoconjugates of the ZP responsible for the primary binding of sperm to be characterised. One specific glycoconjugate that should be targeted for competitive binding in the brushtail possum, and supported by the

results of this thesis as an obvious candidate for one of the glycoconjugates responsible for primary sperm binding, is α -D-GalNAc and/or α -D-Gal. Once such co-culture systems are developed in other Australian marsupials, comparisons can be made about whether any species-specificity exists in the ZP glycoconjugates responsible for sperm binding.

8.4 Conclusions

In this thesis the structure, and possible function, of the marsupial ZP has been investigated under various physiological conditions. While this thesis was being undertaken, the three genes responsible for the ZP in the brushtail possum were characterised and, for the first time in an Australian marsupial, *in vitro* sperm-ZP binding and penetration was achieved. The major focus of this thesis on the glycobiology of the marsupial ZP during its life history, however, provided a unique perspective on its structure and function, and highlights the importance of post-translational modification when considering the product of a gene. The finding in this thesis of the major modification to the coats around the oocyte, particularly the ZP and the PVM, following ovulation further highlight the importance of the oviductal environment in sperm-egg interactions in Australian marsupials. Such reliance by the gametes of Australian marsupials on the oviductal environment for fertilization appears to differ markedly from that of most eutherians. The lack of this appreciation was perhaps one of the primary reasons for the previous failure of researchers to achieve fertilization *in vitro* in this group of mammals.

References

- Abbott AL, and Ducibella T (2001) Calcium and the control of mammalian cortical granule exocytosis. *Frontiers in Bioscience* **6**, d792-d806.
- Abe H (1996) The mammalian oviductal epithelium: regional variations in cytological and functional aspects of the oviductal secretory cells. *Histology and Histopathology* **11**, 743-768.
- Abe H, and Abe M (1993) Immunological detection of an oviductal glycoprotein in the rat. *Journal of Experimental Zoology* **266**, 328-335.
- Abe H, and Oikawa T (1990a) Study of the differentiation of secretory cells in the golden hamster oviductal epithelium by use of a monoclonal antibody. *Journal of Experimental Zoology* **254**, 97-106.
- Abe H, and Oikawa T (1990b) Ultrastructural evidence for an association between an oviductal glycoprotein and the zona pellucida of the golden hamster egg. *Journal of Experimental Zoology* **256**, 210-221.
- Abe H, and Oikawa T (1991) Immunocytochemical localization of an oviductal zona pellucida glycoprotein in the oviductal epithelium of the golden hamster. *Anatomical Record* **229**, 305-314.
- Abou-Haila A, and Tulisani DRP (2000) Mammalian sperm acrosome: formation, contents, and function. *Archives of Biochemistry and Biophysics* **379**, 173-182.
- Adham IM, Nayernia K, and Engel W (1997) Spermatozoa lacking acrosin protein show delayed fertilization. *Molecular Reproduction and Development* **46**, 370-376.
- Akatsuka K, Yoshida-Komiya H, Tulisani DRP, Orgebin-Crist M-C, Hiroi M, and Araki Y (1998) Rat zona pellucida glycoproteins: molecular cloning and characterization of the three major components. *Molecular Reproduction and Development* **51**, 454-467.
- Alcorn GT (1975) Development of the ovary and urogenital ducts in the tammar wallaby *Macropus eugenii* (Desmarest, 1817). Macquarie University.
- Alcorn GT, and Robinson ES (1983) Germ cell development in female pouch young of the tammar wallaby (*Macropus eugenii*). *Journal of Reproduction and Fertility* **67**, 319-325.
- Amanze D, and Iyengar A (1990) The micropyle: a sperm guidance system in teleost fertilisation. *Development* **109**, 495-500.
- Andreuccetti P, Famularo C, Gualtieri R, and Prisco M (2001) Pyriform cell differentiation in *Podarcis sicula* is accompanied by the appearance of surface glycoproteins bearing α -GalNAc terminated chains. *Anatomical Record* **263**, 1-9.

Araki Y, Kurata S, Oikawa T, Yamashita T, Hiroi M, Naiki M, and Sendo F (1987) A monoclonal antibody reacting with the zona pellucida of the oviductal egg but not with that of the ovarian egg of the golden hamster. *Journal of Reproductive Immunology* **11**, 193-208.

Archibong AE, Petters RM, and Johnson BH (1989) Development of porcine embryos from one- and two-cell stages to blastocysts in culture medium supplemented with porcine oviductal fluid. *Biology of Reproduction* **41**, 1076-1083.

Arias EB, Verhage HG, and Jaffe RC (1994) Complementary deoxyribonucleic acid cloning and molecular characterization of an estrogen-dependent human oviductal glycoprotein. *Biology of Reproduction* **51**, 685-694.

Arnold R, and Shorey CD (1985) Structure of the oviductal epithelium of the brush-tailed possum (*Trichosurus vulpecula*). *Journal of Reproduction and Fertility* **73**, 9-19.

Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, and Florman HM (1999) Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proceedings of the National Academy of Sciences (USA)* **96**, 6757-6762.

Avilés M, Okinaga T, Shur BD, and Ballesta J (2000a) Differential expression of glycoside residues in the mammalian zona pellucida. *Molecular Reproduction and Development* **57**, 296-308.

Avilés M, Jaber L, Castells MT, Kan FK, and Ballesta J (1996) Modifications of the lectin binding pattern in the rat zona pellucida after *in vivo* fertilization. *Molecular Reproduction and Development* **44**, 370-381.

Avilés M, El-Mestrah M, Jaber L, Castells MT, Ballesta J, and Kan FWK (2000b) Cytochemical demonstration of modification of carbohydrates in the mouse zona pellucida during folliculogenesis. *Histochemistry and Cell Biology* **113**, 207-219.

Avilés M, Castells MT, Abascal I, Martinez-Menarguez JA, Draber P, Kan FWK, and Ballesta J (1999) Cytochemical localization of GalNAc and GalNAc β 1,4Gal β 1,4 disaccharide in mouse zona pellucida. *Cell Tissue Research* **295**, 269-277.

Baba T, Azuma S, Kashiwabara S-i, and Toyoda Y (1994) Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *Journal of Biological Chemistry* **269**, 31845-31849.

Bachmann L, and Mayer E (1987) Physics of water and ice: implications for cryofixation. In 'Cryotechniques in Biological Electron Microscopy'. (Eds RA Steinbrecht and K Zierold) pp. 1-34. (Springer-Verlag: Berlin)

Bakst MR, and Howarth Jr B (1977a) The fine structure of the hen's ovum at ovulation. *Biology of Reproduction* **17**, 361-369.

Bedford, JM (1977) Sperm/egg interaction: the specificity of human spermatozoa. *Anatomical Record* **188**, 477-487.

Bakst MR, and Howarth Jr B (1977b) Hydrolysis of the hen's perivitelline layer by cock sperm in vitro. *Biology of Reproduction* **17**, 370-379.

Banerjee M, and Chowdhury M (1997) Localization of a 25kDa human sperm surface protein: its role in in-vitro human sperm capacitation. *Molecular Human Reproduction* **3**, 109-114.

Bar-Shira Maymon B, Maymon R, Ben-Nun I, Ghetler Y, Shalgi R, Skutelsky E (1994) Distribution of carbohydrates in the zona pellucida of human oocytes. *Journal of Reproduction and Fertility* **102**, 81-86.

Barber MR, Lee SM, Steffens WL, Ard M, and Fayrer-Hosken RA (2001) Immunolocalization of zona pellucida antigens in the ovarian follicle of dogs, cats, horses and elephants. *Theriogenology* **55**, 1705-1717.

Barr SH, and Oliphant G (1981) Sulfate incorporation into macromolecules produced by cultured oviductal epithelium. *Biology of Reproduction* **24**, 852-858.

Barros C, Bedford JM, Franklin LE, and Austin CR (1967) Membrane vesiculation as a feature of the mammalian acrosome reaction. *Journal of Cell Biology* **34**, 1-5.

Barthelson R, Mobasser A, Zopf D, and Simon P (1998) Adherence of *Streptococcus pneumoniae* to respiratory epithelial cells is inhibited by sialylated oligosaccharides. *Infection and Immunity*, 1439-1444.

Bausek N, Waclawek M, Schneider WJ, and Wohlrab F (2000) The major chicken egg envelope protein ZP1 is different from ZPB and is synthesized in the liver. *Journal of Biological Chemistry* **275**, 28866-28872.

* insert reference (opposite page)

Bedford JM (1982) Fertilization. In 'Reproduction in Mammals, Vol. 1, Germ Cells and Fertilization 2nd Edition'. (Eds CR Austin and RV Short) pp. 128-163. (Cambridge University Press: Cambridge).

Bedford JM (1991) The coevolution of mammalian gametes. In 'A Comparative Overview of Mammalian Fertilization'. (Eds BS Dunbar and MG O'Rand) pp. 3-35. (Plenum Press: New York).

Bedford JM (1996) What marsupial gametes disclose about gamete function in eutherian mammals. *Reproduction Fertility and Development* **8**, 569-580.

Bedford JM (1998) Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. *Biology of Reproduction* **59**, 1275-1287.

Bedford JM, and Breed WG (1994) Regulated storage and subsequent transformation of spermatozoa in the fallopian tubes of an Australian marsupial, *Sminthopsis crassicaudata*. *Biology of Reproduction* **50**, 845-854.

Bedford JM, and Calvin HI (1974) The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to eutherian mammals. *Journal of Experimental Zoology* **188**, 137-156.

Bedford JM, Bernard RTF, and Baxter RM (1998) The 'hybrid' character of the gametes and reproductive tracts of the African shrew, *Myosorex varius*, supports its classification in the Crocidosoricinae. *Journal of Reproduction and Fertility* **112**, 165-173.

Bedford JM, Rodger JC, and Breed WG (1984) Why so many mammalian spermatozoa - a clue from marsupials? *Proceedings of the Royal Society of London B series* **221**, 221-233.

Bellairs R, Harkness M, and Harkness RD (1963) The vitelline membrane of the hen's egg: a chemical and electron microscopical study. *Ultrastructure Research* **8**, 339-359.

Benoff S (1997) Carbohydrates and fertilization: an overview. *Molecular Human Reproduction* **3**, 599-637.

Bennett JH, Breed WG, Hayman DL, and Hope RM (1990) Reproductive and genetical studies with a laboratory colony of the dasyurid marsupial *Sminthopsis crassicaudata*. *Australian Journal of Zoology* **37**, 207-222.

Bennett JH, Smith MJ, Hope RM, and Chesson CM (1982) Fat-tailed dunnart *Sminthopsis crassicaudata*: establishment and maintenance of a laboratory colony. In 'The Management of Australian Mammals in Captivity'. (Ed. DD Evans) pp. 38-44. (Zoological Board of Victoria: Melbourne).

Bergstrom S (1972) Shedding of the zona pellucida of the mouse blastocyst in normal pregnancy. *Journal of Reproduction and Fertility* **31**, 275-277.

Bleau G, and St-Jacques S (1989) Transfer of oviductal proteins to the zona pellucida. In 'The Mammalian Egg Coat: Structure and Function'. (Ed. J Dietl) pp. 99-110. (Springer-Verlag: Berlin).

Bleil JD, and Wassarman PM (1980) Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Developmental Biology* **76**, 185-202.

Bleil JD, and Wassarman PM (1986) Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *Journal of Cell Biology* **102**, 1363-1371.

Bleil JD, and Wassarman PM (1988) Galactose at the nonreducing terminus of O-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. *Proceedings of the National Academy of Sciences (USA)* **85**, 6778-6782.

Bleil JD, Beall CF, and Wassarman PM (1981) Mammalian sperm-egg interaction: fertilization of mouse eggs triggers modification of the major zona pellucida glycoprotein, ZP2. *Developmental Biology* **86**, 189-197.

Bleil JD, Greve JM, and Wassarman PM (1988) Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. *Developmental Biology* **128**, 376-385.

Boatman DE (1997) Responses of gametes to the oviductal environment. *Human Reproduction* **12**, 133-149.

Boatman DE, Felson SE, and Kimura J (1994) Changes in morphology, sperm penetration and fertilization of ovulated hamster eggs induced by oviductal exposure. *Human Reproduction* **9**, 519-526.

Boice ML, McCarthy TJ, Mavrogianis PA, Fazleabas AT, and Verhage HG (1990) Localization of oviductal glycoproteins within the zona pellucida and perivitelline space of ovulated ova and early embryos in baboons (*Papio anubis*). *Biology of Reproduction* **43**, 340-346.

Bonnell BS, and Chandler DE (1996) Egg jelly layers of *Xenopus laevis* are unique in ultrastructure and sugar distribution. *Molecular Reproduction and Development* **44**, 212-220.

Brambell FWR (1956) Ovarian changes. In 'Marshall's Physiology of Reproduction Vol. 1'. (Ed. AS Parkes) pp. 397-457. (Longmans, Green and Co.: London)

Breed WG (1994) How does sperm meet egg? - In a marsupial. *Reproduction Fertility and Development* **6**, 485-506.

Breed WG (1996) Egg maturation and fertilization in marsupials. *Reproduction Fertility and Development* **8**, 617-643.

Breed WG, and Leigh CM (1988) Morphological observations on sperm-egg interactions during in vivo fertilization in the dasyurid marsupial *Sminthopsis crassicaudata*. *Gamete Research* **19**, 131-149.

Breed WG, and Leigh CM (1990) Morphological changes in the oocyte and its surrounding vestments during in vivo fertilization in the dasyurid marsupial *Sminthopsis crassicaudata*. *Journal of Morphology* **204**, 177-196.

Breed WG, Leigh CM, and Bennett JH (1989) Sperm morphology and storage in the female reproductive tract of the fat-tailed dunnart, *Sminthopsis crassicaudata* (Marsupialia: Dasyuridae). *Gamete Research* **23**, 61-75.

Breed WG, Leigh CM, and Ricci M (2001) The structural organisation of sperm head components of the wombat and koala (suborder: *Vombatiformes*): an enigma amongst marsupials. *Journal of Anatomy* **198**, 57-66.

Brewis IA, and Moore HD (1997) Molecular mechanisms of gamete recognition and fusion at fertilization. *Human Reproduction* **12**, 156-165.

Burley, RW, Back, JF, Wellington, JE, and Grigg, GC (1987) Proteins of the albumen and vitelline membrane of eggs of the estuarine crocodile, *Crocodylus porosus*. *Comparative Biochemistry and Physiology, B* **88B**, 863-867.

Brown CR (1983) Purification of mouse sperm acrosin, its activation from proacrosin and effect on homologous egg investments. *Journal of Reproduction and Fertility* **69**, 289-295.

Brown CR, Clarke N, Aiken M, and Bavister BD (1990) Changes in the composition of the hamster zona pellucida after fertilization *in vivo* but not *in vitro*. *Journal of Reproduction and Fertility* **90**, 447-454.

Buhi WC, Alvarez IM, and Kouba AJ (1997) Oviductal regulation of fertilization and early embryonic development. *Journal of Reproduction and Fertility Supplement* **52**, 285-300.

Buhi WC, Alvarez IM, and Kouba AJ (2000) Secreted proteins of the oviduct. *Cells Tissues Organs* **166**, 165-179.

Buhi WC, Vallet JL, and Bazer FW (1989) De novo synthesis and release of polypeptides from cyclic and early pregnant porcine oviductal tissue in explant culture. *Journal of Experimental Zoology* **252**, 79-88.

Buhi WC, Alvarez IM, Sudhipong V, and Dones-Smith MM (1990) Identification and characterization of de novo-synthesized porcine oviductal secretory proteins. *Biology of Reproduction* **43**, 929-938.

Buhi WC, Ashworth CJ, Bazer FW, and Alvarez IM (1992) In vitro synthesis of oviductal secretory proteins by estrogen-treated ovariectomized gilts. *Journal of Experimental Zoology* **262**, 426-435.

Buhi WC, Alvarez IM, Choi I, Cleaver BD, and Simmen FA (1996) Molecular cloning and characterization of an estrogen-dependent porcine oviductal secretory glycoprotein. *Biology of Reproduction* **55**, 1305-1314.

Buhi WC, O'Brien B, Alvarez IM, Erdos G, and Dubois D (1993) Immunogold localization of porcine oviductal secretory proteins within the zona pellucida, perivitelline space, and plasma membrane of oviductal and uterine oocytes and early embryos. *Biology of Reproduction* **48**, 1274-1283.

* insert reference (opposite page)

Campanella C, Carotenuto R, Infante V, Maturi G, and Atripaldi U (1997) Sperm-egg interaction in the painted frog (*Discoglossus pictus*): an ultrastructural study. *Molecular Reproduction and Development* **47**, 323-333.

Caputo M, Infante V, Talevi R, Vaccaro MC, Carotenuto R, and Campanella C (2001) Following passage through the oviduct, the coelomic envelope of *Discoglossus pictus* (Amphibia) acquires fertilizability upon reorganization, conversion of gp42 to gp40, extensive glycosylation, and formation of a specific layer. *Molecular Reproduction and Development* **58**, 318-329.

Casey NP, Martinus R, and Selwood L (2002) Outer egg coats of the marsupial conceptus: secretion and protein composition. *Molecular Reproduction and Development* **62**, 181-194.

Chang YS, Hsu CC, Tsao CC, and Huang FL (1997) Molecular cloning, structural analysis, and expression of carp ZP2 gene. *Molecular Reproduction and Development* **46**, 258-267.

Chapman NR, and Barratt CLR (1996) The role of carbohydrate in sperm-ZP3 adhesion. *Molecular Human Reproduction* **2**, 767-774.

Chapman SA, Bonshek RE, Stoddard RW, Mackenzie KR, and McLeod D (1994) Localisation of $\alpha(2,3)$ and $\alpha(2,6)$ linked terminal sialic acid groups in human trabecular meshwork. *British Journal of Ophthalmology* **78**, 632-637.

Cherr GN, Yudin AL, and Katz DF (1990) Organization of the hamster cumulus extracellular matrix: a hyaluronate-glycoprotein gel which modulates sperm access to the oocyte. *Development Growth and Differentiation* **32**, 353-365.

Cherr GN, Summers RG, Baldwin JD, and Morrill JB (1992) Preservation and visualization of the sea urchin embryo blastocoelic extracellular matrix. *Microscopy Research and Technique* **22**, 11-22.

Chiquoine AD (1960) The development of the zona pellucida of the mammalian ovum. *American Journal of Anatomy* **106**, 149-196.

Clark GF, Oehninger S, and Seppala M (1996a) Role for glycoconjugates in cellular communication in the human reproductive system. *Molecular Human Reproduction* **2**, 513-517.

Clark GF, Oehninger S, Patankar MS, Koistinen R, Dell A, Morris HR, Koistinen H, and Seppala M (1996b) A role for glycoconjugates in human development: the human fetoplacental defence system hypothesis. *Human Reproduction* **11**, 467-473.

Connors SA, Kanatsu-Shinohara M, Schultz RM, and Kopf GS (1998) Involvement of the cytoskeleton in the movement of cortical granules during oocyte maturation, and cortical granule anchoring in mouse eggs. *Developmental Biology* **200**, 103-115.

Cooper NJ, Holland MK, and Breed WG (1998) Extratesticular sperm maturation in the brush-tail possum, *Trichosurus vulpecula*. *Journal of Reproduction and Fertility, Supplement* **53**, 225-226.

Cooper NJ, McClean RV, Leigh CM, and Breed WG (2001) Glycoconjugates on the surface of epididymal spermatozoa in a marsupial, the brushtail possum, *Trichosurus vulpecula*. *Reproduction* **122**, 165-176.

Cornwall GA, Tulisani DRP, and Orgebin-Crist M-C (1991) Inhibition of the mouse sperm surface α -D-mannosidase inhibits sperm-egg binding *in vitro*. *Biology of Reproduction* **44**, 913-921.

Cran DG (1989) Cortical granules during oocyte maturation and fertilization. *Journal of Reproduction and Fertility Supplement* **42**, 177-188.

Cran DG, and Esper CR (1990) Cortical granules and the cortical reaction in mammals. *Journal of Reproduction and Fertility Supplement* **42**, 177-188.

Crawford JL, Shackell GH, Thompson EG, McLeod BJ, and Hurst PR (1997) Preovulatory follicle development and ovulation in the brushtail possum (*Trichosurus vulpecula*) monitored by repeated laparoscopy. *Journal of Reproduction and Fertility* **110**, 361-370.

Cummins JM (1980) Decondensation of sperm nuclei of Australian marsupials: effects of air drying and of calcium and magnesium. *Gamete Research* **3**, 65-75.

Dandekar P, and Talbot P (1992) Perivitelline space of mammalian oocytes: extracellular matrix of unfertilized oocytes and formation of a cortical granule envelope following fertilization. *Molecular Reproduction and Development* **31**, 135-143.

Dandekar P, Aggeler J, and Talbot P (1992) Structure, distribution and composition of the extracellular matrix of human oocytes and cumulus masses. *Human Reproduction* **7**, 391-398.

Dandekar P, Mate KE, and Talbot P (1995) Perivitelline space of marsupial oocytes: extracellular matrix of the unfertilized oocyte and formation of a cortical granule envelope following the cortical reaction. *Molecular Reproduction and Development* **41**, 368-373.

De Martinez NR, and Olavarria JM (1973) Metabolism of sialic acid in toad oviduct. *Biochimica et Biophysica Acta* **320**, 301-310.

De Martinez NR, Mendez BA, and Olavarria JM (1975) Topographical distribution of sialic acids and related enzymes in toad oviduct. *Comparative Biochemistry and Physiology Part B* **50**, 603-607.

Debray H, Decout T, Strecker G, Spik G, and Montreuil J (1981) Specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins. *European Journal of Biochemistry* **117**, 41-55.

Dell A, Morris HR, Easton RL, Patankar M, and Clark GF (1999) The glycobiology of gametes and fertilisation. *Biochimica et Biophysica Acta* **1473**, 196-205.

Demott RP, Lefebvre R, and Suarez SS (1995) Carbohydrates mediate the adherence of hamster sperm to oviductal epithelium. *Biology of Reproduction* **52**, 1395-1403.

Denis-Donini S, and Campanella C (1977) Ultrastructural and lectin binding changes during the formation of the animal dimple in oocytes of *Discoglossus pictus* (Anura). *Developmental Biology* **61**, 140-152.

Denker H-W (2000) Structural dynamics and function of early embryonic coats. *Cells Tissues Organs* **168**, 180-207.

Denker H-W, and Gerdes HJ (1979) The dynamic structure of rabbit blastocyst coverings. I. Transformation during regular preimplantation development. *Anatomy and Embryology* **157**, 15-34.

Didion BA, Martin MJ, and Market CL (1990) Parthenogenetic activation of mouse and pig oocytes matured in vitro. *Theriogenology* **33**, 1165-1175.

- Dietl J (1989) Ultrastructural aspects of the developing mammalian zona pellucida. In 'The Mammalian Egg Coat: Structure and Function'. (Ed. J Dietl) pp. 49-60. (Springer-Verlag: Berlin)
- Donnelly KM, Fazleabas AT, Verhage HG, Mavrogianis PA, and Jaffe RC (1991) Cloning of a recombinant complementary DNA to a baboon (*Papio anubis*) estradiol-dependent oviduct-specific glycoprotein. *Molecular Endocrinology* **5**, 356-364.
- Ducibella T (1991) Mammalian egg cortical granules and the cortical reaction. In 'Elements of Mammalian Fertilization' pp. 205-231. (CRC Press: Boca Raton).
- Ducibella T (1996) The cortical reaction and development of activation competence in mammalian oocytes. *Human Reproduction Update* **2**, 29-42.
- Ducibella T, Anderson E, Albertini DF, Aalberg J, and Rangarajan S (1988) Quantitative studies of changes in cortical granule number and distribution in the mouse oocyte during meiotic maturation. *Developmental Biology* **130**, 184-197.
- Dunbar BS, and Raynor BD (1980) Characterization of porcine zona pellucida antigens. *Biology of Reproduction* **22**, 941-954.
- Dunbar BS, Liu C, and Sammons DW (1981) Identification of the three major proteins of porcine and rabbit zonae pellucidae by high resolution two-dimensional gel electrophoresis: comparison with serum, follicular fluid, and ovarian cell proteins. *Biology of Reproduction* **24**, 1111-1124.
- Dunbar BS, Prasad SV, and Timmons TM (1991) Comparative structure and function of mammalian zonae pellucidae. In 'A Comparative Overview of Mammalian Fertilization'. (Eds BS Dunbar and MG O'Rand) pp. 97-114. (Plenum Press: New York)
- Dunbar BS, Timmons TM, Skinner SM, and Prasad SV (2001) Molecular analysis of a carbohydrate antigen involved in the structure and function of zona pellucida glycoproteins. *Biology of Reproduction* **65**, 951-960.
- Dunbar BS, Avery S, Lee V, Prasad S, Schwahn D, Schwoebel E, Skinner S, and Wilkins B (1994) The mammalian zona pellucida: its biochemistry, immunochemistry, molecular biology, and developmental expression. *Reproduction Fertility and Development* **6**, 331-347.
- El-Mestrah M, and Kan FWK (1999a) Differential distribution of lectin-binding glycoconjugates in the secretory granules of hamster oviductal ampulla during the estrous cycle: a quantitative cytochemical analysis. *Histochemistry and Cell Biology* **111**, 23-32.
- El-Mestrah M, and Kan FWK (1999b) Ultrastructural and ultracytochemical features of secretory granules in the ampullary epithelium of the hamster oviduct. *Anatomical Record* **255**, 227-239.

- El-Mestrah M, and Kan FWK (2001) Distribution of lectin-binding glycosidic residues in the hamster follicular oocytes and their modifications in the zona pellucida after ovulation. *Molecular Reproduction and Development* **60**, 517-534.
- El-Mestrah M, and Kan FWK (2002) Variations in modifications of sugar residues in hamster zona pellucida after *in vivo* fertilization and *in vitro* egg activation. *Reproduction* **123**, 671-682.
- Elinson RP (1986) Fertilization in amphibians: the ancestry of the block to polyspermy. *International Review of Cytology* **101**, 59-100.
- Ellington JE, Carney EW, Farrell PB, Simkin ME, and Foote RH (1990) Bovine 1-2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. *Biology of Reproduction* **43**, 97-104.
- Epifano O, Liang L-f, and Dean J (1995a) Mouse *Zp1* encodes a zona pellucida protein homologous to egg envelope proteins in mammals and fish. *Journal of Biological Chemistry* **270**, 27254-27258.
- Epifano O, Liang L-f, Familiari M, Moos Jr MC, and Dean J (1995b) Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* **121**, 1947-1956.
- Erk I, Nicolas G, Caroff A, and Lepault J (1998) Electron microscopy of frozen biological objects: a study using cryosectioning and cryosubstitution. *Journal of Microscopy* **189**, 236-248.
- Eyestone WH, Jones JM, and First NL (1991) Some factors affecting the efficacy of oviduct tissue-conditioned medium for the culture of early bovine embryos. *Journal of Reproduction and Fertility* **92**, 59-64.
- Falconnier C, and Kress A (1992) Ultrastructural aspects of oocyte growth in the marsupial *Monodelphis domestica* (grey short-tailed opossum). *Journal of Anatomy* **181**, 481-498.
- Familiari G, Nottola SA, Familiari A, and Motta PM (1989) The three-dimensional structure of the zona pellucida in growing and atretic ovarian follicles of the mouse: scanning and transmission electron-microscopic observations using ruthenium red and detergents. *Cell Tissue Research* **257**, 247-253.
- Familiari G, Nottola SA, Micara G, Aragona C, and Motta PM (1988) Is the sperm-binding capability of the zona pellucida linked to its surface structure? A scanning electron microscopic study of human *in vitro* fertilization. *Journal of In Vitro Fertilization and Embryo Transfer* **5**, 134-143.
- Familiari G, Nottola SA, Macchiarelli G, Micara G, Aragona C, and Motta PM (1992) Human zona pellucida during *in vitro* fertilization: an ultrastructural study using saponin, ruthenium red, and osmium-thiocarbohydrazide. *Molecular Reproduction and Development* **32**, 51-61.

Fukuda, Y, Maddock, MB, and Chang, MC (1979) In vitro fertilization of two species of deer mouse eggs by homologous or heterologous sperm and penetration of laboratory mouse eggs by deer mouse sperm. *Journal of Experimental Zoology* **207**, 481-489.

Fazeli A, Duncan AE, Watson PF, and Holt WV (1999) Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biology of Reproduction* **60**, 879-886.

Fazleabas AT, and Verhage HG (1986) The detection of oviduct-specific proteins in the baboon (*Papio anubis*). *Biology of Reproduction* **35**, 455-462.

Florman HM, and Wassarman PM (1985) O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* **41**, 313-324.

Florman HM, Arnoult C, Kazam IG, Li C, and O'Toole CMB (1998) A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. *Biology of Reproduction* **59**, 12-16.

Focarelli R, La Sala GB, Balasini M, and Rosati F (2001) Carbohydrate-mediated sperm-egg interaction and species specificity: a clue from the *Unio elongatulus* model. *Cells Tissues Organs* **168**, 76-81.

Frank J (2001) Cryo-electron microscopy as an investigative tool: the ribosome as an example. *BioEssays* **23**, 725-732.

Frankenberg S, and Selwood L (2001) Ultrastructure of oogenesis in the brushtail possum. *Molecular Reproduction and Development* **58**, 297-306.

Frankenberg S, Newell G, and Selwood L (1996) A light microscopical study of oogenesis in the brushtail possum *Trichosurus vulpecula*. *Reproduction Fertility and Development* **8**, 541-546.

Fraser LR (1982) *p*-Aminobenzamidine, an acrosin inhibitor, inhibits mouse sperm penetration of the zona pellucida but not the acrosome reaction. *Journal of Reproduction and Fertility* **65**, 185-194.

Frigo L, and Woolley PA (1997) Growth and development of pouch young of the stripe-faced dunnart, *Sminthopsis macroura* (Marsupialia: Dasyuridae), in captivity. *Australian Journal of Zoology* **45**, 157-170.

* insert reference (opposite page)

Fukumoto M (1990) Morphological aspects of ascidian fertilization: acrosome reaction, apical processes and gamete fusion in *Ciona intestinalis*. *Invertebrate Reproduction and Development* **17**, 147-154.

Funahashi H, Ekwall H, and Rodriguez-Martinez H (2000) Zona reaction in porcine oocytes fertilized in vivo and in vitro as seen with scanning electron microscopy. *Biology of Reproduction* **63**, 1437-1442.

Funahashi H, Ekwall H, Kikuchi K, and Rodriguez-Martinez H (2001) Transmission electron microscopy studies of the zona reaction in pig oocytes fertilized *in vivo* and *in vitro*. *Reproduction* **122**, 443-452.

Gandolfi F (1995) Functions of proteins secreted by oviduct epithelial cells. *Microscopy Research and Technique* **32**, 1-12.

Gandolfi F, Modina S, Brevini TAL, Galli C, Moor RM, and Lauria A (1991) Oviduct ampullary epithelium contributes a glycoprotein to the zona pellucida, perivitelline space and blastomeres membrane of sheep embryos. *European Journal of Applied Histochemistry* **35**, 383-392.

Gilkey JC (1981) Mechanisms of fertilization in fishes. *American Zoologist* **21**, 359.

Glazier AM, and Molinia FC (1998) Improved method of superovulation in monovulatory brushtail possums (*Trichosurus vulpecula*) using pregnant mares' serum gonadotrophin-luteinizing hormone. *Journal of Reproduction and Fertility* **113**, 191-195.

Goldstein IJ, Hughes RC, Monsigny M, Osawa T, and Sharon N (1980) What should be called a lectin? *Nature* **285**, 66.

Gould KG (1974) Fertilization: a function of the oviduct. In 'The Oviduct and Its Function'. (Eds AD Johnson and CW Foley) pp. 271-300. (Academic Press: New York)

Green DPL (1989) Effect of sodium dodecyl sulphate on the hamster zona pellucida before and after a cortical reaction induced by the ionophore A23187. *Journal of Reproduction and Fertility* **87**, 447-453.

Green DPL (1997) Three-dimensional structure of the zona pellucida. *Reviews of Reproduction* **2**, 147-156.

Green CE, Bredl J, Holt WV, Watson PF, and Fazeli A (2001) Carbohydrate mediation of boar sperm binding to oviductal epithelial cells *in vitro*. *Reproduction* **122**, 305-315.

Greenwald GS (1957) Interruption of pregnancy in the rabbit by the administration of estrogen. *Journal of Experimental Zoology* **135**, 461-465.

Greenwald GS (1958) Endocrine regulation of the secretion of mucin in the tubal epithelium of the rabbit. *Anatomical Record* **130**, 477-495.

Greve JM, and Wassarman PM (1985) Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. *Journal of Molecular Biology* **181**, 253-264.

Grootenhuis AJ, Philipsen HLA, de Breet-Grijsbach JTM, and van Duin M (1996) Immunocytochemical localization of ZP3 in primordial follicles of rabbit, marmoset, rhesus monkey and human ovaries using antibodies against human ZP3. *Journal of Reproduction and Fertility Supplement* **50**, 43-54.

Gulyas BJ, and Schmell ED (1980) Ovoperoxidase activity in ionophore treated mouse eggs. I. Electron microscopic localization. *Gamete Research* **3**, 267-277.

Guraya SS (1968) Histochemical study of developing ovarian oocyte of the American opossum. *Acta Embryologiae et Morphologiae Experimentalis* **10**, 181-191.

Guraya SS (1982) Recent progress in the structure, origin, composition, and function of cortical granules in animal egg. *International Review of Cytology* **78**, 257-360.

Gwatkin RBL, and Williams DT (1977) Receptor activity of the hamster and mouse solubilized zona pellucida before and after the zona reaction. *Journal of Reproduction and Fertility* **49**, 55-59.

Haines BP, Rathjen PD, Hope RM, Whyatt LM, Holland MK, and Breed WG (1999) Isolation and characterisation of a cDNA encoding a zona pellucida protein (ZPB) from the marsupial *Trichosurus vulpecula* (Brushtail possum). *Molecular Reproduction and Development* **52**, 174-182.

Hamazaki T, Nagahama Y, Iuchi I, and Yamagami K (1989) A glycoprotein from the liver constitutes the inner layer of the egg envelope (zona pellucida interna) of the fish, *Oryzias latipes*. *Developmental Biology* **133**, 101-110.

Harding HR, and Aplin KP (1990) Phylogenetic affinities of the koala (Phascolarctidae: Marsupialia): a reassessment of the spermatozoal evidence. In 'Biology of the Koala'. (Eds AK Lee, KA Handasyde and GD Sanson) pp. 1-31. (Surrey Beatty and Sons: Sydney).

Harding HR, Carrick FN, and Shorey CD (1976) Ultrastructural changes in spermatozoa of the brush-tailed possum, *Trichosurus vulpecula* (Marsupialia). Part II. The acrosome. *Cell Tissue Research* **171**, 61-73.

Harding HR, Carrick FN, and Shorey CD (1987) The affinities of the koala *Phascolarctos cinereus* (Marsupialia: Phascolarctidae) on the basis of sperm ultrastructure and development. In 'Possums and Opossums: Studies in Evolution'. (Ed. M Archer) pp. 353-364. (Surrey Beatty & Sons, Royal Zoological Society of NSW: Sydney).

Harper MJK (1982) Sperm and egg transport. In 'Reproduction in Mammals. I. Germ Cells and Fertilization'. (Eds CR Austin and RV Short) pp. 102-127. (Cambridge University Press: Cambridge)

Harris JD, Hibler DW, Fontenot GK, Hsu KT, Yurewicz EC, and Sacco AG (1994) Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families. *DNA Sequence* **4**, 361-393.

Hedrick JL (1996) Comparative structural and antigenic properties of zona pellucida glycoproteins. *Journal of Reproduction and Fertility Supplement* **50**, 9-17.

Hedrick JL, and Katagiri C (1988) *Bufo japonicus* and *Xenopus laevis laevis* egg jellies contain structurally related antigens and cortical granule lectin ligands. *Journal of Experimental Zoology* **245**, 78-85.

- Hedrick JL, and Nishihara T (1991) Structure and function of the extracellular matrix of anuran eggs. *Journal of Electron Microscopy Technique* **17**, 319-335.
- Hedrick JL, Wardrip NJ, and Berger T (1987) Differences in the macromolecular composition of the zona pellucida isolated from pig oocytes, eggs, and zygotes. *Journal of Experimental Zoology* **241**, 257-262.
- Hill JL, Wade MG, Nancarrow CD, Kelleher DL, and Boland MP (1997) Influence of ovine oviducal amino acid concentrations and an ovine oestrus-associated glycoprotein on development and viability of bovine embryos. *Molecular Reproduction and Development* **47**, 164-169.
- Hokke CH, Damm JBL, Kamerling JP, and Vliegenthart JFG (1993) Structure of three acidic O-linked carbohydrate chains of porcine zona pellucida glycoproteins. *FEBS Letters* **329**, 29-34.
- Hoodbhoy T, and Talbot P (1994) Mammalian cortical granules: contents, fate, and function. *Molecular Reproduction and Development* **39**, 439-448.
- Hoodbhoy T, and Talbot P (2001) Characterization, fate, and function of hamster cortical granule components. *Molecular Reproduction and Development* **58**, 223-235.
- Hope RM, and Godfrey GK (1988) Genetically determined variation of pelage colour and reflectance in natural and laboratory populations of the marsupial *Sminthopsis crassicaudata* (Gould). *Australian Journal of Zoology* **36**, 441-454.
- Hope RM, Bennett JH, and Chesson CM (1986) Genetic variation in natural and laboratory populations of the marsupial *Sminthopsis crassicaudata*. *Biochemical Genetics* **24**, 597-613.
- Hoppe PC, and Pitts S (1973) Fertilization in vitro and development of mouse ova. *Biology of Reproduction* **8**, 420-426.
- Horvat B, Vrcic H, and Damjanov I (1992) Changes related to the oestrous cycle in the expression of endometrial and oviductal proteins of mice. *Journal of Reproduction and Fertility* **95**, 191-199.
- Hoshi M, Nishigaki T, Kawamura M, Ikeda M, Gunaratne J, Ueno S, Ogiso M, Moriyama H, and Matsumoto M (2000) Acrosome reaction in starfish: signal molecules in the jelly coat and their receptors. *Zygote* **8 (Supplement)**, S26-S27.
- Huang Jr TTF, Ohzu E, and Yanagimachi R (1982) Evidence suggesting that L-fucose is part of a recognition signal for sperm-zona pellucida attachment in mammals. *Gamete Research* **5**, 355-361.
- Hughes DC, and Barratt CLR (1999) Identification of the true human orthologue of the mouse *Zp1* gene: evidence for greater complexity in the mammalian zona pellucida? *Biochimica et Biophysica Acta* **1447**, 303-306.

- Hughes, DC, and Barratt CLR (2000) The genetics of the mammalian zona pellucida. *Journal of Reproduction and Fertility Abstract Series* **25**, 34 (Abstract 78).
- Hughes RL (1965) Comparative morphology of spermatozoa from five marsupial families. *Australian Journal of Zoology* **13**, 533-543.
- Hughes RL (1974) The tertiary egg membranes of the marsupial *Trichosurus vulpecula*. University of New South Wales.
- Hughes RL (1977) Egg membranes and ovarian function during pregnancy in monotremes and marsupials. In 'Reproduction and Evolution: Proceedings of the 4th Symposium on Comparative Biology of Reproduction'. (Eds JH Calaby and CH Tyndale-Biscoe) pp. 281-291. (Australian Academy of Science: Canberra).
- Hughes RL (1984) Structural adaptations of the eggs and the fetal membranes of monotremes and marsupials for respiration and metabolic exchange. In 'Respiration and Metabolism of Embryonic Vertebrates'. (Ed. RS Seymour) pp. 389-421. (Dr. W. Junk Publishers: Dordrecht)
- Hughes RL, and Shorey CD (1973) Observations on the permeability properties of the egg membranes of the marsupial, *Trichosurus vulpecula*. *Journal of Reproduction and Fertility* **32**, 25-32.
- Hunter RHF (1973) Polyspermic fertilization in pigs after tubal deposition of excessive numbers of spermatozoa. *Journal of Experimental Zoology* **183**, 57-64.
- Hunter RHF (1988) 'The Fallopian Tubes: Their Role in Fertility and Infertility.' (Springer-Verlag: Berlin)
- Hunter RHF (1994) Modulation of gamete and embryonic microenvironments by oviduct glycoproteins. *Molecular Reproduction and Development* **39**, 176-181.
- Hunziker EB, and Schenk RK (1984) Cartilage ultrastructure after high pressure freezing, freeze substitution, and low temperature embedding. II. Intercellular matrix ultrastructure - Preservation of proteoglycans in their native state. *Journal of Cell Biology* **98**, 277-282.
- Hyde BA, and Black DL (1986) Synthesis and secretion of sulphated glycoproteins by rabbit oviduct explants *in vitro*. *Journal of Reproduction and Fertility* **78**, 83-91.
- Hyllner SJ, Westerlund L, Olsson P-E, and Schopen A (2001) Cloning of rainbow trout egg envelope proteins: members of a unique group of structural proteins. *Biology of Reproduction* **64**, 805-811.
- Iino A, and Inoue T (1982) Fine structure of the zona pellucida and the attaching filaments in oocytes from the teleosts. *Okajimas Folia Anatomica Japonica* **58**, 1037-1052.
- Ishihara K, Hosono J, Kanatani H, and Katagiri C (1984) Toad egg-jelly as a source of divalent cations essential for fertilization. *Developmental Biology* **105**, 435-442.

- Iwamoto K, Yonezawa N, Noguchi S, Kudo K, Hamano S, Kuwayama M, and Nakano M (1999) Disulfide formation in bovine zona pellucida glycoproteins during fertilization: evidence for the involvement of cysteine cross-linkages in hardening of the zona pellucida. *Journal of Reproduction and Fertility* **117**, 395-402.
- Jackowski S, and Dumont JN (1979) Surface alterations of the mouse zona pellucida and ovum following in vivo fertilization: correlation with the cell cycle. *Biology of Reproduction* **20**, 150-161.
- Jansen RPS, and Bajpai VK (1982) Oviduct acid mucus glycoproteins in the estrous rabbit: ultrastructure and histochemistry. *Biology of Reproduction* **26**, 155-168.
- Jentoft N (1990) Why are proteins O-glycosylated? *Trends in Biochemical Science* **15**, 291-294.
- Jungnickel MK, Molinia FC, Harman AJ, and Rodger JC (2000) Sperm transport in the female reproductive tract of the brushtail possum, *Trichosurus vulpecula*, following superovulation and artificial insemination. *Animal Reproduction Science* **59**, 213-228.
- Kan FWK, St-Jacques S, and Bleau G (1988) Immunoelectron microscopic localization of an oviductal antigen in hamster zona pellucida by use of a monoclonal antibody. *Cytochemistry* **36**, 1441-1447.
- Kan FWK, St-Jacques S, and Bleau G (1989) Immunocytochemical evidence for the transfer of an oviductal antigen to the zona pellucida of hamster ova after ovulation. *Biology of Reproduction* **40**, 585-598.
- Kan FWK, Roux E, St-Jacques S, and Bleau G (1990) Demonstration by lectin-gold cytochemistry of transfer of glycoconjugates of oviductal origin to the zona pellucida of oocytes after ovulation in hamsters. *Anatomical Record* **226**, 37-47.
- Kang Y-H (1974) Development of the zona pellucida in the rat oocyte. *American Journal of Anatomy* **139**, 535-566.
- Kapur RP, and Johnson LV (1986) Selective sequestration of an oviductal fluid glycoprotein in the perivitelline space of mouse oocytes and embryos. *Journal of Experimental Zoology* **238**, 249-260.
- Kapur RP, and Johnson LV (1988) Ultrastructural evidence that specialized regions of the murine oviduct contribute a glycoprotein to the extracellular matrix of mouse oocytes. *Anatomical Record* **221**, 720-729.
- Kasturi L, Chen H, and Shakin-Eshleman SH (1997) Regulation of N-linked core glycosylation: use of a site-directed mutagenesis approach to identify Asn-Xaa-Ser/Thr sequons that are poor oligosaccharide acceptors. *Biochemical Journal* **323**, 415-419.
- Kasturi L, Eshleman JR, Wunner WH, and Shakin-Eshleman SH (1995) The hydroxy amino acid in an Asn-X-Ser/Thr sequon can influence N-linked core glycosylation efficiency and the

level of expression of a cell surface glycoprotein. *Journal of Biological Chemistry* **270**, 14756-14761.

Katsumata T, Noguchi S, Yonezawa N, Tanokura M, and Nakano M (1996) Structural characterization of the N-linked carbohydrate chains of the zona pellucida glycoproteins from bovine ovarian and fertilized eggs. *European Journal of Biochemistry* **240**, 448-453.

Keefe D, Tran P, Pellegrini C, and Oldenbourg R (1997) Polarized light microscopy and digital image processing identify a multilaminar structure of the hamster zona pellucida. *Human Reproduction* **12**, 1250-1252.

Kellenberger E (1987) The response of biological macromolecules and supramolecular structures to the physics of specimen cryopreparation. In 'Cryotechniques in Biological Electron Microscopy'. (Eds RA Steinbrecht and K Zierold) pp. 35-63. (Springer-Verlag: Berlin)

Kelm S, and Schauer R (1997) Sialic acids in molecular and cellular interactions. *International Review of Cytology* **175**, 137-240.

Kim H, and Schuetz AW (1993) Structural and functional differentiation of follicular and oviductal mouse oocytes visualised with FITC-protein conjugates. *Zygote* **1**, 297-307.

Kim H, Kim H, Kim SR, Kim MK, and Schuetz AW (1996) Oviductal protein produces fluorescence staining of the perivitelline space in mouse oocytes. *Journal of Experimental Zoology* **274**, 351-357.

Kimura J, Sato K, Okano M, and Tsukise A (1994) Localization of ZP3 mRNA in mouse ovary by non-radioactive *in situ* hybridization with digoxigenin-labelled cDNA. *Cellular and Molecular Biology* **40**, 1097-1101.

King RS, Anderson SH, and Killian GJ (1994) Effect of bovine oviductal estrus-associated protein on the ability of sperm to capacitate and fertilize oocytes. *Journal of Andrology* **15**, 468-478.

Kito S, and Bavister BD (1996) Kinetics of sperm penetration and fertilization in vitro in hamster follicular and oviductal ova. *Journal of Experimental Zoology* **274**, 373-383.

Knowling REA, Leigh CM, and Breed WG (1999) A preliminary characterisation of glycosidases in epididymal fluid of the male brushtail possum, *Trichosurus vulpecula*. In 'Proceedings of the 30th Annual Conference of the Australian Society for Reproductive Biology'. Melbourne, Victoria p. 41

Kobata A (1992) Structures and functions of the sugar chains of glycoproteins. *European Journal of Biochemistry* **209**, 483-501.

Kölle S, Sinowatz F, Boie G, Totzauer I, Amselgruber W, and Plendl J (1996) Localization of the mRNA encoding the zona pellucida ZP3 α in the porcine ovary, oocyte and embryo by non-radioactive *in situ* hybridization. *Histochemical Journal* **28**, 441-447.

Kornfeld R, and Kornfeld S (1985) Assembly of asparagine-linked oligosaccharides. *Annual Review of Biochemistry* **54**, 631-664.

Kornfeld K, Reitman ML, and Kornfeld R (1981) The carbohydrate-binding specificity of pea and lentil lectins: fucose is an important determinant. *Journal of Biological Chemistry* **256**, 6633-6640.

Kouba AJ, Atkinson MW, Gandolf AR, and Roth TL (2001) Species-specific sperm-egg interaction affects the utility of a heterologous bovine in vitro fertilization system for evaluating antelope sperm. *Biology of Reproduction* **65**, 1246-1251.

Koyota S, Wimalasiri KMS, and Hoshi M (1997) Structure of the main saccharide chain in the acrosome reaction-inducing substance of the starfish, *Asterias amurensis*. *Journal of Biological Chemistry* **272**, 10372-10376.

Kress A (1996) A comparison of oocyte organelles in *Monodelphis domestica* with those of other marsupials and eutherians. *Reproduction Fertility and Development* **8**, 521-533.

Kress A, Merry NE, and Selwood L (2001) Oogenesis in the marsupial stripe-faced dunnart, *Sminthopsis macroura*. *Cells Tissues Organs* **168**, 188-202.

Kudo K, Yonezawa N, Katsumata T, Aoki H, and Nakano M (1998) Localization of carbohydrate chains of pig sperm ligand in the glycoprotein ZPB of egg zona pellucida. *European Journal of Biochemistry* **252**, 492-499.

Kudo S (1988) Polyspermy-preventing mechanisms in fish eggs. In 'Regeneration and Development'. (Eds S Inoue and e al) pp. 395. (Okada Printing and Publishing: Maebashi, Japan)

Lamont AE, Clarke H, Cooper NJ, Holland MK, and Breed WG (1998) Protein synthesis and secretion by the epididymis of the brush-tail possum, *Trichosurus vulpecula*. *Journal of Reproduction and Fertility* **114**, 169-177.

Lapointe S, Sullivan R, and Sirard M-A (1998a) Binding of a bovine oviductal fluid catalase to mammalian spermatozoa. *Biology of Reproduction* **58**, 747-753.

Lapointe S, Legare C, Gaudreault C, Sullivan R, and Sirard M-A (1998b) cDNA sequence and deduced amino acid sequence of bovine oviductal fluid catalase. *Molecular Reproduction and Development* **51**, 265-273.

Larabell C, and Chandler DE (1991) Fertilization-induced changes in the vitelline envelope of echinoderm and amphibian eggs: self-assembly of an extracellular matrix. *Journal of Electron Microscopy Technique* **17**, 294-318.

Larsson DGJ, Hyllner SJ, and Haux C (1994) Induction of vitelline envelope proteins by estradiol-17 β in 10 teleost species. *General and Comparative Endocrinology* **96**, 445-450.

- Lassalle B, and Testart J (1994) Human zona pellucida recognition associated with removal of sialic acid from human sperm surface. *Journal of Reproduction and Fertility* **101**, 703-711.
- Lauer ME, and McCarthy KJ (2002) In vitro matrix assembly induced by critical assembly concentration (CAC). *Journal of Histochemistry and Cytochemistry* **50**, 1537-1542.
- Lee VH, and Dunbar BS (1993) Developmental expression of the rabbit 55-kDa zona pellucida protein and messenger RNA in ovarian follicles. *Developmental Biology* **155**, 371-382.
- Lee VH, Schwoebel E, Prasad S, Cheung P, Timmons TM, Cook R, and Dunbar BS (1993) Identification and structural characterization of the 75-kDa rabbit zona pellucida protein. *Journal of Biological Chemistry* **268**, 12412-12417.
- Leese HJ (1988) The formation and function of oviduct fluid. *Journal of Reproduction and Fertility* **82**, 843-856.
- Lefebvre R, Lo MC, and Suarez SS (1997) Bovine sperm binding to oviductal epithelium involves fucose recognition. *Biology of Reproduction* **56**, 1198-1204.
- Legge M (1995) Oocyte and zygote zona pellucida permeability to macromolecules. *Journal of Experimental Zoology* **271**, 145-150.
- Léveillé M-C, Roberts KD, Chevalier S, Chapdelaine A, and Bleau G (1987a) Uptake of an oviductal antigen by the hamster zona pellucida. *Biology of Reproduction* **36**, 227-238.
- Léveillé MC, Roberts KD, Chevalier S, Chapdelaine A, and Bleau G (1987b) Formation of the hamster zona pellucida in relation to ovarian differentiation and follicular growth. *Journal of Reproduction and Fertility* **79**, 173-183.
- Liang L-f, Soyal S, and Dean J (1997) FIG α , a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. *Development* **124**, 4939-4947.
- Lin M, and Rodger JC (1999) Acrosome formation during sperm transit through the epididymis in two marsupials, the tammar wallaby (*Macropus eugenii*) and the brushtail possum (*Trichosurus vulpecula*). *Journal of Anatomy* **194**, 223-232.
- Lin M, Sistina Y, and Rodger JC (1995) Electron-microscopic localisation of thiol and disulphide groups by direct mono-maleimido-nanogold labelling in the spermatozoa of a marsupial, the tammar wallaby (*Macropus eugenii*). *Cell and Tissue Research* **282**, 291-296.
- Lindsay LL, and Hedrick JL (1998) Treatment of *Xenopus laevis* coelomic eggs with trypsin mimics pars recta oviductal transit by selectively hydrolyzing envelope glycoprotein gp43, increasing sperm binding to the envelope, and rendering eggs fertilizable. *Journal of Experimental Zoology* **281**, 132-138.
- Lintern-Moore S, and Moore GPM (1977) Comparative aspects of oocyte growth in mammals. In 'Reproduction and Evolution: Proceedings of the 4th Symposium on Comparative Biology

of Reproduction'. (Eds CJ H and CH Tyndale-Biscoe) pp. 215-219. (Australian Academy of Science: Canberra).

Lintern-Moore S, Moore GPM, Tyndale-Biscoe CH, and Poole WE (1976) The growth of the oocyte and follicle in the ovaries of monotremes and marsupials. *Anatomical Record* **185**, 325-332.

Litscher E, and Honegger TG (1991) Glycoprotein constituents of the vitelline coat of *Phallusia mammillata* (Ascidacea) with fertilization inhibiting activity. *Developmental Biology* **148**, 536-551.

Litscher ES, Qi H, and Wassarman PM (1999) Mouse zona pellucida glycoproteins mZP2 and mZP3 undergo carboxy-terminal proteolytic processing in growing oocytes. *Biochemistry* **38**, 12280-12287.

Litscher ES, Juntenen K, Seppo A, Penttilä L, Niemela R, Renkonen O, and Wassarman PM (1995) Oligosaccharide constructs with defined structures that inhibit binding of mouse sperm to unfertilized eggs *in vitro*. *Biochemistry* **34**, 4662-4669.

Liu C, Litscher ES, Mortillo S, Sakai Y, Kinloch RA, Stewart CL, and Wassarman PM (1996) Targeted disruption of the *mZP3* gene results in production of eggs lacking a zona pellucida and infertility in female mice. *Proceedings of the National Academy of Sciences (USA)* **93**, 5431-5436.

Liu DY, Lopata A, Pantke P, and Gordon Baker HW (1991) Horse and marmoset monkey sperm bind to the zona pellucida of salt-stored human oocytes. *Fertility and Sterility* **56**, 764-767.

Lucas H, Bercegeay S, Le Pendu J, Jean M, Mirallie S, and Barriere P (1994) A fucose-containing epitope potentially involved in gamete interaction on the human zona pellucida. *Human Reproduction* **9**, 1532-1538.

Lyne AG, and Hollis DE (1983) Observations on graafian follicles and their oocytes during lactation and after the removal of pouch young in the marsupials *Isodon macrourus* and *Perameles nasuta*. *American Journal of Anatomy* **166**, 41-61.

Lyons CE, Payette KL, Price JL, and Huang RCC (1993) Expression and structural analysis of a teleost homolog of a mammalian zona pellucida gene. *Journal of Biological Chemistry* **268**, 21351-21358.

Maddock MB, and Dawson WD (1974) Artificial insemination of deer-mice (*Peromyscus maniculatus*) with sperm from other rodent species. *Journal of Embryology and Experimental Morphology* **31**, 621-634.

Maegawa M, Kamada M, Yamamoto S, Yamano S, Irahara M, Kido H, and Aono T (2002) Involvement of carbohydrate molecules on zona pellucida in human fertilization. *Journal of Reproductive Immunology* **53**, 79-89.

Malette B, and Bleau G (1993) Biochemical characterization of hamster oviductin as a sulphated zona pellucida-binding glycoprotein. *Biochemical Journal* **295**, 437-445.

Malette B, Paquette Y, Merlen Y, and Bleau G (1995) Oviductins possess chitinase- and mucin-like domains: a lead in the search for the biological function of these oviduct-specific ZP-associating glycoproteins. *Molecular Reproduction and Development* **41**, 384-397.

Maresh GA, and Dunbar BS (1987) Antigenic comparison of five species of mammalian zonae pellucidae. *Journal of Experimental Zoology* **244**, 299-307.

Martinez ML, Fontenot GK, and Harris JD (1996) The expression and localization of zona pellucida glycoproteins and mRNA in cynomolgus monkeys (*Macaca fascicularis*). *Journal of Reproduction and Fertility Supplement* **50**, 35-41.

Martoglio A-M, and Kan FWK (1996) Immunohistochemical localization of oviductin in the endometrial lining of the golden hamster (*Mesocricetus auratus*) during the estrous cycle and early gestation. *Histochemical Journal* **28**, 449-459.

Mate KE (1996) Cytoplasmic maturation of the marsupial oocyte during the periovulatory period. *Reproduction Fertility and Development* **8**, 509-519.

Mate KE (1998) Timing of zona pellucida formation in the tammar wallaby (*Macropus eugenii*) and brushtail possum (*Trichosurus vulpecula*). *Animal Reproduction Science* **53**, 237-252.

Mate KE, and McCartney CA (1998) Sequence and analysis of zona pellucida 2 cDNA (ZP2) from a marsupial, the brushtail possum, *Trichosurus vulpecula*. *Molecular Reproduction and Development* **51**, 322-329.

Mate KE, and Rodger JC (1991) Stability of the acrosome of the brush-tailed possum (*Trichosurus vulpecula*) and tammar wallaby (*Macropus eugenii*) *in vitro* and after exposure to conditions and agents known to cause capacitation or acrosome reaction of eutherian spermatozoa. *Journal of Reproduction and Fertility* **91**, 41-48.

Mate KE, Buist JM, and Duckworth JA (2003) Expression in *Escherichia coli* and immunological characterization of three zona pellucida proteins (ZP1, ZP2, and ZP3) from a marsupial, the brushtail possum (*Trichosurus vulpecula*). *Molecular Reproduction and Development* **64**, 136-143.

Mate KE, Giles I, and Rodger JC (1992) Evidence that cortical granule formation is a periovulatory event in marsupials. *Journal of Reproduction and Fertility* **95**, 719-728.

Mate KE, Molinia FC, and Rodger JC (1998) Manipulation of the fertility of marsupials for conservation of endangered species and control of over-abundant populations. *Animal Reproduction Science* **53**, 65-76.

- Mate KE, Sidhu KS, Molinia FC, Glazier AM, and Rodger JC (2000) Sperm binding and penetration of the zona pellucida *in vitro* but not sperm-egg fusion in an Australian marsupial, the brushtail possum (*Trichosurus vulpecula*). *Zygote* **8**, 189-196.
- Maturi G, Infante V, Carotenuto R, Focarelli R, Caputo M, and Campanella C (1998) Specific glycoconjugates are present at the oolemma of the fertilization site in the egg of *Discoglossus pictus* (Anurans) and bind spermatozoa in an *in vitro* assay. *Developmental Biology* **204**, 210-223.
- Mayr E (1963) 'Animal species and evolution.' (Harvard University Press: Cambridge)
- McCartney CA, and Mate KE (1999) Cloning and characterisation of a zona pellucida 3 cDNA from a marsupial, the brushtail possum *Trichosurus vulpecula*. *Zygote* **7**, 1-9.
- Menghi G, Bondi AM, Accili D, Fumagalli L, and Materazzi G (1985) Characterisation *in situ* of the complex carbohydrates in rabbit oviduct using digestion with glycosidases followed by lectin binding. *Journal of Anatomy* **140**, 613-625.
- Menghi G, Scocco P, Accili D, Bondi AM, and Materazzi G (1989) Attempt to visualize an endogenous lectin recognizing α -L-fucose immunohistochemically in the rabbit oviduct. *Acta Histochemica* **87**, 115-121.
- Menghi G, Scocco P, and Materazzi G (1995) Lectin binding and identification of sialic acid acceptor sugars in rabbit oviduct under hormone administration. *Microscopy Research and Technique* **31**, 488-496.
- Miller CC, Fayrer-Hosken RA, Timmons TM, Lee VH, Caudle AB, and Dunbar BS (1992a) Characterization of equine zona pellucida glycoproteins by polyacrylamide gel electrophoresis and immunological techniques. *Journal of Reproduction and Fertility* **96**, 815-825.
- Miller DJ, Macek MB, and Shur BD (1992b) Complementarity between sperm surface β -1,4-galactosyl-transferase and egg-coat ZP3 mediates sperm-egg binding. *Nature* **357**, 589-593.
- Miller DJ, Gong X, Decker G, and Shur BD (1993) Egg cortical granule N-acetylglucosaminidase is required for the mouse zona block to polyspermy. *Journal of Cell Biology* **123**, 1431-1440.
- Moller CC, Bleil JD, Kinloch RA, and Wassarman PM (1990) Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. *Developmental Biology* **137**, 276-286.
- Monaghan P, Perusinghe N, and Muller M (1998) High-pressure freezing for immunocytochemistry. *Journal of Microscopy* **192**, 248-258.
- Mononen I, and Karjalainen E (1984) Structural comparison of protein sequences around potential N-glycosylation sites. *Biochimica et Biophysica Acta* **788**, 364-367.

- Moore HDM (1996) Gamete biology of the new world marsupial, the grey short-tailed opossum, *Monodelphis domestica*. *Reproduction Fertility and Development* **8**, 605-615.
- Moore HDM, and Taggart DA (1993) *In vitro* fertilization and embryo culture in the grey short-tailed opossum, *Monodelphis domestica*. *Journal of Reproduction and Fertility* **98**, 267-274.
- Mori K, Daitoh T, Kamada M, Maeda N, Maegawa M, Hirano K, Irahara M, and Aono T (1993) Blocking of fertilization by carbohydrates. *human reproduction* **8**, 1729-1732.
- Moritz K, Green RH, and Selwood L (1998) A histological analysis of the reproductive cycle of the common wombat *Vombatus ursinus*. In 'Wombats'. (Eds RT Wells and PA Pridmore) pp. 75-85. (Surrey Beatty & Sons: Chipping Norton).
- Muller-Esterl W, and Fritz H (1981) Sperm acrosin. *Methods in Enzymology* **80**, 621-632.
- Murata K, Sugiyama H, Yasumasu S, Iuchi I, Yasumasu I, and Yamagami K (1997) Cloning of cDNA and estrogen-induced hepatic gene expression for choriogenin H, a precursor protein of the fish egg envelope (chorion). *Proceedings of the National Academy of Sciences (USA)* **94**, 2050-2055.
- Murray MK (1993) An estrogen-dependent glycoprotein is synthesized and released from the oviduct in a temporal- and region-specific manner during early pregnancy in the ewe. *Biology of Reproduction* **48**, 446-453.
- Nagdas SK, Araki Y, Chayko CA, Orgebin-Crist M-C, and Tulisani DRP (1994) O-linked trisaccharide and N-linked Poly-N-Acetylactosaminyl glycans are present on mouse ZP2 and ZP3. *Biology of Reproduction* **51**, 262-272.
- Nakano M, and Yonezawa N (2001) Localization of sperm ligand carbohydrate chains in pig zona pellucida glycoproteins. *Cells Tissues Organs* **168**, 65-75.
- Nakano M, Yonezawa N, Hatanaka Y, and Noguchi S (1996) Structure and function of the N-linked carbohydrate chains of pig zona pellucida glycoproteins. *Journal of Reproduction and Fertility Supplement* **50**, 25-34.
- Nicastro D, Frangakis AS, Typke D, and Baumeister W (2000) Cryo-electron tomography of *Neurospora* mitochondria. *Journal of Structural Biology* **129**, 48-56.
- Nicolson GL, Yanagimachi R, and Yanagimachi H (1975) Ultrastructural localization of lectin-binding sites on the zonae pellucidae and plasma membranes of mammalian eggs. *Journal of Cell Biology* **66**, 263-274.
- Nishigaki T, Chiba K, Miki W, and Hoshi M (1996) Structure and function of asterosaps, sperm-activating peptides from the jelly coat of starfish eggs. *Zygote* **4**, 237-245.

- Noguchi S, and Nakano M (1993) Structural characterization of the *N*-linked carbohydrate chains from mouse zona pellucida glycoproteins ZP2 and ZP3. *Biochemica et Biophysica Acta* **1158**, 217-226.
- Nomura K, and Isaka S (1985) Synthetic study on the structure-activity relationship of sperm activation peptides from the jelly coat of sea urchin eggs. *Biochemical and Biophysical Research Communications* **126**, 974-982.
- O'Day-Bowman MB, Mavrogianis PA, Fazleabas AT, and Verhage HG (1995) A human oviduct-specific glycoprotein: synthesis, secretion, and localization during the menstrual cycle. *Microscopy Research and Technique* **32**, 57-69.
- O'Sullivan CM, Rancourt SL, Liu SY, and Rancourt DE (2001) A novel murine tryptase involved in blastocyst hatching and outgrowth. *Reproduction* **122**, 61-71.
- O'Toole CMB, Arnoult C, Darszon A, Steinhardt RA, and Florman HM (2000) Ca^{2+} entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. *Molecular Biology* **11**, 1571-1584.
- Oehninger S (2001) Molecular basis of human sperm-zona pellucida interaction. *Cells Tissues Organs* **168**, 58-64.
- Oehninger S, Patankar M, Seppala M, and Clark GF (1998) Involvement of selectin-like carbohydrate binding specificity in human gamete interaction. *Andrologia* **30**, 269-274.
- Ohlendieck K, and Lennarz WJ (1995) Role of the sea urchin egg receptor for sperm in gamete interactions. *TIBS* **20**, 29-33.
- Oliphant G, and Ross PR (1982) Demonstration of production and isolation of three sulfated glycoproteins from the rabbit oviduct. *Biology of Reproduction* **26**, 537-544.
- Ozgur K, Patankar MS, Oehninger S, and Clark GF (1998) Direct evidence for the involvement of carbohydrate sequences in human sperm-zona pellucida binding. *Molecular Human Reproduction* **4**, 318-324.
- Parillo F, and Verini-Supplizi A (1999) Glycohistochemical investigation of canine and feline zonae pellucidae of preantral and antral oocytes. *Acta Histochemica* **101**, 127-146.
- Parillo F, and Verini-Supplizi A (2001) Glycohistochemistry of the zona pellucida of developing oocytes in the rabbit and hare. *Research in Veterinary Science* **70**, 257-264.
- Parillo F, Stradaioli G, and Verini-Supplizi A (1998) Glycoconjugates in small antral ovarian follicles of the river buffalo (*Bubalus bubalis* L.). *Acta Histochemica* **100**, 229-243.
- Parillo F, Stradaioli G, Dall'Aglio C, and Verini-Supplizi A (1996) Characterization of the complex carbohydrates in the zona pellucida of mammalian oocytes using lectin histochemistry. *Veterinary Research Communications* **20**, 225-236.

- Patankar MS, Oehninger S, Barnett T, Williams RL, and Clark GF (1993) A revised structure for fucoidan may explain some of its biological activities. *Journal of Biological Chemistry* **268**, 21770-21776.
- Phillips DM, and Shalgi R (1980a) Surface architecture of the mouse and hamster zona pellucida and oocyte. *Journal of Ultrastructure Research* **72**, 1-12.
- Phillips DM, and Shalgi R (1980b) Surface properties of the zona pellucida. *Journal of Experimental Zoology* **213**, 1-8.
- Phillips DM, and Fadem BH (1987) The oocyte of a new world marsupial, *Monodelphis domestica*: structure, formation, and function of the enveloping mucoid layer. *Journal of Experimental Zoology* **242**, 363-371.
- Pilton PE, and Sharman GB (1962) Reproduction in the marsupial, *Trichosurus vulpecula*. *Journal of Endocrinology* **25**, 119-136.
- Pollard JW, Plante C, King WA, Hansen PJ, Betteridge KJ, and Suarez SS (1991) Fertilizing capacity of bovine sperm may be maintained by binding to oviductal epithelial cells. *Biology of Reproduction* **44**, 102-107.
- Prasad SV, Wilkins B, and Dunbar BS (1996) Molecular biology approaches to evaluate species variation in immunogenicity and antigenicity of zona pellucida proteins. *Journal of Reproduction and Fertility Supplement* **50**, 143-149.
- Prasad SV, Mujtaba S, Lee VH, and Dunbar BS (1995) Immunogenicity enhancement of recombinant rabbit 55-kilodalton zona pellucida protein expressed using the baculovirus expression system. *Biology of Reproduction* **52**, 1167-1178.
- Quill TA, and Hedrick JL (1994) Oviductal localization of the cortical granule lectin ligand involved in the block to polyspermy of *Xenopus laevis*. *Development Growth and Differentiation* **36**, 615-620.
- Rankin TL, Tong Z-B, Castle PE, Lee E, Gore-Langton R, Nelson LM, and Dean J (1998) Human ZP3 restores fertility in *Zp3* null mice without affecting order-specific sperm binding. *Development* **125**, 2415-2424.
- Rankin T, Familiar M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, and Dean J (1996) Mice homozygous for an insertion mutation in the *Zp3* gene lack a zona pellucida and are infertile. *Development* **122**, 2903-2910.
- Rapisarda JJ, Mavrogianis PA, O'Day-Bowman MB, Fazleabas AT, and Verhage HG (1993) Immunological characterization and immunocytochemical localization of an oviduct-specific glycoprotein in the human. *Journal of Clinical Endocrinology and Metabolism* **76**, 1483-1488.
- Raychoudhury SS, Suarez SS, and Buhi WC (1993) Distribution of lectin binding sites in the oviducts of cycling and hormone-treated pigs. *Journal of Experimental Zoology* **265**, 659-668.

Revelli A, Costamagna C, Moffa F, Aldieri E, Ochetti S, Bosia A, Massobrio M, Lindblom B, and Ghigo D (2001) Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. *Biology of Reproduction* **64**, 1708-1712.

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208.

Riley CB, and Elhay MJ (1994) Lectin histochemistry. In 'Laboratory Histopathology: A Complete Reference'. (Eds AE Woods and RL Ellis) pp. 8.3-1-8.3-18. (Churchill Livingstone: Edinburgh)

Roberts CT, and Breed WG (1996a) The marsupial shell membrane: an ultrastructural and immunogold localization study. *Cell Tissue Research* **284**, 99-110.

Roberts CT, and Breed WG (1996b) Variation in ultrastructure of mucoid coat and shell membrane secretion of a dasyurid marsupial. *Reproduction Fertility and Development* **8**, 645-648.

Roberts CT, Breed WG, Mayrhofer G (1994) Origin of the oocyte shell membrane of a dasyurid marsupial: an immunohistochemical study. *Journal of Experimental Zoology* **270**, 321-331.

Roberts CT, Selwood L, Leigh CM, and Breed WG (1997) Antiserum to the egg coats of the fat-tailed dunnart (Marsupialia, Dasyuridae) cross-reacts with the egg coats of other marsupial and eutherian species. *Journal of Experimental Zoology* **278**, 133-139.

Robertson L, Wishart GJ, and Horrocks AJ (2000) Identification of perivitelline N-linked glycans as mediators of sperm-egg interaction in chickens. *Journal of Reproduction and Fertility* **120**, 397-403.

Rodger JC (1991) Fertilization of marsupials. In 'A Comparative Overview of Mammalian Fertilization'. (Eds BS Dunbar and MG O'Rand) pp. 117-135. (Plenum Press: New York).

Rodger JC (1994) Prefertilization gamete maturation events in marsupials. *Reproduction Fertility and Development* **6**, 473-483.

Rodger JC (1997) Likely targets for immunocontraception in marsupials. *Reproduction Fertility and Development* **9**, 131-136.

Rodger JC, and Bedford JM (1982) Induction of oestrus, recovery of gametes, and the timing of fertilization events in the opossum, *Didelphis virginiana*. *Journal of Reproduction and Fertility* **64**, 159-169.

Rodger JC, and Mate KE (1988) A PMSG/GnRH method for the superovulation of the monovulatory brush-tailed possum (*Trichosurus vulpecula*). *Journal of Reproduction and Fertility* **83**, 885-891.

Roldan, ER, and Yanagimachi, R (1989) Cross-fertilization between Syrian and Chinese hamsters. *Journal of Experimental Zoology* **250**, 321-328.

Rodger JC, and Mate KE (1993) Marsupial gametes and fertilisation. *Today's Life Science* **5**, 28-33.

Rodger JC, Giles I, and Mate KE (1992) Unexpected oocyte growth after follicular antrum formation in four marsupial species. *Journal of Reproduction and Fertility* **96**, 755-763.

* insert reference (opposite page)

Roux E, and Kan FWK (1995) Stage-specific immunolabeling for oviductin in the secretory granules of the oviductal epithelium of the golden hamster during the estrous cycle. *Anatomical Record* **241**, 369-376.

Roux E, Bleau G, and Kan FWK (1997) Fate of hamster oviductin in the oviduct and uterus during early gestation. *Molecular Reproduction and Development* **46**, 306-317.

Ruddock NT, Machaty Z, Cabot RA, and Prather RS (2001) Porcine oocyte activation: differing roles of calcium and pH. *Molecular Reproduction and Development* **59**, 227-234.

Saling PM (1996) Fertilization: mammalian gamete interactions. In 'Reproductive Endocrinology, Surgery, and Technology'. (Eds EY Adashi, JA Rock and Z Rosenwaks) pp. 403-420. (Lippincott-Raven Publishers: Philadelphia)

Sato Y, Son JH, and Meizel S (2000) The mouse sperm glycine receptor/chloride channel: cellular localization and involvement in the acrosome reaction initiated by glycine. *Journal of Andrology* **21**, 99-106.

Schauer R (1982) Chemistry, metabolism, and biological functions of sialic acids. *Advances in Carbohydrate Chemistry and Biochemistry* **40**, 131-234.

Schauer R (1988) Sialic acids as antigenic determinants of complex carbohydrates. In 'The Molecular Immunology of Complex Carbohydrates'. (Eds AM Wu and LG Adams) pp. 47-72)

Schmell ED, and Gulyas BJ (1980a) Mammalian sperm-egg recognition and binding in vitro. I. Specificity of sperm interactions with live and fixed eggs in homologous and heterologous inseminations of hamster, mouse, and guinea pig oocytes. *Biology of Reproduction* **23**, 1075-1085.

Schmell ED, and Gulyas BJ (1980b) Ovoperoxidase activity in ionophore treated mouse eggs. II. Evidence for the enzyme's role in hardening the zona pellucida. *Gamete Research* **3**, 279-290.

Schmell ED, Gulyas BJ, and Hedrick JL (1983) Egg surface changes during fertilization and the molecular mechanism of the block to polyspermy. In 'Mechanism and Control of Animal Fertilization' pp. 365-413. (Academic Press, Inc)

Schmidt A, Mavrogianis PA, O'Day-Bowman MB, and Verhage HG (1997) Species-specific effect of oviductal glycoproteins on hamster sperm binding to hamster oocytes. *Molecular Reproduction and Development* **46**, 201-207.

- Sellers LA, Allen A, Morris ER, and Ross-Murphy SB (1988) Submaxillary mucins: intermolecular interactions and gel-forming potential of concentrated solutions. *Biochemical Journal* **256**, 599-607.
- Selwood L (1982) A review of maturation and fertilization in marsupials with special reference to the dasyurid: *Antechinus stuartii*. In 'Carnivorous Marsupials'. (Ed. M Archer) pp. 65-76. (Royal Zoological Society of New South Wales: Sydney)
- Selwood L (1992) Mechanisms underlying the development of pattern in marsupial embryos. *Current Topics in Developmental Biology* **27**, 175-233.
- Selwood L (1994) Development of early cell lineages in marsupials: a review of the evidence. *Reproduction Fertility and Development* **6**, 507-527.
- Selwood L (2000) Marsupial egg and embryo coats. *Cells Tissues Organs* **166**, 208-219.
- Selwood L, and McCallum F (1987) Relationship between longevity of spermatozoa after insemination and the percentage of normal embryos in brown marsupial mice (*Antechinus stuartii*). *Journal of Reproduction and Fertility* **79**, 495-503.
- Semino GA, Restani P, and Cerletti P (1985) Effect of bound carbohydrate on the action of trypsin on lupin seed glycoproteins. *Journal of Agricultural Food Chemistry* **33**, 196-199.
- Sendai Y, Abe H, Kikuchi M, Satoh T, and Hoshi H (1994) Purification and molecular cloning of bovine oviduct-specific glycoprotein. *Biology of Reproduction* **50**, 927-934.
- Sendai Y, Komiya H, Suzuki K, Onuma T, Kikuchi M, Hoshi H, and Araki Y (1995) Molecular cloning and characterization of a mouse oviduct-specific glycoprotein. *Biology of Reproduction* **53**, 285-294.
- Shabanowitz RB, and O'Rand MG (1988) Characterization of the human zona pellucida from fertilized and unfertilized eggs. *Journal of Reproduction and Fertility* **82**, 151-161.
- Shackell GH, Norman NG, McLeod BJ, and Hurst PR (1996) A morphometric study of early ovarian development in pouch young of the brushtail possum (*Trichosurus vulpecula*). *Anatomical Record* **246**, 224-230.
- Shakin-Eshleman SH, Spitalnik SL, and Kasturi L (1996) The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. *Journal of Biological Chemistry* **271**, 6363-6366.
- Shalgi R, and Raz T (1997) The role of carbohydrate residues in mammalian fertilization. *Histology and Histopathology* **12**, 813-822.
- Shalgi R, Matityahu A, and Nebel L (1986) The role of carbohydrates in sperm-egg interaction in rats. *Biology of Reproduction* **34**, 446-452.

Slavik, T, Pavlok, A, and Fulka, J (1990) Penetration of intact bovine ova with ram sperm in vitro. *Molecular Reproduction and Development* **25**, 345-347.

Shalgi R, Maymon R, Bar-Shira (Maymon) B, Amihai D, and Skutelsky E (1991) Distribution of lectin receptor sites in the zona pellucida of follicular and ovulated rat oocytes. *Molecular Reproduction and Development* **29**, 365-372.

Shimoda Y, Kitajima K, Inoue S, and Inoue Y (1994) Isolation, structural determination, and calcium-binding properties of the major glycoprotein present in *Bufo japonicus japonicus* egg jelly. *European Journal of Biochemistry* **223**, 223-231.

Sidhu KS, Mate KE, and Rodger JC (1998) Sperm-oviduct epithelial cell monolayer co-culture: an *in vitro* model of sperm-female tract interactions in a marsupial, the tammar wallaby (*Macropus eugenii*). *Journal of Reproduction and Fertility* **114**, 55-61.

Sidhu KS, Mate KE, Molinia FC, Glazier AM, and Rodger JC (1999) Secretory proteins from the female reproductive tract of the brushtail possum (*Trichosurus vulpecula*): binding to sperm and effects on sperm survival *in vitro*. *Reproduction Fertility and Development* **11**, 329-336.

Sinowatz F, Wessa E, Neumuller C, and Palma G (2003) On the species specificity of sperm binding and sperm penetration of the zona pellucida. *Reproduction in Domestic Animals* **38**, 141-146.

Sinowatz F, Amselgruber W, Topfer-Petersen E, Totzauer I, Calvete J, and Plendl J (1995) Immunocytochemical characterization of porcine zona pellucida during follicular development. *Anatomy and Embryology* **191**, 41-46.

Sistina Y, Lin M, Mate KE, Robinson ES, and Rodger JC (1993) The unique stability of the marsupial sperm acrosomal membranes examined by unprotected freeze-thawing and treatment with the detergent Triton X-100. *Reproduction Fertility and Development* **5**, 1-14.

Sitte H, Edelmann L, and Neumann K (1987) Cryofixation without pretreatment at ambient pressure. In 'Cryotechniques in Biological Electron Microscopy'. (Eds RA Steinbrecht and K Zierold) pp. 87-113. (Springer-Verlag: Berlin)

Skutelsky E, Ranen E, and Shalgi R (1994) Variations in the distribution of sugar residues in the zona pellucida as possible species-specific determinants of mammalian oocytes. *Journal of Reproduction and Fertility* **100**, 35-41.

* insert reference (opposite page)

Smith M (1979) Notes on reproduction and growth in the koala, *Phascolarctos cinereus* (Goldfuss). *Australian Wildlife Research* **6**, 5-12.

Smith R, Jenkins A, Loubakos, A, Thompson, P, Ramakrishnan, V, Tomlinson, J, Deshpande, U H, Johnson, D A, Jones, R, Mackie, E J, and Pike, R N (2000) Evidence for the activation of PAR-2 by the sperm protease, acrosin: expression of the receptor on oocytes. *FEBS Letters* **484**, 285-290.

Smith TT, and Nothnick WB (1997) Role of direct contact between spermatozoa and oviductal epithelial cells in maintaining rabbit sperm viability. *Biology of Reproduction* **56**, 83-89.

- Snell WJ, and White JM (1996) The molecules of mammalian fertilization. *Cell* **85**, 629-637.
- Spargo SC, and Hope RM (2003) Evolution and nomenclature of the zona pellucida gene family. *Biology of Reproduction* **68**, 358-362.
- St-Jacques S, Malette B, Chevalier S, Roberts KD, and Bleau G (1992) The zona pellucida binds the mature form of an oviductal glycoprotein (oviductin). *Journal of Experimental Zoology* **262**, 97-104.
- Staros AL, and Killian GJ (1998) *In vitro* association of six oviductal fluid proteins with the bovine zona pellucida. *Journal of Reproduction and Fertility* **112**, 131-137.
- Stewart-Savage J, Grey RD, and Elinson RP (1991) Polarity of the surface and cortex of the amphibian egg from fertilization to first cleavage. *Journal of Electron Microscopy Technique* **17**, 369-383.
- Suarez SS (1998) The oviductal sperm reservoir in mammals: mechanisms of formation. *Biology of Reproduction* **58**, 1105-1107.
- Suarez SS (2001) Carbohydrate-mediated formation of the oviductal sperm reservoir in mammals. *Cells Tissues Organs* **168**, 105-112.
- Suarez SS (2002) Formation of a reservoir of sperm in the oviduct. *Reproduction in Domestic Animals* **37**, 140-143.
- Suarez SS, Revah I, Lo M, and Kolle S (1998) Bull sperm binding to oviductal epithelium is mediated by a Ca^{2+} -dependent lectin on sperm that recognizes Lewis-a trisaccharide. *Biology of Reproduction* **59**, 39-44.
- Suzuki H, Yang X, and Foote RH (1994) Surface alterations of the bovine oocyte and its investments during and after maturation and fertilization in vitro. *Molecular Reproduction and Development* **38**, 421-430.
- Suzuki K, Sendai Y, Onuma T, Hoshi H, Hiroi M, and Araki Y (1995) Molecular characterization of a hamster oviduct-specific glycoprotein. *Biology of Reproduction* **53**, 345-354.
- Swanson WJ, and Vacquier VD (1995) Extraordinary divergence and positive Darwinian selection in a fusogenic protein coating the acrosomal process of abalone spermatozoa. *Proceedings of the National Academy of Sciences (USA)* **92**, 4957-4961.
- Swanson WJ, Yang Z, Wolfner MF, and Aquadro CF (2001) Positive Darwinian selection drives the evolution of several female reproductive proteins in mammals. *Proceedings of the National Academy of Sciences (USA)* **98**, 2509-2514.
- Taggart DA (1994) A comparison of sperm and embryo transport in the female reproductive tract of marsupial and eutherian mammals. *Reproduction Fertility and Development* **6**, 451-472.

- Taggart DA, and Temple-Smith PD (1991) Transport and storage of spermatozoa in the female reproductive tract of the brown marsupial mouse, *Antechinus stuartii* (Dasyuridae). *Journal of Reproduction and Fertility* **93**, 97-110.
- Taggart DA, Leigh CM, and Breed WG (1995) Ultrastructure and motility of spermatozoa in the male reproductive tract of perameloid marsupials. *Reproduction Fertility and Development* **7**, 1141-1156.
- Taggart DA, Johnson JL, O'Brien HP, and Moore HD (1993) Why do spermatozoa of the American marsupial form pairs? A clue from the analysis of sperm pairing in the epididymis of the grey short-tailed opossum, *Monodelphis domestica*. *Anatomical Record* **236**, 465-478.
- Taggart, DA, Breed, WG, Temple-Smith, PD, Purvis, A, and Shimmin, G (1998) Reproduction, mating strategies and sperm competition in marsupials and monotremes. In 'Sperm Competition and Sexual Selection'. (Eds. T.R. Birkhead & A.P. Moller) pp. 623-666, (Academic Press: San Diego).
- Takagi J, Araki Y, Dobashi M, Imai Y, Hiroi M, Tonosaki A, and Sendo F (1989) The development of porcine zona pellucida using monoclonal antibodies: 1. Immunocytochemistry and light microscopy. *Biology of Reproduction* **40**, 1095-1102.
- Takamune K, and Katagiri C (1987) The properties of the oviduct pars recta protease which mediates gamete interaction by affecting the vitelline coat of a toad egg. *Development Growth and Differentiation* **29**, 193-203.
- Takamune K, Yoshizaki N, and Katagiri C (1986) Oviducal pars recta-induced degradation of vitelline coat proteins in relation to acquisition of fertilizability of toad eggs. *Gamete Research* **14**, 215-224.
- Takasaki S, Mori E, and Mori T (1999) Structures of sugar chains included in mammalian zona pellucida glycoproteins and their potential roles in sperm-egg interaction. *Biochimica et Biophysica Acta* **1473**, 206-215.
- Takeuchi Y, Nishimura K, Aoki N, Adachi T, Sato C, Kitajima K, and Matsuda T (1999) A 42-kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC family glycoproteins in mammalian zona pellucida. *European Journal of Biochemistry* **260**, 736-742.
- Takeuchi Y, Cho R, Iwata Y, Nishimura K, Kato T, Aoki N, Kitajima K, and Matsuda T (2001) Morphological and biochemical changes of isolated chicken egg-envelope during sperm penetration: degradation of the 97-kilodalton glycoprotein is involved in sperm-driven hole formation on the egg-envelope. *Biology of Reproduction* **64**, 822-830.
- Talbot P, and DiCarantonio G (1984) Ultrastructure of opossum oocyte investing coats and their sensitivity to trypsin and hyaluronidase. *Developmental Biology* **103**, 159-167.

- Talevi R, Campanella C (1988) Fertilization in *Discoglossus pictus* (Anura): 1. Sperm-egg interactions in distinct regions of the dimple and occurrence of a late stage of sperm penetration. *Developmental Biology* **130**, 524-535.
- Talevi R, and Gualtieri R (2001) Sulfated glycoconjugates are powerful modulators of bovine sperm adhesion and release from the oviductal epithelium in vitro. *Biology of Reproduction* **64**, 491-498.
- Tatemoto H, and Terada T (1999) Analysis of zona pellucida modifications due to cortical granule exocytosis in single porcine oocytes, using enhanced chemiluminescence. *Theriogenology* **52**, 629-640.
- Temple-Smith PD, and Bedford JM (1976) The features of sperm maturation in the epididymis of a marsupial, the brush-tailed possum, *Trichosurus vulpecula*. *American Journal of Anatomy* **147**, 471-500.
- Temple-Smith PD, and Bedford JM (1980) Sperm maturation and the formation of sperm pairs in the epididymis of the opossum. *Journal of Experimental Zoology* **214**, 161-171.
- Temple-Smith PD, and Taggart DA (1990) On the male generative organs of the koala (*Phascolarctos cinereus*): an update. In 'Biology of the Koala'. (Eds AK Lee, KA Handasyde and GD Sanson) pp. 34-54. (Surrey Beatty & Sons: Sydney).
- Tengowski MW, and Schatten G (1997) Low-voltage scanning electron microscopy of mammalian fertilization in vitro: preparation of oocytes. *Microscopy and Microanalysis* **3**, 193-202.
- Tesarik J, Pilka L, and Travník P (1988) Zona pellucida resistance to sperm penetration before the completion of human oocyte maturation. *Journal of Reproduction and Fertility* **83**, 487-495.
- Thaler CD, Cardullo RA (1996) Defining oligosaccharide specificity for initial sperm-zona pellucida adhesion in the mouse. *Molecular Reproduction and Development* **45**, 535-546.
- Thillai-Koothan P, van Duin M, and Aitken RJ (1993) Cloning, sequencing and oocyte-specific expression of the marmoset sperm receptor protein, ZP3. *Zygote* **1**, 93-101.
- Tokuyasu KT (1997) Immuno-cytochemistry on ultrathin cryosections. In 'Cells, a Laboratory Manual. Vol 3. Subcellular Localization of Genes and Their Products'. (Eds DL Spector, RD Goodman and LA Leinwand) pp. 131.1-131.27. (Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY)
- Topfer-Petersen E (1999) Carbohydrate-based interactions on the route of a spermatozoon to fertilization. *Human Reproduction Update* **5**, 314-329.
- Topper EK, Kruijt L, Calvete J, Mann K, Topfer-Petersen E, and Woelders H (1997) Identification of bovine zona pellucida glycoproteins. *Molecular Reproduction and Development* **46**, 344-350.

VandeVoort, CA, Tollner, TL, and Overstreet, JW (1992) Sperm-zona pellucida interaction in cynomolgus and rhesus macaques. *Journal of Andrology* **13**, 428-432.

Tsubamoto H, Hasegawa A, Inoue M, Yamasaki N, and Koyama K (1996) Binding of recombinant pig zona pellucida protein 1 (ZP1) to acrosome-reacted spermatozoa. *Journal of Reproduction and Fertility Supplement* **50**, 63-67.

Tsuda T, Kohno Y, Nishimura K, Aoki N, Kitajima K, and Matsuda T (2000) Structure and differently regulated expression of chicken egg-envelope glycoproteins, ZPA, ZPB, ZPC, and ZP1. In 'The Third International Symposium on The Molecular and Cellular Biology of the Egg and Embryo Extracellular Matrix'. Lake Tahoe, California p. Abstract no. I2

Tsuiki A, Hoshiai H, Takahashi K, Suzuki M, and Hoshi H (1986) Sperm-egg interactions observed by scanning electron microscopy. *Archives of Andrology* **16**, 35-47.

Tulsiani DRP, Yoshida-Komiya H, and Araki Y (1997) Mammalian fertilization: a carbohydrate-mediated event. *Biology of Reproduction* **57**, 487-494.

Tyndale-Biscoe CH, and Renfree MB (1987) 'Reproductive Physiology of Marsupials.' (Cambridge University Press: New York)

Ullmann SL, and Butcher L (1996) Mammalian oocyte organelles with special reference to pleomorphic mitochondria and vacuole formation in marsupials. *Reproduction Fertility and Development* **8**, 491-508.

Uribe MCA, and Guillette Jr LJ (2000) Oogenesis and ovarian histology of the American alligator *Alligator mississippiensis*. *Journal of Morphology* **245**, 225-240.

Vacquier VD, Porter DC, Keller SH, and Aukerman M (1989) Egg jelly induces the phosphorylation of histone H3 in spermatozoa of the sea urchin *Arbacia punctulata*. *Developmental Biology* **133**, 111-118.

* insert reference (opposite page)

van Duin M, Polman JE, De Breet IT, van Ginneken K, Bunschoten H, Grootenhuis A, Brindle J, and Aitken RJ (1994) Recombinant human zona pellucida protein ZP3 produced by chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. *Biology of Reproduction* **51**, 607-617.

Verhage HG, Fazleabas AT, and Donnelly K (1988) The *in vitro* synthesis and release of proteins by the human oviduct. *Endocrinology* **122**, 1639-1645.

Verhage HG, Boice ML, Mavrogianis P, Donnelly K, and Fazleabas AT (1989) Immunological characterization and immunocytochemical localization of oviduct-specific glycoproteins in the baboon (*Papio anubis*). *Endocrinology* **124**, 2464-2472.

Verhage HG, Mavrogianis PA, Boice ML, Li W, and Fazleabas AT (1990) Oviduct epithelium of the baboon: hormonal control and the immuno-gold localization of oviduct-specific glycoproteins. *American Journal of Anatomy* **187**, 81-90.

Verhage HG, Fazleabas AT, Mavrogianis PA, O'Day-Bowman MB, Donnelly KM, Arias EB, and Jaffe RC (1997a) The baboon oviduct: characteristics of an oestradiol-dependent oviduct-specific glycoprotein. *Human Reproduction Update* **3**, 541-552.

Verhage HG, Mavrogianis PA, Boomsma RA, Schmidt A, Brenner RM, Slayden OV, and Jaffe RC (1997b) Immunologic and molecular characterization of an estrogen-dependent glycoprotein in the rhesus (*Macaca mulatta*) oviduct. *Biology of Reproduction* **57**, 525-531.

Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG, and Kopf GS (1999) Cholesterol efflux-mediated signal transduction in mammalian sperm. *Journal of Biological Chemistry* **274**, 3235-3242.

Vitaioli L, Ricci R, Bellini L, Baldoni E, Antuzzi D, and Bolognani L (1990) Sialic acid and neuraminidase activity in the frog oviduct: comparative biochemical investigation in the different tracts during the reproductive cycle. *Comparative Biochemistry and Physiology Part B* **95**, 35-38.

von Weymarn N, Guggenheim R, and Muller H (1980) Surface characteristics of oocytes from juvenile mice as observed in the scanning electron microscope. *Anatomy and Embryology* **161**, 19-27.

Voyle RB, Haines BP, Loffler KA, Hope RM, Rathjen PD, and Breed WG (1999) Isolation and characterisation of zona pellucida A (ZPA) cDNAs from two species of marsupial: regulated oocyte-specific expression of ZPA transcripts. *Zygote* **7**, 239-248.

Waclawek M, Foisner R, Nimpf J, and Schneider WJ (1998) The chicken homologue of zona pellucida protein-3 is synthesized by granulosa cells. *Biology of Reproduction* **59**, 1230-1239.

Wagner A, Ekhlasi-Hundrieser M, Hettel C, Petrunkina A, Waberski D, Nimtz M, and Topfer-Petersen E (2002) Carbohydrate-based interactions of oviductal sperm reservoir formation - studies in the pig. *Molecular Reproduction and Development* **61**, 249-257.

Wakayama T, Ogura A, Suto J, Matsubara Y, Kurohmaru M, Hayashi Y, and Yanagimachi R (1996) Penetration by field vole spermatozoa of mouse and hamster zonae pellucidae without acrosome reaction. *Journal of Reproduction and Fertility* **107**, 97-102.

Walter I, and Bavdek S (1997) Lectin binding patterns of porcine oviduct mucosa and endometrium during the oestrous cycle. *Journal of Anatomy* **190**, 299-307.

Wassarman PM (1987a) Early events in mammalian fertilization. *Annual Review of Cell Biology* **3**, 109-142.

Wassarman PM (1987b) The biology and chemistry of fertilization. *Science* **235**, 553-560.

Wassarman PM (1987c) The zona pellucida: a coat of many colors. *BioEssays* **6**, 161-166.

Wassarman PM (1988) Zona pellucida glycoproteins. *Annual Review of Biochemistry* **57**, 415-442.

Wassarman PM (1990) Profile of a mammalian sperm receptor. *Development* **108**, 1-17.

Wassarman PM (1995) Towards molecular mechanisms for gamete adhesion and fusion during mammalian fertilization. *Current Opinion in Cell Biology* **7**, 658-664.

Wassarman PM, and Albertini DF (1994) The mammalian ovum. In 'The Physiology of Reproduction. Second Edition'. (Eds E Knobil and JD Neill) pp. 79-122. (Plenum Press: New York)

Wassarman PM, and Josefowicz WJ (1978) Oocyte development in the mouse: an ultrastructural comparison of oocytes isolated at various stages of growth and meiotic competence. *Journal of Morphology* **156**, 209-236.

Wassarman PM, and Litscher ES (1995) Sperm-egg recognition mechanisms in mammals. *Current Topics in Developmental Biology* **30**, 1-19.

Wassarman PM, and Litscher ES (2001) Towards the molecular basis of sperm and egg interaction during mammalian fertilization. *Cells Tissues Organs* **168**, 36-45.

Wassarman PM, and Mortillo S (1991) Structure of the mouse egg extracellular coat, the zona pellucida. *International Review of Cytology* **130**, 85-110.

Wassarman PM, Liu C, and Litscher ES (1996) Constructing the mammalian egg zona pellucida: some new pieces of an old puzzle. *Journal of Cell Science* **109**, 2001-2004.

Wassarman PM, Liu C, and Litscher ES (1997) Building the mammalian egg zona pellucida: an organelle that regulates fertilization. In 'Microscopy of Reproduction and Development: A Dynamic Approach'. (Ed. PM Motta) pp. 169-176. (Antonio Delfino Editore: Rome).

Wassarman PM, Chen J, Cohen N, Litscher ES, Liu C, Qi H, and Williams Z (1999) Structure and function of the mammalian egg zona pellucida. *Journal of Experimental Zoology* **285**, 251-258.

Wassarman P, Bleil J, Fimiani, C, Florman, H, Greve, J, Kinloch, R, Moller, C, Mortillo, S, Roller, R, Salzmann, G, and Vazquez, M (1989) The mouse egg receptor for sperm: a multifunctional zona pellucida glycoprotein. In 'The Mammalian Egg Coat: Structure and Function'. (Ed. J Dietl) pp. 18-37. (Springer-Verlag: Berlin).

Wegner CC, and Killian GJ (1991) In vitro and in vivo association of an oviduct estrus-associated protein with bovine zona pellucida. *Molecular Reproduction and Development* **29**, 77-84.

Wegner CC, and Killian GJ (1992) Origin of oestrus-associated glycoproteins in bovine oviductal fluid. *Journal of Reproduction and Fertility* **95**, 841-854.

- Wiebkin OW (1994) Mucosubstances and structurally related macromolecular carbohydrates. In 'Laboratory Histopathology: A Complete Reference'. (Eds AE Woods and RL Ellis) pp. 6.6-1-6.6-43. (Churchill Livingstone: Edinburgh)
- Willis P, Sekhar KN, Brooks P, and Fayrer-Hosken RA (1994) Electrophoretic characterization of equine oviductal fluid. *Journal of Experimental Zoology* **268**, 477-485.
- Wilson MT, Farmer MA, and Karwoski CJ (1998) Ultrastructure of the frog retina after high-pressure freezing and freeze substitution. *Journal of Microscopy* **189**, 219-235.
- Witton CJ, Swann K, Carroll J, and Moore HDM (1999) Injection of boar sperm factor causes calcium oscillations in oocytes of the marsupial opossum, *Monodelphis domestica*. *Zygote* **7**, 271-277.
- Woolley P (1990a) Reproduction in *Sminthopsis macroura* (Marsupialia: Dasyuridae) I. The female. *Australian Journal of Zoology* **38**, 187-205.
- Woolley P (1990b) Reproduction in *Sminthopsis macroura* (Marsupialia: Dasyuridae) II. The male. *Australian Journal of Zoology* **38**, 207-217.
- Wu AM, Sugii S, and Herp A (1988) A guide for carbohydrate specificities of lectins. In 'The Molecular Immunology of Complex Carbohydrates'. (Eds A Wu and LG Adams) pp. 819-847. (Plenum Press: New York)
- Wu T-C, Lee M-C, Wan Y-J, and Damjanov I (1984) Lectin binding sites of the mouse ovary, intraovarian and ovulated ova. *Histochemistry* **80**, 527-533.
- Yamagata K, Murayama K, Kohno N, Kashiwabara S-i, and Baba T (1998a) *p*-Aminobenzamidine-sensitive acrosomal protease(s) other than acrosin serve the sperm penetration of the egg zona pellucida in mouse. *Zygote* **6**, 311-319.
- Yamagata K, Murayama K, Okabe M, Toshimori K, Nakanishi T, Kashiwabara S-i, and Baba T (1998b) Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. *Journal of Biological Chemistry* **273**, 10470-10474.
- Yamasaki N, Richardson RT, and O'Rand MG (1995) Expression of the rabbit sperm protein Sp17 in COS cells and interaction of recombinant Sp17 with the rabbit zona pellucida. *Molecular Reproduction and Development* **40**, 48-55.
- Yamasaki N, Tsubamoto H, Hasegawa A, Inoue M, and Koyama K (1996) Genomic organization of the gene for pig zona pellucida glycoprotein ZP1 and its expression in mammalian cells. *Journal of Reproduction and Fertility Supplement* **50**, 19-23.
- Yanagimachi R (1994) Mammalian Fertilization. In 'The Physiology of Reproduction, Second Edition'. (Eds K E and JD Neill) pp. 189-317. (Raven Press Ltd: New York)
- Yang C-H, and Yanagimachi R (1989) Differences between mature ovarian and oviductal oocytes: a study using the golden hamster. *Human Reproduction* **4**, 63-71.

- Yonezawa N, Hatanake Y, Takeyama H, and Nakano, M (1995). Binding of pig sperm receptor in the zona pellucida to the boar sperm acrosome. *Molecular Reproduction and Development* **40**, 48-55.
- Yonezawa N, Mitsui S, Kudo K, and Nakano M (1997) Identification of an N-glycosylated region of pig zona pellucida glycoprotein ZPB that is involved in sperm binding. *European Journal of Biochemistry* **248**, 86-92.
- Yudin AL, Cherr GN, and Katz DF (1988) Structure of the cumulus matrix and zona pellucida in the golden hamster: a new view of sperm interaction with oocyte-associated extracellular matrices. *Cell Tissue Research* **251**, 555-564.
- Yudin AL, Vandervoort CA, Li M-W, and Overstreet JW (1999) PH-20 but not acrosin is involved in sperm penetration of the macaque zona pellucida. *Molecular Reproduction and Development* **53**, 350-362.
- Yurewicz EC, Hibler D, Fontenot GK, Sacco AG, and Harris J (1993a) Nucleotide sequence of cDNA encoding ZP3 alpha, a sperm-binding glycoprotein from zona pellucida of pig oocyte. *Biochimica et Biophysica Acta* **1174**, 211-214.
- Yurewicz EC, Pack BA, Armant DR, and Sacco AG (1993b) Porcine zona pellucida ZP3 alpha glycoprotein mediates binding of the biotin-labeled M(r) 55,000 family (ZP3) to boar sperm membrane vesicles. *Molecular Reproduction and Development* **36**, 382-389.
- Yurewicz EC, Sacco AG, Gupta SK, Xu N, and Gage DA (1998) Hetero-oligomerization-dependent binding of pig oocyte zona pellucida glycoproteins ZPB and ZPC to boar sperm membrane vesicles. *Journal of Biological Chemistry* **273**, 7488-7494.

Appendix 1

Preparation of Oocytes with Ruthenium Red and Saponin

1. Wash oocytes three times in 0.1M cacodylate buffer, pH 7.2 for 10 minutes.
2. Wash oocytes in 0.02% saponin and 1% ruthenium red (RR), made in 0.1M cacodylate buffer, for 30 minutes.
3. Fix oocytes in 3% glutaraldehyde plus 1% RR and 0.02% saponin, made in 0.1M cacodylate buffer, at 4°C overnight.
4. Wash oocytes three times in 0.02% saponin and 1% ruthenium red (RR), made in 0.1M cacodylate buffer, for 10 minutes.
5. Post-fix oocytes with 1% osmium tetroxide in 0.1M cacodylate buffer containing 0.75% RR and 0.02% saponin, for 1 hour.
6. Wash oocytes three times in 0.1M cacodylate buffer, pH 7.2 for 10 minutes.
7. Process oocytes as normal for either transmission or scanning electron microscopy, as described in section 2.3 and 2.4.

Appendix 2

Preparation of Oocytes for Rapid Freezing and Freeze Substitution

1. Pre-cool liquid propane within the Leica plunge freezing unit (Leica Microsystems Pty Ltd, Austria) with liquid nitrogen.
2. Following the collection of oocytes into culture media (see section 2.2), pipette oocytes onto 1-2mm wide by 1cm long piece of 150 μ m Nitex nylon mesh that rests on filter paper (to absorb excess culture media).
3. Grip the mesh containing the oocytes with reverse-grip forceps and attach to the Leica plunge freezing unit and plunge freeze into the pre-cooled liquid propane.
4. Place the mesh containing the oocytes into a vial containing liquid nitrogen and transfer to a vial containing a solution of molecular sieve 100% dry methanol with 1% tannic acid and 5% dimethoxypropane at -90°C within the Leica Freeze Substitution Unit (Leica Microsystems Pty Ltd, Austria).
5. The mesh containing the oocytes remains within the Freeze Substitution Unit for 3-5 days with daily changes of the freeze substitution solution.
6. After 3-5 days, wash the mesh containing the oocytes four times in molecular sieve 100% dry methanol at -90°C for 1 hour and post-fix the mesh containing the oocytes in 1% osmium tetroxide in molecular sieve 100% dry methanol at -90°C for 2 hours.
7. Wash the mesh containing the oocytes four times in molecular sieve 100% dry methanol at -90°C for 30 minutes and bring up to room temperature (from -90°C to -20°C over 2 hours, then from -20°C to 4°C over 2 hours).
8. Once at room temperature, process oocytes as normal for either transmission or scanning electron microscopy, as described in section 2.3 and 2.4.

Appendix 3

Publications and Presentations arising from this thesis

Chapman, J.A., Wiebkin, O.W. & Breed, W.G. (2000) Interspecific variation of zona pellucida glycoconjugates in several species of marsupial.
Journal of Reproduction and Fertility, v. 119(May), pp. 111-120

NOTE:

This publication is included on pages 302-311 in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://doi.org/10.1530/jrf.0.1190111>

Breed, W.G., Hope, R.M., Wiebkin, O.W., Spargo, S.C. & Chapman, J.A. (2002) Structural organization and evolution of the marsupial zona pellucida.
Reproduction, v. 123(January), pp. 13-21

NOTE:

This publication is included on pages 312-320 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1530/rep.0.1230013>

Chapman, J.A., Wiebkin, O.W. & Breed, W.G. (1999) Interspecific variation in glycoconjugates of the zonae pellucidae of several marsupial species.

Presented at: 31st Annual Conference for the Australian Society for Reproductive Biology, 26-29 September, Melbourne, Australia

NOTE:

This publication is included on page 322 in the print copy of the thesis held in the University of Adelaide Library.

Chapman, J.A. & Breed, W.G. (2003) The oviduct of the brushtail possum, *Trichosurus vulpecula*: the glycoconjugate environment at ovulation and its probable contribution to the post-ovulatory zona pellucida.

Presented at: 21st New Zealand Conference on Microscopy, 7-12 February, Wellington, New Zealand,

NOTE:

This publication is included on pages 323-324 in the print copy of the thesis held in the University of Adelaide Library.

Chapman, J.A., Wiebkin, O.W. & Breed, W.G. (2002) Structure and composition of the zona pellucida of a marsupial, the brushtail possum *Trichosurus vulpecula*, following ovulation and egg activation. *Presented at: 17th Australian Conference on Electron Microscopy, 4-8 February, Adelaide, South Australia*

NOTE:

This publication is included on pages 325-326 in the print copy of the thesis held in the University of Adelaide Library.

Chapman, J.A. & Breed, W.G. (2001) What prevents polyspermy in a marsupial egg? A study in the brushtail possum.

Presented at: 47th Scientific Meeting and Possum and Glider Symposium, 2-6 July, Brisbane, Queensland

NOTE:

This publication is included on pages 327-328 in the print copy of the thesis held in the University of Adelaide Library.

Chapman, J.A., Wiebkin, O.W. & Breed, W.G. (2000) How similar is the molecular structural organization of the marsupial zona peillucida to that of Eutherians?.

Presented at: 3rd International Symposium on the Molecular and Cellular Biology of the Egg and Embryo Extracellular Matrix, 27 August- 1 September, Lake Tahoe, California

NOTE:

This publication is included on pages 329-330 in the print copy of the thesis held in the University of Adelaide Library.

Chapman, J.A., Wiebkin, O.W. & Breed, W.G. (2000) Structure of the zona pellucida of a model marsupial, the brushtail possum (*Trichosurus vulpecula*: family *Phalangeridae*).
Presented at: Fertility 2000, 31 July-2 August, Edinburgh, Scotland

NOTE:

This publication is included on pages 331-332 in the print copy
of the thesis held in the University of Adelaide Library.

Chapman, J.A., Wiebkin, O.W. & Breed, W.G. (1999) Interspecific variation in glycoconjugates of the zonae pellucidae of several marsupial species.

Presented at: 30th Annual Conference for the Australian Society for Reproductive Biology, 26-29 September, Melbourne, Australia

NOTE:

This publication is included on pages 333-334 in the print copy of the thesis held in the University of Adelaide Library.