WOMEN IN REPRODUCTIVE SCIENCE

Reproduction down under

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Abstract

Australia is home to a unique assembly of mammals – the marsupials and monotremes. Despite this uniqueness, they have been largely ignored by the biomedical scientific community, and yet study of marsupials has contributed to modern research on reproduction, development, evolution, conservation, molecular and comparative genomics. My lifetime passion for these long-neglected Australian fauna has led to unexpected discoveries and insights that challenged assumptions and opened up new areas of international research. I used a range of disciplinary expertise to pursue the study of these unique mammals. My main experimental species has been the tammar wallaby that I have used as a model species to investigate and understand not only biomedical problems but also to provide knowledge that is critical for the continued conservation and management of Australia's dwindling native mammals. This model provided more than a few surprises for me and my wonderful team of students, post-docs and collaborators about how hormones, genes and signalling molecules control reproductive biology and development in a wider context as well as how the interactions of the environment with mother and conceptus, with mother and fetus and mother and young ultimately control most aspects of successful reproduction in mammals.

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Introduction: marsupial models

Marsupials are best known for their cute young in pouches (Fig. 1), but not all marsupials have a pouch and of course no male marsupial has a pouch. The way in which marsupials differ most from their eutherian mammal counterparts is in their mode of reproduction (Tyndale-Biscoe & Renfree 1987). They have a lengthy post-natal development during which the highly altricial young completes its development like an exteriorised fetus, dependent on their amazing milk that changes dynamically throughout the whole of lactation. It is these characteristics that have made them so attractive as experimental models for answering questions of the control and evolution of mammalian reproduction and development, although research granting bodies did not always see that research on Australia's interesting and exceptional mammals is worth funding!

Tackling these problems has necessitated field studies and collection largely from remote locations on South Australia's Kangaroo Island together with the establishment and maintenance of the largest marsupial breeding colony in the world in Melbourne, very much a hands-on activity, annually replenishing it with animals caught in the wild (at night) with my team of students,

research fellows and colleagues from all over the world. This has required the development of knowledge about their handling and husbandry, and support (and extensive grant writing) to ensure the colony's long-term viability.

Overturing entrenched ideas: the marsupial placenta and conceptus

The term 'placental mammal' has been the bugbear of my career, because it gave people the impression that marsupials were aplacental mammals. Even textbooks to this day reiterate this erroneous conclusion. The placenta in most marsupials is an epitheliochorial placenta (like that of the pig), not an invasive chorio-allantoic (haemochorial) placenta (like that of mouse and man), but some, especially bandicoots, do have an allantoic placenta, and it is highly invasive, albeit for a very short time. During my PhD I was able to demonstrate that the relationship between the marsupial embryo and its mother is based on a fully functional placenta and that there is maternal recognition of pregnancy in which the embryo stimulates the endometrium to increase in size and to remain in a highly secretory condition, suggesting that the fetal effect may be either an endocrine factor or

My life with marsupials and other mammalian friends

Early days

I was born in Brisbane after the war, and enjoyed climbing up jacaranda trees, eating pawpaw (papaya in the US) and being taken to the beach with my mum and dad and older siblings, Beverley and Neil. I think I must have been imprinted with marsupials from an early age. One of my earliest memories was visiting Lone Pine Sanctuary in Brisbane at around the age of 2, with my parents and being given a koala to cuddle. I'm sure this experience imprinted marsupials upon my subconscious! Not long after that, we moved to Canberra, the Australian capital (known still as the 'Bush Capital'). Canberra was tiny in those days but was a wonderful place to grow up – wonderful bush and animals, only 2 h from some of the best beaches in Australia, surfing and scuba diving, and two hours to the snow for skiing (both cross country and down-hill), and three-four hours from Sydney (for partying!).

Mentors

I have been so lucky with my mentors. First, Hugh Tyndale-Biscoe, a scientist of considerable intellect, who constantly challenged me and who taught me to think outside the box was my honours and then PhD Supervisor. He was then (and still is now) demanding of high standards, and taught me a never ending curiosity about these Australian mammals. One of the most enjoyable phases of my association with Hugh was co-authoring our book, *Reproductive Physiology of Marsupials* (written, incidentally between daughter number 1 and daughter number 2). This book is known as 'The Bible' amongst my students, and despite its 1987 publishing date is still consulted on a frequent basis. Hugh taught me all things reproduction, sophisticated surgery and field work. He also taught me the value of clear writing and presentation. I met my other three amazing mentors Emmanuel Ciprian Amoroso, ('Amo' as he was universally known), Anne McLaren and Roger Short at an international conference in Canberra while I was a PhD student. Amo was the father of comparative placentation – a Trinidadian by origin based in London and Cambridge and a Fellow of the Royal Society – what an inspiration! I co-authored his very last review paper when I was on sabbatical in Babraham in Cambridge with Brian Heap. Little did I know then that of the other two, also Fellows of the Royal Society, one (Anne) would become a lifelong friend and surrogate grandmother to my children, and that the other (Roger) would become my husband and father of our two wonderful daughters, Dr Tamsin Short (clinical psychologist, especially forensic psychology) and Dr Kirsty Short (virologist, especially influenza).

I am a keen advocate for women in science, having received my fair share of discrimination when I was a younger researcher in my early academic positions. I have mentored (and kept in touch with) a large and enthusiastic cadre of former students and postdoctoral fellows who all occupy important positions in academic, research and pharmaceutical organizations throughout Australia and internationally. My closest colleague, Professor Geoff Shaw was my fourth PhD student, over 40 years ago, and we are still collaborating!

The one big advantage of working on unconventional animals is that you have to learn everything about these wild animals, from the anatomy, physiology, ecology, behaviour, molecular biology, genetics and genomics because there has been so much less information available than on common laboratory species like mice or domestic species like pigs and cows. It also meant that we had to work closely as a team, for it is very difficult to catch a wallaby by yourself!

Career

I have worked at six Universities in Australia, USA and UK: the Australian National University in Canberra, the University of Tennessee, the University of Edinburgh, Murdoch University in Perth, Monash University and Melbourne University both in Melbourne. Each endowed me with great experiences. After completion of my PhD at ANU supervised by Hugh Tyndale-Biscoe, I was awarded a Fulbright Fellowship to work with Joe Daniel at the University of Tennessee in Knoxville (where I became known as the 'possum lady from Australia, learnt how to write a research grant and revised some of Carl Hartman's conclusions on opossum reproduction). I then was lucky enough to work on a Ford Foundation fellowship with Anne McLaren in Edinburgh (on mice!) at the ARC Institute of Animal Genetics where the famous Waddington was head of the Institute. In 1974, from Edinburgh, I was appointed as a foundation member of staff at the then brand-new University on Perth, Western Australia, Murdoch University, where I was a foundation Lecturer in Vertebrate Biology (only the third junior, non-Professorial member of staff) and where I established a 12 hectare (30 acres) Native Fauna Research Unit with tammars, agile wallabies and quokkas. Murdoch was truly a baptism by fire. We had no buildings, no campus, no laboratories, only temporary facilities in the Noalimba Migrant Centre in the suburb of Bullcreek, just down the road from the 600 acres of the Applecross Pine plantation that was to become Murdoch University. After almost ten years at Murdoch in early 1981, I became engaged to Roger Short, put my lab on hold in Murdoch and moved to Edinburgh once again, on a Royal Society Fellowship where I worked in the MRC Reproductive Biology Unit (led by Roger). We were married in Canberra in January 1982 and returned to Edinburgh. We were fortunate and forever grateful to both be recruited to Australia in May 1982 by Professors David de Kretser and Geoff Thorburn to the Department of Anatomy (Marilyn) and Physiology (Roger) at Monash University where I became an NHMRC Research Fellow. I held that fellowship until I moved to the University of Melbourne as Head of the Department of Zoology (now School of BioSciences) in 1991, where I remain to date.

Concluding remarks

I have, in the words of the Australian author Albert Lacey had 'A Fortunate Life'. I was lucky to have a supportive extended family, husband and children and supportive mentors. I was lucky that I could study and research my passion for Australian animals, and lucky that some discoveries have been translated into clinical medicine while others have been important for conservation. I've been lucky to be able to grab opportunities when they arose (together with the hard work that always accompanied these). Most of all I've been lucky in the fantastic cohort of national and international colleagues, students and Research Fellows who all have made this journey immensely exciting and rewarding with discoveries, mateship and adventure in some remote locations in Australia and internationally. They have all become part of my extended family.

My lifelong philosophy is summed up by my motto, a quotation from Johann Wolfgang von Goethe 'Whatever you can do or dream you can, begin it; Boldness has genius, power, and magic in it.'

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Figure 1 Tammar wallaby with her 9 month old pouch young, soon to leave the pouch.

an inflammatory effect (Renfree 1972). In the decades since, this has been amply confirmed and this observation extended (Renfree 2010), including the demonstration that the placenta is also hormone-secreting. As well as the incipient progesterone synthesis identified earlier (Heap *et al.* 1980), we now know the placenta produces prostaglandins E_2 and $F_{2\alpha}$ (Shaw *et al.* 1999) as well as the pituitary hormones GH, GH-R, IGF-2, PRL and LH β (Menzies *et al.* 2011), in contrast to earlier dogma (Fig. 2).

Marsupial blastocysts have no inner cell mass, so we were intrigued as to how the trophoblast cells (that

will form the placenta) are differentiated from those cells that will form the embryo proper. Investigating the development of macropodid conceptuses from ovulation through cleavage stages to the early blastocyst *in vivo* and *in vitro* (Renfree & Lewis 1996) did not tell us where on the single cell layered blastocyst the embryo would develop, but hinted at polarisation of the cells. Later studies using immunocytochemistry of the molecular analysis of mechanisms of early cell lineage specification in the wallaby showed us that while the same genes are involved in early development (e.g. *POU5F1*, *CDX2*, *NANOG* and *GATA6*), there is considerable evolutionary plasticity in the mechanisms regulating early lineage specification in mammals and marsupials differ markedly from mice in this regard – for example the pluriblast-trophoblast differentiation coincided with a global nuclear-to-cytoplasmic transition of *CDX2* localisation, and the Hippo pathway factors YAP and WWTR1 showed mutually distinct localisation patterns that suggest non-redundant roles (Frankenberg *et al.* 2010, 2013). In this way they may be more akin to the pattern seen in bovine embryos (Frankenberg *et al.* 2016).

How do you stop an embryo growing and then start it again: the phenomenon of embryonic diapause

Once in the blastocyst stage, some marsupial blastocysts have a hesitation in their development known as embryonic diapause. These are the kangaroos and wallabies, and the small possums including the feather-tailed glider and the diminutive honey possum. In the tammar, over many years, Geoff Shaw and I and others, especially from Hugh Tyndale-Biscoe's lab, demonstrated the environmental and physiological signals for entry into and exit from diapause (reviewed in Renfree & Shaw 2000, Renfree & Fenelon 2017). These include melatonin, mediated by photoperiod beginning on the longest day, prolactin, that inhibits the growth of the corpus luteum, and progesterone that reactivates the secretory endometrium and blastocyst. We always knew lactation and the sucking stimulus was a key factor, but I decided to do some heroic surgery to test this by removing all neural signals from the teat while allowing lactation to continue. The results were unequivocal: it is neural inputs from the teat that control lactational embryonic diapause in marsupials (Renfree 1979). This work provided the background for our extensive study (with my husband Roger Short and Geoff Shaw) on the contraceptive effects of breastfeeding in women, reinforcing the idea that breastfeeding is Nature's contraceptive, even in women (Short *et al.* 1991).

To investigate the proximal signals for the seasonal diapause, I made another difficult surgical approach. Removal of the superior cervical ganglion, that lies right on top of the internal carotid artery, showed the sympathetic innervation of the pineal gland controls

Figure 2 Tammar fetus and placenta. A full term fetus (day 25 of the 26.5 day pregnancy) still attached to the uterus and within its fetal membranes: the amnion, the allantois and the yolk sac (choriovitelline) placenta.

melatonin secretion and in turn the remarkable highly synchronised reactivation from seasonal diapause in the tammar wallaby that occurs on the longest day in the Southern hemisphere, December 22nd (Renfree *et al.* 1981). An even more difficult surgical approach – pinealectomy – by Steve McConnell in Hugh Tyndale-Biscoe's lab gave the same result (McConnell & Hinds 1985, Tyndale-Biscoe *et al.* 1986). Thus melatonin is critical for the control of seasonal breeding, as it is also in the red deer (Lincoln & Short 1980, Lincoln *et al.* 1984). These results from two very different species gave Roger the idea that melatonin might ameliorate jet lag (Short 1993), an idea that he subsequently proved and for which he held the use patent – now used all over the world for jet lag, including for shift workers and astronauts.

Conception and contraception

While more and more marsupials are becoming extinct due to human clearing of habitat and climate change, there are some species that are overabundant. Roger had a friend and colleague in the company 'Leiras' in Finland, who marketed a levonorgestrel contraceptive implant. When they were taken over, Roger obtained a number of their implants. With Geoff Shaw, Kath Handasyde and our students, we used these to reverse-translate this human contraceptive into an effective management tool for overabundant populations of kangaroos (Nave *et al.* 2000, 2002) and koalas (Hynes *et al.* 2011), now being used in the field for management of overabundant koalas by State authorities. On the flip side, my lab developed assisted reproductive techniques for marsupials, including the first *in vitro* fertilised marsupial embryo (Richings *et al.* 2004), the first conceptus produced by artificial insemination (AI) (Paris *et al.* 2005) and the first maturation of follicles *in vitro* after cold storage (Richings *et al.* 2006). Injection through the shell coat that surrounds all marsupial conceptuses was challenging, as was attempting AI via the long median vagina of marsupials. In the end, AI success was only achieved by bypassing the median and lateral vaginae (marsupials have three vaginae) and injection into the uterine cavity (Paris *et al.* 2005).

Can milk composition control growth? Lactation and growth acceleration

Marsupials have the remarkable ability to alter the composition of their milk in tune with the growth of the young. Some continuously-breeding macropodid marsupials have concurrent, asynchronous lactation, in which a new tiny young is drinking milk of a different composition from that of its older sibling ('young-at-foot') (Green *et al.* 1980, Nicholas 1998, Nicholas *et al.* 1997). How the marsupial teat and mammary gland behaves independently from the adjacent teat to synthesise milks

of different composition depending upon the age of the pouch young has been a key question in lactational biology. We established that the neuroendocrine control of milk ejection in marsupials by oxytocin differs between these glands (Lincoln & Renfree 1981a,b), and it appears to be an aging phenomenon of the gland and not related to the sucking stimulus (Trott *et al.* 2003). This means that there is a strong maternal control of pouch young growth. We exploited this to show the effects of providing different milk compositions at different stages of growth. We fostered young from an early stage of lactation to a more advanced stage of lactation and caused an astonishingly rapid growth acceleration with early maturation and shortened time to pouch exit and independence of the young (Trott *et al.* 2003, Menzies *et al.* 2007, Hetz *et al.* 2015). Interestingly, the marsupial mammary gland and placenta share 77 genes with the early-mid eutherian placenta. These genes (e.g. *GATA3*, *MSH2*, and *GCM1*) are associated with nutrient transport (Guernsey *et al.* 2017) – demonstrating again the importance of lactation and maternal control of growth during development – marsupials have truly traded the umbilical cord for the teat.

The development of males and females: sexual differentiation

The long post-natal period of development provided a unique opportunity to understand how hormones, genes and signalling molecules control sex determination and sexual differentiation. Pondering over some scanning electron micrographs, I serendipitously observed that there were scrotal bulges in the embryo before the differentiation of the testis and production of testosterone. I called Roger to tell him what I had seen. He was immediately sceptical! I determined to show that marsupials really did differentiate their scrotum before they have functional testes capable of synthesising testosterone. Geoff Shaw had recently returned to my lab and with our sabbatical visitor, Dr Wai-Sum O and Roger we established that these structures were hormone-independent and that there was indeed a direct genetic control by a gene or genes on the X chromosome (O *et al.* 1988, Renfree & Short 1988, Fig. 3). This novel finding has been amply confirmed and has directed research from multiple laboratories to an entirely new and expanding field of investigation. Direct X and Y chromosome effect, (and Z/W effects in birds), have now been demonstrated in eutherian mammals, particularly in mice, and almost certainly in humans (Arnold 1997, Burgoyne *et al.* 2001, Arnold & Burgoyne 2004, Renfree *et al.* 2014).

These studies overturned entrenched dogma and the Jost paradigm of hormonally-controlled sexual differentiation in all mammals (O *et al.* 1988, Renfree & Short 1988, Shaw *et al.* 1988). We demonstrated that these sexually dimorphic characters differentiate

Figure 3 Fetal gonads and developing scrotum and pouch. Some sexual dimorphisms (namely the scrotum and the mammary glands) are controlled by genes on the X chromosome and differentiate before the testis begins secreting androgens. Female fetuses at day 25 of gestation, 1.5 days before birth have indifferent gonads (top, left hand panel) but have already developed paired mammary primordia (MP; top, middle panel), while males have indifferent gonads (bottom left hand panel) yet have paired scrotal bulges (SB; bottom, middle and right hand panel). The phallus (Ph) is likewise not yet sexually dimorphic and the hind limbs (HL) are merely a paddle. WD: Wolffian duct. Redrawn from Renfree *et al.* (1996).

independently of hormones, and we went on to show that these are linked to X-chromosome dosage. At about the same time, we discovered that we could induce testis-to-ovary sex reversal with exogenous oestrogen (Coveney *et al.* 2001), the first demonstration of oestrogen-induced gonadal sex reversal in any mammal. Surprisingly, we sex reversed not only the somatic cells but also the germ cells after oral treatment with oestrogen alone. This led on to our demonstration with Andrew Pask, then a research fellow in my lab, that oestrogen prevents the nuclear shuttling of SOX9 (Pask *et al.* 2010), suggesting a mechanism by which oestrogens are necessary to help to maintain ovarian development in other mammals, and how environmental oestrogen analogues might contribute to sex reversal in amphibians and the decline of male fertility in humans. Understanding how environmental oestrogen analogues contribute to sex reversal and the decline of male fertility coupled with increase in the frequency of disorders of sex development in humans is critical to understand the role of environmental endocrine disruptors (EEDs) in development. We are now using this marsupial model to investigate EEDs (see end of text).

Another serendipitous event led me to the next phase of our studies on sexual differentiation. I was fortunate that a famous endocrinologist, Jean D Wilson (from University of Texas Medical School in Dallas) was visiting Monash just after he had published a paper on gonadal steroids in the North American opossum (George *et al.* 1985). In conversation, I asked Jean if he might be interested in the tammar, and so began a thirty-year collaboration that continues to this day. We puzzled over the fact that the timing of the virilisation of structures of the male reproductive system in marsupials could not be accounted for by the classical pathways producing testosterone and dihydrotestosterone. After many hours in the wallaby yards, much fine dissection, numerous incubations with radioactive steroids and the help of Jean's Dallas colleagues with

HPLC, Jean, Geoff Shaw and I identified a previously unrecognised androgen biosynthetic pathway that forms dihydrotestosterone via an alternate pathway in which the potent androgen androstenediol is the key circulating androgen. This alternate pathway appears to be essential for the development of normal male tammar as well as mouse and man (Shaw *et al.* 2000, Leihy *et al.* 2001, Wilson *et al.* 2003). It has been translated into a new clinical guideline by the US Endocrine Society and has again spawned a whole new field of clinical studies (Arlt *et al.* 2004, Homma *et al.* 2006, Fluck *et al.* 2011, Fukami *et al.* 2013, Reisch *et al.* 2013, Marti *et al.* 2017, Speiser *et al.* 2018). It explains the virilisation seen in girls with P450 oxidoreductase deficiency (Arlt *et al.* 2004, Homma *et al.* 2006) and explains virilisation of female infants with 21-hydroxylase deficiency (Reisch *et al.* 2013). Burgeoning international studies of this pathway now implicate the alternate pathway not only in sexual development, but also is implicated in the hyperandrogenism seen in polycystic ovarian syndrome (Marti *et al.* 2017).

In more recent studies, again using a surgical approach in which I transplanted gonads from the neonatal male tammar under the skin of female tammar, we induced a penis-like phallus in females and after castration, early post-natal male young developed a phallus with an open urethral canal, providing a model for hypospadias. Hypospadias is the second most common birth defect occurring in 1:125 boys in the Western world and is increasing in frequency (Kalfa *et al.* 2010, 2011), so marsupials provide a unique model for understanding this increasingly common disorder of sex differentiation. Because these critical phases of phallus development occur after birth of the pouch young in marsupials, they can be readily manipulated, unlike in eutherian embryos that complete these stages *in utero*. Our most recent studies are now investigating the effects of oestrogens as well as androgens on phallus development.

Transcriptomics

In recent years we have completed a new genome of the tammar wallaby that has a 200× coverage. While it is yet to be published, the new genome does include over 150 transcriptomes of various reproductive tissues, including an amazing set of transcriptome data completed for us by Prof. Asao Fujiyama and his colleagues at the National Institute of Informatics and the National Institute of Genetics in Japan of the developing phallus after we treated the young with androgens, oestrogens and after castration. These data have shown us just how sensitive the genes of the developing phallus are to exogenous androgens and oestrogens, highlighting their steroid hormone sensitivity (Chew *et al.* 2014, Chen *et al.* 2018a,b). Furthermore, there are hundreds of long non-coding genes (lncRNAs) that are also up-or down-regulated by the steroid treatments, apparently in concert with their coding genes (Chen *et al.* 2019). lncRNAs have conserved functions, but do not have conserved sequences between mammals, so they are often difficult to fully characterise. Nevertheless it is clear that both coding and non-coding genes could be direct targets of exogenous hormones, and underpins many of the effects observed with EEDS. With research fellow Gerard Tarulli we are now investigating the relative importance of the androgens, oestrogens and their inhibitors on phallus differentiation.

Father's influence on the offspring: genomic imprinting

In 1996, I was fortunate to be one of only two Australians to be invited to attend the award of the Japan prize to Prof. Ryuzo Yanagamachi. At the symposium that followed the award ceremony (where I met and spoke with Emperor Akihito), I met a young Japanese scientist (Dr, now Professor) Fumi Ishino, who had a very interesting poster on genomic imprinting in mice. Genomic imprinting results from a parent-of-origin mark (usually methylation) on certain genes, many of which appear to control growth of the fetus. Our discussion around Fumi's poster led to a 22+ year ongoing collaboration on marsupial genomic imprinting, first focusing on the placentally-imprinted genes and developing new concepts on the evolution of imprinting. With these wonderful Japanese colleagues and research fellow Shunsuke Suzuki, we showed that retrotransposon insertion could drive the acquisition of imprinting in mammalian evolution via differential methylation, proving for the first time that a differentially methylated region (DMR) also occurred in marsupials, contrary to the assumptions of the time (Suzuki *et al.* 2007). A second marsupial gene with a DMR resulted from collaboration with Wolf Reik's laboratory at Babraham UK where Guillaume Smits identified the first ncRNA (*H19*) in the tammar wallaby (Smits *et al.* 2008). We also began collaborating with

another key Cambridge imprinting laboratory, that of Anne Ferguson-Smith that continues today. We have now examined 14 genes that are imprinted in the eutherian placenta in the marsupial placenta, but of these only 8 are imprinted, and only three have a DMR (*PEG10*, *H19* and *IGF2R*) (Renfree *et al.* 2013, Suzuki *et al.* 2018).

Postgraduate students working in our lab were able to visit the Ishino and the Ferguson-Smith labs and vice versa. This has been a wonderful unexpected consequence of these collaborations. We identified genomic imprinting of insulin and IGF2 in the tammar placenta, and another surprising finding was that these two genes are imprinted in the marsupial mammary gland and pouch young post-natally, reinforcing the parallels between eutherian placentation and marsupial nutritional control via lactation. Most recently, continuing our collaboration with Shunsuke Suzuki we showed that there is a lncRNA (*ALID*) in the marsupial *IGF2R* locus. This gene is the best candidate factor for establishing the paternal silencing of marsupial *IGF2R* but without the transcriptional overlap that occurs in the mouse *Igf2r* locus with its lncRNA *Airn* (Suzuki *et al.* 2018). The tammar also shares the same process of global methylation of germ cells as other mammals, although as expected, the timing differs, being post-natal in the tammar and pre-natal in eutherians (Ishihara *et al.* 2019).

All this from a chance encounter at an international conference!

Australian Mammal Genomes

In a parallel universe, Jenny Graves (then at La Trobe University in Melbourne) had spent much of her career working on marsupial chromosomes. Our work first intersected during the search for the sex-determining gene in mammals. Jenny asked me if I could provide her with some marsupial tissue. I gladly did so, not realising it would lead to another amazing long term and productive collaboration. These tissues helped show that the *ZFY* gene was NOT the marsupial sex determining gene and led to the identification of *SRY* in marsupials (Foster *et al.* 1992). Later, Jenny invited me, Peter Temple-Smith and others to propose the construction of the platypus genome to NIH. This bid was successful, and led to an important paper (Warren *et al.* 2008). Sadly, when we tried to get NIH funding for the first marsupial genome of the tammar wallaby, we were pipped at the post by colleagues in the US who made a case for the South American opossum genome as an 'American' marsupial. The opossum genome was therefore the first marsupial genome sequenced and assembled. In response, Jenny and I proposed and were awarded an ARC Centre of Excellence (C of E) grant in Kangaroo Genomics (KanGO) (though with reduced funding to the other centres, so we called it the 'Centre of Very Goodness!'). This was a fantastic collaborative effort with the late

Professor Des Cooper from Macquarie University, and Partner investigators Dr Sue Forrest (Australian Genome Research Facility) and Prof Terry Speed of the Walter and Eliza Hall Institute of Medical Research, with some 50 investigators. Jenny led KanGO for the first 5 years, and I led for the last three. The 8 years funding allowed us to make huge leaps and bounds in the field of comparative genomics. We succeeded in getting the tammar genome published as the first Australian marsupial genome, largely as a result of this C of E (Renfree *et al.* 2011). We now have additional marsupial genomes – the Tasmanian devil (Murchison *et al.* 2012) and the koala (Johnston *et al.* 2018). Andrew Pask has just published the extinct Tasmanian Tiger genome (Feigin *et al.* 2018; see more below) and the dunnart genome is virtually finished (AJ Pask, MB Renfree and SR Frankenberg, unpublished results). We are currently working on updating the platypus genome and including the echidna genome, led by Guojie Zhang.

We used comparative genomic approaches to define the developmental processes common to all mammals and to identify the genes conserved among the monotreme, marsupial, and eutherian genomes. From our genome studies we now know that marsupials provide a suite of essential mammalian characteristics – controlling pregnancy, placentation, lactation and sex determination and differentiation including the *SRY* gene that is critical for testis and subsequently brain development – to help understand the mammalian genome, including the human genome. However, the monotremes continue to fascinate and confound, with their multiple chromosome (5Xs and 5 Ys: platypus and 5Xs and 4 Ys: echidna) and their lack of the male sex-determining gene *SRY*. These studies have shed light on the evolution of mammalian reproduction and development.

Extinct and threatened mammals: Tasmanian tigers and elephants

My colleague, Joan Dixon at the Museum Victoria who was curator of mammals allowed me to sample some of the extinct thylacine pouch young samples that were in the Museum. I took the tissue to Texas to Richard Behringer's laboratory, where several years later Andrew Pask cloned a regulatory sequence from the *Col2a* gene from this extinct marsupial, and Richard Behringer then made the transgenic mouse where it gave appropriate expression. This was the first example of the expression of a gene from an extinct animal into a living organism (the mouse embryo). This proof of principle study (Pask *et al.* 2008) generated great international interest since it presages a way of bringing the genes of extinct species 'back to life'.

Using another iconic mammal, we had the good fortune to obtain a series of 10 embryos and fetuses of the African elephant resulting from a cull at Kruger

Figure 4 Elephant development. Gestation of the elephant is 22 months. This fetus is 167 days old, shown with an ultrasound image of a similar aged fetus *in utero*. Crown-rump-length of the fetus was 150 mm. Based on images from Hildebrandt *et al.* (2007). With thanks to Prof. Geoff Shaw for creating the montage.

National Park. With our postgraduate student (Gaeth *et al.* 1999) (Fig. 4) we described the early development of these embryos. A surprising observation was that these fetuses had nephrostomes in the developing embryo mesonephric kidneys, confirming the palaeontological conclusions that elephants have an aquatic origin of elephants. Putting the detailed anatomy together with the amazing ultrasound images of our colleague from Berlin, Prof. Thomas Hildebrandt, we continued the study and provided the first (and still the only) accurate growth curves of any elephant during the first trimester of the 22 month pregnancy (Hildebrandt *et al.* 2007).

A prickly problem

With the help and support of Queensland's Currumbin Wildlife Sanctuary (Gold Coast) and Steve Johnston at University of Queensland and our research fellow Jane Fenelon, we have now embarked on the world's first morphological and molecular study of the complete development of the echidna embryo (from egg-laying, when the embryo is at the early somite stage to the newly hatched egg 10 days later) and the early stage pouch young, as well as a detailed study of the adult reproductive physiology and endocrinology. Currumbin have created a small breeding colony (Wallage *et al.* 2015) and have partnered with us to study these remarkable animals, a unique opportunity to learn about the reproduction and development of these egg-laying mammals, the

Figure 5 An echidna young and its egg. A newly hatched echidna pouch young and the leathery egg from which it hatched (to the same scale). The diameter of the egg is 15–16 mm – about the same size as an Australian 5 cent piece that also features the echidna!

Monotremata. This monotreme (one of the two types of egg-laying mammals) is abundant in Australia, but until very recently the echidna had rarely been bred in captivity. After 18 days of *in utero* development, the young hatches from a leathery egg with the help of an egg tooth on the upper jaw and a caruncle on their nose (Fig. 5). The stage of development at hatching, with its altricial body, well developed fore-limbs and hind limbs that are just limb paddles, is morphologically remarkably like the stage of development reached by a new-born tammar wallaby. However, as their name implies, they differ from therian mammals in having only one exterior opening, the cloaca, for all their reproductive and excretory activities.

Next steps

Future research will use many different model species as it is increasingly recognised that there is no one model that will answer questions about human reproduction. Marsupials will surely be amongst them with their delayed post-natal development that provides access to development that only occurs *in utero* in us and other eutherian mammals. We are increasingly exposed to multiple chemical and compounds in the environment. These environmental endocrine disruptors (EEDs) are a pressing problem for reproduction not only in mammals but in most other species and including fish and amphibians. Transgenerational effects of these EEDs are also an increasing concern. Infertility and errors like hypospadias are increasing in men and boys respectively. We need to change government policy on the use of compounds now known to be environmental toxins, especially in the face of additional disruption from climate change. And then there is the enigma of embryonic diapause! How exciting if we could discover the molecular signals that put that embryo to sleep, and wake it up, and maybe there will be factors that might

help us control cell growth in other areas where cell stasis will be critical, including cancer. Reproduction is a wonderful field in which to do research – as Roger always says, ‘Reproduction is the science of the transmission of life. What could be more important than that!’.

Conclusion

My studies have incorporated very many diverse biological and biomedical questions, in some cases establishing new fields of research. Providing new insights through the study of the unique evolutionary innovations in the reproductive systems of marsupials and monotremes has been challenging, but there is no doubt that study of these remarkable animals still has so much to tell us about ourselves and the world in which they evolved. All this work was a result of amazing collaborations and hard work of my colleagues, students and research fellows – without them progress would have ground to a halt and it would not have been as much fun as it has been and continues to be.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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