ISSN 0370-2731/99 Proceedings of the New Zealand Society of Animal Production 59: 239-244

Fertility control of possums: The search for regulators of gonadal development and pituitary function.

D.C. ECKERY, L.E. COLBOURNE, D.A. HEATH, S.B. LAWRENCE, S. LUN, W. NG CHIE, L. WHALE, J.L. JUENGEL, A.E. FIDLER, L.G. MOORE AND K.P. MCNATTY

AgResearch, Wallaceville Animal Research Centre, PO Box 40063, Upper Hutt, New Zealand

ABSTRACT

The Possum Research Programme of the Reproductive Biology Group at the Wallaceville Animal Research Centre is focused in four specific areas, namely: (a) identifying growth factors and hormones associated with gonadal development during the first 200 days of life; (b) searching for possum homologues of a riboflavin carrier protein (RCP) and a gut epithelial receptor/transporter protein (FcRn); (c) elucidating the role of gonadotrophin-releasing hormone (GnRH) on pituitary gonadotrophin secretions and; (d) producing and testing reagents which might interfere with gonadal formation and function of the pituitary gland. Possum specific reagents and *in vitro* test systems have been developed and will be used to develop strategies for fertility control.

Keywords: possum; development; growth factors; pituitary; fertility control.

INTRODUCTION

The purpose of this review is to summarise recent research findings and to highlight future directions in the development of strategies to control reproductive function in possums. The Possum Research Programme of the Reproductive Biology Group at the Wallaceville Animal Research Centre is focused in four specific areas, namely: (a) identifying growth factors and hormones associated with gonadal development during the first 200 days of life; (b) searching for possum homologues of a riboflavin carrier protein (RCP) and a gut epithelial receptor/transporter (FcRn); (c) elucidating the role of gonadotrophin-releasing hormone (GnRH) on pituitary gonadotrophin secretions and; (d) producing and testing reagents which might interfere with gonadal formation or function of the pituitary gland.

Gonadal development during the first 200 days of life

Events relating to germ cell maturation and follicular development were determined during the first 200 days of life post-partum. Data were collected from 69 female pouch young/joeys ranging in age from 8 to 200 days. These data are summarised in Figure 1 with respect to the number of germ cells, the proportion of oogonia, oocytes (germ cells in prophase I of meiosis), primordial follicles and growing follicles and the proportion of mitotic and atretic germ cells. These results define more precisely the timing of key events referred to in previous publications (Eckery *et al*., 1996; Frankenberg *et al*., 1996; Shackell *et al*., 1996; Ullmann, 1996). During the first 19 days of life, all germ cells were present as primordial germ cells or oogonia with ~12% undergoing mitosis and ~2% undergoing atresia. The first oogonia to enter meiosis (oocytes) were observed around day 35; thereafter oogonia either enter meiosis and develop into primordial follicles (i.e. around day 50) or undergo atresia. Granulosa cells, which surround the oocytes to form primordial follicles, appear to originate from cord-like structures in the medulla (Figure 2) referred to as medullary cords (Ullmann, 1996). The highest total numbers of germ cells were observed between days 60-99 of pouch life, however

FIGURE 1: Germ cells in female brushtail possums (n=69) during the first 200 days of life. (A) Total number of germ cells (mean ± sem) and number normalised to ovarian volume (mm^3). (B) Proportions of oogonia, oocytes, primordial follicles and primary follicles. (C) Proportions of meiotic and atretic germ cells (mean ± sem). (Modified from Eckery *et al*., 1998b with permission.)

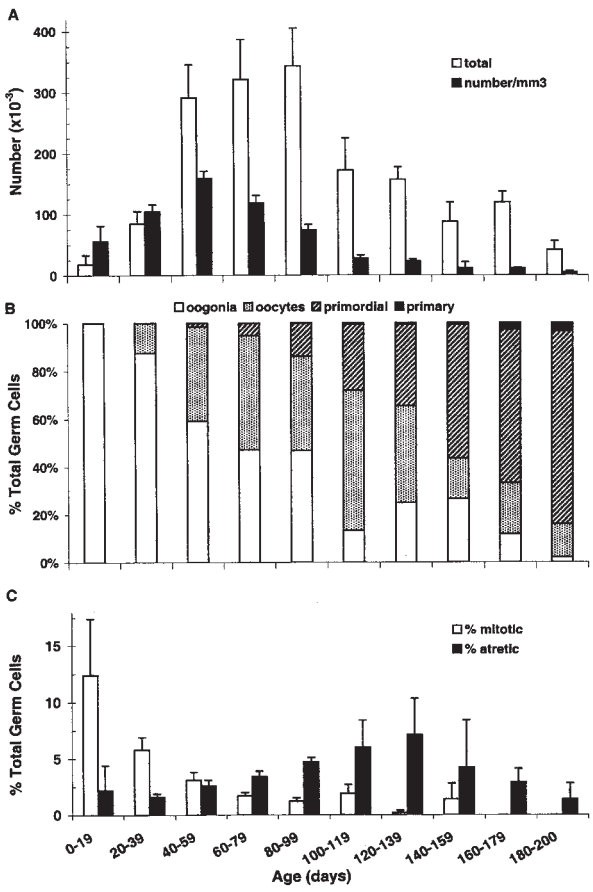
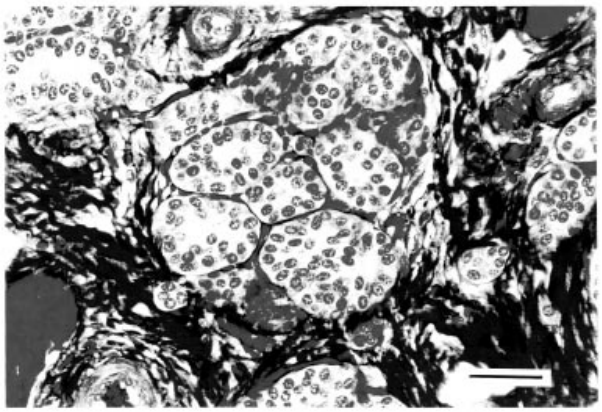


FIGURE 2: Medullary cords located in the ovarian medulla of a day 140 pouch young. Scale bar, 50μm.



when normalised to ovarian volume the maximum was reached between days 40-59. From day 99 until day 199 more than 80% of germ cells disappeared from the ovary without developing into primordial follicles. This massive loss of germ cells can be attributed to the high incidence of atresia (i.e. oogonia, oocytes). Evidence of follicular growth was first noted around day 65 when primary follicles were observed in the innermost regions of the ovarian cortex. Subsequently secondary and tertiary (antral) follicles were noted around day 95 and day 160 respectively.

Collectively these results suggest that reagents used to disrupt follicular formation and/or early follicular growth would need to access the ovaries of pouch young animals before day 100 of pouch life.

Growth factors and ovarian development

There is growing evidence for the importance of local growth factors and steroids during both gonadal development and early follicular growth (for reviews see Byskov and Hoyer, 1994; Greenwald and Roy, 1994). Several growth factors known to be associated with these processes in eutherians have been identified in possums. Studies are underway to determine if the function of these factors can be disrupted during pouch life such that pouch young are made permanently infertile.

C-kit and stem cell factor (SCF)

In eutherians, the tyrosine kinase receptor, c-kit and its ligand SCF are involved in the differentiation of primordial germ cells and both oocyte and follicular growth (Colombre & Russell, 1954; Kuroda *et al*., 1988; Besmer *et al*., 1993; Clark *et al*., 1996). The roles of these growth factors in possums are unknown. We have amplified, cloned and sequenced an 822 base pair (bp) cDNA of SCF which corresponds to the entire coding region (Greenwood *et al*., 1996; Table 1). This sequence has ~75% and ~66% identity to SCF of eutherian mammals at the nucleotide and predicted amino acid levels respectively. Likewise we have cloned a 282 bp cDNA of c-kit from possum ovarian poly $(A)^+$ RNA. (A. Fidler, unpublished data; Table 1). The 282 bp sequence of c-kit corresponds to a portion of the extracellular domain, and has ~80% and ~64% identity to c-kit of eutherian mammals at the nucleotide and predicted amino acid levels respectively. A 618 bp portion of the above

TABLE 1: Reagents developed and/or validated for research in possum reproduction.

Reagent	w/v [a]	Purpose [b]
possum (p) stem cell factor (SCF) cDNA	w	GE
recombinant possum (rp) SCF	w	ICC, S
rabbit anti-rp SCF	w	ICC
p c-kit cDNA	w	GE
rp c-kit	w	ICC, S
rabbit anti-rp c-kit	w	ICC
rabbit anti-human (aa 955-976) c-kit	v	ICC
p β_A-activin/inhibin subunit cDNA	w	GE
p β_B-activin/inhibin subunit cDNA	w	GE †
p α-inhibin subunit cDNA	w	GE
p Oct 3/4 cDNA	w	GE †
p growth differentiation factor-9 cDNA	w	GE
ro follistatin	w	ICC, S
rabbit anti-ro follistatin	w	ICC
mouse anti-human (aa 88-114) b_A -activin/inhibin subunit	v	ICC
mouse anti-human (aa 1-32) α-inhibin subunit	v	ICC
rabbit anti-ovine StAR	v	ICC
rabbit anti-human 3β-hydroxysteroid dehydrogenase	v	ICC
p FcRn cDNA	v	GE
p follicle-stimulating hormone (FSH)	w	RIA, B, RA, S
p luteinising hormone (LH)	w	RIA, B, RA, S
rabbit anti-human FSH	v	RIA
rabbit anti-ovine LH	v	RIA
p α-gonadotrophin subunit cDNA	w	GE
p FSHβ subunit cDNA	w	GE
p LHβ subunit cDNA	w	GE
p gonadotrophin-releasing hormone receptor cDNA	w	GE
p LH receptor cDNA	W	GE

[a] - w = developed at Wallaceville Animal Research Centre; v = validated at Wallaceville

[b] - RIA = radioimmunoassay; B = bioassay; RA = receptor assay; ICC = immunocytochemistry; S = standard; GE = gene expression

† = in collaboration with S. Frankenberg and Dr. L. Selwood (La Trobe University, Victoria, Australia)

SCF cDNA encoding the entire extracellular domain and a portion of the transmembrane domain, and the aforementioned 282 bp sequence of c-kit have been expressed in *E. coli*. The resulting recombinant proteins have been used to generate antisera for both immunohistochemical and *in vivo* studies. Using these antisera as immunohistochemical probes, both SCF and c-kit have been localised to possum germ cells and granulosa cells (Figure 3) suggesting as with eutherians, these growth factors are important for germ cell maturation, follicular formation and growth. In addition, i*n situ* hybridisation studies using the c-kit cDNA have shown that gene expression for c-kit is specific to germ cells and not found in any other ovarian cell types (e.g. granulosa, theca and interstitial cells).

To determine whether these growth factors affect gonadal development, the next step in these studies will be to determine whether the antisera to SCF or c-kit are capable of neutralising the bioactivity of the native reagents. Antisera are currently being screened using a cell-line which responds to the addition of SCF and undergoes cell division. Those antisera which are effective in the bioassay will be used to test whether germ cell maturation and/or follicular formation and growth can be disrupted *in vivo*. For these studies, antisera will be administered to pouch

FIGURE 3: Photomicrographs showing localisation of (A) stem cell factor to germ cells (g) in the ovary of a day 99 pouch young and (B) c-kit to germ cells (g), granulosa cells (gc) and surface epithelium (se) in the ovary of an adult possum. Scale bars, (A) 50 μm; (B) 100 μm. (From Eckery *et al*., 1998b with permission.)

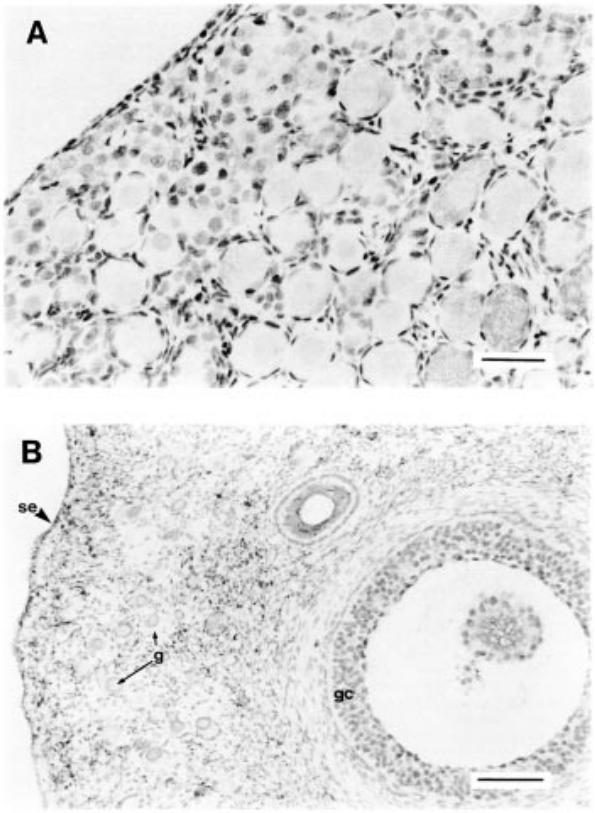
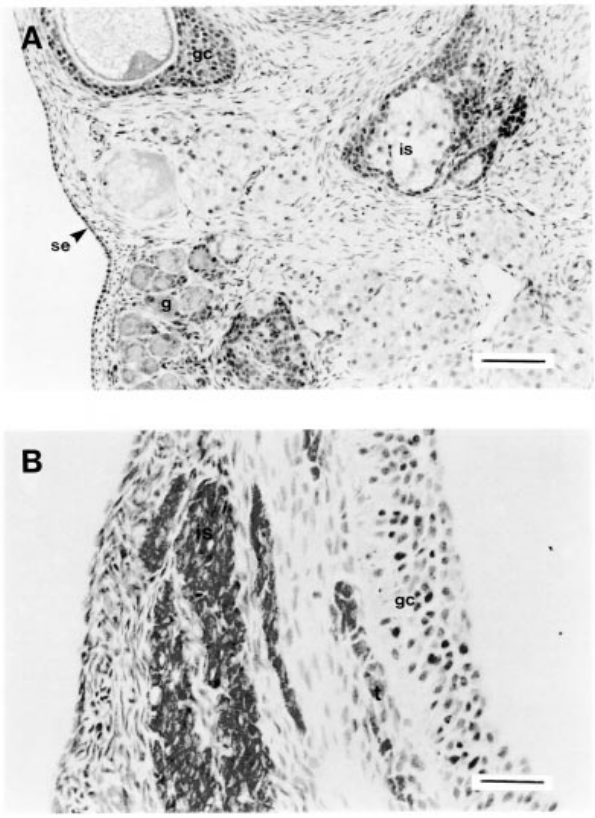


FIGURE 4: Photomicrographs showing localisation of (A) StAR to germ cells (g), granulosa cells (gc), interstitial cells (is) and surface epithelium in the ovary of an adult possum and (B) 3β-hydroxysteroid dehydrogenase to granulosa cells (gc), theca (t) and interstitial cells (is) in the ovary of a juvenile (backrider) possum. Scale bars, (A) 50 μm; (B) 100 μm. (From Eckery *et al*., 1998b with permission.)



young possums over varying intervals from day 3 until day 50 of life and the gonads recovered to determine germ cell numbers using stereology (Gundersen, 1988; Smith *et al*., 1997) and the degree of ovarian development.

Activins, inhibins, follistatin, transcription factors and steroidogenic reagents

In the sheep gonad, gene expression for steroidogenic factor-I occurs from the time of gonadal formation and the genes for steroid acute regulatory protein (StAR) and the cytochrome P450 enzymes for cholesterol side chain cleavage, 17α-hydroxylase and aromatase are expressed around the time of morphological sexual differentiation (McNatty *et al*., 1998). Also in sheep, the genes for β_B-activin/inhibin, follistatin and α-inhibin are expressed early in follicular growth, namely during primary and secondary development (Braw-Tal *et al*., 1994; Tisdall *et al*., 1994; Tisdall *et al*., 1995; McNattty *et al*., 1999). Similarly in sheep the peptides for follistatin, β-activin/inhibin, α-inhibin and β_A-activin/inhibin have been localised to either oocytes, zona pellucida, granulosa cells and/or the rete ovarii (McNatty *et al*., 1999). Given the potential importance of these factors in ovarian development and function, we are in the process of isolating and cloning these genes from possums for *in situ* hybridisation studies. Peptides and antisera for these factors are also being validated for immunohistochemical studies. A summary of those reagents which have been developed and/or validated are summarised in Table 1. Preliminary results have shown that the cells expressing these genes and the cellular localisations of these peptides are similar in most instances to those described in eutherians. For example, both StAR and 3β-hydroxysteroid dehydrogenase are localised to steroidogenic cell types in the possum ovary (Figure 4), namely granulosa, theca and/or interstitial cells. StAR was also localised to germ cells but the significance of this remains obscure at present. We are now examining the temporal expression of these genes and peptides during gonadal development and follicular growth in possums.

Novel proteins

Riboflavin-carrier protein (RCP)

RCP has been demonstrated in some species to be a pregnancy specific protein which enables riboflavin to be transferred from mother to fetus (Dancis, 1962; Clarke, 1977). Riboflavin is a water soluble vitamin and like thiamine and ascorbic acid is selectively transported and concentrated by mammalian fetuses. Evolutionarily, RCP is highly conserved and its presence has been demonstrated in a variety of species including chickens, rats, mice, guinea pigs, cows, sheep, monkeys and humans (Adiga & Murty, 1983). RCP is a phosphoglycoprotein of maternal origin and essential for the maintenance of pregnancy (Natraj *et al*., 1987). Immunoneutralisation of endogenous RCP results in rapid resorption of the placenta and/or fetal wastage and is being investigated as a possible fertility control

method for several mammalian species (Adiga *et al*., 1997). Currently we are collecting plasma from pregnant and lactating possums to search for possum homologues of RCP using affinity chromatography and a radiolabelled riboflavin probe. If present in possums, we intend to test whether neutralisation of endogenous RCP leads to the death of fetuses or pouch young.

Gut IgG receptor/transporter (FcRn)

During the first three weeks after birth, suckling rats and mice selectively absorb maternal IgG across the gut epithelium and thereby acquire passive immunity (Simister & Mostov, 1989). This absorption process is mediated by a specific receptor, FcRn, which binds the constant (Fc) region of IgG molecules. Binding is pH dependent being optimal in the gut lumen (pH of 6.0-6.5) and dramatically reduced in serosal fluid (pH of 7.4). Given the extensive period during which marsupial pouch young absorb maternal immunoglobulin from milk we postulated that possums have a homologous IgG transport system.

A cDNA for possum FcRn has now been cloned (Dr. F. Adamski, AgResearch, Ruakura Research Centre, Hamilton, New Zealand; pers. comm.) and in situ hybridisation studies are being performed on pouch young possum tissues to determine which cells produce FcRn and the ages when it is produced. Future studies in our lab will investigate the feasibility of recombinant variants of FcRn as biocontrol agents.

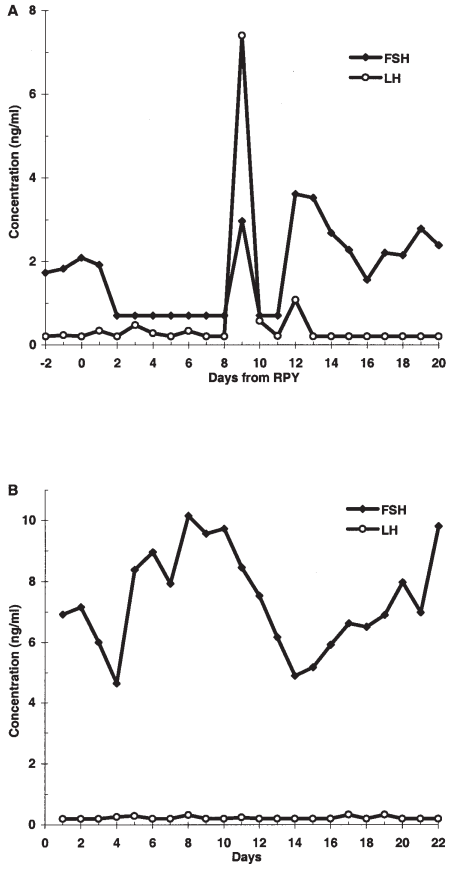
Pituitary gonadotrophins

In eutherians, the central role of the gonadotrophins, FSH and LH, in gonadal function has been well established. The secretion of gonadotrophins is primarily regulated by the hypothalamic hormone GnRH. Unlike in most mammalian species a second type of GnRH, namely chicken type II GnRH, has been found in possums. The function of this second type of GnRH is not known and the relationship between GnRH and gonadotrophin synthesis/secretion in possums is poorly understood. Our research is focused towards elucidating the role of GnRH in possum reproductive endocrinology and devising ways to disrupt pituitary function in this species.

Development of radioimmunoassays

We have reported the purification of both possum FSH and LH and the development of sensitive radioimmunoassays (RIA) for both these hormones (Moore *et al*., 1997a; 1997b; Table 1). When assessed by receptor and bioassay, the purified possum FSH had ~21% of the potency of ovine FSH (USDA-oFSH-19-SIAFP-RP2). The RIA was developed using ^{125}I-possum FSH, possum FSH standard and an antiserum raised against human FSH (generously supplied by Dr A. McNeilly, University of Edinburgh, UK). The FSH RIA has a sensitivity of 0.3 ng/ml, a 50% displacement of 2.7 ng/ml and a cross-reactivity of 0.05% against possum LH. The purified possum LH had ~20% of the potency of ovine LH (NIDDK-oLH-26) in a receptor assay using possum testicular receptors. The possum LH was also able to stimulate cyclic 3',5'-adenosine monophosphate when added to bovine granulosa cells *in vitro*. The RIA for possum LH was developed using ^{125}I-possum LH, possum LH standard and an antiserum raised against ovine LH (McNatty *et al*., 1987). The LH RIA has a sensitivity of 0.15 ng/ml, a 50% displacement of 1.9 ng/ml and a cross-reactivity of <0.02% against possum FSH.

FIGURE 5: Plasma gonadotrophin concentrations in possums. (A) Profile of a female bled daily following removal of pouch young (RPY). (B) Profile of a male bled daily during the breeding season (May).



Gonadotrophin concentrations

We have determined gonadotrophin profiles in both female and male possums during different reproductive states (Eckery, *et al*., 1998a). In females bled daily following removal of pouch young (Figure 5), levels of FSH were generally elevated until the week before ovulation when they fell below the sensitivity of the assay. A surge of FSH occurred the day before ovulation and in the 1-2 days following ovulation FSH levels returned to elevated levels. LH remained at basal levels (at or below the sensitivity of the assay) before and after ovulation except when a preovulatory surge of LH occurred the day before ovulation concomitant with the FSH surge. Frequent sampling from females revealed that LH secretion was pulsatile whereas FSH secretion was not.

In male possums bled daily (Figure 5), FSH levels remained elevated and were much higher than in females. Interestingly in most males the pattern of FSH secretion was episodic with a period of 9-10 days. LH remained at basal levels with only occasional small increases in concentration. As in females, LH secretion was found to be pulsatile whereas FSH secretion was not.

Regulation of gonadotrophin secretion

To test whether the gonadotrophin concentrations in plasma of possums were influenced by GnRH, both male and female possums were injected with either mammalian GnRH or chicken type II GnRH and the gonadotrophin responses monitored by RIA. Both forms of GnRH were found to be capable of stimulating gonadotrophin release in possums; whereas chronic exposure of possums to a potent GnRH agonist (Deslorelin; Peptech, NSW, Australia) caused suppression of both FSH and LH. Collectively these data provide evidence that gonadotrophin secretion is, at least in part, regulated by GnRH. Methods for culturing pituitary cells *in vitro* have been established and will be used to further evaluate the effects of GnRH analogues on pituitary function.

To test whether gonadal factors influence gonadotrophins in possums both male and female animals were gonadectomised and the consequences monitored by RIA. Plasma concentrations of gonadotrophins increased in both sexes following gonadectomy indicating that as with other mammals the gonads of possums produce hormones that suppress the release of both gonadotrophins.

Sites of gonadotrophin synthesis and GnRH action

To test whether the pituitary is the major if not only site of GnRH action and gonadotrophin synthesis the genes for GnRH-receptor (~600 bp), α-gonadotrophin, β-FSH and β-LH have been cloned and sequenced (Table 1). The nucleotide sequences of the coding regions for GnRH-receptor, α-gonadotrophin, β-FSH and β-LH subunits demonstrate ~83%, ~86%, ~77% and 71% identity with the ovine genes respectively. Moreover as with the ovine genes two N-linked glycosylation sites are present on each gonadotrophin subunit. From *in situ* hybridisation and northern blotting studies, the pituitary but not the brain has been shown to be the site of gonadotrophin subunit gene expression.

In another study to determine the sites of GnRH action, cell membranes and RNA were prepared from various tissues from adult male and female possums (Eckery, *et al*., 1998c). A single class of high affinity receptors for GnRH (Kd, 0.63nM; Bmax, 51fM/mg protein) was identified. Moreover, GnRH receptor gene expression and ^{125}I-GnRH binding were found only in the pituitary. Thus, binding of GnRH is exclusive to the pituitary gland in possums.

GnRH-toxins

In mammals, abolition of LH and FSH synthesis leads to hypogonadism and sterility. Recent studies have demonstrated that GnRH conjugated to plant toxins specifically inhibited gonadotrophin secretion and caused sterility in rodents; whereas the toxins without conjugation to GnRH were ineffective (Dr. T. Nett, Colorado State University, USA, pers. comm.). Although not proven, it is thought that once GnRH-toxin conjugate binds to a GnRH receptor, the receptor-GnRH-toxin complex is internalised thus allowing intracellular access to the toxin which then acts to inhibit protein synthesis and cause cell death. In collaboration with Dr. T. Nett, we will test the efficacy of certain GnRH-toxin conjugates to sterilise possums. The effects of the GnRH-toxin conjugates will be monitored from measurements of FSH and LH in plasma following GnRH challenge and both GnRH-receptor and gonadotrophin gene expression by *in situ* hybridisation.

SUMMARY

In possums we have shown that the growth factors SCF and c-kit are associated with the ovary and specifically with germ cell development and follicular formation and growth. Antibodies to these factors have been produced and will be tested for their ability to disrupt gonadal development in pouch young animals. A search is underway for a possum homologue of an important vitamin transporter protein, namely RCP. A second transporter protein, FcRn, has now been identified in possums and variants of this protein will be tested as biocontrol agents. We have shown that administration of GnRH stimulates gonadotrophin secretion and that chronic exposure to a potent GnRH agonist inhibits gonadotrophin secretion in possums. Moreover, our studies suggest the pituitary gland as the exclusive site of GnRH binding and gonadotrophin synthesis. Toxins which inhibit protein synthesis will be chemically conjugated to GnRH in an attempt to direct the toxin to pituitary gonadotrophes to inhibit the synthesis of FSH and LH and thus induce sterility.

ACKNOWLEDGEMENTS

We thank the National Hormone and Pituitary Program, Maryland, USA for the provision of hormone standards; Dr Nigel Groome, Oxford Brookes University, Oxford, UK for the supply of monoclonal antisera to α-inhibin and β_A-activin/inhibin subunit; Professor Alan McNeilly, MRC Unit of Reproductive Biology, Edinburgh, UK for the supply of antisera to FSH; Dr Doug Stocco, Texas Tech University, Texas, USA for the reagents for StAR; Professor Iain Mason, University of Edinburgh, Edinburgh, UK for the antisera to 3β-hydroxysteroid dehydrogenase and Dr. Fran Adamski, AgResearch, Ruakura Research Centre, Hamilton, New Zealand for FcRn cDNA. Financial support for this programme was provided by the Foundation for Research, Science and Technology and Ministry of Agriculture and Fisheries Policy Division.

REFERENCES

Adiga, P.R.; Murty, C.V.R. 1983. Vitamin carrier proteins during embryonic development in birds and mammals. *CIBA Foundation Symposium* **98:** 111-136.

Adiga, P.R.; Subramanian, S.; Rao, J.; Kumar, M. 1997. Prospects of riboflavin carrier protein (RCP) as an antifertility vaccine in male and female mammals. *Human Reproduction Update* **3:** 325-334.

Besmer, P.; Manova, K.; Duttlinger, R.; Huang, E.J.; Packer, A.; Gyssler, C.; Bachvarova, R.F. 1993. The kit ligand (steel factor) and its receptor c-kit/w: pleiotropic roles in gametogenesis and melanogenesis. *Development* (Suppl.): 125-137.

Braw-Tal, R.; Tisdall, D.J.; Hudson, N.L.; Smith, P.; McNatty, K.P. 1994. Follistatin but not α or β_A inhibin subunit mRNA is expressed in ovine fetal ovaries in late gestation. *Journal of Molecular Endocrinology* **13:** 1-9.

Byskov, A.G.; Hoyer, P.E. 1994. Embryology of mammalian gonads and ducts. *In*: The Physiology of Reproduction, Second Edition. Ed. E. Knobil and J.D. Neill. New York, Raven Press Ltd. pp. 487-540.

Clark, D.E.; Tisdall, D.J.; Fidler, A.E.; McNatty, K.P. 1996. Localisation of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries. *Journal of Reproduction and Fertility* **106:** 329-335.

Clarke, H.C. 1977. Distribution of riboflavin in blood: in women and in prenates. *International Journal of Vitamins and Nutrition Research* **47:** 361-363.

Colombre, J.L.; Russell, E.S. 1954. Analysis of the pleiotropism at the W-locus in the mouse: the effects of W and W^o substitution upon postnatal development of germ cells. *Journal of Experimental Zoology* **126:** 277-296.

Dancis, J. 1962. The placenta in fetal nutrition and excretion. *American Journal of Obstetrics & Gynecology* **84:** 1749-1755.

Eckery, D.C.; Colbourne, L.; Lun, S.; McNatty, K.P. 1998a. Pulsatile secretion of gonadotrophins in brushtail possums (Trichosurus vulpecula). *Proceedings of the New Zealand Society of Endocrinology*: NZ3.

Eckery, D.; Lawrence, S.; Greenwood, P.; Stent, V.; Ng Chie, W.; Heath, D.; Lun, S.; Vanmontfort, D.; Fidler, A.; Tisdall, D.; Moore, L.; McNatty, K.P. 1998b. The isolation of genes, novel proteins, and hormones and the regulation of gonadal development and pituitary function in possums. *In*: Biological control of possums. *The Royal Society of New Zealand Miscellaneous Series* **45:** 100-110.

Eckery, D.C.; Lawrence, S.; Lun, S.; McNatty, K.P. 1998c. Characterisation of receptors for gonadotrophin-releasing hormone (GnRH) in brushtail possums (Trichosurus vulpecula). *Proceedings of the New Zealand Society of Endocrinology*: NZ14.

Eckery, D.C.; Tisdall, D.J.; Heath, D.A.; McNatty, K.P. 1996. Morphology and function of the ovary during fetal and early neonatal life: a comparison between sheep and brushtail possum (*Trichosurus vulpecula*). *Animal Reproduction Science* **42:** 551-561.

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

Greenwald, G.S.; Roy, S.K. 1994. Follicular development and its control. *In*: The Physiology of Reproduction, Second Edition. Ed. E. Knobil and J.D. Neill. New York, Raven Press Ltd. pp. 629-724.

Greenwood, P.J.; Seamer, C.; Tisdall, D.J. 1996. Cloning, sequencing and expression of stem cell factor (c-kit ligand) cDNA of Brushtail Possum (*Trichosurus vulpecula*). *Reproduction, Fertility and Development* **8:** 789-795.

Gundersen, H.J.G. 1988. The new stereological tools. *ARMIS* **96:** 857-881.

Kuroda, H.; Terada, N.; Nakayama, H.; Matsumoto, K.; Kitamura, Y. 1988. Infertility due to growth arrest of ovarian follicles in *sl/sl*^{l} mice. *Developmental Biology* **126:** 71-79.

McNatty, K.P.; Heath, D.A.; Lundy, T.; Fidler, A.E., Quirke, L.D.; Stent, V.C.; O'Connell, A.; Smith, P.R.; Groome, N.; Tisdall, D.J. 1999. Control of early ovarian follicular development. *Journal of Reproduction and Fertility (Supplement)*: in press.

McNatty, K.P.; Hudson, N.L.; Henderson, K.M.; Gibb, M.; Morrison, L.; Ball, K.; Smith, P. 1987. Differences in gonadotrophin concentrations and pituitary responsiveness to GnRH between Booroola ewes which were homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing their ovulation rate. *Journal of Reproduction and Fertility* **80:** 577-588.

McNatty, K.P.; Quirke, L.; Lun, S.; Smith, P.; Lundy, T.; Tisdall, D.J. 1998. Ontogeny of steroidogenesis in the fetal sheep ovary around sexual differentiation. *Biology of Reproduction* **58**(suppl. 1)**:** A450.

Moore, L.G.; Ng Chie, W.; Lun, S.; Lawrence, S.B.; Young, W.; McNatty, K.P. 1997a. Follicle stimulating hormone in the brushtail possum (*Trichosurus vulpecula*): purification, characterisation and radioimmunoassay. *General and Comparative Endocrinology 106*: 30-38.

Moore, L.G.; Ng Chie, W.; Lun, S.; Lawrence, S.B.; Heath, D.A.; McNatty, K.P. 1997b. Isolation, characterisation and radioimmunoassay of luteinizing hormone in the brushtail possum. *Reproduction, Fertility and Development* **9:** 419-425.

Natraj, U.; Kumar, A.R.; Kadam, P. 1987. Termination of pregnancy with antiserum to chicken riboflavin carrier protein. *Biology of Reproduction* **36:** 677-685.

Shackell, G.H.; Norman, N.G.; McLeod, B.J.; Hurst, P.R. 1996. A morphometric study of early ovarian development in pouch young of the brushtail possum (*Trichosurus vulpecula*). *The Anatomical Record* **246:** 224-230.

Simister, N.E.; Mostov, K.E. 1989. An Fc receptor structurally related to MHC class I antigens. *Nature* **337:** 184-187.

Smith, P.; Hudson, N.L.; Corrigan, K.A.; Shaw, L.; Smith, T.; Phillips, D.J.; McNatty, K.P. 1997. Effects of the Booroola gene ($FecB^B$) on body mass, testis development and hormone concentrations during fetal life. *Journal of Reproduction and Fertility* **108:** 253-261.

Tisdall, D.J.; Hudson, N.L.; Smith, P.; McNatty, K.P. 1994. Localization of ovine follistatin and α and β_A inhibin subunit mRNA in the sheep ovary during the oestrous cycle. *Journal of Molecular Endocrinology* **12:** 181-193.

Tisdall, D.J.; Smith, P.; Leeuwenberg, B.; McNatty, K.P. 1995. FSH-receptor, β_B inhibin subunit, follistatin, β_A and α inhibin subunits and IGF-1 genes are expressed sequentially in ovine granulosa cells during early follicular development. *Proceedings of the UK Society for the Study of Fertility*, Dublin: A28.

Ullmann, S.L. 1996. Development of the ovary in the brushtail possum *Trichosurus vulpecula* (Marsupialia). *Journal of Anatomy* **189:** 651-665.