SEASONAL CHANGES IN PITUITARY AND PLASMA PROLACTIN CONCENTRATIONS, AND THE ROLE OF PROLACTIN IN THE CONTROL OF DELAYED IMPLANTATION IN FEMALE *Miniopterus schreibersii*

THESIS

Submitted in fulfilment of the

requirements for the Degree of

DOCTOR OF PHILOSOPHY

of Rhodes University

by

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DECEMBER 1993

ADDENDUM

The following paragraphs are added to or changed in the text as follows:

1. Chapter 2, page 19, line 12: Add 'All plasma samples were run in one assay.'

2. Chapter 2, page 19, line 18: Instead of 'Plasma prolactin concentrations measured ranged from 57 to 206 ng/ml', it should read 'The assay measured high levels of prolactin in lactating bats, and low levels in bats treated with bromocryptine, a chemical which inhibits prolactin secretion in most mammals. The results therefore strongly suggest that the assay was measuring prolactin, and since the data are interpreted qualitatively, they represent an acceptable form of validation.'

3. Chapter 3, page 29, line 7: The text should read 'three to six pituitaries' instead of 'three pituitaries.'

4. Chapter 3, page 29, line 18: Instead of 'immunoreactivity of the tissue', the text should read 'immunoreactivity of the tissue, embedded in Araldite/Agar 100 resin.'

5. Chapter 3, page 35, line 5: Instead of 'n was always $\geq$ 6)', it should read ' n = 3 - 6).'

6. Chapter 3, page 37, line 11: Instead of 'The secretory granules were smaller in size (140 - 490 nm) than those present at other times of the year', the text should read 'The secretory granules were smaller (140 - 490 nm), approximately half the size of those present at other times of the year, and even if granule size is controlled for, the maximum level of immunoreactivity (4.4 probes/granule) is lower than that recorded during pregnancy and lactation.'

7. Chapter 4, page 49: Change first paragraph to read: 'It was not possible to determine whether the adult females had mated at the time of collection and seven were found to be not pregnant at the end of the experiments. Furthermore a number of bats died during the experiments reducing the final sample size (Table 1).'

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ACKNOWLEDGEMENTS

I am very grateful to my supervisor, Professor Ric Bernard for all his help and encouragement throughout this project.

I would like to thank Dr. Dave Gray for helping me with the prolactin radioimmunoassay, and thanks to Professor Bernard and Dr. Gray for doing the progesterone assay.

Thank you to Dr. Gareth Griffiths, for helping me with the immunogold double-labelling, and for donating the protein A gold probes and the fetal calf serum.

To Rob Cross, Neil Cannon and Shirley Pinchuck, thank you for your assistance with the electron microscope and photography work.

A very big thanks to Dr. Dave Gray, Johnny Rischbiter and Ute Simon for proof-reading the manuscript, and to all my friends for their support. To Andrew Murdoch, thanks for printing my graphs.

A special thanks to my parents for all their encouragements and support.

I am grateful for the funding supplied by the Foundation of Research and Development, and Rhodes University.

DEDICATION

This is dedicated to my Mum and Dad,

who made it possible for me to study,

and who always believed in me.

ABSTRACT

Mammotropes were successfully identified in the anterior pituitary gland of *Miniopterus schreibersii* using immunocytochemical (ICC) staining at the light and electron microscopy level. Mammotropes were distributed throughout the gland, were polygonal in shape and during secretory activity contained numerous large secretory granules (350 - 800 nm). Using double ICC labelling, prolactin and growth hormone were never co-localiszed and found in individual cells only. Plasma prolactin levels were successfully measured on a monthly basis using radioimmunoassay and monthly pituitary prolactin levels were quantified using morphometric analysis of immunogold ICC staining and densitometry with polyacrylamide gels.

Seasonal changes in the ultrastructure of mammotropes, and pituitary and plasma prolactin concentrations in female *Miniopterus schreibersii* indicated that there was an increase in prolactin secretion during the second half of the period of delayed implantation and that prolactin secretion remained elevated during normal embryonic development and lactation. This suggests that prolactin may be part of the luteotropic and lactogenic complex, and that the hormone might be responsible for terminating the period of delayed implantation. The latter is supported by experiments, where exogenous prolactin initiated precocious implantation during early delayed implantation, and treatment with

bromocryptine (which inhibits prolactin synthesis) retarded implantation.

Activation of mammotropes to synthesise prolactin and an increase of plasma prolactin levels occurred shortly after the winter solstice (21 June), suggesting that increasing daylength may be the environmental cue, which terminates the period of delayed implantation in *Miniopterus schreibersii*.

CHAPTER 1

GENERAL INTRODUCTION

Bats are one of the largest, most widely distributed groups of mammals (Hill & Smith, 1984), and show a wide variety of reproductive patterns, which are linked to their environment. Some tropical bats show aseasonal reproduction and breeding is polyoestrous, due to reduced seasonal fluctuations in climate and food availability (Myers, 1977). However in temperate climates, reproduction is strictly seasonal and monoestrous (Racey, 1982), with parturition usually coinciding with optimal temperatures and food abundance. In hibernating species reproduction is further restricted to the summer months and this period may be too short to accommodate a complete reproductive cycle (Bernard, 1989). To overcome this problem, many hibernating bats make use of reproductive delayed phenomena. Delayed fertilization is common in most species of Vespertilionid bats from temperate latitudes (Wilson, 1979), and delayed development has been described for *Hipposideros caffer* (Bernard & Meester, 1982), *Macrotus californicus* (Bradshaw, 1962; Bleier, 1975; Burns & Easley, 1977; Richardson, 1979), *Artibeus jamaicensis* (Flemming, 1971) and *Haplonycteris fischeri* (Heideman, 1989). Obligate delayed implantation, which

occurs widely in mammals (bear: Wimsatt, 1963; roe deer: Aitken, 1974 and Sempéré, 1977; mink: Papke *et al.*, 1980; European badger: Canivenc & Bonnin, 1981; ferret: Agu *et al.*, 1986; spotted skunk: Berria *et al.*, 1989; seal: Temte, 1985 and Boyd, 1991), has been reported in the following bat species: *Natalus stramineus* (Mitchell, 1965, in Burns, 1981), *Eidolon helvum* (Mutere, 1965), *Rhinolophus rouxi* (Rammakrishna & Rao, 1977) and *Miniopterus* species (Dwyer, 1963; Wallace, 1978; Richardson, 1979; Bernard, 1980).

The bat species used in the present study, Schreibers' long-fingered bat *(Miniopterus schreibersii),* makes use of delayed implantation during winter, at which time they may undergo short periods of torpor. Follicular development starts at the end of summer and ends just prior to winter, when copulation, ovulation and fertilization take place. Implantation is delayed for about four months and only takes place at the end of winter. This is followed by a period of normal embryonic development (about four months) and the young are born during summer. A common characteristic in most female mammals that make use of delayed implantation is that during the delay the corpus luteum is secretorily inactive and plasma progesterone levels are low. This is also the case in the long-fingered bat (Peyre & Herlant, 1963; Kimura *et al.*, 1987; Crichton *et al.*, 1989; Bernard *et al.*, 1991).

The role of the pituitary gland in mammalian reproduction has long been established (Karsch, 1984). In particular three hormones (prolactin, follicle stimulating and luteinizing hormones), secreted by mammotropes and two types of gonadotropes in the anterior pituitary, are important in regulating reproductive cycles (Tougard & Tixier-Vidal, 1988).

Prolactin is a biologically important hormone, as it has numerous physiological functions. In fish prolactin plays a role in conserving electrolytes, fat gain or loss, locomotor activity, inhibition of metamorphosis, and it has an antigonadal effect (Spieler

et al., 1976; Hall & Chadwick, 1978). In amphibians (Ishii *et al.*, 1989; Matsuda *et al.*, 1990b) and birds (Arnason & Skadhauge, 1991; Seiler *et al.*, 1992) the hormone is important in osmoregulation and reproduction. In reptiles it is important in growth, metamorphosis, osmoregulation and reproduction (Thompson & Trimble III, 1975; Pearson & Licht, 1987). Prolactin in mammals plays a role in the stimulation of developmental and lactational processes in the mammary gland (Rillema *et al.*, 1988). Furthermore in some mammalian species prolactin is luteotropic (Niswender & Nett, 1988; Okkens *et al.*, 1990; Taverne *et al.*, 1990) and in mustelids the hormone is required to terminate the period of delayed implantation (Papke *et al*, 1980; Canivenc & Bonnin, 1981; Berria *et al.*, 1989).

In view of the small amount of work done on the hormonal control of reproduction in bats, in particular on the control of delayed implantation, and considering the established importance of prolactin, the aims of this project were:

1. to identify prolactin secreting cells and to monitor their seasonal changes in abundance, ultrastructure and prolactin concentrations;
2. to monitor seasonal changes in plasma prolactin concentrations;
3. to relate these seasonal changes to the reproductive cycle of delayed implantation; and
4. using experimental hormone manipulations to establish the role of prolactin in terminating delayed implantation in female *Miniopterus schreibersii*.

CHAPTER 2

IDENTIFICATION OF MAMMOTROPES AND DETERMINATION OF PITUITARY AND PLASMA PROLACTIN CONCENTRATIONS

INTRODUCTION:

Numerous studies have been done on the structure of the anterior pituitary gland (adenohypophysis) in various animals, in particular on the location and abundance of the various pituitary cell types (mouse: Barnes, 1962 and Sano, 1962; rabbit & cat: Dawson & Friedgood, 1938; reptiles: Licht & Pearson, 1978; mongoose: Bhiwgade & Gadegone, 1981; squirrel: Bhiwgade & Gulhane, 1980; rat: Kurosumi, 1968 and Kurosumi & Inoue, 1986; human: Pearse, 1952 and Pearse & van Noorden, 1963; bat: Siegel, 1955; Azzali, 1971; Patil, 1974; Richardson, 1979; Caxton-Martins *et al.*, 1984 and Badwaik, 1988 & 1989). Since pituitary cells appear very similar, differential identification based on morphology has not been very successful and morphology has to be combined with tinctoral affinities to separate the individual cell types (Childs, 1986). An easier and more

accurate method of identifying the pituitary cells was achieved with the development of immunocytochemistry (ICC) by Coons in 1941, a technique in which an antiserum to an antigen (eg. a hormone) specifically labels the cells containing the particular antigen (Polak & van Noorden, 1987).

At least five different cell types have been identified in the anterior pituitary: somatotropes, which produce growth hormone; mammotropes, which produce prolactin; corticotropes, which produce adrenocorticotropin; thyrotropes, which produce thyroid stimulating hormone and gonadotropes, which produce luteinizing hormone and follicle stimulating hormone. The location of mammotropes within the anterior pituitary has been established in fish (Fairbridge *et al.*, 1990; Yan & Thomas, 1991), shrew (Ishibashi & Shiino, 1988), salamander (Thompson & Trimble III, 1975), newt (Vellano *et al.*, 1973), mouse (Baker & Gross, 1978), rat (Nakane, 1970) and bats (Richardson, 1978 & 1981; Mikami *et al.*, 1988; Ishibashi & Shiino, 1989b). In some studies workers discovered an additional cell type (mammosomatotropes), which synthesises and secretes both growth hormone and prolactin (cow: Fumagalli & Zanini, 1985 & rat: Nikitovitch-Winer, 1987), however these cells are not always found (cow: Dacheux & Dubois, 1976 & frog: Gustalla *et al.*, 1993).

Prolactin, which is the hormone secreted by the mammotropes, is a small polypeptide and its primary structure has been determined for humans and a number of other mammals (Pankov & Butnev, 1986). Prolactin is heterogenic in structure, thus displaying different biological, chemical and immunological characteristics (Nicoll & Nichols, 1971). Due to its wide range of activities, prolactin has been studied extensively and many papers have emphasized techniques for its separation, isolation and detection in the fields of endocrinology (Merchant, 1974; Kugu *et al.*, 1988; Caillol *et al.*, 1990),

medicine (Touitou *et al.*, 1988), and even phylogenetic studies (Noso *et al.*, 1993).

This chapter deals with the methods used and the identification and location of mammotropes and the determination of pituitary and plasma prolactin concentrations in the anterior pituitary of Schreibers' long-fingered bat, *Miniopterus schreibersii.*

METHODS & RESULTS:

Collection of bats and removal of the pituitary gland and plasma samples:

Between six and nine adult female *Miniopterus schreibersii* were collected on a monthly basis between January 1990 and September 1992 from an abandoned mine adit in the Eastern Cape Province of South Africa (Maitland Mines; 33°58'S, 25°17'E). Additional specimens were collected from the stormwater drains in Grahamstown (33°19'S, 26°32'E), Howiesons' Poort (33°22'S, 26°22'E) and Table Farm (33°17'S, 26°25'E), the latter two locations being situated just outside Grahamstown. Bats with a body weight greater than 45 grams, a forearm longer than 45 mm and blunted teeth were assumed to be adults. Collection of bats was usually during mornings and the tissue and plasma samples were taken in the afternoon, to avoid circadian variation in prolactin concentrations, which have been demonstrated for humans (Stern & Reichlin, 1990), a number of other mammalian species (ferret: Agu *et al.*, 1986; rat: Bondarenka & Pesotskaya, 1987 and Pitman *et al.*, 1989; ewe: McMillen & Walker, 1991), in fish (mullet: Spieler *et al.*, 1976) and in gallinaceous birds (El Halawani *et al.*, 1984).

The bats were sacrificed by asphyxiation with CO_2 and blood (0.2-0.4 ml) was collected directly from both ventricles using a 1.0 ml syringe. The samples were

centrifuged (1600g x 5 minutes) and the plasma (0.1 - 0.2 ml) was frozen and stored at -20°C for radioimmunoassay. The bats were then decapitated, and to ensure rapid fixation of the pituitary gland, the fixative was injected into the brain through the foramen magnum using a 1.0 ml syringe. The pituitary gland was exposed by separating the lower jaw and the basisphenoid from the skull and then removed, immersed in about 1.0 ml of fixative, and prepared for light and electron microscopy. For LM the entire pituitary was fixed, but for TEM the pars intermedia and the posterior pituitary were removed from the anterior pituitary.

Light Microscopy: (Appendix 1)

The pituitary glands (n= 2-3 per month) were fixed in 10% neutral buffered formalin for 2 hours, dehydrated using increasing ethanol concentrations and embedded in paraffin (solidification point 46-48°C) at a temperature of 57°C (to prevent protein denaturation). 5 µm sections were cut on a rotary microtome, placed on slides coated with 0.01% w/v poly-L-lysine (Sigma), and dried in an oven at 37°C overnight. The sections were dewaxed, hydrated and stained using immunocytochemistry (ICC). The antiserum used was a polyclonal anti-ovine prolactin (NIDDK-anti-oPRL-IC-1 [rabbit, dilution 1/600]; kindley donated by Dr A.F. Parlow, Director of Pituitary Hormones and Antisera Center, Harbor-ULCA Medical Center, Torrance, California) together with an IgG-conjugated 1 nm colloidal gold probe (dilution 1/40; Janssen, Làmmerdines, Olen, Belgium) and silver enhancement (IntenSE M, Amersham, UK). No counterstaining was performed. Standard controls and specificity tests were included, and are discussed later in this chapter.

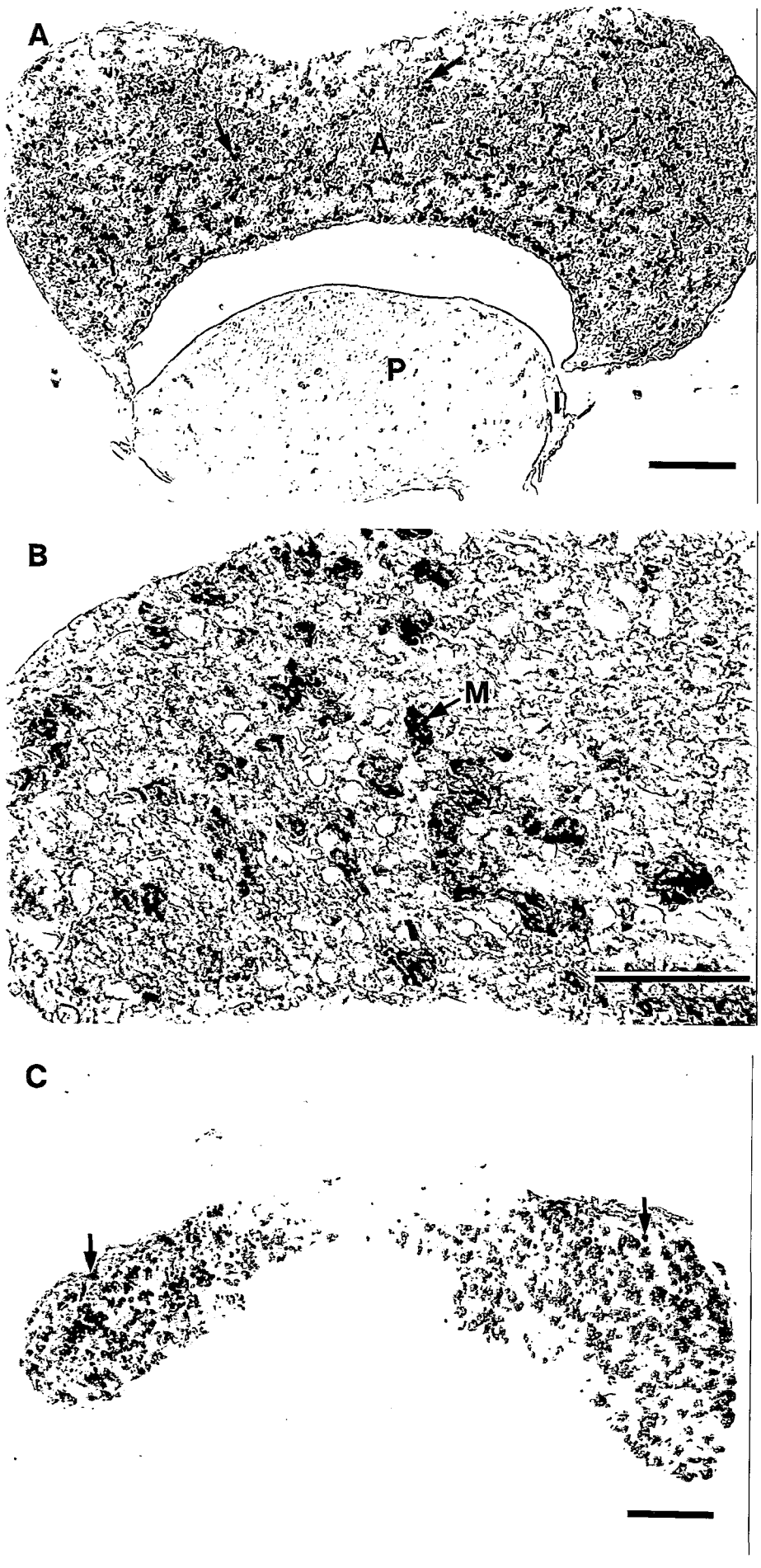
The pituitary gland comprises the anterior pituitary, the posterior pituitary and the pars intermedia (Fig. 1A). Using ICC the mammotropes were successfully stained and

Figure 1. Light micrograph sections of the pituitary gland of female *Miniopterus schreibersii*.

A. Section of the pituitary gland, showing the anterior pituitary (A), the posterior pituitary (P) and the pars intermedia (I). Mammotropes (arrows) are distributed throughout the anterior pituitary. Scale bar = 200 µm.

B. Section of the anterior pituitary, showing the mammotropes (M) stained black, due to the silver enhancement. Scale bar = 100 µm.

C. Section of the pituitary gland, showing the mammotropes (arrows) concentrated in the lateral lobes of the anterior pituitary. Scale bar = 200 µm.


A
A
P
B
M
C

appear black, due to the silver enhancement. Mammotropes usually occur in groups and are polygonal in shape (Fig. 1B), and are generally distributed throughout the anterior pituitary (Fig. 1A), but may be concentrated in the lateral lobes (Fig. 1C).

Electron Microscopy: (Appendix 2)

Pituitary glands (n= 3-6 per month) were fixed by immersion in cold 4% paraformaldehyde in 0.2 M Hepes (pH 7.4) for 2 hours, dehydrated in increasing ethanol concentrations, and embedded in Araldite/Agar 100 or LR-White. The blocks were polymerised at 37°C for ca. 10 days and at 64°C for 22.5 hours respectively. 70 nm sections were cut on an LKB 8800 A Ultratome III, collected on nickel grids and immunocytochemically stained. The prolactin antiserum used was the same as for LM-ICC (NIDDK-anti-oPrl-IC-1; optimal dilution 1/10000), and the secondary antibody was an IgG-conjugated 20 nm colloidal gold probe. The sections were counterstained using methanolic uranyl acetate (Stempak & Ward, 1964). Again control and specificity tests were performed and are discussed later in this chapter.

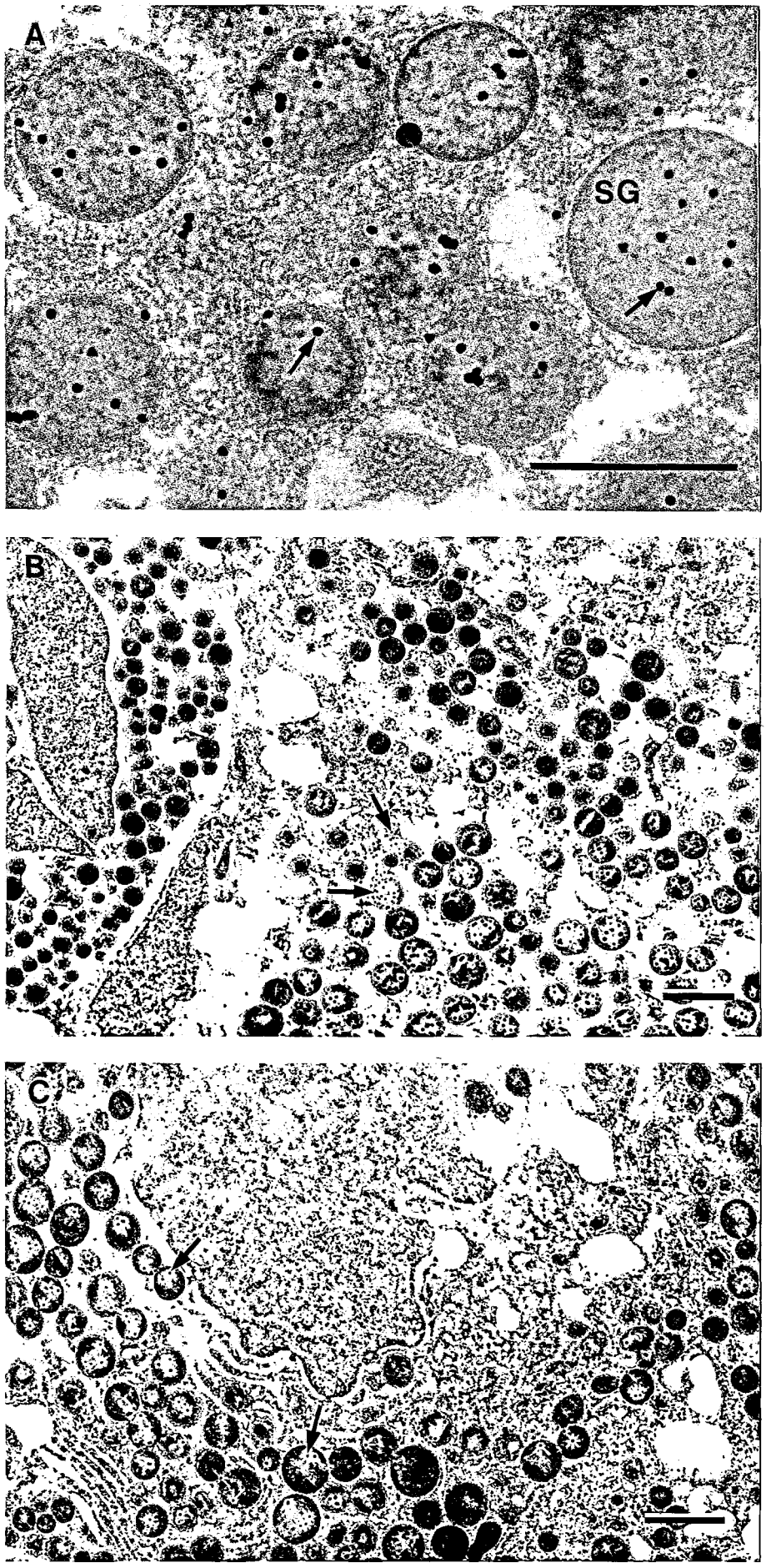
Prolactin in the cells was demonstrated by the presence of the gold probes on the secretory granules (Fig. 2A), and the two different embedding media used, resulted in different levels of immunoreactivity. LR-White showed a higher immunoreactivity to the PRL antibody (ie. more gold probes were detected on the secretory granules), but the ultrastructural resolution was poorer than with the Araldite/Agar 100 embedding medium (Figs 2B & 2C). The use of LR-White was advantageous as the prolactin antibody showed a decrease in immunoreactivity over the three years of the study and during months of increased mammotrope activity in the last year (especially October to January) PRL immunoreactivity using LR-White was nearly double that of Araldite/Agar 100, which

Figure 2. Electron micrographs of sections through mammotropes of the anterior pituitary of female *Miniopterus schreibersii*.

A. Section through a mammotrope, showing different sized secretory granules (SG). The presence of prolactin is demonstrated by the gold probes (arrows) on the granules. Scale bar = 0.5 µm.

B. Section through a mammotrope of the anterior pituitary embedded in LR-White, showing a very high immunoreactivity, which is indicated by numerous gold probes (arrows) on the secretory granules. Scale bar = 1.0 µm.

C. Section through a mammotrope of the anterior pituitary embedded in Araldite/Agar 100, showing a lower immunoreactivity (indicated by few gold probes [arrows] on the granules) compared to the LR White embedded tissue. Scale bar = 1.0 µm.


A
SG
B
C

showed little difference in immunoreactivity between months.

Mammotropes are polygonal in shape, and contain an irregular shaped nucleus, a well developed Golgi body and rough endoplasmic reticulum. Numerous electron-dense secretory granules, which vary in shape (round to irregular) are present throughout the cytoplasm. The granules occurred in two overlapping size classes, one present during secretory inactivity (140-490 nm) and the other during secretory activity (350-830 nm).

Double Labelling: (Appendix 3)

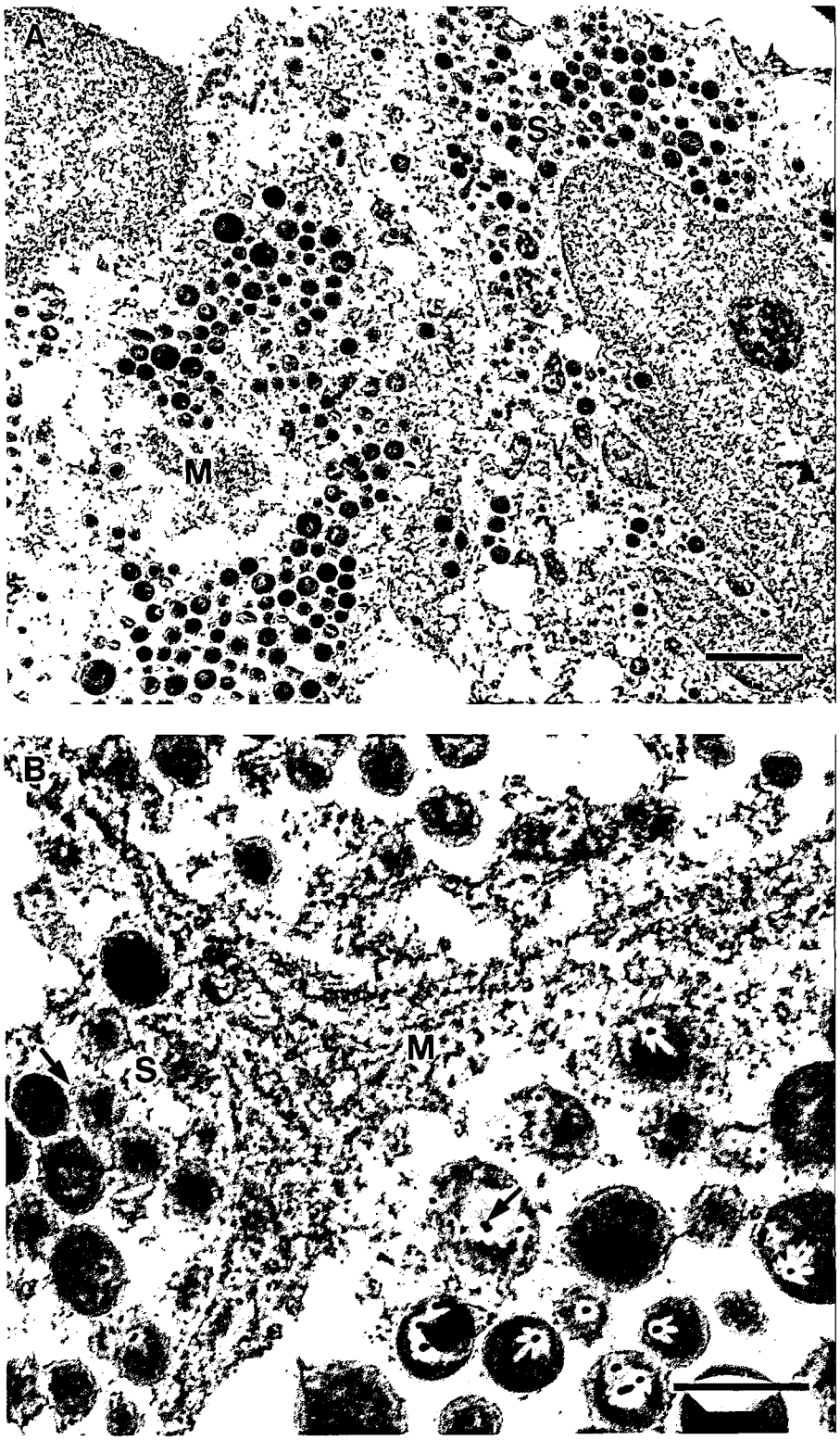
Sections of pituitary (from months where the mammotropes were secretorily active) were double labelled using the anti-oPRL-IC-1 and an anti-murine growth hormone (monkey; Pituitary Hormones and Antisera Center, Harbor-ULCA Medical Center, Torrance, California). The dilutions were 1/10000 and 1/2000 respectively. To visualise the primary antisera two different secondary antisera were used. Firstly the anti-mGH was labelled using a Protein A-6 nm colloidal gold probe, and for the anti-oPRL a Protein A-9 nm colloidal gold probe was used. The other method involved a goat-anti-monkey IgG-10 nm gold probe for the anti-mGH and a goat-anti-rabbit IgG-20 nm gold probe for the anti-oPRL.

Double labelling using Protein A-colloidal gold probes showed nonspecific staining (ie. label present in the cytoplasm and on cell organelles other than the secretory granules) and both the 6 and 9 nm gold probes were present in the same cells. However the IgG-colloidal gold probes showed no nonspecific label and the GAM-IgG-10 nm gold probe selectively stained a cell type with small to medium sized secretory granules, while the GAR-IgG-20 nm gold probe only labelled cells with larger secretory granules. The different gold probes were never found to be present in the same cells (Figs 3A & 3B).

Figure 3. Electron micrographs of sections through the anterior pituitary of female *Miniopterus schreibersii*, showing ICC double labelling.

A. Section through the anterior pituitary, showing a mammotrope (M) and a somatotrope (S). Scale bar = 1.0 μm.

B. Section through a mammotrope (M; showing 20 nm gold probes [arrow] on the granules) and somatotrope (S; showing 10 nm gold probes [arrow] on the granules). Scale bar = 0.5 μm.


A
S
M
B
S
M

Specificity Tests & Controls:

The specificity or crossreactivity of the antisera was tested by absorbing the diluted antisera (1/10000 anti-oPRL or 1/2000 anti-mGH respectively) with a 10 nmol/ml prolactin or growth hormone solution. The absorbed antisera-antigen solution was incubated at 4°C for 24 hours and then used in place of the primary antisera in the ICC labelling procedure. At both the light and electron microscope level, the PRL-anti oPRL and the GH-anti mGH complexes showed no staining. This is expected as the prolactin/growth hormone forms a complex with its respective antibody and no free antibody is present to react with the hormones (PRL or GH) in the cells, indicating a high degree of specificity of the antibody. Switching the prolactin antibody with the growth hormone antibody (PRL-anti mGH and GH-anti oPRL) resulted in normal staining in both cases, as with the unabsorbed antibody, thus very little or no cross-reactivity between the PRL and the GH antibody takes place, nor between the GH and the PRL antibody.

A tissue control was performed by immunocytochemically staining the pars nervosa, which contains no mammotropes, resulting in negative staining.

Method controls involved the substitution of the primary or secondary antisera with buffer (PBS or Hepes, pH 7.4). For the single-labelling technique this resulted in no staining, whereas substitution of one of the primary or secondary antibodies during the double-labelling resulted in the staining of one cell type only.

Electrophoretic Separation of Pituitary Hormones using SDS-Gel Electrophoresis: (Laemmili, 1970)

Anterior pituitary glands (n= 2-3 per month) were each homogenized in 100 µl sample buffer (appendix 4) containing 20 µl of 0.1 M phenylmethylsulphonyl fluoride (an

enzyme inhibitor). Homogenization took place by alternating 10 minutes sonication of the sample in a waterbath (at room temperature) and cooling the sample on ice, until the pituitary was completely homogenized. 20 µl of the homogenate was added to 20 µl dissociation buffer (appendix 4), boiled for 5 minutes and then placed onto a discontinuous polyacrylamide gel.

The gel was run at 40 volts for ca. 12 hours. The gels were stained in Coomassie blue for 2 hours and destained for ca. 4 hours.

Analysis of the gels was done using the UVP gel documentation software package (Ultra-Violet Products Ltd, Science Park, Milton Road, Cambridge, UK). To determine the molecular weight and concentration of the bat prolactin, homogenised bat anterior pituitaries were run with molecular weight markers and different concentrations of pure prolactin (luteotropic hormone from sheep pituitary, Sigma L-6520, 31 IU/mg).

Seven marker proteins with molecular weights of 66000 (bovine albumin), 45000 (egg albumin), 36000 (glyceraldehyde-3-phosphate dehydrogenase), 29000 (carbonic anhydrase), 24000 (trypsinogen), 20100 (trypsin inhibitor) and 14200 (α-lactalbumin) were used (Fig. 4). The smallest marker (α-lactalbumin), migrated the furthest distance towards the positive electrode. In all gels run, the sheep prolactin showed an R_f value of about 0.58, which was the same R_f value as trypsinogen, and thus indicated a molecular weight of about 24000. One band, which separated from the homogenised bat anterior pituitary and which was assumed to be the bat prolactin, had a slightly higher R_f value (ca. 0.6) giving it a slightly lower molecular weight of about 23000. In some pituitaries a second band was present just above the prolactin band (Fig. 4). This hormone had an R_f value of 0.57, therefore its molecular weight is ca. 25000. Calculations of concentrations were difficult. The UVP software package measures the density of each band which is related to the

concentration of the hormone and often the staining and destaining of the gel were not uniform. This resulted in differential staining, and sometimes the prolactin bands disappeared completely. However using the results obtained the prolactin concentration varied on a monthly basis between 0.62 (June) and 19.75 µg (October) per pituitary, and are discussed further in chapter 3.

In spite of the drawbacks of this technique, it was used to support the results obtained using the ICC staining technique. The ICC results showed seasonal changes in pituitary prolactin immunoreactivity and the gel electrophoresis was used as an alternative measure of pitutiary prolactin concentration. It should be noted that the PRL radioimmunoassay was not available in the first two years of the study and this method of assessing mammotrope secretory activity was only used in 1993.

Prolactin Radioimmunoassay: (Appendix 5)

Bat plasma was extracted using acetone precipitation (Husain *et al.*, 1973). To 100 µl plasma, 900 µl of distilled water and 2 ml of chilled acetone (-20°C) was added, mixed and centrifuged at 5000 g for 10 minutes. The pellet was discarded and the supernatant was dried down under air at 35°C, and resuspended in 150 µl of RIA buffer.

NIDDK-oPRL-I-2 was used for iodination and as an assay standard, and the antisera (raised in a rabbit) was NIDDK-anti-oPRL-2, which showed negligible cross-reactivity with oGH (0.054%) and other pituitary hormones (< 0.015%) (all hormones and antisera were kindly donated by Dr. A.F. Parlow, Director of Pituitary Hormones and Antisera Center, Harbor-ULCA Medical Center, Torrance, California). The oPRL was labelled with ^{125}I using the chloramine-T method, with purification by column chromatography on Sephadex G50. The bound tracer was diluted with RIA buffer to give ca. 200000 cpm per ml and

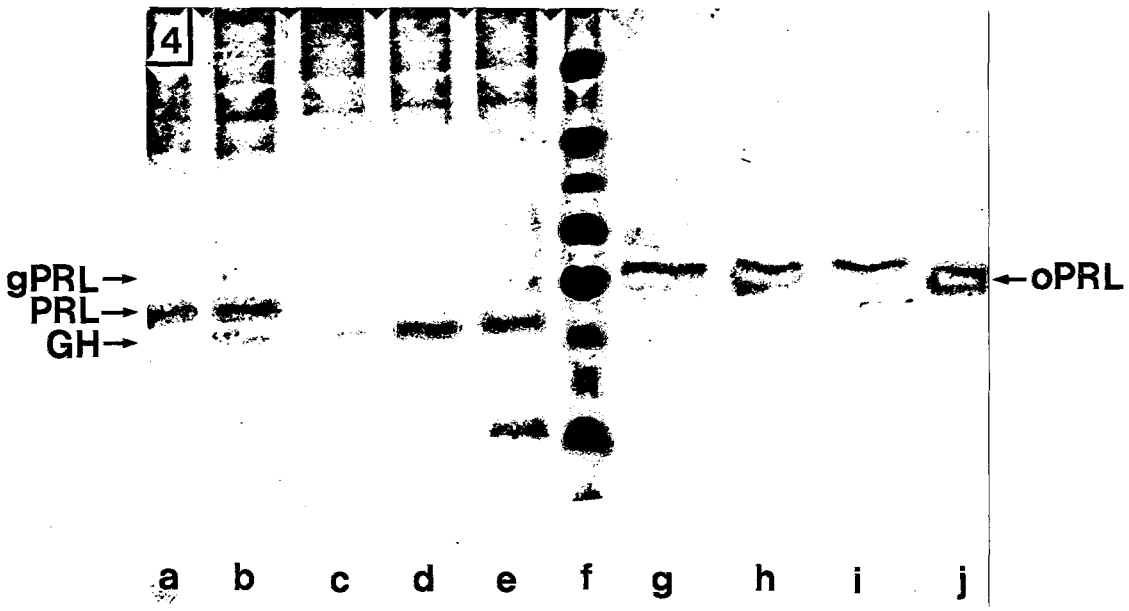
stored at -20°C.

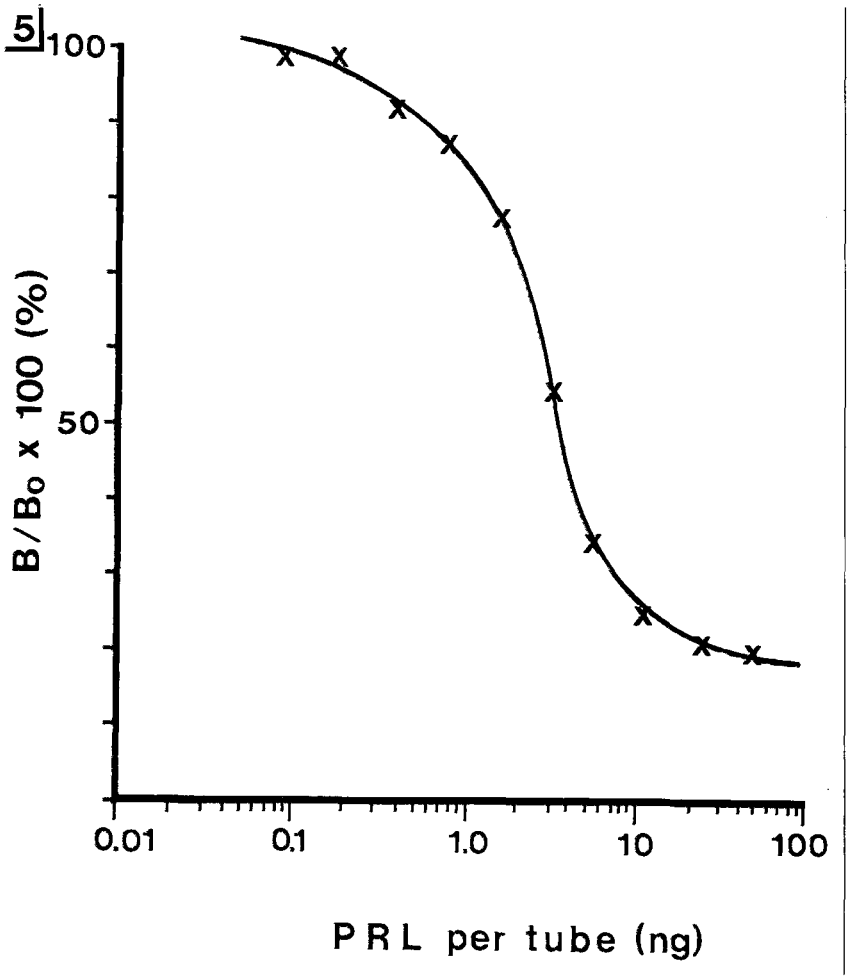
To 100 µl of extracted bat plasma 300 µl of anti-oPRL (at a final dilution of 1:60000) was added, mixed and left for 30 minutes. Then 100 µl of iodinated oPRL (ca. 20000 cpm) was added, mixed and incubated at room temperature overnight.

For the standard curve, the bat plasma was substituted with prolactin standards and controls included B_0 (plasma is replaced with buffer) and the antibody blank (Abl: 400 µl buffer and 100 µl of labelled tracer). Separation of antibody bound from free tracer was achieved by the rapid addition of 1 ml absolute ethanol, followed by mixing and centrifugation at 3000 g for 10 minutes at 4°C. The supernatants were removed by aspiration and radioactivity in the pellets was recorded with a Packard Auto-Gamma Scintillation Spectrometer 5120. Sensitivity of the assay was 0.19 ng/0.1 ml (Fig. 5). Extraction of the bat plasma was necessary as unextracted plasma often produced tracer binding far in excess of the maximum possible, presumably due to the presence of interfering plasma proteins. This problem was eliminated using acetone extraction, which precipitated all larger proteins from the plasma. The extraction recovery of prolactin added to a pool of bat plasma was only 36%, however it was consistent. Measured prolactin concentrations were corrected for this recovery. Plasma prolactin concentrations measured ranged from 57 to 206 ng/ml (to be discussed in chapter 3).

Figure 4. Photograph of a polyacrylamide gel: well a & b - homogenised pituitaries from December 1991; well c, d & e - homogenised pituitaries from February 1992; well f - marker proteins; well g, h, i & j - increasing concentrations of ovine prolactin. GH - bat growth hormone; PRL - bat prolactin; gPRL - glycosylated bat prolactin; oPRL - ovine prolactin.

Figure 5. Typical standard curve for ovine prolactin.


4
gPRL→
PRL→
GH→
←oPRL
a b c d e f g h i j


5
100
50
B/Bo x 100 (%)
0.01
0.1
1.0
10
100
PRL per tube (ng)

DISCUSSION:

The gross morphology of the pituitary gland of *Miniopterus schreibersii* is the same as described for other bat species (Guthrie, 1933; Sawyer, 1936; Siegel, 1955; Herlant, 1956; Patil, 1974; Anthony & Gustafson, 1984; Mikami *et al.*, 1988). Using conventional histological staining techniques, mammotropes have been identified as type II acidophils or ε-cells, which occur in clusters, are large, and irregular to polygonal in shape (Herlant, 1964; Azzali, 1971; Patil, 1974; Bhiwgade & Gulhane, 1980; Bhiwgade & Gadegone, 1981; Badwaik, 1988), and are most abundant in pregnant and lactating females, the cells being hypertrophied during these stages (Barnes, 1962; Bhiwgade & Gulhane, 1980; Bhiwgade & Gadegone, 1981). With the introduction of immunofluorescence and immunocytochemistry, these acidophils were positively identified as mammotropes (Vellano *et al.*, 1973; Thompson & Trimble III, 1975; Baker & Gross, 1978; Hansen & Hansen, 1981; Yan & Thomas, 1991). Reports on the distribution of mammotropes vary greatly from being concentrated in the rostral region of the anterior pituitary (Licht & Pearson, 1978; Fairbridge *et al.*, 1990; Yan & Thomas, 1991), in the dorsocentral area (close to the pars intermedia; Nakane, 1970; Vellano *et al.*, 1973), to an even distribution throughout the gland (Dacheux & Dubois, 1976; Baker & Gross, 1978; Neill, 1988; Gustalla *et al.*, 1993; present study). However distribution and numbers of mammotropes vary seasonally (this will be further discussed in chapter 3), which could explain this variation.

Electron microscopy has shown that the mammotropes contain large, electron dense secretory granules, which have a maximum diameter of between 600 - 900 μm (Sano, 1962; Kurosumi, 1968; Tesar *et al.*, 1969; Nakane, 1970; Licht & Pearson, 1978; Kurosumi & Inoue, 1986; Ishibashi & Shiino, 1988; present study), a well developed Golgi body and

extensive rough endoplasmic reticulum (Herlant, 1964; Nakane 1970; Kurosumi & Inoue, 1986; Ishibashi & Shiino, 1988). These cells usually showed a high prolactin immunoreactivity (present study), indicating a high pituitary prolactin concentration. A second, atypical mammotrope has been described, which contains smaller, less electron dense secretory granules which have a diameter of 150 - 250 μm (Herlant, 1964; Kurosumi & Inoue, 1986). These cells are less often found in mature females and most often found during winter, and it is speculated that they are inactive mammotropes, as they are also found in juveniles and males (Nogami & Yoshimura, 1980; Kurosumi & Inoue, 1986; present study). In addition, these cells show a low prolactin immunoreactivity, thus suggesting a reduced level of prolactin synthesis.

The occurrence of mammosomatotropes seems to vary greatly between and within species. These cells have been described for the cow (Fumagalli & Zanini, 1985 & Kineman *et al.*, 1991), rat (Nikitovitch-Winer, 1987; Porter *et al.*, 1990 & 1991), rat and shrew (Ishibashi & Shiino, 1989a) and the Japanese house bat (Ishibashi & Shiino, 1989b), where the workers found growth hormone and prolactin label in separate granules within the same cell, and also within the same granules. However mammosomatotropes were reported to be absent in the cow (Dacheux & Dubois, 1976), porcine (Dacheux, 1980), rat (Kurosumi & Inoue, 1986), newt (Campantico & Gustalla, 1992) and the frog (Gustalla *et. al.*, 1993). In the present study, protein A labelling showed gold label for GH and PRL to be present in the same cells and even the same granules, however a lot of non-specific label was present throughout the cell, rendering the results unreliable. The GAM and GAR-IgG gold probes gave the opposite result, in that the different size gold probes labelled different cell types, with little or no non-specific label, indicating that GH and PRL are synthesised and secreted by individual cells. Most workers reporting the presence of

mammosomatotropes have used Protein A-colloidal gold probes to label both the growth hormone and the prolactin, and in view of the results from the present study such results should be interpreted with care. However, Nikitovitsch-Winer (1987) also used GAM and GAR-IgG colloidal gold and found GH and PRL to be present in the same cell and even in the same secretory granules in the rat anterior pituitary. Clearly therefore, mammosomatotropes are present in some and absent from other anterior pituitaries. In the case of *Miniopterus schreibersii* mammosomatotropes seem to be absent from the anterior pituitary (Mikami *et al.*, 1988 & present study).

Separation of pituitary hormones and determination of pituitary prolactin concentration was only partially successful. The band, assumed to be bat pituitary prolactin, had a molecular weight of about 23000 daltons, which falls within the range of molecular weights (22000 to 26500 daltons) reported for other vertebrate prolactins (Harrow & Mazur, 1955; Lewis *et al.*, 1984; Pankov & Butnev, 1986; Martinat *et al.*, 1990; Matsuda *et al.*, 1990a; Soares *et al.*, 1991; Suzuki *et al.*, 1991; Berghman *et al.*, 1992; Rand-Weaver & Kawauchi, 1992; Noso *et al.*, 1993). Two additional prominent bands were visible on the gel, one with a lower molecular weight (21000), probably corresponding to growth hormone, and one with a molecular weight of about 25000, which could be the glycosylated form of prolactin, as described by Lewis *et al.* (1984) and Pankov & Butnev (1986). Various techniques have been used to measure pituitary prolactin levels, such as different types of bioassays (Clarke, 1971; March & McKeown, 1973), radioimmunoassay (Fernandes *et al.*, 1987; Ishii *et al.*, 1989; Yamamoto *et al.*, 1989), and densitometric measurements of PAGE prolactin bands (Nicoll *et al.*, 1969; Yanai & Nagasawa, 1969; Clarke, 1973). Pituitary prolactin concentrations for the toad were reported to be between 0.5 and 4.5 µg PRL/gland (Ishii *et al.*, 1989), for the canine 2.0 to 5.0 µg PRL/mg tissue

(Fernandes *et al.*, 1987), for the rat between 6.2 and 7.2 µg/mg tissue (Nicoll *et al.*, 1969) and for tilapia and average of 58 µg/mg tissue (Clarke, 1973). Bat pituitary prolactin concentrations show similar values, ranging between 0.62 and 19.75 µg/pituitary (pituitary weights in female *Miniopterus schreibersii* ranges between 0.9 and 1.6 mg; Bojarski, 1990).

Plasma prolactin concentrations, determined using radioimmunoassay, showed very high basal levels (57 ng/ml), although similarly high levels have been reported for the turkey (60 ng/ml; Youngren *et al.*, 1993) and the mullet (85 ng/ml; Spieler *et al.*, 1976). Most basal levels for prolactin however range between 2.0 ng/ml in gilts (Diekman *et al.*, 1991) and 30.0 ng/ml in goats (Prandi *et al.*, 1988). Elevated prolactin levels also show a wide range from 12 to 700 ng/ml (Diekman *et al.*, 1991; Youngren *et al.*, 1993). The highest plasma prolactin level in the bat was recorded during pregnancy and the concentration was 206 ng/ml. For the PRL-RIA it was necessary to extract the prolactin from the plasma using chilled acetone. This procedure removed larger plasma proteins (albumin and globulins), which are known to interfere with the antibody-antigen binding (in this case Ab-PRL), thus giving misleading results. This interference varies between and within species, for example the Boer and Angora goats. Goat prolactin concentrations were measured using RIA in unextracted and extracted plasma, and prolactin concentrations in the Boer goat were found to be higher in the extracted compared to the unextracted plasma. However in the Angora goat no prolactin was detected in the unextracted plasma, whereas the hormone could be detected in the extracted plasma. This indicates a higher interference of plasma proteins in the Ab-PRL binding in the Angora goat compared to the Boer goat (D. Gray, pers. comm.). None of the other workers have used extraction methods prior to measuring PRL with RIA, and the different degree of interference of large plasma proteins

could explain the large variation in plasma prolactin levels reported in the literature.

SUMMARY:

Using immunocytochemistry, mammotropes were positively identified in the anterior pituitary gland, as being large irregular to polygonal shaped cells which contain two different sized classes of secretory granules. These granules are the storage site for prolactin and are a typical characteristic of protein secreting cell types. A band, assumed to be prolactin, was successfully separated from other pituitary hormones using polyacrylamide gel electrophoresis, and its molecular weight was determined to be about 23000. Pituitary and plasma prolactin concentrations were measured using densitometry and radioimmunoassay respectively, and were found to be similar to values reported in the literature.

CHAPTER 3

SEASONAL CHANGES IN MAMMOTROPE ULTRASTRUCTURE AND PITUITARY AND PLASMA PROLACTIN CONCENTRATIONS IN RELATION TO THE REPRODUCTIVE CYCLE

INTRODUCTION:

In most mammals the activation of the corpus luteum to produce progesterone is controlled mainly by luteinizing hormone (LH), to a lesser extent by prolactin (PRL) and in some cases by follicle stimulating hormone and estradiol (Niswender & Nett, 1988). In most mammals that use delayed implantation, implantation coincides with the activation of the corpus luteum (Stoufflet *et al.*, 1989), and in small mustelid carnivores prolactin is required to activate the corpus luteum and thus terminate the period of delay (Berria *et al.*, 1989; Sundqvist *et al.*, 1989).

Although seasonal changes in mammotrope activity have been described for *Myotis myotis* (Herlant, 1962), *Vesperugo savi* and *V. piccolo* (Azzali, 1971), *Macrotus californicus*

(Richardson, 1978, 1979 & 1981), *Taphozous melanopogan* (Badwaik, 1988), *Miniopterus schreibersii fulinginosus* (Mikami *et al.*, 1988) and *Pipistrellus abramus* (Ishibashi & Shiino, 1989b), the role of prolactin in delayed implantation has not yet been investigated in bats. However Peyre & Herlant (1963) noted that the mammotropes of *Miniopterus schreibersii* were hypertrophied at the time of implantation and activation of the corpus luteum, and therefore concluded that the hormone plays a role (direct or indirect) in these two processes.

I have monitored the activity pattern of mammotropes (which secrete PRL) by estimating seasonal changes in mammotrope abundance and ultrastructure, PRL immunoreactivity and pituitary prolactin concentrations. Since pituitary prolactin concentrations only represent the balance between prolactin synthesis and secretion and may not reflect plasma prolactin concentrations, seasonal plasma prolactin levels have been measured.

MATERIALS & METHODS:

Adult (n = 6-9) and occasionally a juvenile female *Miniopterus schreibersii* were collected on a monthly basis between January 1990 and September 1992 and the pituitary glands were prepared for light and electron microscopy. Sections were stained using immunocytochemistry (as described in chapter 2), and were analyzed morphometrically as follows:

1. The abundance of mammotropes was estimated from one midsagittal, immunogold stained light microscope section of two to three pituitary glands per month.

The areas of the whole pituitary (P) and positively stained mammotropes (M) were measured using a digitizer tablet and the SigmaScan software package, a scientific measurement system. The percentage of the total area of the pituitary, occupied by mammotropes was calculated using the following formula: %M=(M/P)x100, and was taken as an indication of mammotrope abundance.

2. The abundance of positively stained granules present in the cytoplasm of mammotropes for three pituitaries each month, was estimated using a standard point counting technique (Weibel *et al.*, 1966). A grid consisting of 110 test points (Pt), each 0.5 mm apart, was drawn on the screen of the transmission electron microscope onto which the image of the tissue was superimposed. The working magnification was x 29000. The number of test points falling on a positively stained secretory granule (hits) was recorded. The areas examined were randomly chosen, but restricted to the cytoplasm of the mammotrope. The procedure was repeated for each pituitary gland (three to six pituitaries per month) in which 10 mammotropes were examined, each time recording the number of hits (Pg). The abundance (A in %) of positively stained secretory granules in mammotropes was then calculated using the following formula: %A=(Pg/Pt)x100.

3. Changes in the amount of prolactin present in the secretory granules was estimated from changes in immunoreactivity of the tissue. Changes in immunoreactivity were assessed by counting the number of gold particles on 25 randomly selected granules in 10 mammotropes for each pituitary gland during the year, and changes in the abundance of gold particles were taken as a measure of changes in pituitary prolactin content (Posthuma *et al.*, 1987).

Pituitary and plasma prolactin concentrations were measured (as described in chapter 2) on a monthly basis. Monthly data from different years were pooled and

presented as means ± standard errors. Means were compared using one way analyses of variance and the Tukey multiple range test.

RESULTS:

Seasonal changes in the abundance of mammotropes:

Mammotrope abundance in the anterior pituitary showed statistically significant changes during the year ($P < 0.01$; Fig. 6B). During follicular development and delayed implantation, mammotropes were scarce, and occupied between 2.03% ± 0.39 (April; n=2) and 4.42% ± 0.58 (July; n=2) of the volume of the anterior pituitary (Figs 6B & 7A). At implantation (during August) mammotrope abundance increased, reaching a peak in December at the time of parturition (24.53% ± 0.80, n=3; Fig. 6B & 7B), and decreasing but remaining relatively high during lactation in January (11.05% ± 0.84, n=4); Fig. 6B). In comparison, in two juvenile female bats collected during July and August, mammotrope abundance was low (3.33 and 2.18% respectively).

Seasonal changes in the ultrastructure and immunoreactivity of mammotropes:

Mammotropes displayed statistically significant seasonal changes in the abundance of labelled secretory granules, the size of the granules and the prolactin immunoreactivity ($P < 0.01$ for all). Just prior to implantation pituitary prolactin immunoreactivity increased from 1.79 ± 0.12 (July; n=6) to 4.09 ± 0.58 (August; n=7 [Fig. 8B]) gold probes/granule and the abundance of labelled secretory granules increased from 3.62% ± 0.84 (July; n=6) to 12.78% ± 2.40 (August; n=7 [Fig. 8C]).

Figure 6. Reproductive cycle (A; COF - copulation, ovulation and fertilisation; IMP - implantation; PAR - parturition) of, and monthly changes in mammotrope abundance (B) in female *Miniopterus schreibersii* (bars are mean values and the lines on the bars are ± standard errors; n was always ≥ 2). Mammotrope abundance during follicular development and delayed implantation are significantly lower than during normal embryonic development and lactation ($P < 0.01$).

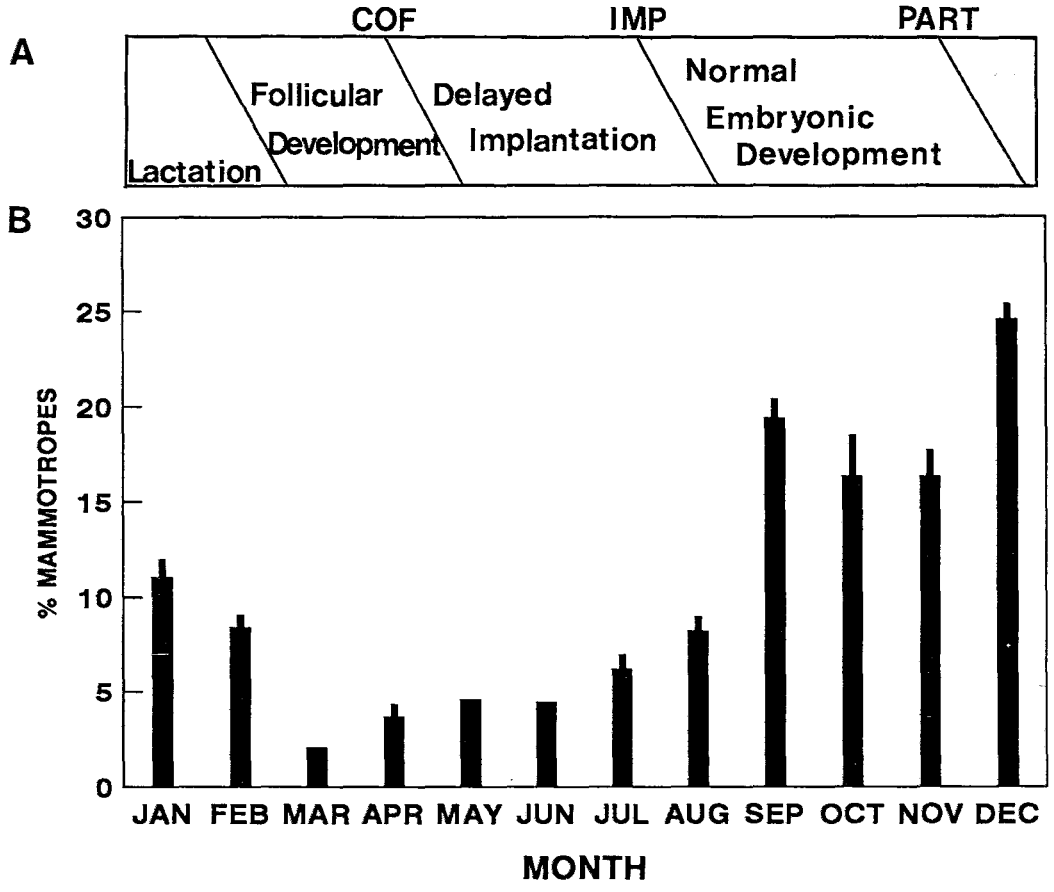

A
COF
IMP
PART
Lactation
Follicular Development
Delayed Implantation
Normal Embryonic Development
B
% MAMMOTROPES
30
25
20
15
10
5
0
JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTH

Figure 7. Light micrographs showing sections of the anterior pituitary of female *Miniopterus schreibersii.*

A. Section of the anterior pituitary, showing the mammotrope (arrows) abundance during June. Scale bar = 100 µm.

B. Section of the anterior pituitary, showing the increased mammotrope (arrows) abundance during December. Scale bar = 100 µm.

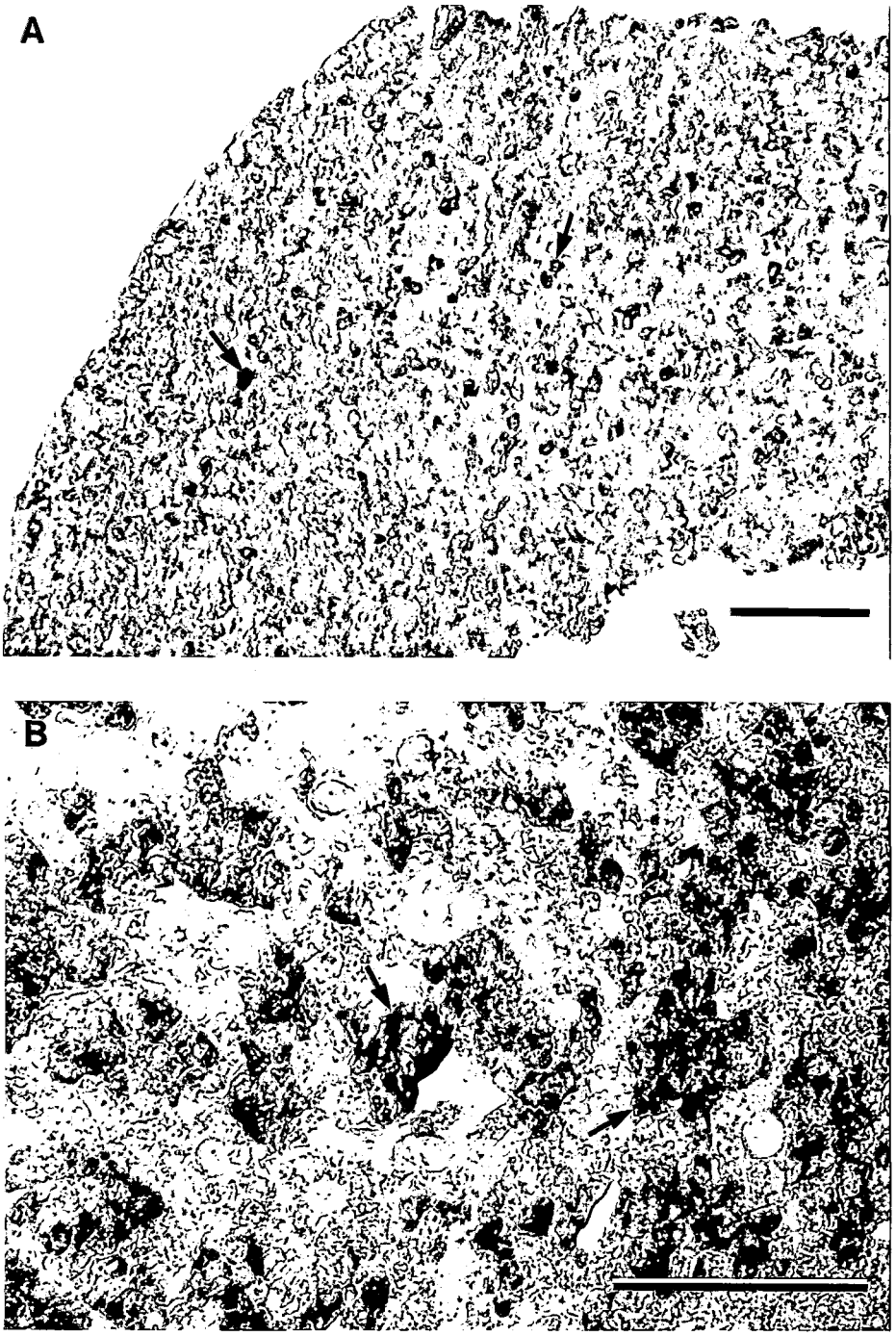
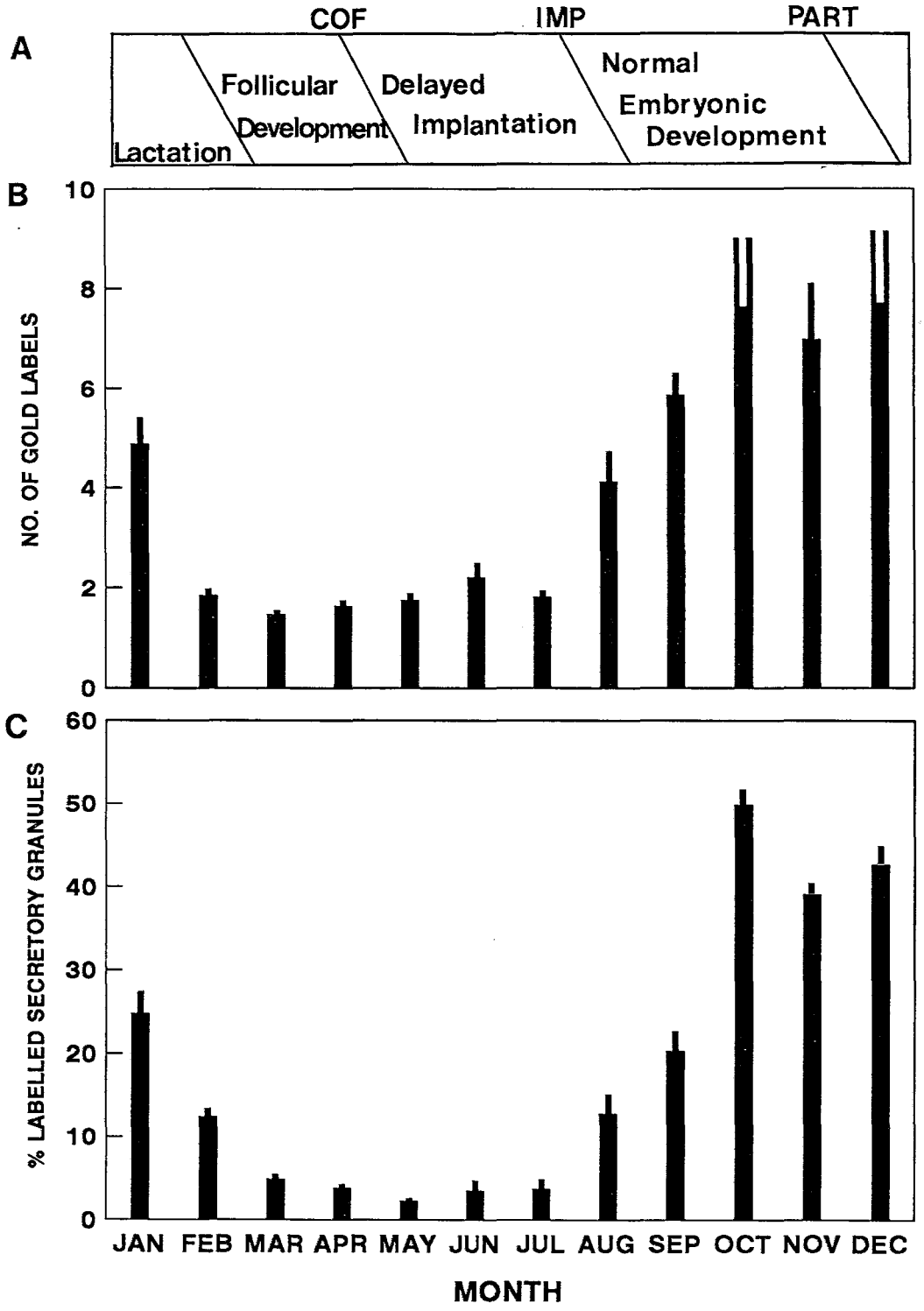

A
B

Figure 8. Reproductive cycle (A; COF - copulation, ovulation and fertilisation; IMP - implantation; PAR - parturition) of, and monthly changes in pituitary immunoreactivity (B; as assessed by number of gold probes per granule) and in the abundance of labelled secretory granules in the mammotropes (C) in female *Miniopterus schreibersii* (bars in the graphs represent means and the lines on the bars are ± standard errors; n was always ≥ 6). Numbers of gold probes and % labelled secretory granules during follicular development and delayed implantation were significantly lower than levels during normal embryonic development and lactation ($P < 0.01$).


A
COF
IMP
PART
Lactation
Follicular Development
Delayed Implantation
Normal Embryonic Development
B
NO. OF GOLD LABELS
0
2
4
6
8
10
C
% LABELLED SECRETORY GRANULES
0
10
20
30
40
50
60
JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTH

During pregnancy and lactation mammotropes contained numerous labelled secretory granules (between 20.32% ± 2.32 [September; n=14] and 49.82% ± 1.75 [October; n=9]), which showed a very high immunoreactivity to the prolactin antibody (between 4.85 ± 0.56 [January; n=13] and 9.14 ± 1.44 [December; n=9] gold probes/granule) (Figs 8B, 8C & 9A). The size of secretory granules ranged between 350 - 830 nm (however larger sized granules were dominant; Fig. 9A), and the cells possessed very prominent Golgi bodies (Fig. 9B). In contrast, during follicular development and delayed implantation, the cells appeared inactive, containing few labelled secretory granules (between 2.23% ± 0.16 [May; n=5] and 4.82% ± 0.45 [March; n=10]; Figs 8C & 9C), which showed a low prolactin immunoreactivity (between 1.45 ± 0.08 [March; n=10] and 2.19 ± 0.26 [June; n=7] gold probes/granule; Figs 8B & 9C). The secretory granules were smaller in size (140 - 490 nm) than those present at other times of the year. Prolactin immunoreactivity of the mammotropes from the two juveniles in July and August was low (1.73 and 2.24 gold probes per secretory granule respectively).

Seasonal changes in pituitary prolactin concentrations:

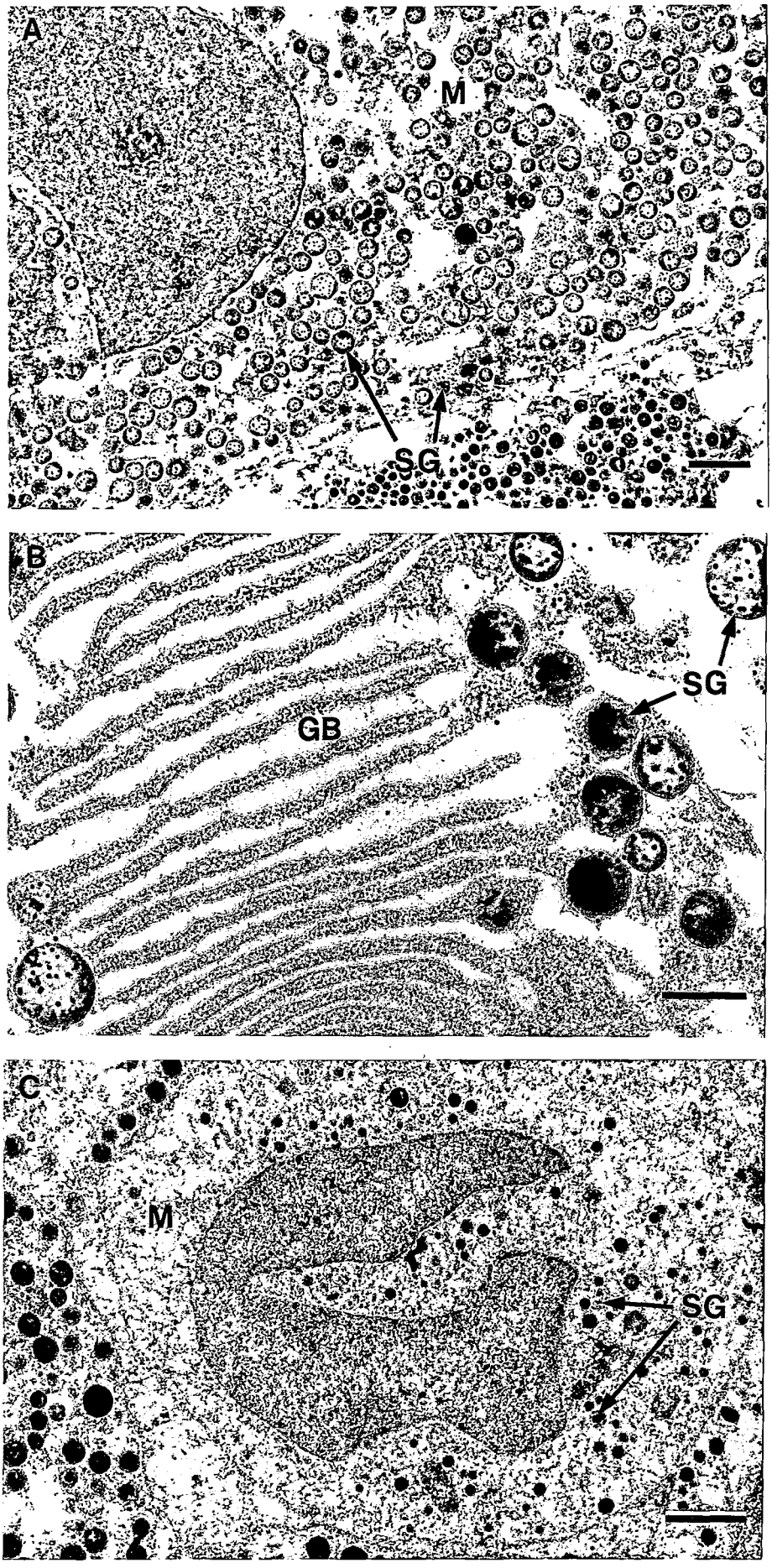
During June (delayed implantation) pituitary prolactin concentration was very low (0.62 µg PRL/pituitary; n=1). Prolactin concentrations in October (during normal embryonic development) were high (15.49 ± 6.03 µg PRL/pituitary; n=2) and then decreased in January during lactation (2.11 ± 0.82 µg PRL/pituitary; n=3). No prolactin bands were detected in the gels from February to May, which coincides with follicular development.

Figure 9. Electron micrographs of sections through mammotropes in the anterior pituitary of female *Miniopterus schreibersii*.

A. Section through a mammotrope (M) during December, showing a high prolactin content and different sized secretory granules (SG). Scale bar = 1.0 μm.

B. Section through a mammotrope, showing a prominent Golgi body (GB), surrounded by numerous secretory granules (SG), containing prolactin. Scale bar = 1.0 μm.

C. Section through a mammotrope (M) during May, showing a very low prolactin content and few secretory granules (SG). Scale bar = 1.0 μm.


A
M
SG
B
GB
SG
C
M
SG

Seasonal changes in plasma prolactin concentrations:

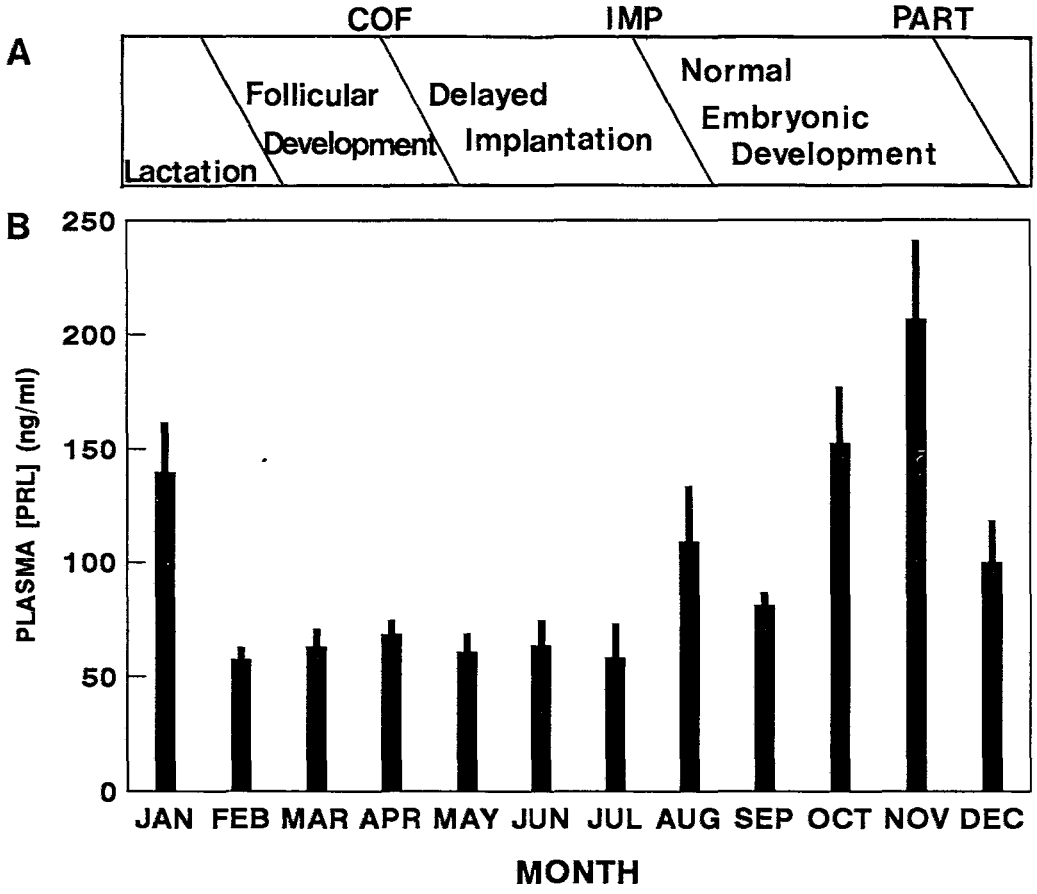
Plasma prolactin concentrations showed statistically significant changes during the year, ranging from 57 to 206 ng/ml ($P < 0.01$). The lowest prolactin concentrations (57.06 ± 14.21 to 68.27 ± 6.59 ng/ml) were recorded during follicular development (February to May) and delayed implantation (May to July; Fig. 10B). Plasma prolactin concentrations increased at implantation and reached a peak in November (206.32 ± 34.60 ng/ml) just prior to parturition. In December (late pregnancy), plasma prolactin concentrations dropped to 99.68 ± 18.41 ng/ml, but remained elevated during lactation in January (139.38 ± 22.05 ng/ml).

DISCUSSION:

Changes in mammotrope abundance, prolactin immunoreactivity, and pituitary and plasma prolactin concentrations in the long-fingered bat all showed the same seasonal trend, with low levels during delayed implantation, and elevated levels at implantation and during normal embryonic development and lactation.

Delayed implantation in Schreibers' long-fingered bat coincides with winter, when the bats undergo short periods of hibernation (Bernard *et al.*, 1991). During this time the corpus luteum is inactive and progesterone concentrations are low, and implantation coincides with corpus luteum activation and elevated plasma progesterone concentrations (Bernard *et al.*, 1991). Luteal inactivity and low progesterone concentrations are a common feature in mammals that make use of delayed implantation (seal: Temte, 1985; mink: Stoufflet *et al.*, 1989 and Murphy *et al.*, 1990; badger: Canivenc & Bonnin, 1979; skunk:

Figure 10. Reproductive cycle (A; COF - copulation, ovulation and fertilisation; IMP - implantation; PAR - parturition) of, and monthly changes in plasma prolactin concentrations (B) in female *Miniopterus schreibersii* (bars are mean values and the lines on the bars are ± standard errors; n was always ≥ 6). Plasma prolactin levels during follicular development and delayed implantation are significantly lower than levels during normal embryonic development and lactation ($P < 0.01$).


A
COF
IMP
PART
Lactation
Follicular Development
Delayed Implantation
Normal Embryonic Development
B
PLASMA [PRL] (ng/ml)
250
200
150
100
50
0
JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTH

Berria *et al.*, 1989; *Miniopterus schreibersii*: Crichton *et al.*, 1989) and delayed development (*Macrotus californicus*: Burns & Easley, 1977; Burns, 1981).

Mead (1981), working on mustelids, found that the increase in luteal function observed before implantation is totally dependent upon hormonal cues received from the pituitary. This finding was based on experimental work done on skunks (Mead, 1975) and mink (Murphy & Moger, 1977), where hypophysectomy inhibited luteal activity and implantation at the end of the period of delay. However different pituitary hormones or combinations of them have been reported to be responsible for the activation of the corpus luteum at the end of the period of delayed implantation. The role of prolactin in terminating delayed implantation has been established in the ferret (Agu *et al.*, 1986), mink (Murphy *et al.*, 1981 & 1990; Sundqvist *et al.*, 1989) and skunk (Berria *et al.*, 1989), and the preimplantation increase in pituitary and plasma prolactin suggests that prolactin may play the same role in *Miniopterus schreibersii* (present study). Prolactin also seems to be important in the control of delayed development, as an increase in the abundance and size of mammotropes and plasma prolactin levels during the transition from delayed to normal development in *Macrotus californicus* indicate that this hormone may be responsible for the termination of the period of delay (Richardson, 1979).

In addition to prolactin, plasma LH levels in the long-fingered bat also increase prior to implantation and during gestation (Bernard *et al.*, 1991), suggesting that both LH and PRL may play a role in terminating delayed implantation. Plasma LH levels have been found to gradually increase in the spotted skunk as the time of implantation approaches (Foresman & Mead, 1974), and increasing plasma LH levels in combination with an increased LH-gonadotrope activity is found in the European Badger (Canivenc & Bonnin, 1981). Furthermore studies on the endocrine control of facultative delayed implantation in

mice and rats has shown that prolactin in combination with luteinizing hormone or follicle stimulating hormone is needed to initiate implantation (Gidley-Baird, 1981). Ultimately experimental manipulation of PRL and LH during the period of delay will ascertain their role in initiating implantation and this will be discussed in chapter 4.

Pituitary and plasma prolactin levels increase further during postimplantation gestational development, indicating that the hormone may be required for luteal maintenance. In October/November the placenta takes over progesterone production and the corpus luteum degenerates (van der Merwe & van Aarde, 1989; Bernard *et al.*, 1991). However prolactin synthesis and secretion remains elevated, probably to meet the prolactin requirements of lactation. Increased mammotrope activity during pregnancy and lactation, measured by an increase in the number and size of prolactin secreting cells has also been described for other mammals (mouse: Barnes, 1962; vole: Clarke & Forsyth, 1964; palm squirrel: Bhiwgade & Gulhane, 1980; grey mongoose: Bhiwgade & Gadegone, 1981) and several bat species (*Myotis myotis*: Herlant, 1962; *Miniopterus schreibersii*: Peyre & Herlant, 1963 and Mikami *et al.*, 1988; *Macrotus californicus*: Richardson, 1979; *Taphozous melanopogon*: Badwaik, 1988).

SUMMARY:

Seasonal changes in mammotrope activity were successfully related to the reproductive cycle of female *Miniopterus schreibersii*. Mammotrope prolactin immunoreactivity, and plasma and pituitary prolactin levels were low during delayed implantation, increased at the time of implantation and remained elevated during parturition

and lactation. This indicated that prolactin may be required for terminating delayed implantation and for luteal maintenance.

CHAPTER 4

EXPERIMENTAL MANIPULATION OF IMPLANTATION IN FEMALE *Miniopterus schreibersii*

INTRODUCTION:

Extensive experimental work has been done on the hormonal control of delayed implantation in small mustelid carnivores, such as the spotted skunk (Foresman & Mead, 1974; Mead, 1981; Berria *et al.*, 1989), the European badger (Canivenc & Bonnin, 1981) and the mink (Martinet *et al.*, 1981). In most mammals that make use of delayed implantation, implantation was found to coincide with activation of the corpus luteum, elevated plasma prolactin and progesterone levels and in some cases increasing plasma luteinizing hormone levels. In female long-fingered bats, mammotropes (present study) and gonadotropes (Bernard *et al.*, 1991) show an increase in activity towards the end of delayed implantation, coinciding with elevated plasma prolactin (present study), progesterone and luteinizing hormone concentrations (Bernard *et al.*, 1991).

In this chapter the effect of prolactin, human chorionic gonadotropin (a luteinizing

hormone analog) and bromocryptine (a dopamine agonist that inhibits prolactin synthesis in mammals) on delayed implantation is examined in the female long-fingered bats.

MATERIALS & METHODS:

Pregnant female bats were collected one day prior to the start of the experiments and kept under natural photoperiod in an outside flight cage, which contained two roost boxes. The bats were fed daily by hand on mealworm larvae and were given water.

Experiment 1. Effect of prolactin in early delayed implantation: Thirty female bats were collected on the 5th of May 1991 of which twenty received daily subcutaneous injections between the shoulder blades for 10 days. Ten specimens received 0.01 mg ovine prolactin (Sigma, St Louis, USA) in 0.1 ml of saline per day, ten were injected with 0.1 ml of saline only, and ten served as untreated controls.

Experiment 2. Effect of human chorionic gonadotropin (hCG) and prolactin in early delayed implantation: Treatment with prolactin was a repetition of the previous experiment to increase the sample size for the experiment. Twenty-four female bats were collected on the 3rd of May 1992, of which eight received 10 I.U. of hCG (Pregnyl, Donmed Pharmaceuticals, South Africa) in 0.1 ml saline per day for 10 days, eight received 0.01 mg ovine prolactin in 0.1 ml saline per day and eight served as untreated controls. Saline controls were omitted because in the previous experiment no significant difference in progesterone concentrations or development of the embryo could be seen

between the saline and untreated controls. Furthermore the results of the prolactin experiments in 1991 and 1992 were pooled as no significant difference between the plasma progesterone concentrations were found between the controls and between the experimental bats.

Experiment 3. Effect of prolactin in late delayed implantation: Sixteen bats were collected on the 19th of July 1990, of which eight received daily injections of 0.01 mg prolactin (Sigma, St Louis, USA) per day for seventeen days and eight served as untreated controls. The experiment was terminated at the time of normal implantation.

Experiment 4. Effect of bromocryptine (dopamine agonist) in late delayed implantation: Sixteen female bats were collected on the 21st of August 1992 (no bats were available just prior to implantation, as the bats moved to an unknown location and only returned at the end of August), of which eight received 0.05 mg of 2 bromo-α-ergocryptine (Sigma, St Louis, USA) in 0.1 ml saline per day for eight days and eight served as untreated controls.

At the end of each experiment the bats were sacrificed by asphyxiation with CO_2. Blood was taken directly from both ventricles, centrifuged (1600 g for 5 minutes) and the plasma stored at -20°C for progesterone (experiment 1, 2 & 3) and prolactin (experiment 4) radioimmunoassays. The right uterine horn (in which implantation occurs) was removed and prepared for standard light microscopy (Bernard *et al.*, 1991). In addition, for experiment 4, the pituitary gland was removed, prepared for TEM and stained using ICC (as described in chapter 2).

As it was impossible to determine whether the adult females had mated at the time of collection, a few female bats were found to be not pregnant at the end of the experiment, reducing the final sample size (Table 1).

Table 1. Final sample size of pregnant bats used in all experiments:

experiment / treatment	1	2	3	4
+ prolactin	8	5	4	-
+ hCG	-	5	-	-
+ bromocryptine	-	-	-	5
saline controls	7	-	-	-
untreated controls	8	4	2	5

Progesterone assay: Progesterone was measured using a standard competitive radioimmunoassay as previously described (Bernard *et al.*, 1991). Cross reactivities of the progesterone antiserum were 11β-hydroxyprogesterone (53%), 11α-hydroxyprogesterone (25%), 5 pregnane-3-20-one (22%), 17_{β}-hydroxyprogesterone (3%), pregnenolone (2%), 11 deoxycorticosterone (2%), 11 deoxycortisol (2%), 20 hydroxy-4-pregnane-3-one (0.4%), 3 hydroxy-5-pregnane-20-one (0.3%), cortisol (<0.1%), testosterone (<0.001%), Δ-4-

androstenedione (<0.001%), 17β-oestradiol (<0.001%) and oestrone (<0.001%). Sensitivity was 0.31 ng/ml. The intra- and interassay coefficients of variation were <5% and <8% respectively.

Prolactin assay: Prolactin was measured using a standard radioimmunoassay as described in chapter 2.

RESULTS:

The influence of prolactin:

Daily injections of prolactin at the beginning of delayed implantation resulted in significantly elevated plasma progesterone concentrations (6.24 ± 2.44 ng/ml) compared to the controls (2.15 ± 0.30 ng/ml; $P < 0.01$), which in turn were not significantly different from concentrations previously established for delayed implantation (1.67 ± 0.24 ng/ml; $P > 0.05$; Bernard *et al.*, 1991).

All control bats showed the embryo at the bilaminar blastocyst stage with regions of the trophoblast in contact with the intact uterine epithelium (Fig. 11A). Three of the experimental bats (which received exogenous prolactin daily) showed the embryo at the same stage as the control bats. However in another three experimental bats the embryo was further developed and implantation was initiated (Fig. 11B). The yolk sac cavity had swollen and as a result, the trophoblast was in contact with the uterine epithelium throughout the circumference of the uterine lumen (Fig. 11B). Although the uterine epithelium was intact in places, elsewhere it had degenerated and the syncytiotrophoblast was proliferating and invading the maternal tissue. The endometrium appeared stratified.

In the remaining seven experimental bats implantation had progressed even further, the primitive amnion had degenerated and the embryonic plate had straightened out.

Daily injections of prolactin at the end of delayed implantation increased plasma progesterone concentrations to 4.40 ± 0.53 ng/ml, which was significantly higher than the controls (2.45 ± 0.07 ng/ml; $P < 0.01$).

Implantation in the control bats was at a very early stage (Fig. 11C): the columnar epithelium of the uterus was still present and the endometrial cells were cuboidal. The embryo was at the bilaminar blastocyst stage, the primitive amnion was present and the embryonic plate was highly curved. In the experimental bats implantation was more advanced and the embryo more developed (Fig. 11D). The columnar epithelium of the uterus had degenerated, the trophoblast had begun to invade the endometrium and the endometrial cells were stratified. In the embryo, the primitive amnion had degenerated and the embryonic plate had straightened out.

The influence of hCG:

Daily treatment with hCG significantly increased plasma progesterone levels in the experimental bats (40.42 ± 11.11 ng/ml) compared to the controls (2.15 ± 0.30 ng/ml; $P < 0.01$). However, in all bats the embryo was at the free floating bilaminar blastocyst stage, thus hCG did not initiate implantation.

The effect of bromocryptine:

As the collection date of the bats was very late (towards the end of August), implantation had already started to take place. However bromocryptine had a significant

Figure 11. Light micrographs of sections through the uterus of female *Miniopterus schreibersii.*

A. Section through the uterus of a control bat during early delayed implantation (May), showing a free floating bilaminar blastocyst. E - entoderm; T - trophoblast; B - blastocyst. Scale bar = 300 µm.

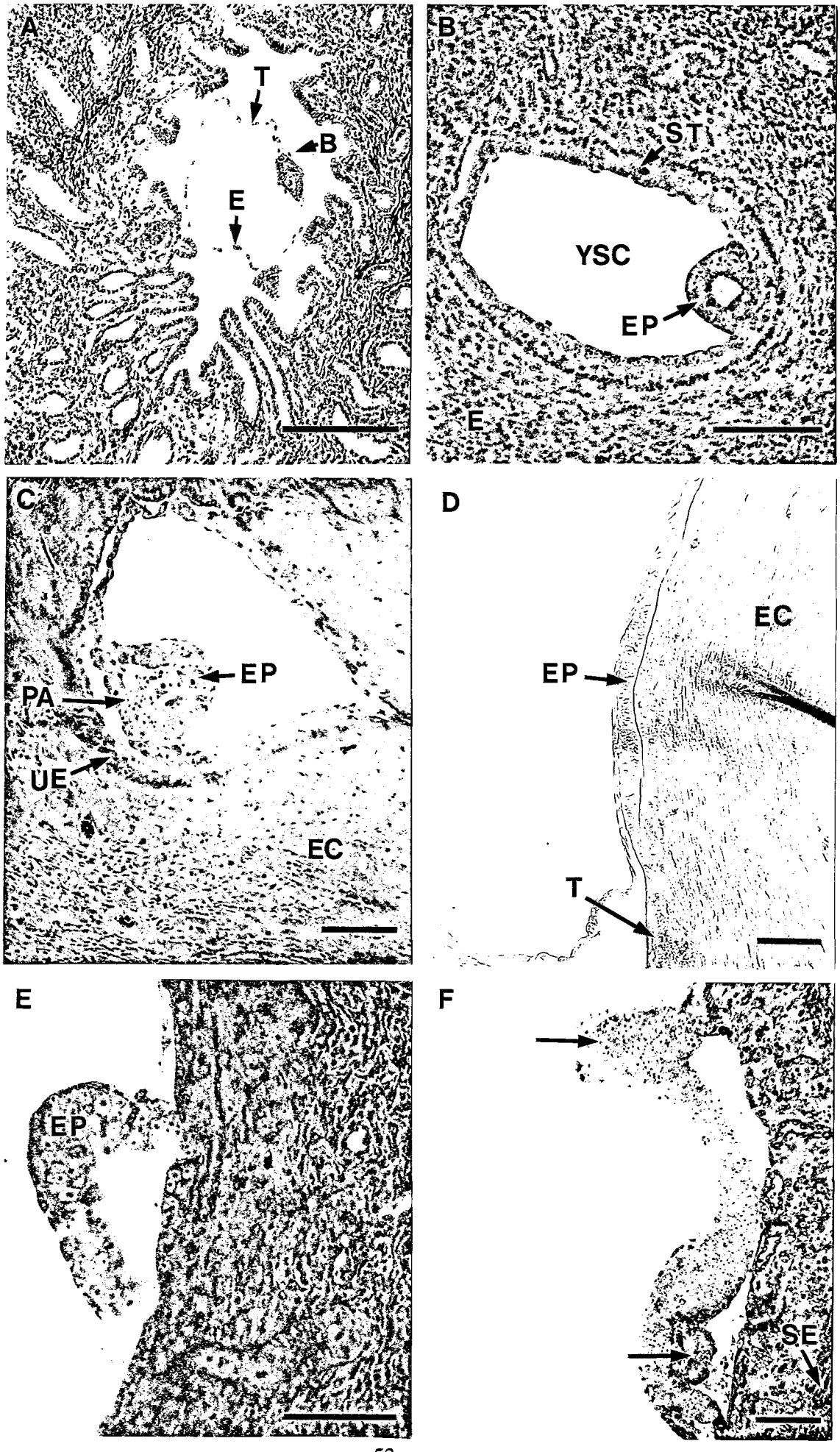
B. Section through the uterus of an experimental bat (+ prolactin) during early delayed implantation (May). Implantation had started, but the embryonic plate (EP) was still highly curved. E - endometrium. ST - syncytiotrophoblast. YSC - yolk sac cavity. Scale bar = 200 µm.

C. Section through the uterus of a control bat at the time of normal implantation (August). The uterine epithelium (UE) and the primitive amnion (PA) were still present, the endometrial cells (EC) were still cuboidal, and the embryonic plate (EP) was highly curved. Scale bar = 300 µm.

D. Section through the uterus of an experimental bat (+ prolactin) during late delayed implantation (August), showing the embryo at a far more advanced developmental stage. The trophoblast (T) had started to burrow into the endometrium, the endometrial cells (EC) were stratified and the embryonic plate (EP) had straightened out. Scale bar = 200 µm.

E. Section through the uterus of an experimental bat (+ bromocryptine) at implantation (end of August). Implantation was still at a very early stage, seen by the highly curved embryonic plate (EP). Scale bar = 200 µm.

F. Section through the uterus of a control bat during normal embryonic development (end of August), showing the advanced development of the embryo. SE - stratified endometrium; arrows - folds of somatopleure. Scale bar = 300 µm.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effect on the pituitary and plasma prolactin levels and on implantation.

Prolactin immunoreactivity was significantly reduced in the bromocryptine treated bats (2.37 ± 0.03 gold probes/granule) in comparison to the control bats (3.45 ± 0.25 gold probes/granule; $P < 0.01$). The abundance of labelled secretory granules was lower in the experimental bats (6.37% ± 0.39), although not significantly different from the control bats (9.68% ± 1.73; $P > 0.05$). Furthermore, plasma prolactin levels were significantly lower in the experimental group (50.91 ± 4.82 ng/ml) than in the controls (81.16 ± 4.76 ng/ml; $P < 0.01$).

Implantation had been initiated in both the control and experimental groups. However implantation in the bromocryptine treated bats was at a very early stage (Fig. 11E), with the embryonic plate still being highly curved. In the control bats the embryo had developed much further (Fig. 11F), and amniogenesis had started to occur by the upgrowth of lateral folds of somatopleure.

DISCUSSION:

Treatment of female bats with exogenous prolactin during early delayed implantation, when endogenous LH concentrations are low, resulted in increased plasma progesterone concentrations and initiation of implantation. The developmental stage of the embryo after ten days of prolactin treatment during early delayed implantation was similar to the developmental stage in August at normal implantation. These findings are in accordance with results of experiments on mustelids that make use of delayed implantation, where prolactin terminates the period of delayed implantation (ferret: Murphy, 1979; mink:

Papke *et al.*, 1980, Martinet *et al.*, 1983 and Murphy *et al.*, 1981; spotted skunk: Berria *et al.*, 1989 and Kaplan *et al.*, 1991).

In addition, treatment with bromocryptine (which inhibits prolactin synthesis) prevents the normal increase of plasma prolactin concentrations, and retards blastocyst implantation, thus identifying prolactin as the sole factor which initiates the activity of the corpus luteum and blastocyst implantation in the ferret (Agu *et al.*, 1986), the mink (Murphy *et al.*, 1981) and the skunk (Berria *et al.*, 1989). However, Shaban & Terranova (1986) found that in the rat bromocryptine inhibits luteinizing hormone as well as prolactin secretion and increasing plasma LH concentrations have been observed during the second half of delayed implantation in the spotted skunk (Foresman & Mead, 1974), the mink (Martinet *et al.*, 1981), the ferret (Donovan, 1967), and in the long-fingered bat, *Miniopterus schreibersii* (Bernard *et al.*, 1991). This suggests that LH might also play a role in terminating delayed implantation. However, although treatment of female long-fingered bats with hCG during early implantation significantly increased plasma progesterone levels, it failed to initiate implantation (present study). Thus LH does not play a role in initiating implantation, and increasing pituitary and plasma LH levels in *Miniopterus schreibersii* in August and September probably indicate that LH is part of the luteotropic complex, as has also been reported in the mink (Martinet *et al.*, 1981).

Therefore, as in the mustelids, prolactin alone seems to be the hormone responsible for terminating delayed implantation in the long-fingered bat, *Miniopterus schreibersii.*

It is still unclear how prolactin acts to initiate implantation but it could be important in stimulating the synthesis and secretion of a still unidentified luteal protein that is necessary in combination with progesterone to initiate implantation (Mead *et al.*, 1988). This protein seems to be essential as subcutaneous injections of progesterone failed to

initiate implantation in *Miniopterus schreibersii* (Bernard & Bojarski, in press), the ferret (Mead *et al.*, 1988), the spotted skunk (Mead, 1981) and the northern fur seal (Daniel, 1981).

Mead (1981) questioned the role of prolactin in implantation in the European badger, as this species (exhibiting a post-partum oestrus) possesses unimplanted blastocysts in the uterus during lactation, when plasma prolactin concentrations are high. However the length of lactation is only about four months, coinciding with the early part of delayed implantation (Canivenc & Bonnin, 1981). During this time plasma LH levels are low and the corpus luteum is inactive. This could suggest that in the European badger both LH and PRL together are needed to activate the corpus luteum and that high prolactin levels alone are not effective.

Furthermore hormones act on receptor sites which have to be either formed or activated (Rillema *et al.*, 1988). Using a homologous radioreceptor assay, prolactin receptors have been localised in the endometrium of the porcine uterus (Young & Bazer, 1989), and they also have been identified on rat (Shirota *et al.*, 1990) and mink ovary (Rose *et al.*, 1986). Prolactin receptor sites have been shown to alter their numbers under a variety of physiological conditions, which correlate with functions of prolactin. Seasonal changes in the concentration of testicular receptors for prolactin have been observed in male rodents, in which during testicular regression in winter the numbers of these receptors were very low and increased gradually at the beginning of spring (Fuentes *et al.*, 1993). In female mink, prolactin receptors on the ovaries increase significantly during embryonic diapause, but their concentration decreases soon after plasma prolactin levels increase and the blastocyst becomes reactivated (Rose *et al.*, 1986). However since the receptor assay, which was used, detects only free or unbound sites, the reduction of prolactin receptor sites

is probably due to endogenous prolactin occupying these sites. As prolactin is a luteotropin in the mink (Sundqvist *et al.*, 1989), prolactin receptor sites should increase and a five fold increase was found during early pregnancy (Rose *et al.*, 1986), thus the apparent reduction in receptor sites is not real. It is thus quite possible that suitable receptor sites for prolactin are not functional in early delayed implantation in the European badger and consequently the high plasma prolactin concentrations do not initiate implantation.

Hormone concentration is another important factor, as a too dilute or too concentrated hormone might not elicit a response from the receptor sites. Plasma prolactin levels during lactation in the European badger might not be at the optimal concentration to initiate implantation, as prolactin levels are much higher (9.5 - 16.5 ng/ml) than at implantation (4.5 ng/ml; Maurel *et al.*, 1989).

SUMMARY:

Results from the experimental manipulation of delayed implantation in the long-fingered bat, *Miniopterus schreibersii*, showed that prolactin in early and late delayed implantation resulted in increased plasma progesterone concentrations and early implantation, while bromocryptine suppressed pituitary and plasma prolactin concentrations and retarded development. Although hCG caused elevated plasma progesterone concentrations, it did not influence the unimplanted blastocyst. It is concluded from these results that prolactin is the pituitary hormone responsible for terminating delayed implantation at the end of winter.

CHAPTER 5

GENERAL DISCUSSION & CONCLUSION

Prolactin has numerous important physiological functions and in mammals it is one of the main lactogenic hormones (Tucker, 1988), sometimes being involved in the maintenance of the corpus luteum (Niswender & Nett, 1988; Szafranska & Tilton, 1993). The elevated plasma prolactin levels during post-implantational development and lactation in *Miniopterus schreibersii* suggest that in this species prolactin may be part of the luteotropic and lactogenic complex. Furthermore an increase in pituitary and plasma prolactin levels during the second half of delayed implantation indicates that prolactin is probably involved in terminating the period of delay. This is supported by experiments, where exogenous prolactin during early delayed implantation initiated implantation, and treatment with bromocryptine (which inhibits prolactin synthesis) during late delayed implantation retarded implantation. Similarly, prolactin initiates implantation in small mustelid carnivores such as mink (Sundqvist *et al.*, 1989) and spotted skunk (Berria *et al.*, 1989), that make use of delayed implantation.

Mammotropes in the anterior pituitary of *Miniopterus schreibersii* were secretorily

inactive and plasma prolactin levels were low during follicular development and the first half of delayed implantation, which coincided with winter. Wimsatt (1960) suggested that the low metabolic rate during hibernation may be responsible for reduced secretory activity or inactivity of the anterior pituitary and consequently the reproductive delay. However colonies of *Miniopterus schreibersii* in the Eastern Cape do not hibernate throughout winter (Bernard & Bester, 1988). As temperatures during winter are mild (mean temperatures reported for July range between a minimum of 7°C and a maximum of 19°C; Bernard 1989), the bats undergo short periods of torpor during particularly cold periods only (Bernard & Bester, 1988). Thus a low metabolic rate can not be responsible for the control of the reproductive delay in *Miniopterus schreibersii.*

Studies on delayed implantation in mustelid carnivores have indicated that inadequate prolactin secretion (Martinet *et al.*, 1983) and high levels of melatonin during winter may be responsible for preventing implantation (Murphy & James, 1974; Sundqvist *et al.*, 1989). Melatonin implants inhibit prolactin secretion, thus delaying implantation in mink (Martinet *et al.*, 1983; Murphy *et al.*, 1990) and spotted skunk (Kaplan *et al.*, 1991). Melatonin exerts its control via hypothalamic dopamine (Rao & Mager, 1987) and treatment with dopamine inhibits prolactin secretion in mink (Sundqvist *et al.*, 1989), ferret (Agu *et al.*, 1986) and wallaby (Pearce-Kelly *et al.*, 1992), while lesions to the anterior hypothalamus in the spotted skunk remove the inhibitory control on the pituitary and prolactin secretion increases (Kaplan *et al.*, 1991). Photoperiod has been established to be the proximate cue which terminates the period of delayed implantation in small carnivores (Duby & Travis, 1972; Murphy & James, 1974; Canivenc & Bonnin, 1979 & 1981; Renfree *et al.*, 1981; Murphy *et al.*, 1981 & 1990; Temte, 1985; Berria *et al.*, 1989; Boyd, 1991), whereby an increase in daylength lowers melatonin production from the pineal

gland. This decreases dopamine secretion from the hypothalamus, thus removing the block on the pituitary and the mammotropes synthesise and secrete prolactin, which is required to initiate implantation.

The data presented in the present study suggest that the control of delayed implantation in *Miniopterus schreibersii* is similar to that established for the mustelid carnivores. In all members of the genus *Miniopterus* from temperate latitudes implantation takes place when daylength is ten to twelve hours and increasing, and increasing daylength is thought to be the environmental stimulus that initiates implantation (Racey, 1982 for review). The reduced secretory activity of mammotropes and low plasma prolactin levels in winter (present study) are probably associated with elevated plasma melatonin levels caused by the short photoperiod. It is suggested that increasing daylength would lower melatonin levels, reduce hypothalamic dopamine secretion and remove the block on prolactin synthesis by the mammotropes. The results from the present study clearly show that endogenous pituitary and plasma prolactin concentrations increase shortly after the winter solstice (21 June), and the hormone manipulation experiments have shown that prolactin is necessary for implantation to occur.

As *Miniopterus schreibersii* is nocturnal and cave dwelling, the pineal has to be extremely sensitive to low light levels, to perceive changes in daylength. There are no published reports on the light sensitivity or photoresponsiveness of the bat pineal, however preliminary experiments testing the light sensitivity of the pineal of *Rhinolophus capensis* (occurring in sympatry with *Miniopterus schreibersii* in the Eastern Cape) showed that melatonin synthesis was inhibited under very low light levels (photosafe red light: 1.87 lux at one meter [Bernard & Carter, pers. comm.]).

In conclusion, the pattern of change in pituitary and plasma prolactin levels, and the results of the hormone manipulation experiments suggest that prolactin is the pituitary hormone that terminates delayed implantation. In view of the established link between daylength and prolactin secretion, it is likely that increasing daylength is the environmental cue that initiates implantation in *Miniopterus schreibersii*.

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APPENDICES

Appendix 1. Protocol for immunogold labelling with silver enhancement for light microscopy:

SOLUTION	TIME (minutes)
Xylene	3x2
100% Ethanol	2x2
95% Ethanol	2
70% Ethanol	2
PBS (pH 7.3)	2x5
0.2 M glycine in PBS (pH 7.3)	5
Blocking solution[1]	30
Washing buffer[2]	5
1/600 anti-oPRL[3]	60
Washing buffer	3x10
1/40 GAR IgG-colloidal gold[4] (1 nm)	180
Washing buffer	3x10
PBS (pH 7.3)	3x5
2.5% Glutaraldehyde in PBS (pH 7.3)	10
Distilled water	2x5
Initiator & Enhancer solution[5]	6-18
Distilled water	2x5
Mount using water-soluble mounting medium	

1. Phosphate Buffered Saline (PBS) (pH 7.3), containing 0.8% w/v Bovine Serum Albumin (BSA), 0.1% v/v IGSS Gelatin, 0.1% v/v Triton X, 5% v/v Normal Goat Serum (NGS) and 0.01% w/v Sodium Azide.

2. PBS (pH 7.3), containing 0.8% w/v BSA, 0.1% v/v IGSS Gelatin, 0.1% v/v Triton X and 0.01% w/v Sodium Azide.

3. The antibody was diluted in PBS (pH 7.3), containing 0.1% BSA and 0.01% Sodium Azide.

4. The colloidal gold was diluted in PBS (pH 8.2), containing 0.1% BSA.

5. Reagents A & B of the IntenSE M kit (Amersham, UK).

Appendix 2. Protocol for immunogold labelling for electron microscopy:

SOLUTION	TIME (minutes)
1/10 Normal goat serum	10
1/10000 anti-oPRL[1]	120
Tris buffer (pH 7.3)	4x1
Tris buffer (pH 7.3) + 0.1% BSA	4x1
Tris buffer (pH 8.2) + 0.1% BSA	4x1
1/10 Normal goat serum	10
1/50 GAR IgG-colloidal gold[2] (20 nm)	120
Tris buffer (pH 7.3) + 0.1% BSA	4x1
Tris buffer (pH 7.3)	4x1
Distilled water	4x1
2% Methanolic uranyl acetate	5
Distilled water	4x2

1. The antibody was diluted in TRIS (pH 7.3), containing 0.1% Bovine Serum Albumin (BSA) and 0.01% Sodium Azide.

2. The colloidal gold was diluted in TRIS (pH 8.2), containing 0.1% BSA.

Appendix 3. Immunogold double-labelling protocol:

A. Double-labelling using Protein A-colloidal gold:

SOLUTION	TIME (minutes)
PBS (pH 7.3) + 10% FCS + 0.12% glycine	15
1/2000 anti-mGH[1]	60
PBS (pH 7.3) + 0.12% glycine	4x4
1/50 Protein A-colloidal gold[1] (6nm)	60
PBS (pH 7.3) + 0.12% glycine	6x4
4% Paraformaldehyde[2]	5
Phosphate Buffered Saline (PBS; pH 7.3)	2x4
PBS (pH 7.3) + 0.12% glycine	2x4
1/10000 anti-oPRL[1]	60
PBS (pH 7.3) + 0.12% glycine	4x4
1/50 Protein A-colloidal gold[1] (9nm)	60
PBS (pH 7.3) + 0.12% glycine	6x4
Distilled water	5x2
2% Methanolic uranyl acetate	5
Distilled water	5x2

1. The antibodies and colloidal gold probes were diluted in 5% fetal calf serum (FCS) and 0.12% glycine.

2. Paraformalde renders the Protein A gold probe (6 nm) inert by fixing it, thus preventing any crossreactions with the second Protein A gold probe (9 nm).

B. Double-labelling using GAM- and GAR-colloidal gold:

SOLUTION	TIME (minutes)
1/10 Normal goat serum	10
1/2000 anti-mGH[1]	120
Tris buffer (pH 7.3)	4x1
Tris buffer (pH 7.3) + 0.1% BSA	4x1
Tris buffer (pH 8.2) + 0.1% BSA	4x1
1/10 Normal goat serum	10
1/50 GAM IgG-colloidal gold[2] (10nm)	120
Tris buffer (pH 7.3) + 0.1% BSA	4x1
Tris buffer (pH 7.3)	4x1
1/10 Normal goat serum	10
1/10000 anti-oPRL[1]	120
Tris buffer (pH 7.3)	4x1
Tris buffer (pH 7.3) & 0.1% BSA	4x1
Tris buffer (pH 8.2) & 0.1% BSA	4x1
1/10 Normal goat serum	10
1/50 GAR IgG-colloidal gold[2] (20nm)	120
Tris buffer (pH 7.3) & 0.1% BSA	4x1
Tris buffer (pH 7.3)	4x1
Distilled water	4x1
2% Methanolic uranyl acetate	5
Distilled water	4x2

1. The antibodies were diluted in TRIS buffer (pH 7.3), containing 0.1% Bovine Serum Albumin (BSA) and 0.01% Sodium Azide.

2. The colloidal gold probes were diluted in TRIS buffer (pH 8.2), containing 0.1% BSA.

Appendix 4. Solutions and buffers used in SDS-PAGE:

A. Sample buffer:

Sodium lauryl sulphate (SDS)	5.0 g
Mercaptoethanol (ME)	5.0 ml
Glycerol	7.5 ml
1.0 M Tris-HCl (pH 6.8)	6.3 ml
Distilled water	31.2 ml

B. Dissociation buffer

SDS	5.0 g
ME	5.0 ml
Glycerol	7.5 ml
1.0 M Tris-HCl (pH 6.8)	6.3 ml
Distilled water	28.7 ml
Bromophenol blue (0.2%)	2.5 ml

C. Resolving gel (12%)

30% Acrylamide:bis stock solution	32.0 ml
1.0 M Tris-HCl (pH 8.8)	30.0 ml
Distilled water	13.2 ml
10% SDS	0.8 ml
1.5% fresh ammonium persulphate	4.0 ml
Temed	10 µl

D. Stacking gel (4%)

30% Acrylamide:bis stock solution	2.0 ml
1.0 M Tris-HCl (pH 6.8)	1.9 ml
Distilled water	9.5 ml
80% Glycerol	1.0 ml
10% SDS	0.2 ml
1.5% fresh ammonium persulphate	0.7 ml
Temed	20 µl

E. Staining solution

Methanol	45.0 ml
Glacial acetic acid	10.0 ml
Coomassie brilliant blue	0.4 g
Distilled water	45.0 ml

F. Destaining solution

Methanol	45.0 ml
Glacial acetic acid	10.0 ml
Glycerol	1.0 ml
Distilled water	44.0 ml

Appendix 5. Solutions and buffers used for the RIA:

A. Basic buffer:

$NaH_2PO_4.2H_2O$	6.2 g
Sodium Chloride	9.0 g
EDTA (Di-Sodium Salt)	3.7 g
Sodium Azide	1.0 g
Distilled Water	1.0 l

B. Gel-PBS (RIA buffer):

Basic buffer	1.0 l
Gelatin	1.0 g
Triton X	1.0 ml

C. Iodination of prolactin:

i. To 5 µg Prolactin add:
ii. 20 µl Phosphate Buffer
iii. 10 µl ^{125}I (1.0 mCi)
iv. 10 µl Chloramine-T (5.7 mg Chl-T in 10 ml $PO_{4\text{-}B}$uffer)
v. Mix for 5 seconds
vi. Add 200 µl 1% Potassium Iodide
vii. Add directly to column