IMMUNOLOCALISATION OF, AND ULTRASTRUCTURAL CHANGES IN THE LH GONADOTROPES OF *Miniopterus schreibersii* and *Rhinolophus capensis* (MAMMALIA: CHIROPTERA) IN RELATION TO THEIR REPRODUCTIVE CYCLES

Thesis

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ERRATA SHEET

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ABSTRACT

LH gonadotropes are successfully identified in the anterior pituitary of *Miniopterus schreibersii* and *Rhinolophus capensis*, using immunogold labelling at the light and electron microscopy level. The gonadotropes are oval to polygonal in shape and possess numerous small secretory granules, which contain LH throughout the year. Their distribution is similar in both species, although the abundance varies slightly between species and sexes. Seasonal changes are detected in gonadotrope ultrastructure, pituitary LH levels, and plasma hormone levels, and activity pattern of LH gonadotropes generally coincide with reproductive activity in both bat species.

In female *M. schreibersii* gonadotrope activity, and high pituitary LH, and plasma LH levels coincide with follicular development, implantation and gestation. During the period of delayed implantation gonadotropes are inactive and plasma LH levels are low, coinciding with corpus luteum inactivity and low plasma progesterone levels. Implantation coincides with increased activity of the gonadotrope activity, increased plasma LH levels, reactivation of the corpus luteum and elevated plasma progesterone levels. Activation of LH gonadotropes towards the end of hibernation may be initiated by the winter solstice, which marks the change to increasing daylength.

In female *R. capensis* gonadotrope activity and high pituitary and plasma LH levels occur towards the end of follicular development. During hibernation gonadotrope activity and plasma LH levels decrease. Ovulation coincides with increasing

gonadotrope activity (which follows the winter solstice), although a preovulatory peak in plasma LH is not detected.

In male *M. schreibersii* and *R. capensis*, gonadotrope activity and high plasma LH and testosterone levels coincide with spermatogenesis, except during a period of reproductive inactivity in spring in male *M. schreibersii*, where gonadotropes appear active and plasma LH and testosterone levels are high. The reason for this apparent activity is not known. Male sperm storage during hibernation in male *R. capensis* coincides with low gonadotrope activity and low plasma LH and testosterone levels. Factors initiating gonadotrope activity and hence spermatogenesis are probably decreasing daylength (summer solstice) in *M. schreibersii* and increasing ambient temperatures and food abundance (following the hibernation period) in *R. capensis*.

CHAPTER 1: GENERAL INTRODUCTION

Bats, being one of the largest, most widely distributed groups of mammals (Hill & Smith, 1984), show a wide variety of reproductive patterns, which are linked to their environment. Some tropical bats show aseasonal breeding, due to reduced seasonal fluctuations in climate and food availability (Myers, 1977). However in temperate climates, reproduction is strictly seasonal (Racey, 1982). Most bats form temperate latitudes belong to the families Rhinolophidae and Vespertilionidae and it is only members of these families that can hibernate (Gustafson, 1979). Hibernation restricts reproduction to the summer months and this period is too short to accommodate a complete reproductive cycle (Bernard, 1989). To overcome this problem, hibernating bats make use of one of the reproductive delay phenomena, where gametogenesis occurs before the hibernation period, and during winter either ovulation or implantation is delayed. At the end of winter ovulation or implantation occurs and this is followed by a four month gestation period and the young are born during summer, when food is abundant (Bernard, 1989). The two bat species examined in this study are the long-fingered bat, *Miniopterus schreibersii* (family: Vespertilionidae), and the Cape horseshoe bat *Rhinolophus capensis* (family: Rhinolophidae), which make use of delayed implantation, and delayed ovulation and male sperm storage respectively (Richardson, 1977; Bernard, 1985).

Reproduction in mammals is controlled by hormones (luteinizing hormone, follicle stimulating hormone and prolactin) which are secreted from the anterior pituitary (Anthony, 1987). In this study I examined the role of luteinizing hormone (LH), which in the male

stimulates the Leydig cells to produce testosterone, and in the female activates the thecal cells to produce oestrogen precursors and initiates ovulation and development of the corpus luteum.

The hormonal control of delayed implantation has mainly been studied in small carnivores (Canivenc & Bonnin, 1981; Mead, 1981; Berria *et al.*, 1989; Sundqvist *et al.*, 1989), but is still poorly understood and very little work has been done in bats. A common feature in female mammals is, that during the delay, the corpus luteum is inactive and plasma progesterone levels are low (Mead, 1981). Since corpus luteum activity appears to be dependent on LH from the anterior pituitary, a study of seasonal changes in LH gonadotrope activity is an essential part in our overall understanding of the hormonal control of delayed implantation. The hormonal control of delayed ovulation has been examined in *Myotis lucifugus*. During the delay, a single Graafian follicle is present in the ovary, but ovulation does not occur until arousal from hibernation in spring (Wimsatt, 1960; Anthony, 1987), and this is possibly due to the absence of sufficient LH to initiate ovulation prior to the hibernation period. Hence a study of LH gonadotrope activity may indicate a possible role of LH in the hormonal control of delayed ovulation. In the males of both species, the ultimate interest is in the cues that initiate the onset of spermatogenesis. Since LH, through its action on the Leydig cells, is responsible for spermatogenesis, a study of seasonal changes in LH gonadotrope activity will bring us closer to determining the time during which external factors initiate the reproductive cycle.

The aims of this study are therefore:

1. To identify the LH gonadotropes in the anterior pituitary of

Miniopterus schreibersii and *Rhinolophus capensis*.

2. To monitor seasonal, ultrastructural changes in the gonadotropes in both male and female bats of both species.

3. To relate these seasonal changes to the reproductive cycles of *M. schreibersii* and *R. capensis*, in particular to the hormonal control of delayed implantation and delayed ovulation respectively.

CHAPTER 2: MATERIALS AND METHODS

Collection sites and sampling of bats

A minimum of 3 female and 3 male Schreibers' long-fingered bats (*M. schreibersii*) were collected on a monthly basis from June 1988 to May 1989 (Table 1) from Maitland Mines in the Eastern Cape Province of South Africa (33° 58' S, 25° 17' E). Additional specimens of *M. schreibersii* were collected from the stormwater drains in Grahamstown (33° 19' S, 26° 32' E). Cape Horseshoe bats (*R. capensis*) were collected on a monthly basis from May 1988 to April 1989 from a tunnel on Table Farm (33° 17' S, 26° 25' E), close to Grahamstown. Four males were collected each month, however, due to a depletion of the colony and a change in the sex ratio during spring and summer, female *R. capensis* were not always available (Table 1).

The specimens were killed by asphyxiation with CO_2, weighed and the forearm length was measured. Bats with a body weight greater than 9.0 or 11.0 grams and a forearm longer than 44 or 48 mm for *M. schreibersii* and *R. capensis* respectively, and with blunted teeth were assumed to be adults.

Removal of pituitary gland

The bats were decapitated and to ensure rapid fixation the pituitary glands were initially fixed, by injecting either 10% neutral buffered formalin or 0.1% glutaraldehyde and 3% paraformaldehyde (for light and electron microscopy respectively) into the brain through the foramen magnum using a 1 ml syringe. The

pituitary gland was exposed by removing the lower jaw and the basisphenoid. The tissue was then removed, weighed to the nearest 0.5 grams and prepared for light and electron microscopy. In the embedding procedure for the electron microscope, the pars nervosa and pars intermedia were removed from the anterior pituitary.

Table 1: Summary of the specimens of the Schreibers' long-fingered bat and Cape horseshoe bat that have been used in this study:

MONTH		*M. schreibersii*		*R. capensis*	
		male	female	male	female
MAY	1988	-	-	4	4
JUNE	1988	4	4	4	4
JULY	1988	4	4	4	4
AUGUST	1988	4	4	4	1
SEPTEMBER	1988	4	4	4	1
OCTOBER	1988	4	4	4	1
NOVEMBER	1988	4	4	4	1
DECEMBER	1988	3	3	4	-
JANUARY	1989	3	3	4	-
FEBRUARY	1989	4	4	4	-
MARCH	1989	4	4	4	4
APRIL	1989	4	4	4	4
MAY	1989	4	4	-	-

Light microscopy

The pituitary glands of one male and one female bat per species (about 2 mm long and 1 mm wide) were fixed by immersion in 10% neutral buffered formalin, dehydrated and embedded in paraplast. 5 μm section were cut on a rotary microtome. The sections were dewaxed, hydrated and then stained with either the Mallory-Heidenhain rapid one-step method (Cason, 1950), or a combination of stains, comprising aldehyde fuchsin, periodic acid, Schiff's reagent and orange G (Elftman, 1959). Both staining procedures differentially stain basophils (gonadotropes and thyrotropes) and acidophils (somatotropes and mammotropes) (Humanson, 1967). The slides were viewed under a Nikon microscope and pictures were taken on an Olympus Vanex microscope using an Olympus C 35A camera. In order to positively identify the LH gonadotropes, immunocytochemistry (ICC) using a monoclonal antibody, was applied. Due to the importance of ICC and its methodological complexity, this technique is discussed separately in chapter 3.

Electron microscopy

One anterior pituitary of each sex and species was fixed by immersion in cold (4° C) phosphate buffered (pH 7.3) glutaraldehyde (2.5%) for approximately 28 hours, and secondarily fixed in 1% buffered (pH 7.3) osmium tetroxide (Millonig) for 90 minutes. The tissue was washed in phosphate buffered saline (pH 7.3), dehydrated using increasing alcohol concentrations and

embedded in a mixed embedding medium of Araldite and Agar 100 (Cross, 1989). Ultrathin sections (70 nm) were cut on an LKB 8800 A Ultratome III, and collected on 300 mesh copper grids. The sections were stained with 5% aqueous uranyl acetate (Watson, 1958) for 25 minutes and concentrated lead citrate (Reynolds, 1963) for 5 minutes. The section were examined under a JEOL JEM 100 CX II transmission electron microscope. The tissue was prepared for and stained using ICC also and this is discussed in chapter 3.

Experimental work

Experimental manipulation using luteinizing hormone-releasing hormone (LHRH) was used as an additional means of identifying the gonadotropes, the assumption being that the cell type that responded to the treatment would be the gonadotropes. Using a 1 ml syringe, 0.1 ml of an LHRH solution (2.5 μg of LHRH in 0.5 ml physiological saline, 0.9% NaCl; Shiino, 1982) was injected into the abdomen of reproductively active male *M. schreibersii*. The animals were left for 15, 30 and 60 minutes. Two control animals were included, one recieved no treatment, the other was injected with phosphate buffered saline (pH 7.3) for 15 minutes. The animals were then killed by asphyxiation, the pituitary glands were removed, prepared for EM and stained using ICC.

Blood sampling

Blood (0.15 - 0.30 ml) was collected from the ventricles using a 1 ml syringe, immediately after the specimens were

sacrificed. The samples were centrifuged at 1600 *g* for 5 minutes and the plasma (0.10 - 0.15 ml) was frozen and stored at -20° C, until assayed.

Plasma hormone assays

A. Luteinizing hormone assay: LH was measured in bat plasma samples by conventional double-antibody radioimmunoassay (NIADDK kit) using NIADDK-rLH-RH-2 as standard. Intra- and interassay coefficients of variation were less than 10%. Sensitivity was 0.5 ng/ml.

B. Testosterone assay: Testosterone determinations were performed on extracts of plasma using TRK 402 titrated testosterone (Radiochemical Centre, Amersham, U.K.) and a highly specific antiserum raised against testosterone-3-carboxymethyl- oxime-bovine serum albumin (Millar & Newley, 1976). Cross-reaction with all major naturally-occurring steroids was < 0,1% except for dihydrotestosterone for which it was 5.1%. The intra- and interassay coefficients of variation were 3.1 and 8.4% respectively. Sensitivity was 1.44 ng/ml.

C. Progesterone assay: 0.1 ml of plasma was extracted with 5.0 ml petroleum ether (40 - 60), evaporated to dryness, and reconstituted in 0.8ml benzene of which 0.1 ml aliquots were transferred to borosilicate glass tubes and dried down. 0.1 ml of radioimmunoassay buffer was added along with 0.1 ml of progesterone antiserum 1521 (progesterone-21-bovine serum albumen conjugate; titre 1:20000 final; cross-reactions were 11 β hydroxyprogesterone (53%), 11 α 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%), 17 β estradiol (<0.001%), estrone (<0.001%)) and incubated at room temperature for 30 minutes. 0.1 ml of tritiated progesterone in radioimmunoassay buffer (10,000 cpm, TRK 413, Radiochemical Centre, Amersham) was added and incubated for 1 hour at 37° C. Separation was achieved using dextran-charcoal and the supernatant counted. The intra- and interassay coefficients of variation were less than 10% respectively. Sensitivity was 0.31 ng/ml.

These hormone assays were done in the laboratory of Professor R. P. Millar (Department of Chemical Pathology, Medical School, Observatory, 7925, Cape Town) by his staff.

Morphometric analyses

A. The abundance of LH gonadotropes was estimated from one midsaggital, immunogold stained light microscope section of each pituitary gland per month. The areas of the whole pituitary (P) and positively stained gonadotropes (G) were measured using a digitizing tablet and the Sigma Scan software package, a scientific measurement system. The percentage of the total area of the pituitary, occupied by LH gonadotropes was calculated using the following formula: %G=(G/P)x100, and was taken as an indication of LH gonadotropes abundance.

B. The abundance of positively stained granules present in the cytoplasm of LH gonadotropes for 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 gonadotrope. The procedure was repeated for each pituitary gland (one pituitary of each sex and species per month) in which 10 LH gonadotropes were examined, each time recording the number of hits (Pg). The abundance (A in %) of positively stained secretory granules in LH gonadotropes was then calculated using the following formula: $A=(Pg/Pt)x100$.

C. Changes in the amount of luteinizing hormone 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 granules in 10 LH gonadotropes for each pituitary gland during the year, and changes in the abundance of gold particles are taken as a measurement of changes in pituitary LH content.

Statistical analysis

Where suitable, Pearson's correlation coefficient and one way analyses of variance have been used to compare the data obtained from the morphometry and from the measurement of bat weight, pituitary weight and forearm length.

CHAPTER 3: IMMUNOCYTOCHEMISTRY (ICC)

Introduction

Immunocytochemistry, which originated with A.H. Coons in 1941 (Polak & Van Noorden, 1987), is an important and rapidly developing staining method that has many fields of application, such as histopathology (Burns, 1982), endocrinology and cell biology (Polak & Van Noorden, 1987). The principle involved in ICC, is the detection of biological substances (antigens, e.g. pituitary hormones) using specific primary antibodies, which attach to the particular antigen. This antigen-primary antibody complex may be visualised in a number of ways using fluorescence, a colour reaction between the enzyme peroxidase and a substrate, and electron dense colloidal gold (Burns, 1982). Such markers may be attached to the primary antibody or a secondary antibody which will recognise the antigen-primary antibody complex. In the case of the anterior pituitary gland, ICC can be used to specifically identify the different hormone secreting cell types (Petrusz & Ordronneau, 1983).

Tissues may be stained by ICC either before or after embedding, both methods having advantages for the detection of a particular antigen. Pre-embedding labelling is mainly used for the detection of solvent labile antigens (e.g. steroids), and overcomes the problem of hormone extraction by alcohols during the embedding process (Franklin, 1982). Some advantages of post-embedding labelling are that the antibody does not have to penetrate the tissue blocks, and it allows the localisation of two different antigens by staining adjacent sections from the same block (Ordronneau, 1982).

In the present study the post-embedding labelling procedure is employed at both LM and TEM level. Initially, the unlabelled antibody peroxidase-anti-peroxidase method (PAP; Sternberger, 1969) was used, which yielded unsatisfactory results, for reasons that are discussed later in this chapter. Following this, immunogold labelling (IG; Faulk & Taylor, 1971) was applied.

PAP labelling method

This ICC labelling method involves the attachment of an unlabelled primary antibody to the antigen in the tissue. The enzyme peroxidase-anti-peroxidase, raised in the same host as the primary antibody, binds indirectly to the primary antibody-antigen complex using, as a bridge, a secondary antibody (Burns, 1982). The addition of standard peroxidase substrates (e.g. diaminobenzidene), which are activated using hydrogen peroxide, render the antibody-peroxidase complex visible. The endproduct is a particular colour, depending on the substrate used, and is the product of a reaction between the enzyme peroxidase and the substrate. For electron microscopy the electron density of the substrate can be further enhanced by the use of metal compounds (e.g. hydrogen gold chloride), which bind to the substrate (Fairen *et al.*, 1977; Siegesmund *et al.*, 1979; Newman *et al.*, 1983). Some tissues contain endogenous peroxidase, which will react with the substrate, to give false positive results. This can be avoided by blocking the peroxidase molecules in the tissue, using for example hydrogen peroxide, leaving no free peroxidase to react with the substrate.

IG labelling method

This method comprises two main steps. Firstly, the attachment of the primary antibody to the antigen, and secondly, a secondary antibody conjugated with colloidal gold particles, binds to the primary antibody (Polak & Van Noorden, 1987). Different sizes of gold particles are available (normal range: 1 - 30 nm), but even the larger particle size is too small to be detected at the light microscope level. Hence the signal of the label is enhanced by the addition of silver particles, which bind to the secondary antibody conjugated colloidal gold particles, and result in a larger structure which can be seen with a light microscope (Holgate *et al.*, 1983). The choice of gold probe size plays a role in the labelling density. Large gold probes (20 - 30 nm), which can be easily detected at lower magnifications, are affected by steric hinderance, resulting in a low density labelling. This problem does not occur with small gold probes (1 - 5 nm), which produce a high density of label, hence increasing the sensitivity of the immunoreaction (Slot & Geuze, 1981). The main advantage of IG labelling compared to the PAP labelling method is, that this method can be used for subcellular quantitation (Childs *et al.*, 1986).

Problems in ICC

A number of problems concerning the antibody and antigen arise during the fixation, embedding and staining processes. For successful ICC staining, tissue ultrastructure and antigens have to be fixed adequately, yet the antigenic sites must still be available to the antibody (Polak & Van Noorden, 1987). Conventional aldehyde fixatives cause a strong cross-linking of

tissue proteins, which greatly reduces the antigen availability. Hence these fixatives are used at very low concentrations (0.5 - 2.0%; Ordronneau, 1982), in combination with a second fixative which allows rapid penetration (e.g. paraformaldehyde; Brandzaeg, 1982).

The temperature of polymerisation of the resin blocks is important, because pituitary hormones, being glycoproteins (Childs *et al.*, 1985) are heat labile, and are denatured at high temperatures (e.g. 63° C, which is common for polymerisation of epon resins). To overcome this problem polymerisation at 37° C for longer periods (Van Noorden & Polak, 1985) or low temperature resins (Fryer & Wells, 1983) can be used.

The choice of monoclonal or polyclonal antibodies and to which donor antigen the antibody was raised is important. Monoclonal antibodies, being specific to a single sequence on an antigen, reduce the possibility of cross-reactions with other polypeptides present in the same tissue (Van Noorden & Polak, 1985). But this does not rule out the possibility of cross-reaction, since the monoclonal antibody can react to an antigenic sequence which is shared by more than one biological substance (Polak & Van Noorden, 1987). This is the case with luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) in the pituitary gland. These glycoproteins have two subunits; an identical α-subunit, and different β-subunits (Tougard *et al.*, 1980). Hence many antibodies to pituitary hormones are raised against the β-subunit (Childs *et al.*, 1985), although this does not rule out the possibility of cross-reactions between common sequences in the β-subunits. Furthermore there is very little free β-subunit in the pituitary, hence whole gonadotropin molecules are used to raise

β-subunit antibodies and it is likely that these antibodies recognise whole LH, FSH and TSH sequences (Childs *et al.*, 1985). Taking these factors into account, the extent of cross-reactivity of the antibody to different antigens has to be determined using specificity tests (Polak & Van Noorden, 1987). These tests involve the incubation of an antibody with both its specific antigen and other antigens with which the antibody may cross-react. This antibody-antigen complex is then used to stain the tissue. Using the antibody/specific antigen complex there should be no staining. When combined with other antigens, successful staining of the tissue indicates, that the antibody does not recognise the antigen and the former is free to react with the antigen in the tissue. Little or no staining indicates a reaction between the antibody and the antigen and therefore cross-reactivity is high.

The choice of a suitable donor antigen which is used to raise the antibody is significant, particularly if the donor antigen and the antigen in the tissue (which is to be used for ICC staining) are not the same. Some monoclonal human antibodies, for example, have a low avidity (stickiness) to antigens in animal tissue (Polak & Van Noorden, 1987), and hence are lost during the washing process.

The dilution of the antibodies is another important factor because a concentrated antibody solution will result in high background staining, therefore masking the specific label. If the antibody solution is too dilute, not enough antibody is available to bind all antigens in the tissue, resulting in reduced specific label. It is therefore important that a range of antibody dilutions should be tried and the particular antibody dilution which eliminates all non-specific staining, but still retains all

the specific label, is the optimal dilution. During the staining process, blocking stages (using normal sera) have to be included to help eliminate non-specific binding of the antibodies to the tissue and the resin or wax block caused by hydrophobic or electrostatic forces (Polak & Van Noorden, 1987). To reduce any possibility of misinterpreting non-specific binding, tissue and method controls are included into the staining procedure (Ordronneau, 1982). Tissue controls involve the staining of a particular tissue, which does not contain an antigen to the antibody used, hence no staining should occur. Method controls involve the substitution of the antibodies with phosphate buffered saline, resulting in no staining.

Methods & Results

Preparation of tissue for LM-ICC

After removal, the pituitaries of one male and one female bat per species were fixed by immersion in 10% phosphate buffered (pH 7.3) formalin for two hours (which satisfactorily preserved the cell structure), dehydrated using increasing ethanol concentrations and embedded in parafin (solidification point of 46° - 48° C) at a temperature of 57° C. Sections of 5 μm were cut on a rotary microtome, collected on slides (coated with 0.01% w/v poly-L-lysine), and dried in an oven (37° C) overnight.

Preparation of tissue for EM-ICC

The anterior pituitary gland of one male and one female of

each species was fixed by immersion in a solution of 0.1% glutaraldehyde and 3% paraformaldehyde for two hours, dehydrated using increasing ethanol concentrations and embedded in Araldite-Agar 100. The blocks were polymerised at 37° C for about 10 days. The low glutaraldehyde concentration in the fixative gave a reduced ultrastructural preservation compared to conventional aldehyde fixatives, but the tissue ultrastructure was sufficiently preserved for the purpose of this study. 70 nm sections were cut on an LKB 8800A Ultratome III and collected on either gold or nickel grids. Copper grids can not be used, as the gold chloride and the colloidal gold react with the copper, resulting in contaminated grids and sections (Ordronneau, 1982).

Types of antisera used and their dilutions

Three monoclonal antibodies, two raised against human β-LH and β-FSH, the third against bovine β-LH, and all raised in mouse, were used. The polyclonal antibodies used, were raised in mouse against human β-FSH, ovine prolactin and human growth hormone. The unlabelled secondary antibody (for PAP) and the secondary antibody conjugated colloidal gold probe were raised in goat against mouse IgG. Different sizes of colloidal gold were used for LM (1 & 10 nm) and EM (10 & 20 nm). The PAP was raised in the same host as the primary antibody to recognise the anti-mouse secondary antibody.

Series of dilutions were tested for the antisera, which are summarised in Table 2. All other antisera and staining components were used at dilutions provided by the suppliers or recommended by other workers (Table 3).

The antisera were diluted with buffer (pH 7.3; Tris or PBS for EM and LM respectively) containing sodium azide (a preservative), bovine serum albumin (BSA) and Triton X (a detergent), the last two to prevent non-specific binding of the protein to the section. The DAB, gold chloride and hydrogen peroxide were diluted using TRIS or PBS buffer (pH 7.3) for EM and LM respectively. The AEC was diluted in 0.05 M acetate buffer (pH 5.2).

Table 2: Dilutions of antisera:

Antiserum	Dilution
monoclonal bovine β-LH	1/100 to 1/1000
monoclonal human β-FSH	1/100 to 1/1000
polyclonal human β-FSH	1/400 to 1/1000
polyclonal ovine prolactin	1/500 & 1/1000
polyclonal human growth hormone	1/100, 1/500 & 1/1000
PAP	1/100 to 1/300
NGS	1/10 & 1/20

Table 3: Dilutions of antisera and staining components:

Antiserum/Staining component	Dilution	Reference
monoclonal human β-LH	1/100	as supplied in the Amersham LH - ICC staining kit
secondary antibody coated colloidal gold probes	1/40	Serotec
gold chloride	1/15	Fairen *et al.*, 1977
3,3'diaminobenzidene,4-tetrahydrochloride (DAB)	1/20	Van Noorden & Polak, 1985
0.04% 3-amino-9-ethylcarbazole in N,N-dimethyl-formamide (AEC)	1/50	Graham *et al.*, 1965

ICC staining for LM (Appendix I & II)

The slides were placed in a closed damp chamber, to prevent the sections from drying out, and staining was performed by placing 50 μl of the staining solution onto each section. The sections of pituitary glands were stained with the LH antibody only. No staining was achieved with the monoclonal human β-LH antibody, whereas the monoclonal bovine β-LH antibody successfully stained the anterior pituitary. The optimal dilutions for the antisera were 1/200 bovine primary LH antibody (for PAP & IG), 1/50 secondary antibody (for PAP), and 1/100 and 1/10 for the PAP and the NGS respectively.

For the PAP labelling method, the two different peroxidase substrates used (DAB and AEC), produced a brown and red colour change respectively (Figs 1a & b). The sections were counterstained with Mayr's hematoxylin. The colour change in the LH gonadotropes shows little contrast to the other cells in the anterior pituitary, even when the sections are not counterstained.

IG staining, using 10 nm gold probe resulted in little or no staining, whereas 1 nm gold probes produced satisfactory results (Fig. 1c). The signal of the gold label was enhanced by the addition of silver particles (IntenSE M kit, Jansen Biotech N. V.), staining the LH gonadotropes a brown-black colour, which show a clear contrast to the other cells in the anterior pituitary. No counterstaining was performed.

ICC staining for EM (Appendix III & IV)

Staining was performed by placing an inverted grid onto a drop (20 μl) of the staining solution. The optimal dilutions for the

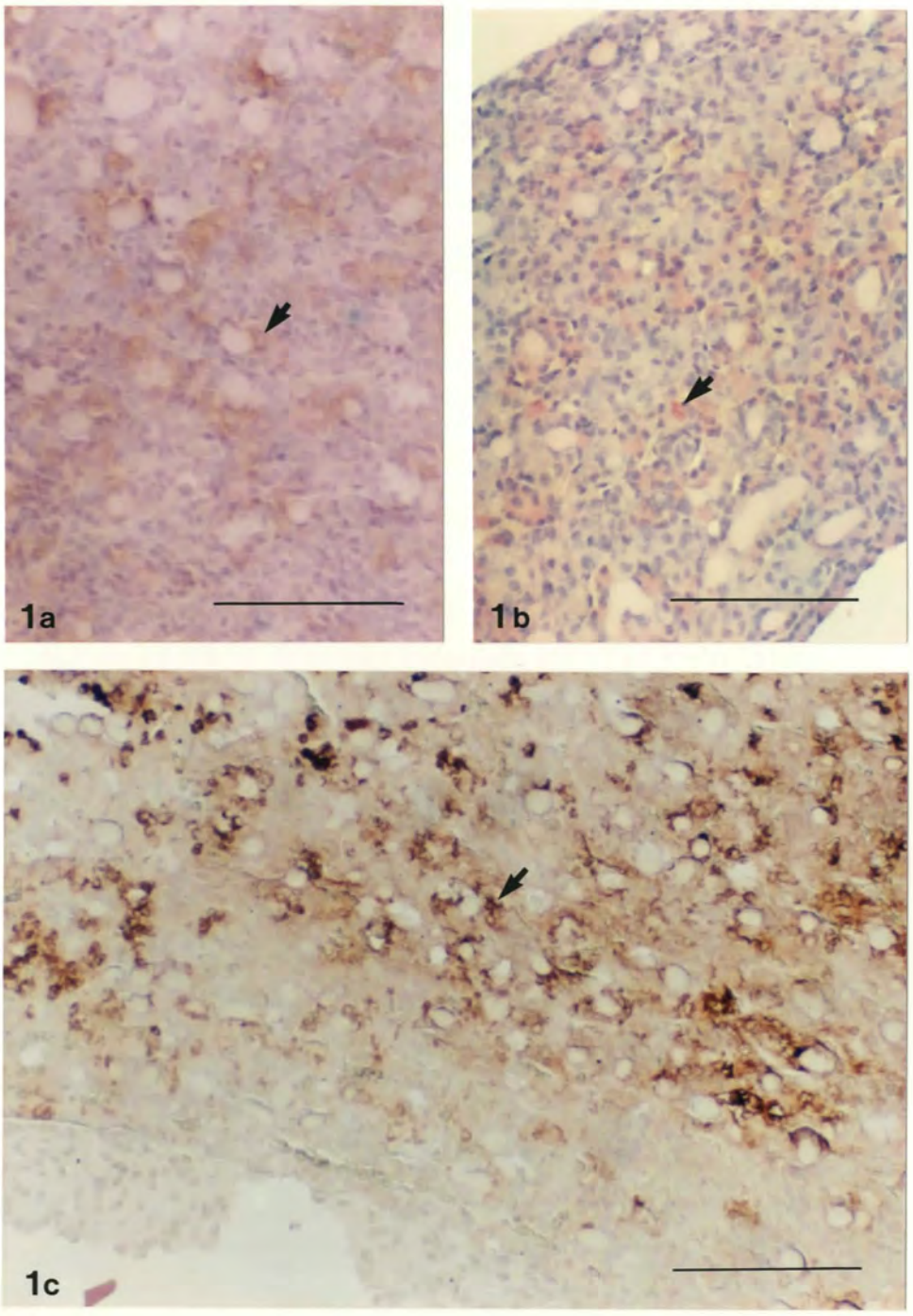


Figure 1: Sections of the anterior pituitary of female *M. schreibersii* from April, stained using the PAP labelling method (with DAB (a) and AEC (b) as a substrate) and the immunogold-silver enhanced labelling method (c). The arrows indicate positively stained cells. (Scale bars represent 100 μm).

antisera were 1/400 monoclonal bovine β-LH antibody (for PAP & IGG), 1/1000 polyclonal ovine prolactin antibody (for IGG), 1/20 polyclonal human growth hormone (for IGG), 1/1000 polyclonal human β-FSH (for IGG), 1/100 secondary antibody (for PAP), 1/300 PAP and 1/10 NGS.

Although the PAP-gold chloride complex accumulates on the secretory granules containing the antigen, thus increasing their electron density (Childs *et al.*, 1986) (Fig. 2a), individual PAP molecules are distributed throughout the cytoplasm. At high magnifications, the outline of the secretory granules is difficult to define, due to effusion of the PAP molecules across their borders (Childs *et al.*, 1986) (Fig. 2b). The PAP molecules are irregular shaped and vary in size.

The IG labelling method, using 10 and 20 nm gold probes and 2% methanolic uranyl acetate (Stempak & Ward, 1964) as a counterstain, gave excellent results using the bovine β-LH, ovine prolactin and human growth hormone antibodies (Figs 3a & b). The polyclonal human β-FSH antibody stained the secretory granules, but there was very little label (Fig. 4). Distinct uniformly-sized gold particles are present on the secretory granules and little or no label is found in the cytoplasm of the LH gonadotropes. Individual 20 nm gold particles can still be detected at lower magnifications (Fig. 5).

Specificity tests and controls

The specificity of the antibody is usually tested by absorbing a 0.1-10 nmol/ml antigen solution with the diluted primary antibody

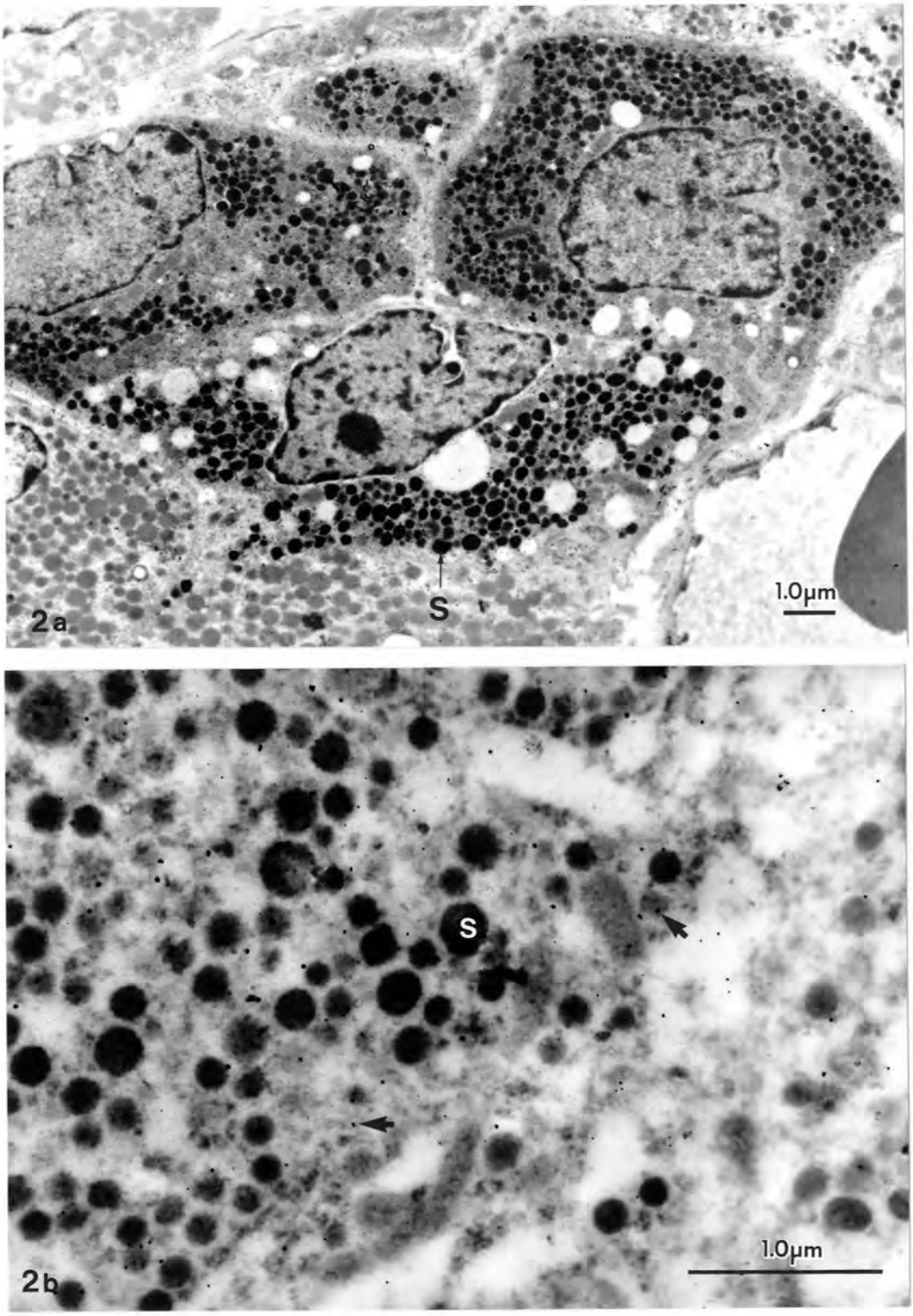


Figure 2: Sections of LH gonadotropes of female *R. capensis* from August, at low (a) and high (b) magnifications, stained with the PAP labelling method. Note the higher electron density of the LH gonadotropes compared to other surrounding cell types. Individual PAP molecules (arrows) can be seen on secretory granules (S) and in the cytoplasm.

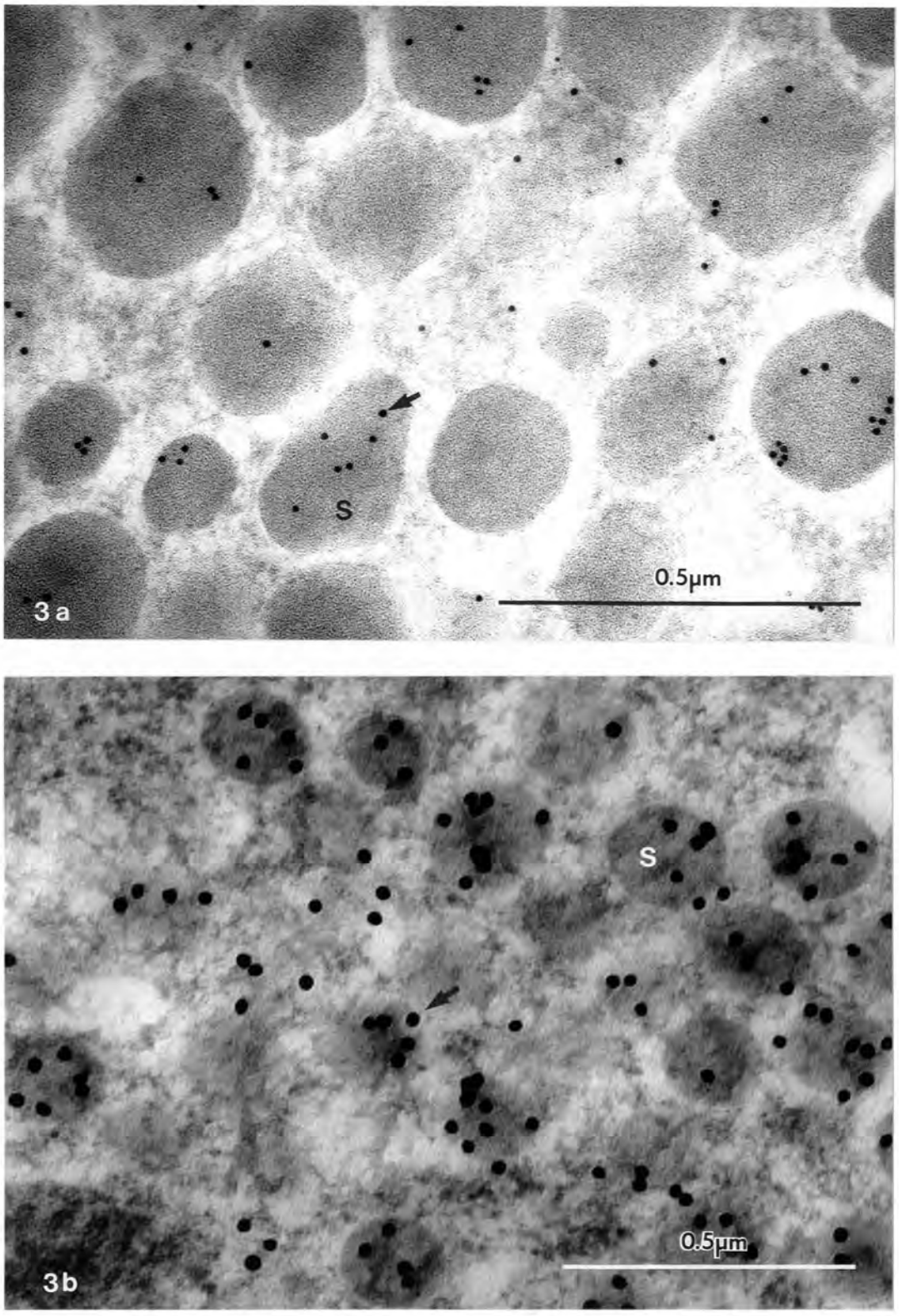
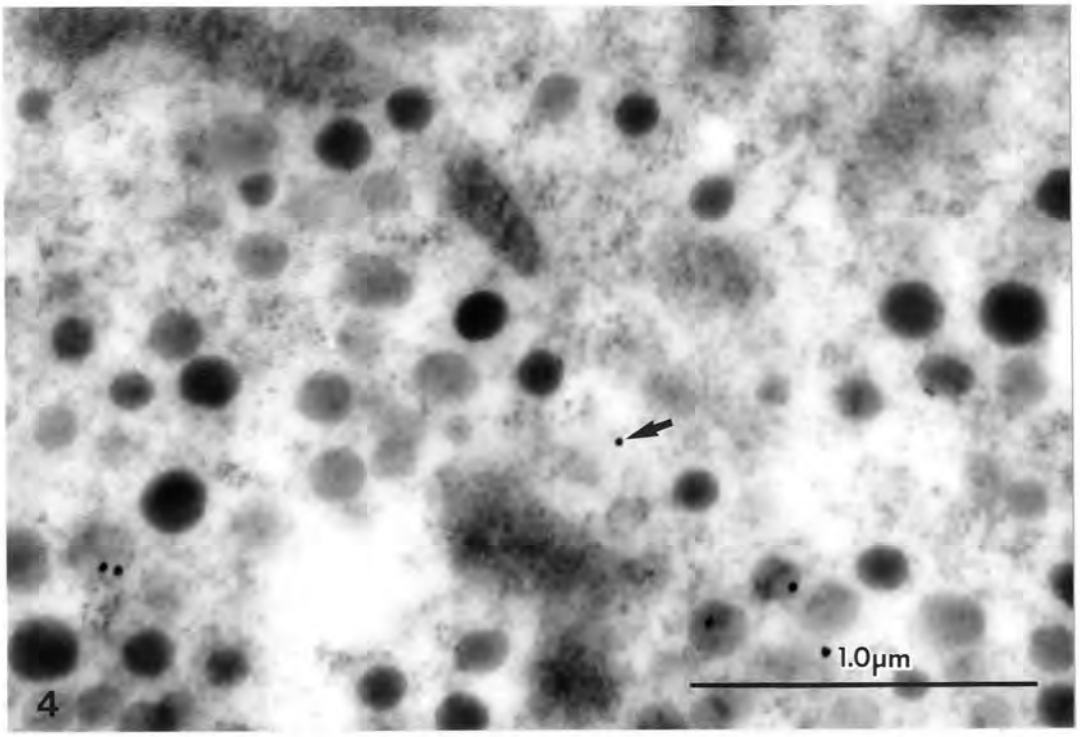


Figure 3: Section of an LH gonadotrope of male *M. schreibersii* from December, showing the secretory granules (S) labelled with 10 nm (a) and 20 nm (b) gold particles (arrows).



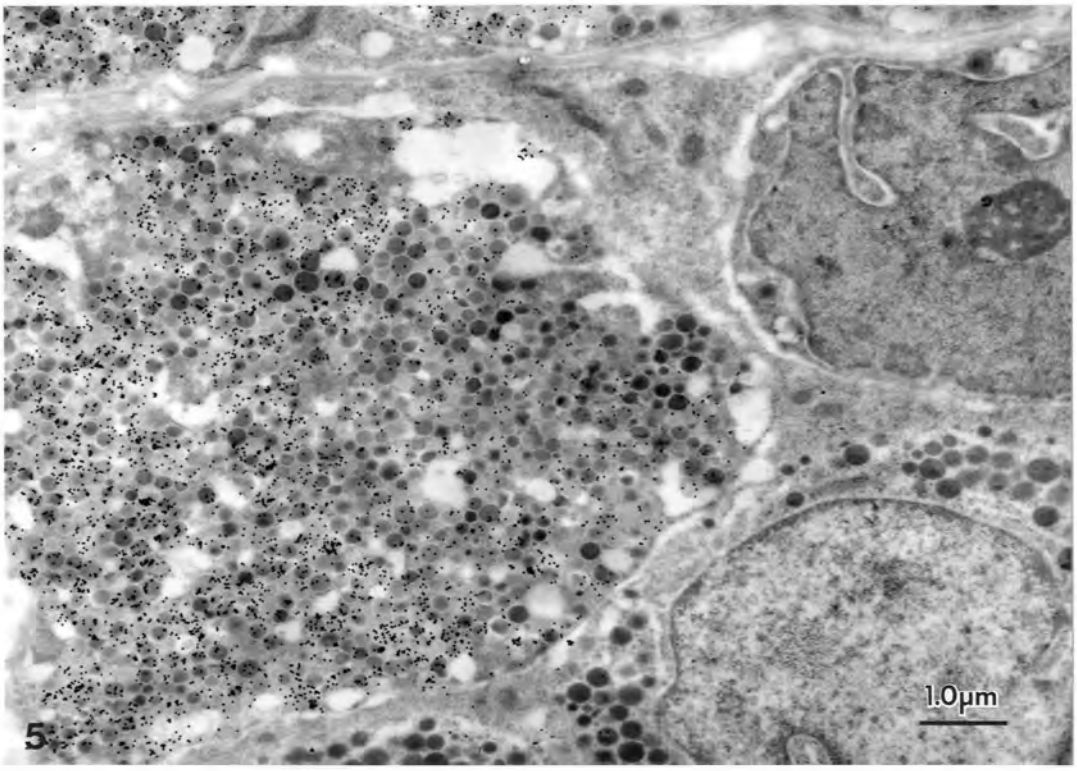
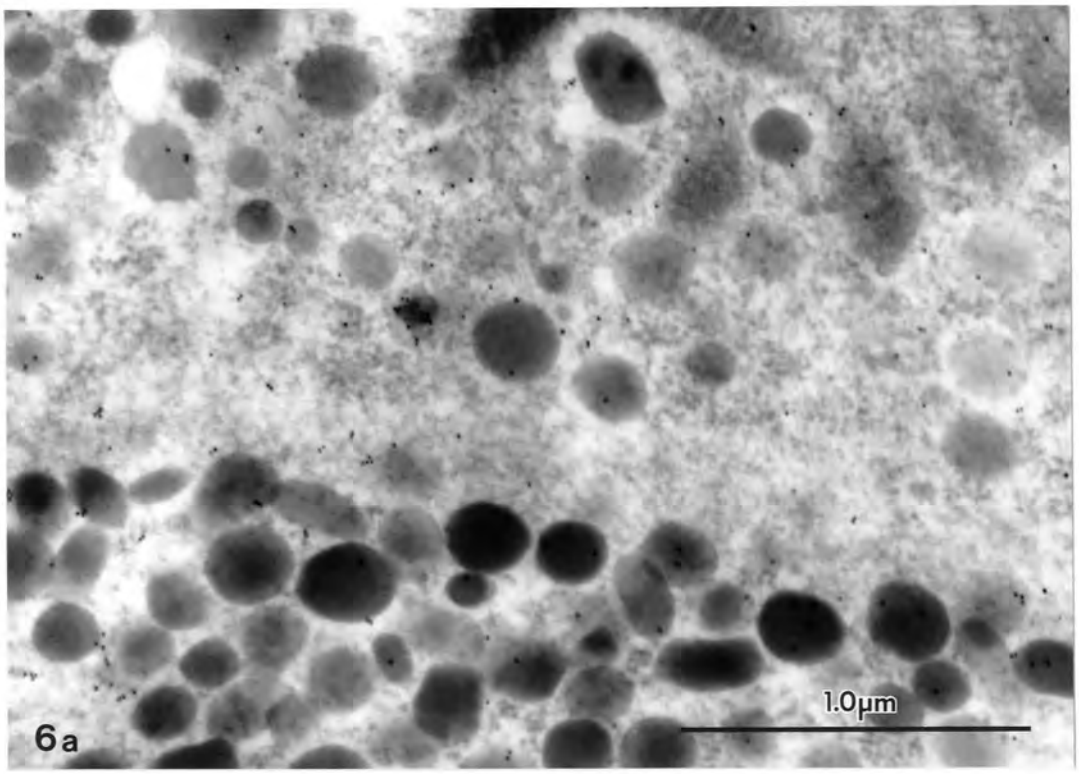


Figure 4: Section of the anterior pituitary of male *M. schreibersii* from March, showing a gonadotrope containing FSH, which is indicated by the presence of gold label (arrow). Note the small amount of label present. (Stain: IG labelling using 10 nm gold particles).

Figure 5: Section of the anterior pituitary of female *M. schreibersii* from October, showing LH gonadotropes at low magnification.(Stain: IG labelling using 20 nm gold particles).

(Polak & Van Noorden, 1987). To test the specificity of the LH, a 10 nmol/ml antibody-antigen solution was prepared by absorbing 0.01 mg human β-LH (molecular weight 28500: Pierce, 1988) with 34 μl 1:400 bovine β-LH antibody. Cross-reactivity with TSH and FSH was tested using a 10 nmol/ml antibody-antigen solution, for which 0.025 mg human β-TSH (molecular weight 30000: Oser, 1965) and 0.05 mg human β-FSH (molecular weight 28500: Pierce, 1988) were absorbed with 83 μl and 172 μl of 1:400 bovine β-LH antibody respectively. The absorbed antibody-antigen solution was incubated at 4° C for 24 hours and then used in place of the primary antibody in the immunogold labelling procedure. The LH antibody-LH antigen complex should give no staining (Polak & Van Noorden, 1987). However, results obtained (Fig. 6a), showed staining on all cells and on the resin. The test was repeated three times with the same result. Since the gold label was distributed uniformly over the resin and tissue section, and did not stain one cell type more than another, it was assumed no immunological reaction took place. The reason for this non-specific binding is unknown. The LH antibody-TSH antigen complex stained the tissue (Fig. 6b), indicating little or no cross-reaction between the β-TSH and the β-LH antibody, which is free to react with the LH antigen in the tissue. Positive staining was obtained with the human β-FSH and bovine β-LH antibody complex as well, but staining was slightly reduced compared to the control (normal staining with the FSH antibody), hence some cross-reactivity does take place.

A tissue control was performed by leaving the pars nervosa (which contains no gonadotropes) attached to the anterior



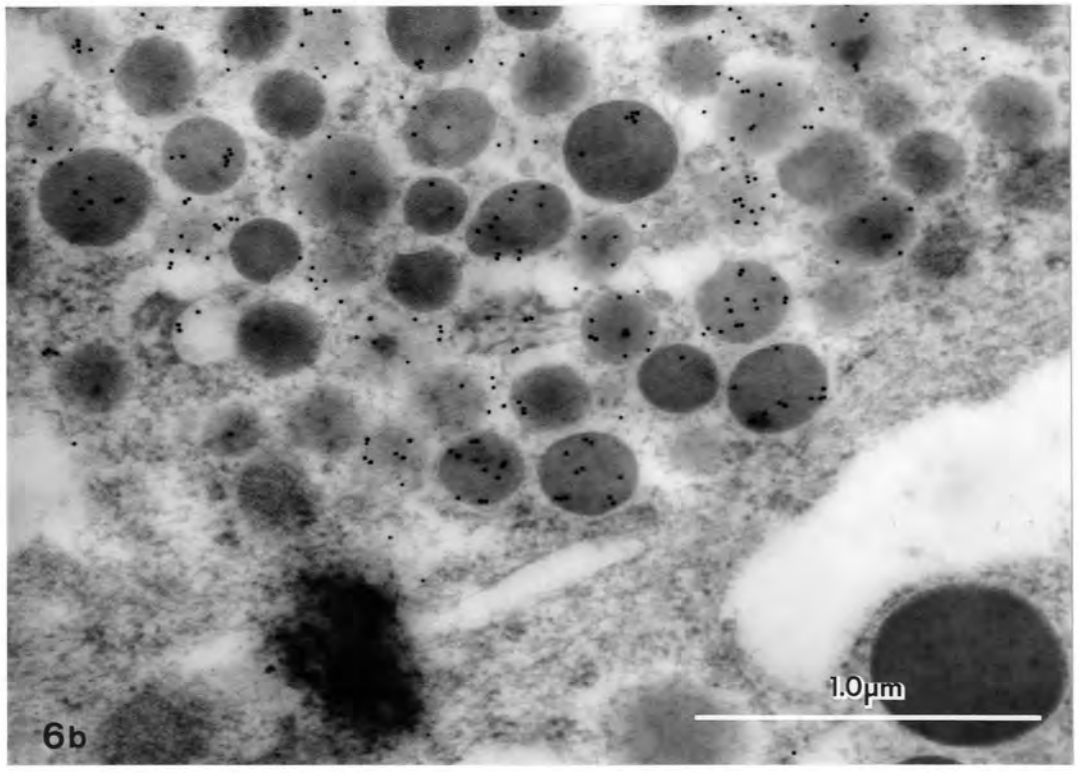


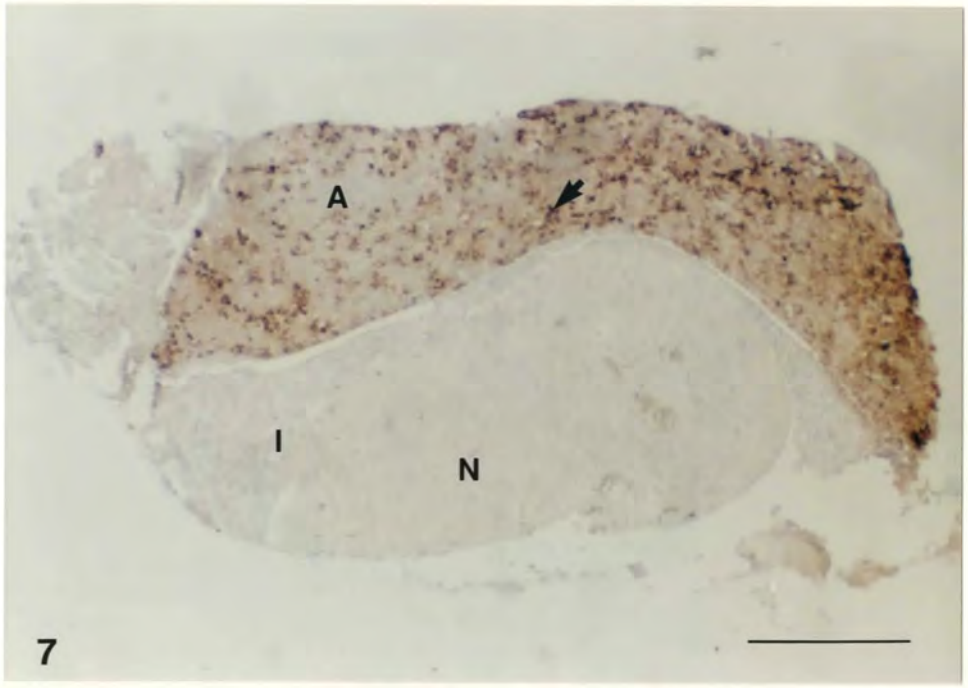
Figure 6: Control sections of an LH gonadotrope of female *M. schreibersii* from November, stained with the LH antibody - LH antigen (a) and LH antibody - TSH antigen (b) complex. Note the nonspecific staining in 6a and the typical staining in 6b. (Stain: IG labelling using 20 nm gold particles).

pituitary. No staining was found in the pars nervosa, whereas the anterior pituitary stained positively (Fig. 7). Method controls involved the substitution of the primary antisera with buffer pH 7.3 (PBS or Tris), resulting in no staining (Fig. 8).

Discussion

Post-embedding labelling is widely used in pituitary immunocytochemistry (Nakane, 1970; Herbert, 1976; Dacheux, 1980; Anthony & Gustafson, 1984; Peute *et al.*, 1986). Its main disadvantage is the possibility of losing or denaturing the antigen in the tissue during fixation and embedding (Ordronneau, 1982). In the present study, two different fixatives were used for LM and EM, both adequately fixing the tissue ultrastructure, while retaining a high antigenicity. The use of parafin with a low temperature solidification point for LM, and polymerisation of resin blocks at 37^{o} C for EM, eliminated the problem of denaturing the heat labile antigens.

LH gonadotropes were successfully identified, using the bovine primary antibody. According to the manufacturers the bovine β-LH antibody has a 14% cross-reactivity with human β-TSH however, the cross-reactivity test with TSH was negative or at least minimal, as no reduction in staining occurred. Staining was slightly reduced in the test for cross-reactivity with β-FSH, indicating a degree of recognition between the human β-FSH antigen and the LH antibody. Since FSH and LH are present in the same cell



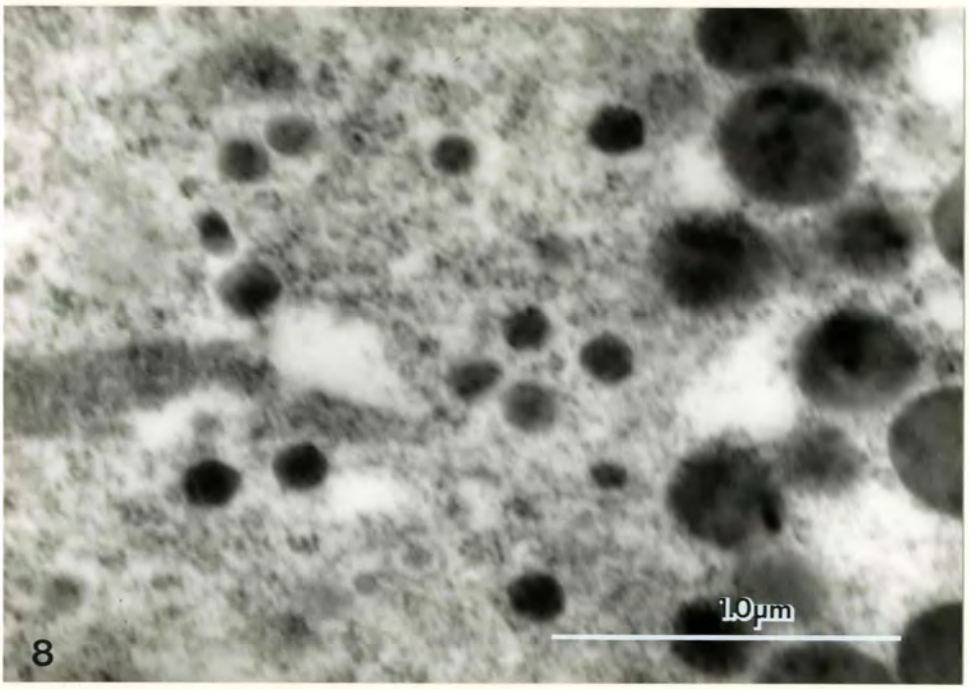


Figure 7: Horizontal section of the pituitary gland of male *M. schreibersii* from May, stained with the LH antibody. Note the absence of stain in the pars nervosa (N) and the pars intermedia (I). Positively stained cells (arrow) are present in the anterior pituitary (A). (Stain: IGSS; scale bar represents 200 µm).

Figure 8: Control section of an LH gonadotrope of female *M. schreibersii* from December, showing no staining.

type, identification of gonadotropes is not influenced. For subcellular quantification of LH, the degree of cross-reactivity does play a role, however since the staining was only slightly reduced, the amount of cross-reactivity is probably not significant. It is assumed that the non-specific staining during the LH specificity test was a result of a non-immunological attraction between the LH antibody-LH complex and the tissue and resin. It appears, that the LH antibody did recognise the human β-LH antigen and for the purpose of this study, it is assumed that the bovine β-LH antibody, being a monoclonal antibody, is specific to β-LH as stated in the product specification from Serotec. No staining was obtained with the human β-LH antibody, which either failed to recognise the LH antigen in the bat tissue (due perhaps to a different amino acid sequence in the β-subunit) or it is possible that the antibody and antigen share a few amino acids only, resulting in a low avidity and causing the loss of the primary antibody during the washing process. The same problem occurred with the monoclonal human β-FSH antibody, resulting in no staining. The polyclonal human β-FSH antibody (which is less specific compared to the monoclonal antibody) stained the pituitary, although immunoreactivity between the antibody and antigen was very low. Both the ovine prolactin and human growth hormone antibodies successfully stained the mammotropes and somatotropes respectively.

Both ICC staining methods (PAP & IG) specifically stain LH gonadotropes in the anterior pituitary at LM and EM levels, leaving other cell types unlabelled. At the LM level, IG labelling and silver enhancement produces a higher contrast of the label than

the PAP method. This was found to be advantageous for the morphometric analysis, as the LH gonadotropes were more easily distinguished from other cell types. The advantage of 1 nm over 10 nm gold probes is that the 1 nm gold probe is not affected by steric hinderance, resulting in high density labelling (Slot & Geuze, 1981).

The PAP labelling method at the EM level shows non-specific labelling, which was reduced using higher dilutions of the antisera, but this also caused a reduction in specific label. This non-specific staining could be due to the effusion of the peroxidase-substrate complex or may be caused by the displacement of the antigen during fixation or embedding (Childs *et al.*, 1986). Little non-specific label was obtained, using the IG labelling method. The electron dense gold particles clearly stand out against their background, even if the tissue is counterstained with uranyl acetate. Both, the 10 and 20 nm gold probes, specifically label the secretory granules in the LH cells, and can be easily detected at high magnifications. However, at the low magnifications required to photograph whole LH gonadotropes, only the 20 nm gold probe was visible and consequently this size gold probe was used throughout this study.

Summary

ICC is a valuable technique which permits the identification and localisation of the different cell types within the pituitary (Nakane, 1970; Moriarty, 1975). The development of ICC led many

workers to review the classical tinctorial cell types in the anterior pituitary, and to compare them with the ICC stained functional cell types (Nakane, 1970; Baker & Gross, 1978; Richardson, 1979; Tougard, 1980; Petrusz & Ordronneau, 1983; Childs, 1986). It was found, that the one cell-one hormone theory, generally accepted for pituitary cells types (Richardson, 1979), did not apply to gonadotropes, which synthesise two different hormones. These hormones, LH and FSH, are found in the same cell and sometimes even in the same secretory granule (Herbert, 1975 & 1976; Batten & Hopkins, 1978; Campbell *et al.*, 1987).

Furthermore, the accurate localisation of a particular antigen, allows the quantification at the cellular (using PAP and IG labelling) and subcellular level (using IG labelling only) (Childs *et al.*, 1985). For example, local antigen concentrations can be measured, by assessing the immunoreactivity of the antigen to the antibody, which is quantified by counting the number of gold particles (Posthuma *et al.*, 1987).

CHAPTER 4: HISTOLOGY OF THE ANTERIOR PITUITARY AND ULTRASTRUCTURE OF THE LH GONADOTROPES

Introduction

There have been numerous studies on the anterior pituitary of mammals such as the rat (Nakane, 1970; Herbert, 1975; Kurosumi & Inoue, 1986; Hata & Watanabe, 1989), mouse (Baker & Gross, 1978; Payette *et al.*, 1987), rabbit (Mazur & Younglai, 1986), lizard (Licht & Pearson, 1978; Pearson & Licht, 1987), dog (El Etreby *et al.*, 1977) and human (Pearse & Van Noorden, 1963; Robyn *et al.*, 1973). Early light microscope studies, attempting to identify and localise the different types of glandular cells, were based on morphological criteria alone (Childs, 1986), involving the cells' affinity for tinctorial stains, and observations of cytological changes upon alteration of target organ hormonal feedback (Purves & Griesbach, 1954). Six different cell types were identified, each secreting one hormone: somatotropes (growth hormone), mammotropes (prolactin), corticotropes (adrenocorticotropin), thyrotropes (thyroid stimulating hormone) and two types of gonadotropes (luteinizing hormone and follicle stimulating hormone) (Kurosumi, 1968). However, the gonadotropic cells exhibit very similar morphological characteristics, and a differentiation, based on ultrastructure was not possible (Rennels *et al.*, 1971; Shiino *et al.*, 1972).

The introduction of ICC provided a means of accurately identifying the different cell types in the anterior pituitary, using antibodies raised against the hormones secreted by the cells. Using ICC it was found, that gonadotropes contain both FSH

and LH, and that only one type of gonadotrope exists in the anterior pituitary (Nakane, 1970; Herbert, 1975 & 1976; Moriarty, 1975; Moriarty & Garner, 1977; El Etreby *et al.*, 1977). However, work by Richardson (1979) indicates, that there are three different cell populations within the gonadotropes, of which two contain either LH or FSH, and the third contains both hormones. It is still not certain, how many different types of gonadotropes are present in the anterior pituitary gland.

To date, little work has been done on the anterior pituitary of the Chiroptera using ICC (Richardson, 1979, 1981a & b; Anthony & Gustafson, 1984; Mikami *et al.*, 1988), but the ultrastructure and distribution of gonadotropes were found to be similar to other mammals (rat: Nakane, 1970; rhesus monkey: Herbert, 1976 & 1978; mouse: Baker & Gross, 1978).

This chapter deals with the morphology and ultrastructure of the anterior pituitary gland of *M. schreibersii* and *R. capensis*, and the identification of different cell types with emphasis on the LH gonadotropes.

Results

Morphology and histology of the pituitary gland:

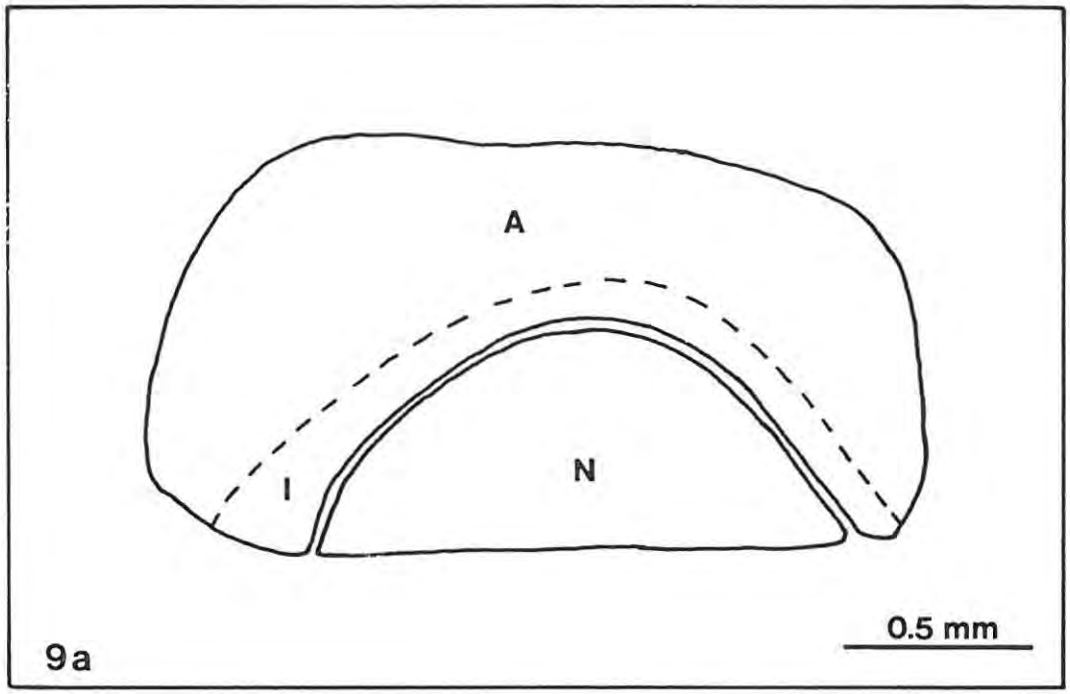
The pituitary gland, situated at the base of the brain, comprises the anterior pituitary (pars distalis), the pars intermedia and the pars nervosa. In both species the gland is elongate in shape (about 2 mm long and 1 mm wide), dorso-ventrally compressed, and is surrounded by a thin, transparent membrane. In *R. capensis*, the

pars nervosa is in a posterior-dorsal position, while in *M. schreibersii*, it is located posteriorly (Figs 9a & b).

Horizontal sections of the pituitary gland of both species show the anterior pituitary to have an elongate, slightly concave shape. Situated posteriorly is the oval pars nervosa, which is separated from the anterior pituitary by a very narrow pars intermedia (Fig. 7).

The anterior pituitary of both species consists either of acini or chords of parenchymatous cells, which are surrounded by a thin layer of connective tissue and interspersed with numerous sinusoids (Fig. 10). Identification of cell types using tinctorial methods, showed the distribution of basophils (thyrotropes and gonadotropes) mainly in the middle region, and of acidophils (somatotropes and mammotropes) in the lateral regions of the anterior pituitary (Fig. 11). The basophils are oval to polyhedral and sometimes stellate in shape; the acidophils tend to be larger than the basophils and are oval to angular in shape (Fig. 12).

The LH gonadotropes, identified using ICC, are distributed throughout the anterior pituitary gland (Fig. 7) and are sometimes concentrated in the middle region (Fig. 13). The cells occur either singly or in groups and are often situated close to or around sinusoids (Fig. 14). The relative abundance of LH gonadotropes is greater in male *M. schreibersii* than in females. For example in May, LH gonadotropes in the female constitute 6.1% of the pituitary volume and in the male 10.7% (Figs 15a & b). The LH gonadotropes in the female occur mainly as single cells and are scattered throughout the pituitary, whereas in the male, the cells form small groups which are distributed throughout the gland. The



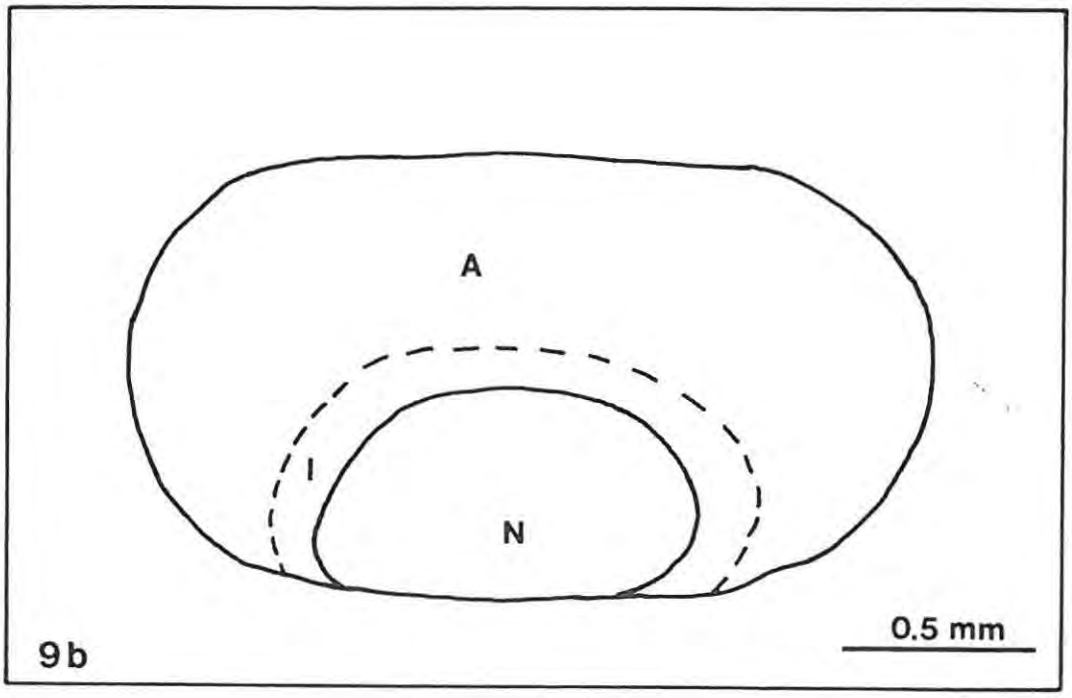
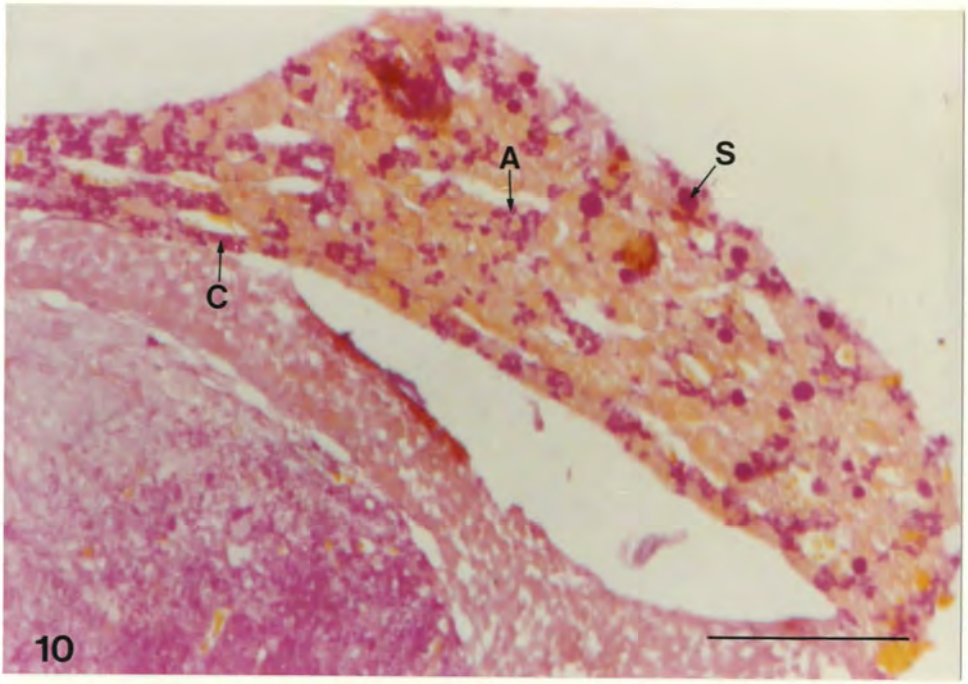


Figure 9: Diagrams of the pituitary gland of *Miniopterus schreibersii* (a) and *Rhinolophus capensis* (b), showing the position of the pars nervosa. A - anterior pituitary; N - pars nervosa; I - position of the pars intermedia.



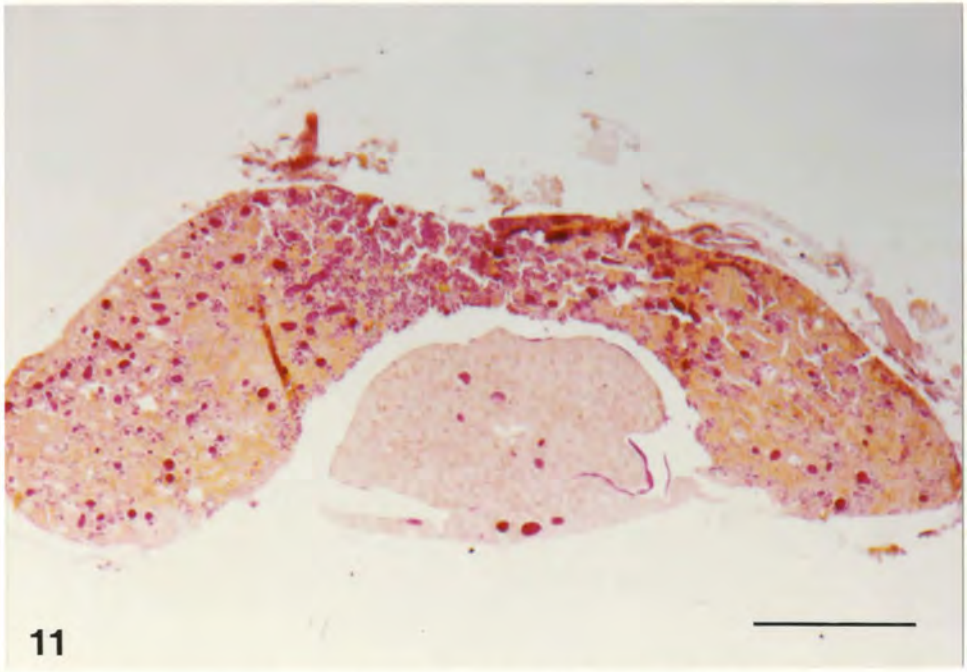


Figure 10: Section of the pituitary gland of female *M. schreibersii* from March, showing the cells of the anterior pituitary arranged in acini (A) or chords (C). S - sinusoids. (Stain: Aldehyde fuchsin, periodic acid, Schiff's reagent and orange G; scale bar represents 100 μm).

Figure 11: Horizontal section of the pituitary gland of male *M. schreibersii* from March, showing the distribution of acidophils (in yellow) and basophils (in red). Note the acidophils are present mainly in the lateral regions and the basophils in the middle region of the anterior pituitary. (Stain: Aldehyde fuchsin, periodic acid, Schiff's reagent and orange G; scale bar represents 200 μm).

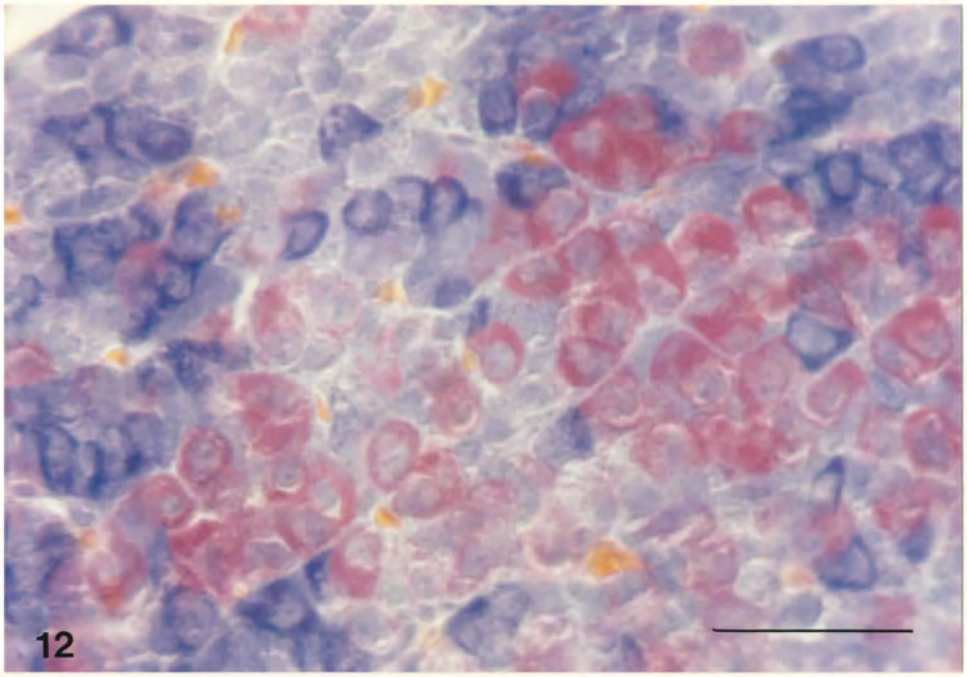
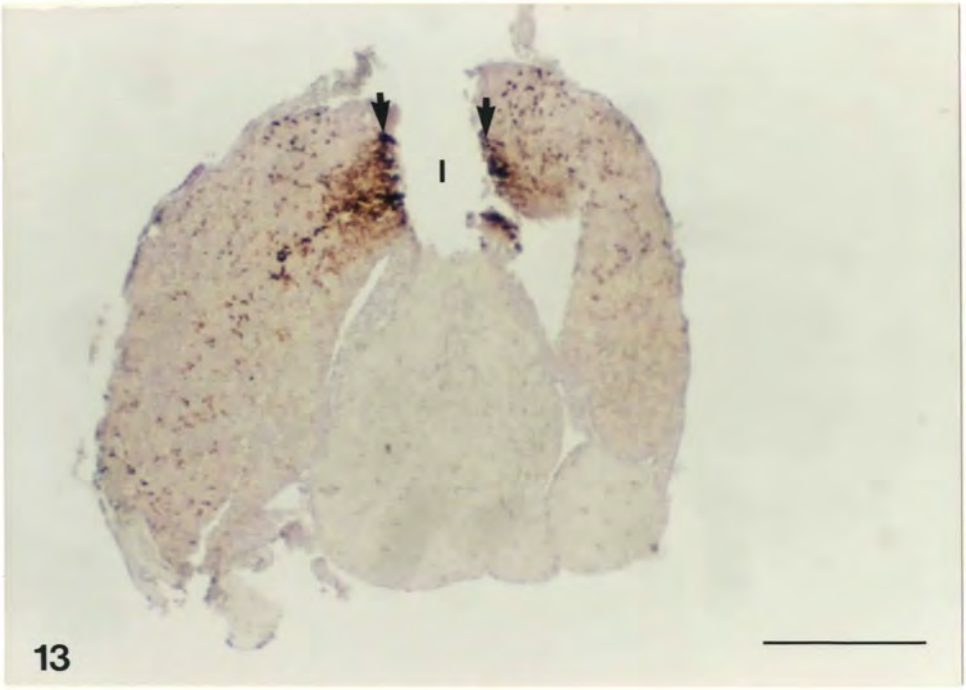


Figure 12: Section of the anterior pituitary of male *M. schreibersii* from March, showing the acidophils (in red) and basophils (in blue). (Stain: Mallory - Heidenhain; scale bar represents 50 μm).



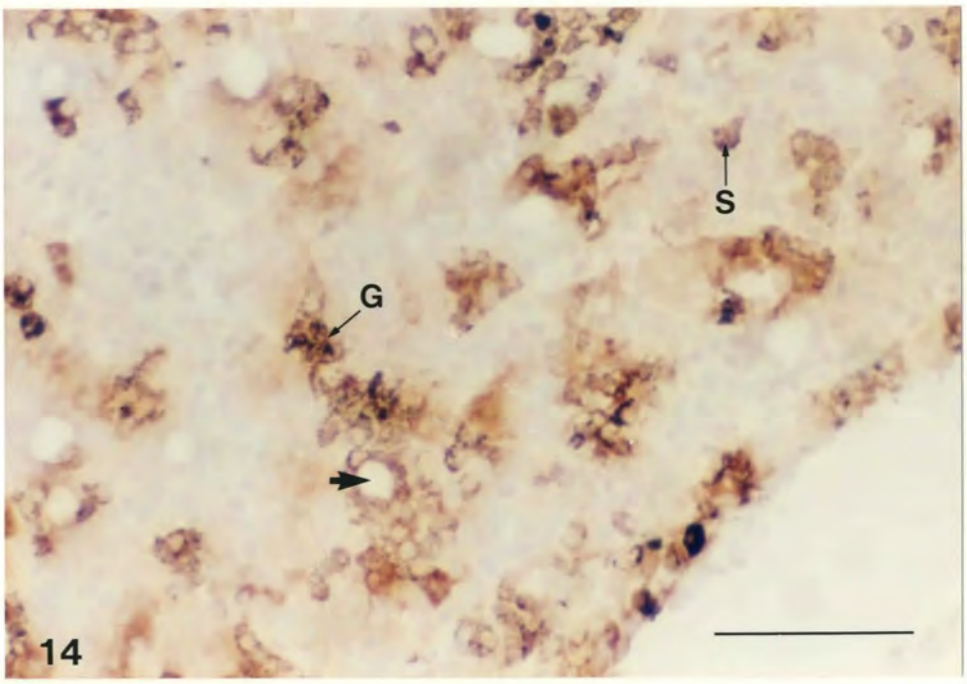
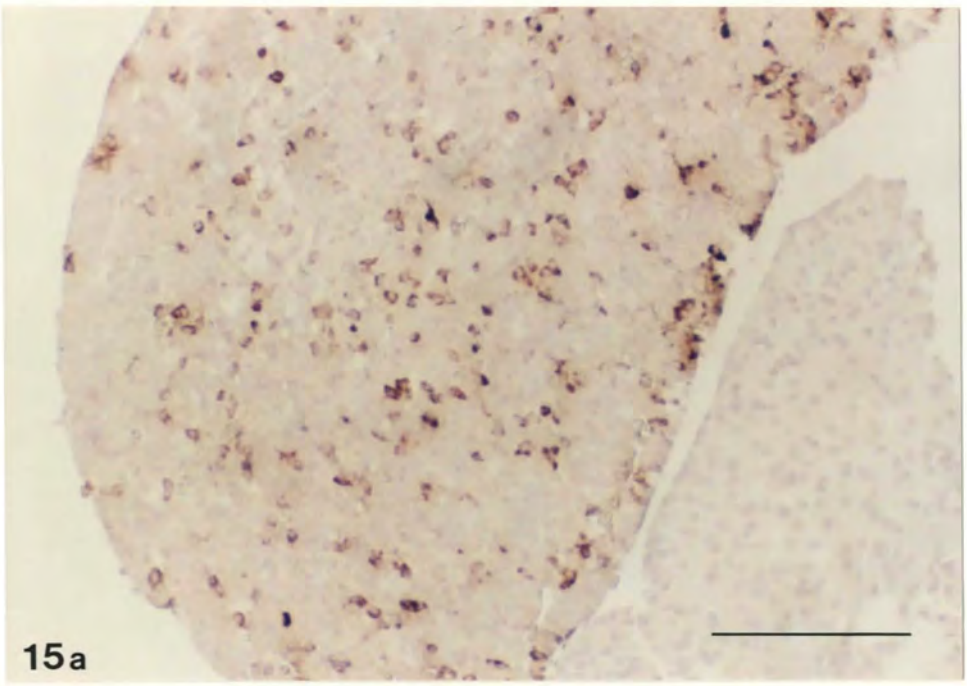


Figure 13: Horizontal section of the pituitary gland of female *M. schreibersii* from January, showing the concentration of LH gonadotropes (arrows) in the middle region of the gland. I - position of the infundibular stalk. (Stain: IGSS; scale bar represents 200 μm).

Figure 14: Section of the anterior pituitary of male *M. schreibersii* from March, showing single (S) and groups (G) of LH gonadotropes. Arrow - sinusoid. (Stain: IGSS; scale bar represents 50 μm).



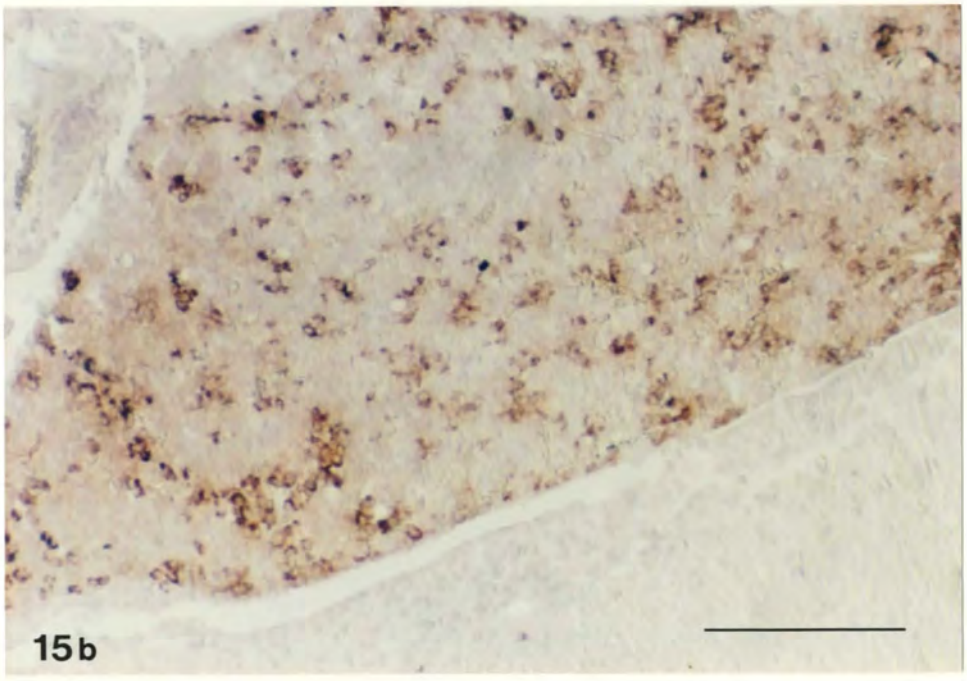


Figure 15: Sections of the anterior pituitary, showing the abundance of LH gonadotropes in female (a) and male (b) *M. schreibersii* in May. (Stain: IGSS; scale bars represent 100 μm).

distribution of LH gonadotropes is similar in *R. capensis*, where, although data are limited, the relative abundance of LH gonadotropes is greater in the female. For example in April, LH gonadotropes in the male comprise 7.7% (Fig. 16a), and in the female it is 13.7% (Fig. 16b).

Ultrastructure of the anterior pituitary

Cells in the anterior pituitary contain numerous secretory granules, well developed rough endoplasmic reticulum and Golgi body, and typically irregularly shaped nuclei (Figs 17a & b). The main difference between the cells can be seen in their shape and the size of the secretory granules. The cell shape varies between oval, polyhedral or stellate (Figs 18a & b). The majority of cells are oval or polyhedral in shape and contain on average either large (200 - 500 nm) or small (100 - 250 nm) secretory granules (Fig. 18b). The stellate cell type contains electron dense secretory granules ranging in size from 130 nm to 350 nm (Fig. 18a). The secretory granules vary in electron density between and within the cells (Fig. 19) and some cells are devoid of secretory granules. In spite of these small differences, the ultrastructure of the cells of the anterior pituitary is very similar and LH gonadotropes can not be identified on morphological characteristics alone.

LH gonadotropes, stained using the bovine LH antibody, are either oval (Fig. 20a) or polyhedral (Fig. 20b) in shape. They are characterised by secretory granules (most are 100 - 250 nm in diameter and few are 350 - 400 nm in diameter) which vary in electron density although many are electron lucent (Fig. 21).

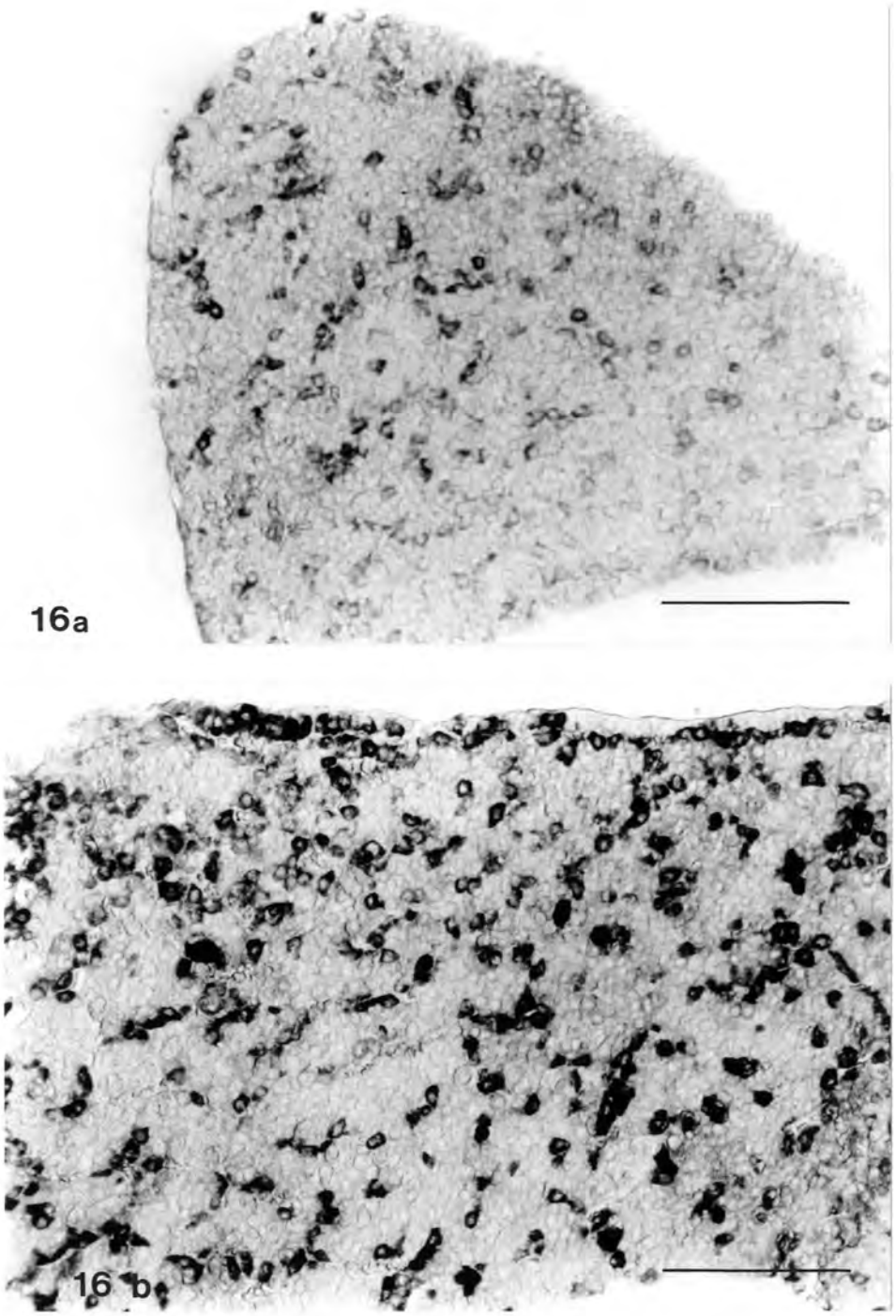
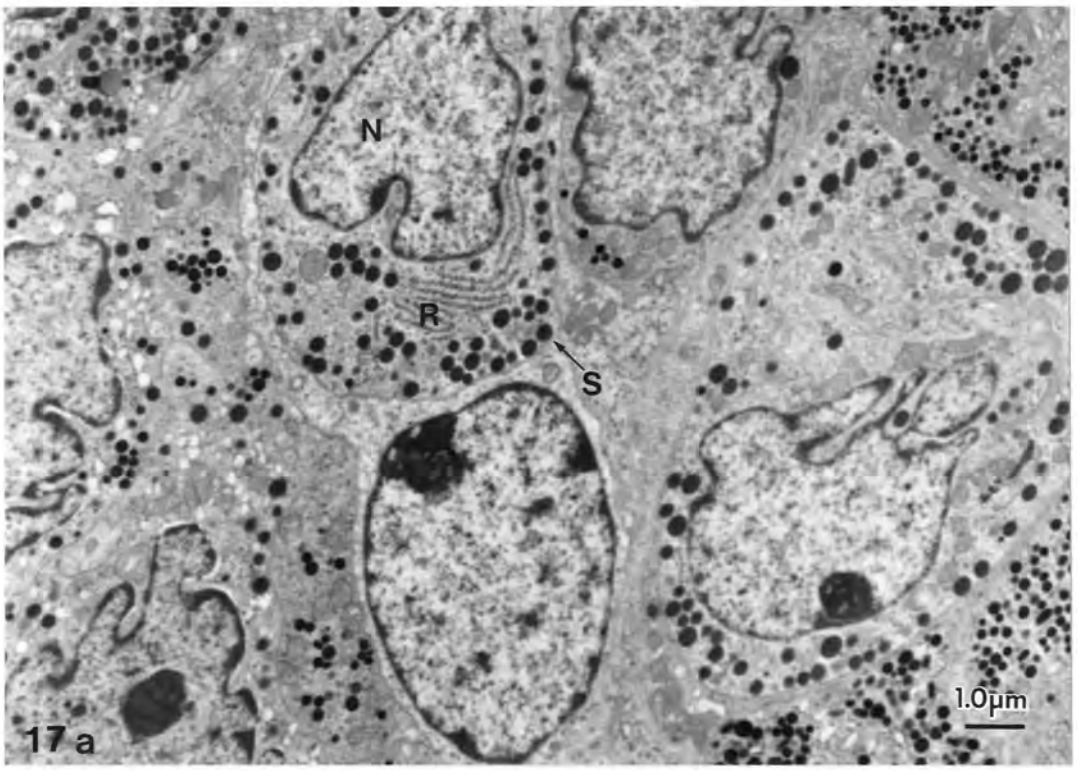


Figure 16: Sections of the anterior pituitary, showing the abundance of LH gonadotropes in male (a) and female (b) *R. capensis* in April. (Stain: IGSS; scale bars represent 100 μm).



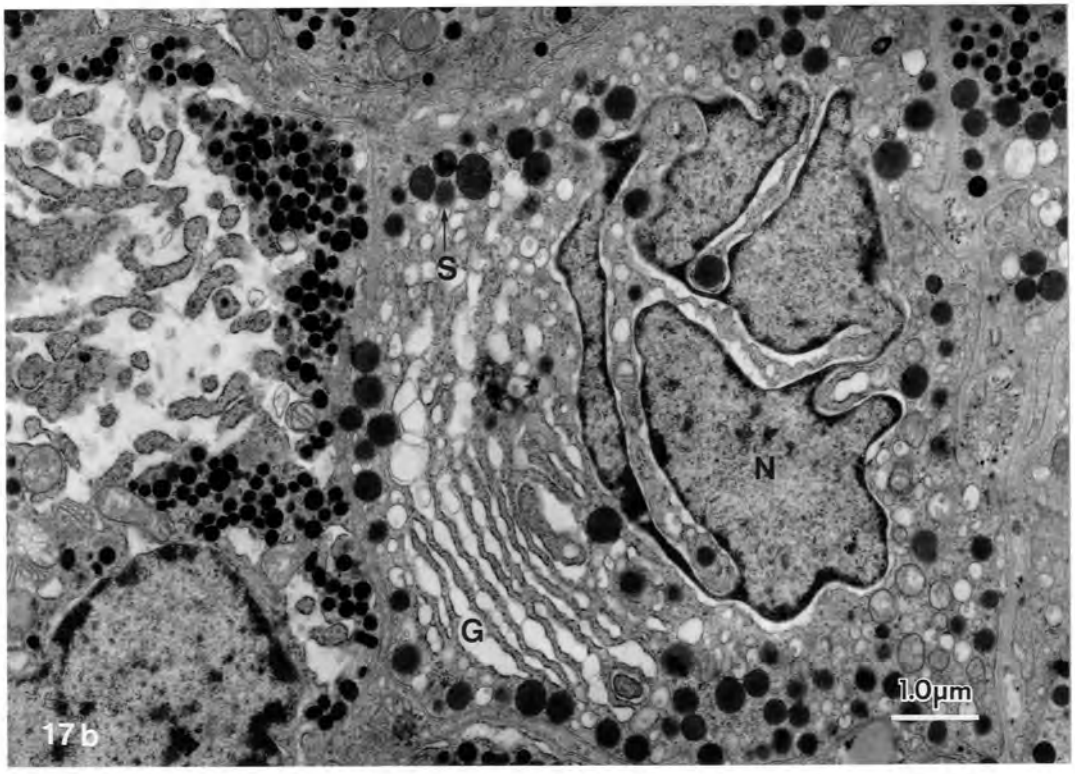


Figure 17: Sections of anterior pituitary cells of male *M. schreibersii* from March, showing numerous secretory granules - S (a & b), well developed rough endoplasmic reticulum - R (a) and Golgi body - G (b), and irregular shaped nuclei - N (a & b). (Stain: Uranyl acetate & lead citrate).

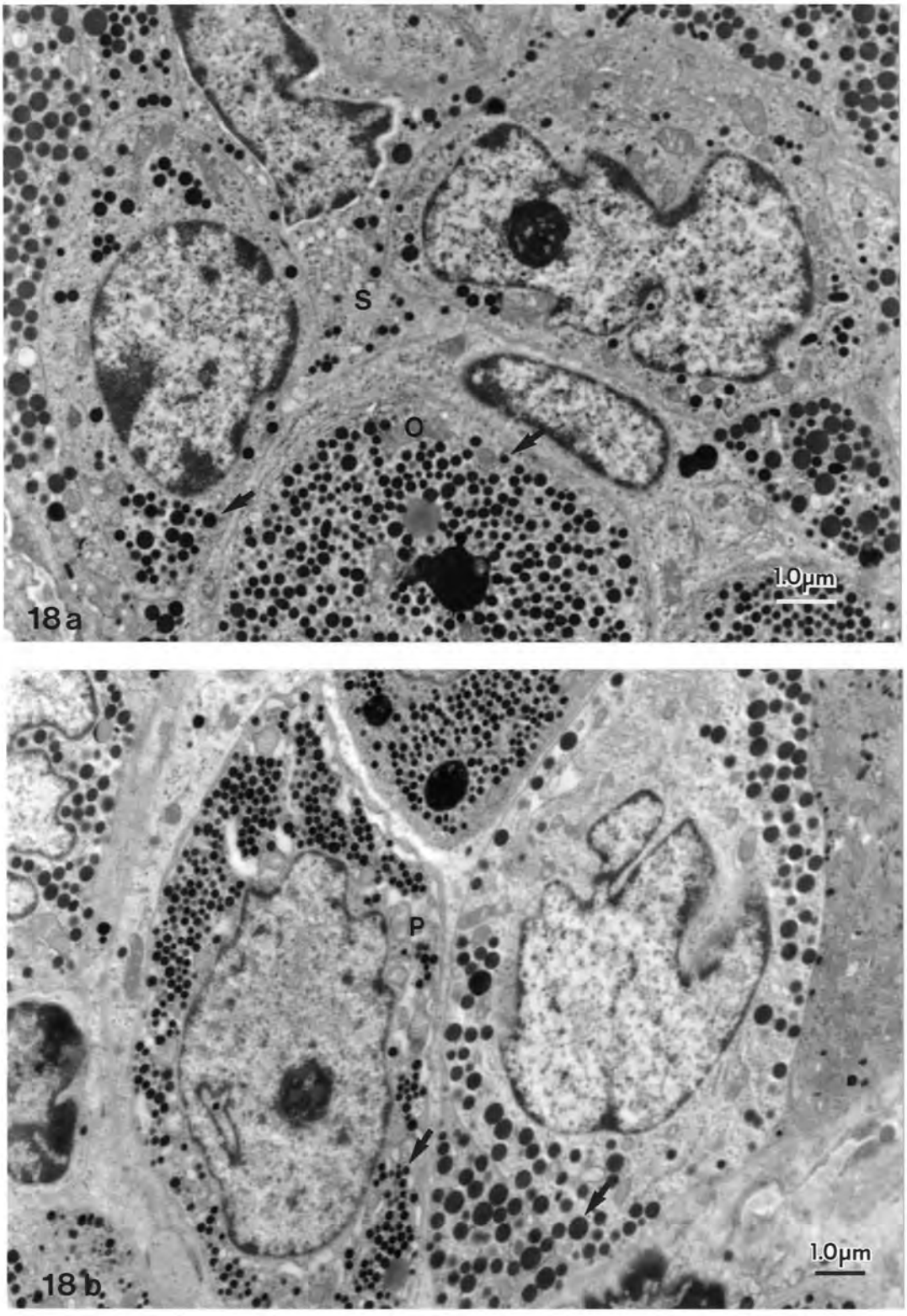


Figure 18: Sections of the anterior pituitary of female *R. capensis* from February, showing differently shaped cells. S - stellate (a); O - oval (a); P - polyhedral (b); arrows - secretory granules of different sizes. (Stain: Uranyl acetate & lead citrate).

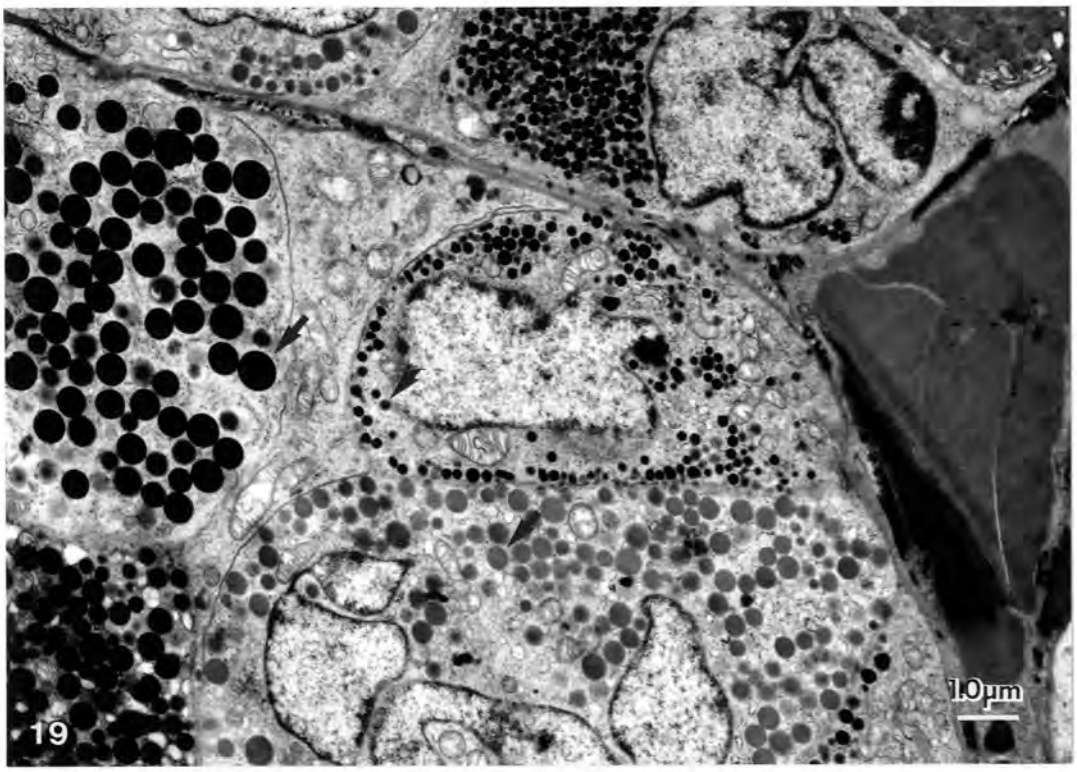


Figure 19: Section of anterior pituitary cells of female *M. schreibersii* from December, containing secretory granules, which vary in size and electron density (arrows). (Stain Uranyl acetate & lead citrate).

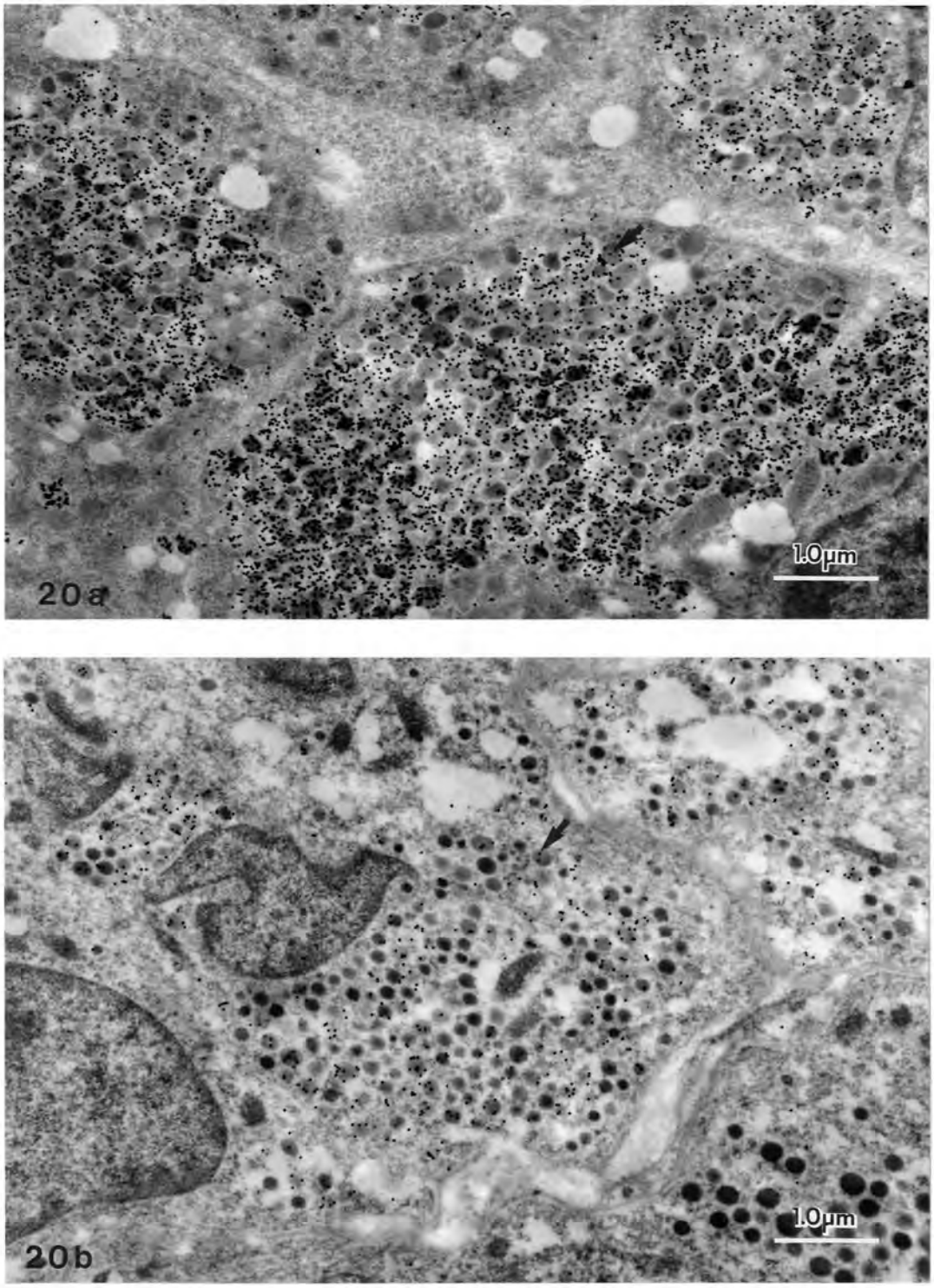
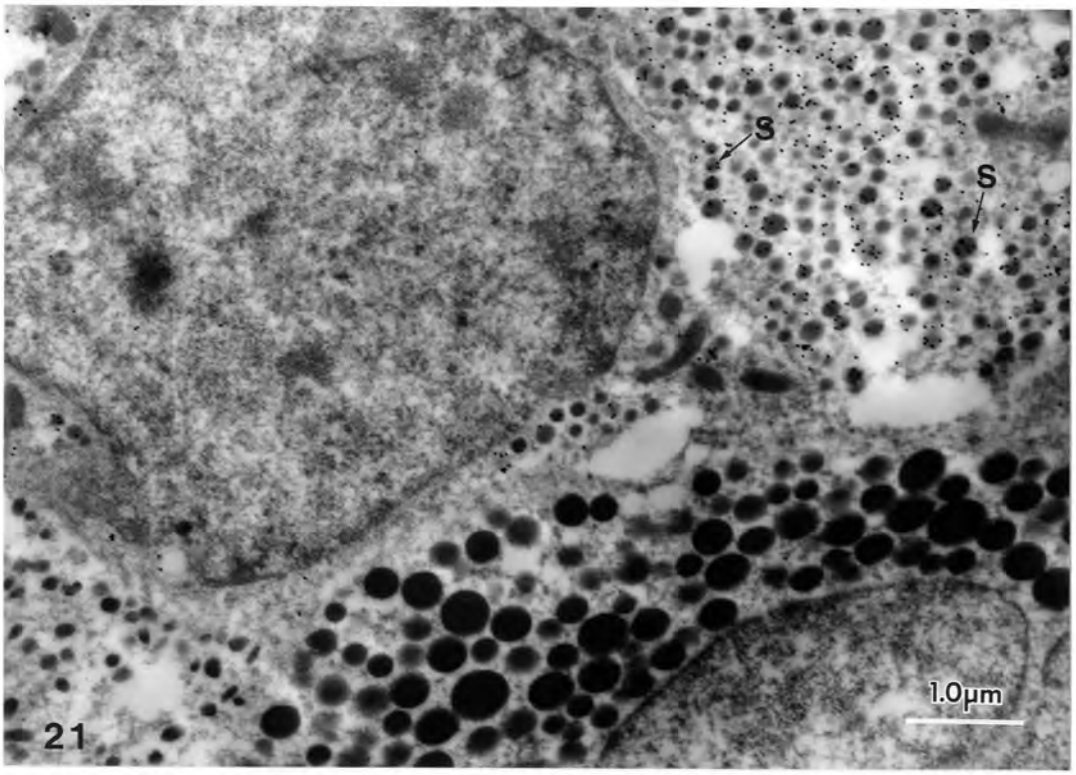


Figure 20: Sections of the anterior pituitary of male *M. schreibersii* from March (a) and December (b), showing oval (a) and polyhedral (b) shaped LH gonadotropes. The gold particles (arrows) on the secretory granules indicate the presence of LH. (Stain: IG labelling using 20nm gold particles).



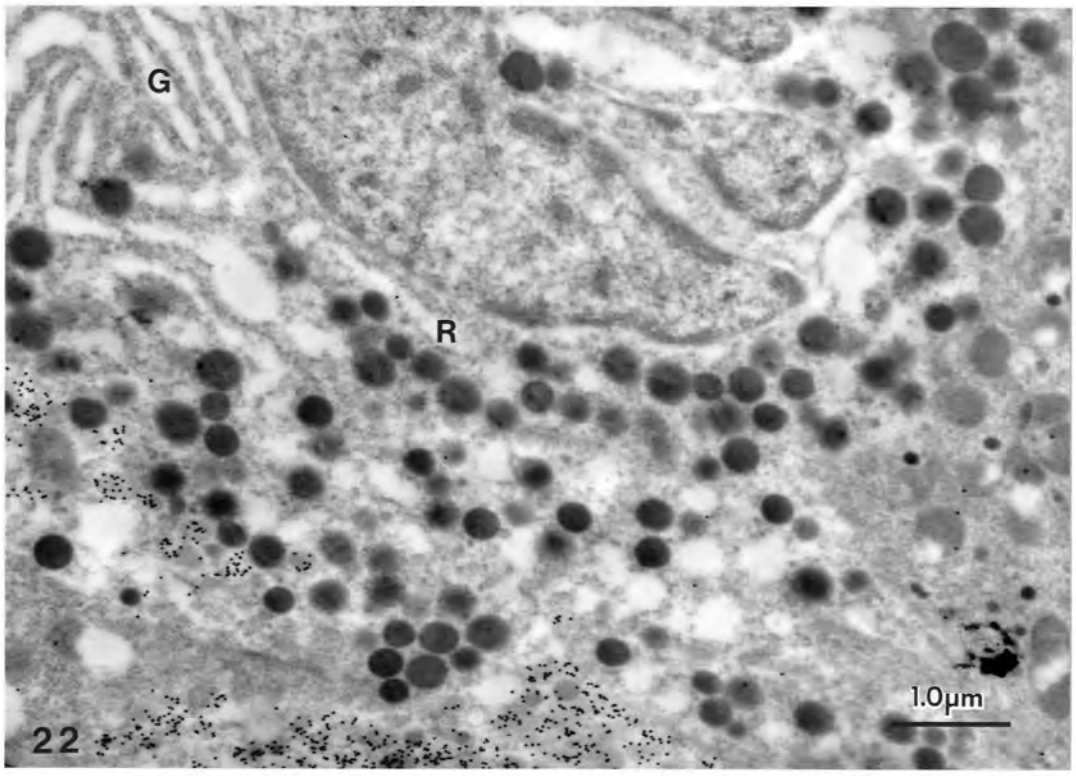


Figure 21: Section of a LH gonadotrope of male *R. capensis* from April, showing secretory granules (S) varying in size and electron density. (Stain: IG labelling using 20 nm gold particles).

Figure 22: Section of an active LH gonadotrope of male *M. schreibersii* from April. G - Golgi body; R - rough endoplasmic reticulum. (Stain: IG labelling using 20 nm gold particles).

During active secretion, the LH cells contain a well developed Golgi body, its cisternae greatly dilated (Fig. 22) and the rough endoplasmic reticulum appears as elongate or oval electron translucent structures, due to dilation of the cisternae (Fig. 22). Identification of FSH gonadotropes, using the polyclonal human FSH antibody, was only partially successful since little labelling was achieved. However, the same cell type (based on ultrastructure) was stained as labelled by the LH antibody (Fig. 23).

The mammotropes, identified using ICC and the prolactin primary antibody, are characterised by numerous electron dense secretory granules (Fig. 24a), ranging in size from 170 - 520 nm, dilated rough endoplasmic reticulum cisternae, and irregularly shaped nuclei. The somatotropes, identified using the growth hormone primary antibody, have slightly smaller (170 - 440 nm) and less electron dense secretory granules (Fig. 24b) compared to the mammotropes. Both cell types have a high proportion of larger secretory granules.

Experimental work

The injection of LHRH into male *M. schreibersii* in April, induced a response from the LH gonadotropes. There was a marked decrease in the relative abundance of labelled secretory granules per cell between the control bat (58%) (no injection) and the bat treated with LHRH for 15 minutes (9%) (Fig. 25a). The pituitary LH content (number of gold particles per granule) decreased as well from an average of 4.2 to 3.0. The treatment control, injected with PBS for 15 minutes, showed a decrease in relative abundance

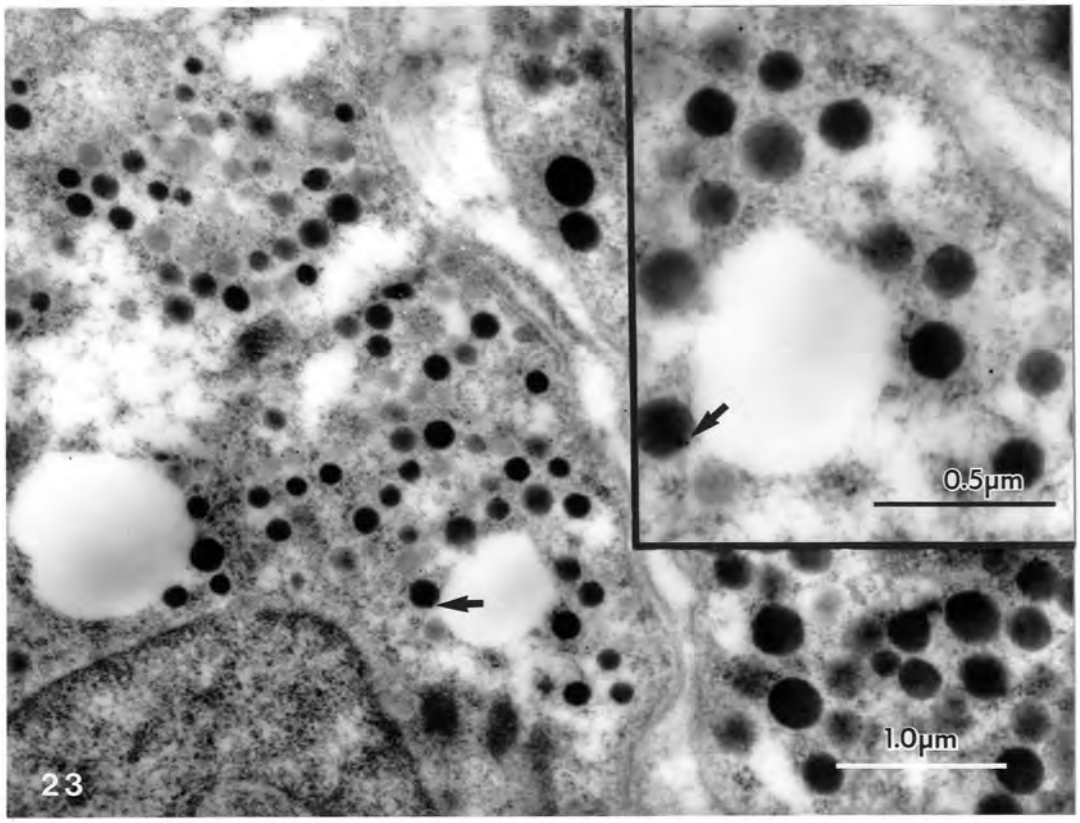
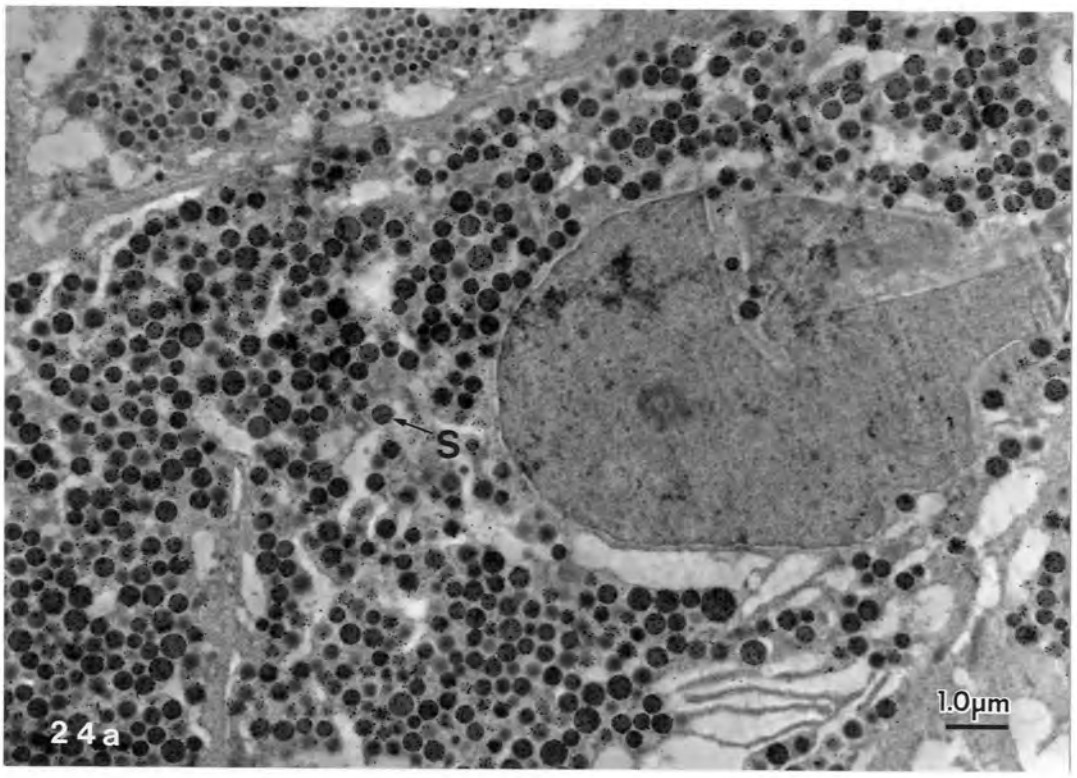


Figure 23: Section of a gonadotrope of female *M. schreibersii* from March, containing FSH, indicated by the presence of the gold particle (arrows) on some secretory granules. (Stain: IG labelling using 10 nm gold particles).



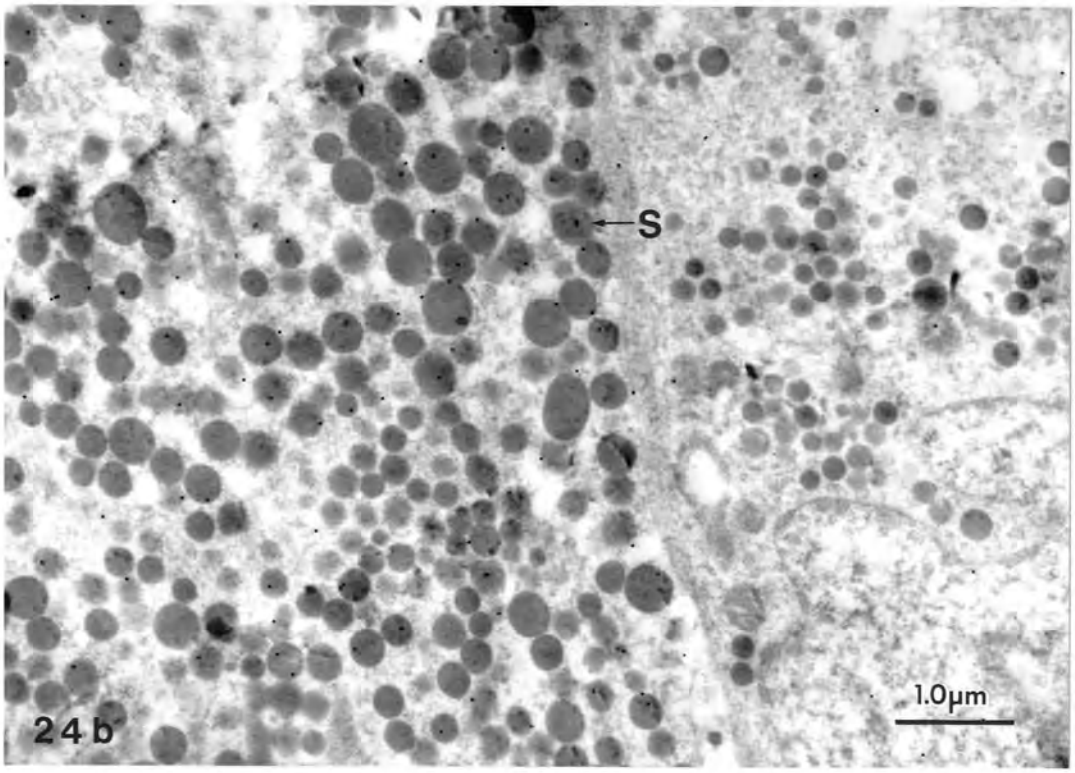


Figure 24: Sections of a mammotrope of female *M. schreibersii* from December (a) and a somatotrope of male *M. schreibersii* from March (b), containing numerous larger secretory granules (S), ranging in size from 170 - 520 nm and 170 - 440nm respectively. (Stain: IG labelling using 20 nm gold particles).

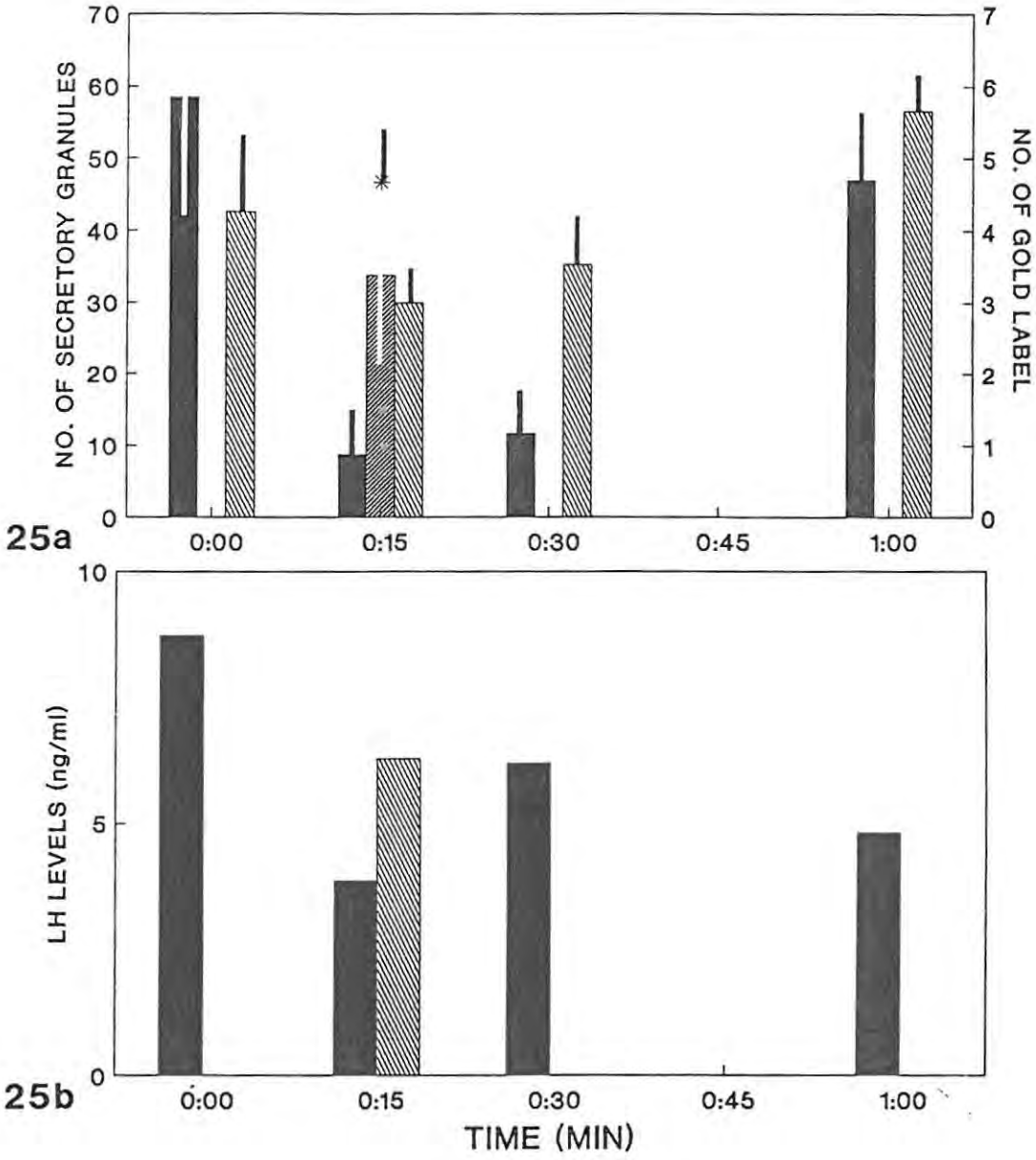


Figure 25: Changes in pituitary LH content (number of gold label per granule; a), abundance of secretory granules (a) and plasma LH levels (b) in male *M. schreibersii* after LHRH treatment. (Key to fig. 25a: ▒ - no. of gold particles, ▬ - no. of sec. gran., * - no. of gold particles in the 15 min. PBS treated bat, ▨ - no. of sec. gran. in the 15 min. PBS treated bat; key to fig. 25b: ▬ - LH levels, ▒ - LH levels in the 15 min. PBS treated bat; lines on/in the bars indicate standard deviations).

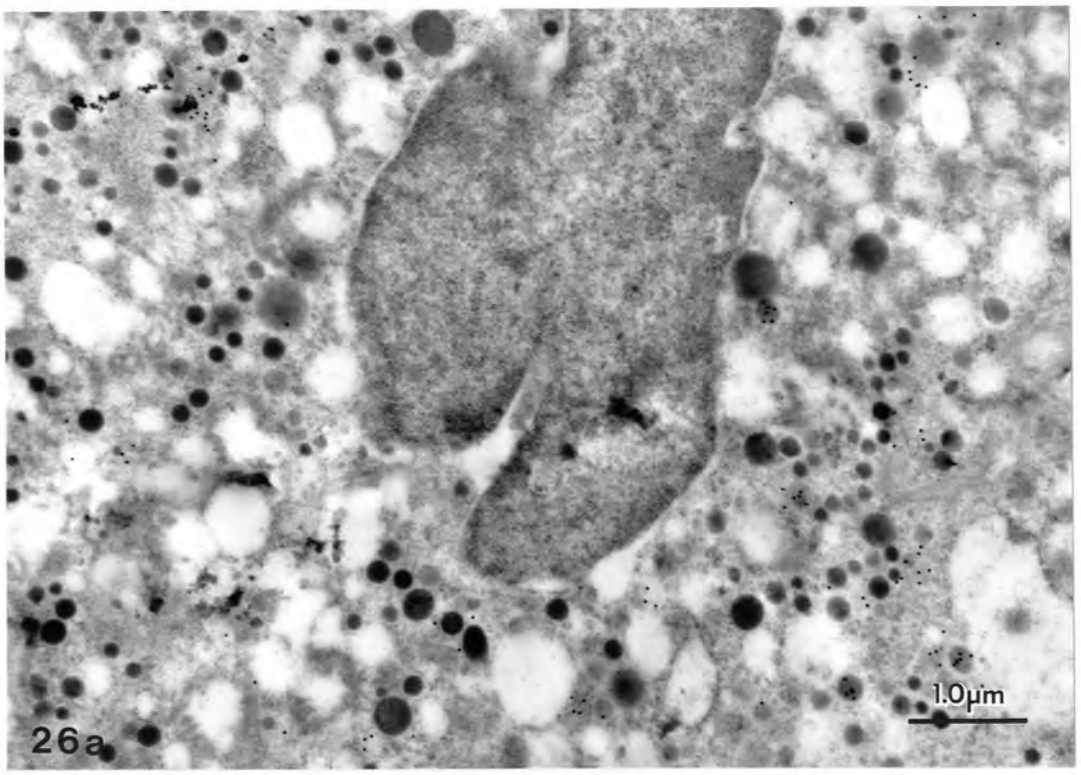
of secretory granules (58 to 34%), but the pituitary LH content showed a small increase (4.2 to 4.7 gold particles per granule). 30 minutes after LHRH treatment, there was a slight increase in both the relative abundance of secretory granules (9 to 12%) and pituitary LH content (3.0 to 3.5 gold particles per granule). These increases continued, and one hour after LHRH treatment, the relative abundance of secretory granules was slightly less than the control (47%), and pituitary LH content was greater than the control bat (5.7 gold particles per granule).

Gonadotropes from all specimens treated with LHRH show extensive dilation of the rough endoplasmic reticulum, and a marked difference can be seen in the number of secretory granules per cell and gold particles per granule between the 15 and 60 minute LHRH treatment (Figs 26a & b).

Plasma LH concentrations are lower in the experimental animals 15 minutes after injection with LHRH, and thereafter LH concentrations return to approximately normal values (Fig. 25b).

Secretion of hormones

Numerous sinusoids are distributed throughout the anterior pituitary, by which the hormones are transported in the blood to the target organs (Fig. 14). Cells close to the sinusoids are sometimes seen secreting granules into the capillaries by exocytosis (Fig. 27). An alternative mechanism is provided by the extensive dilations of the rough endoplasmic reticulum, which form channels that are continuous with the perivascular space (Fig. 28). Labelled secretory granules have been observed within



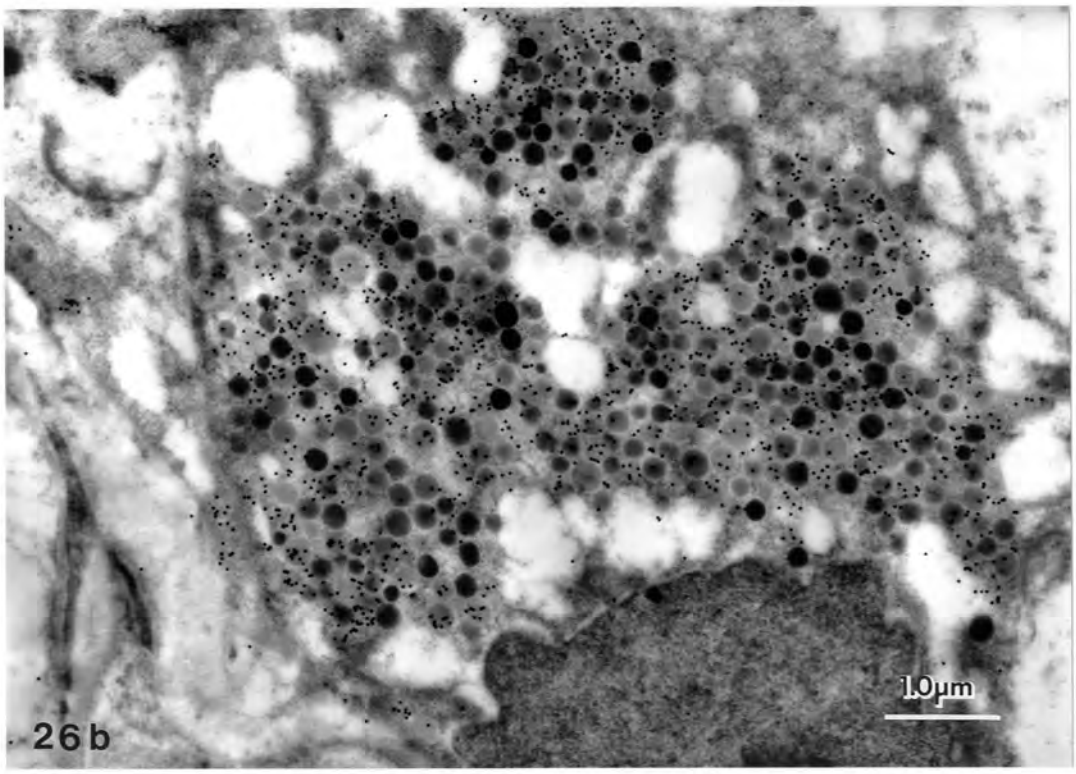
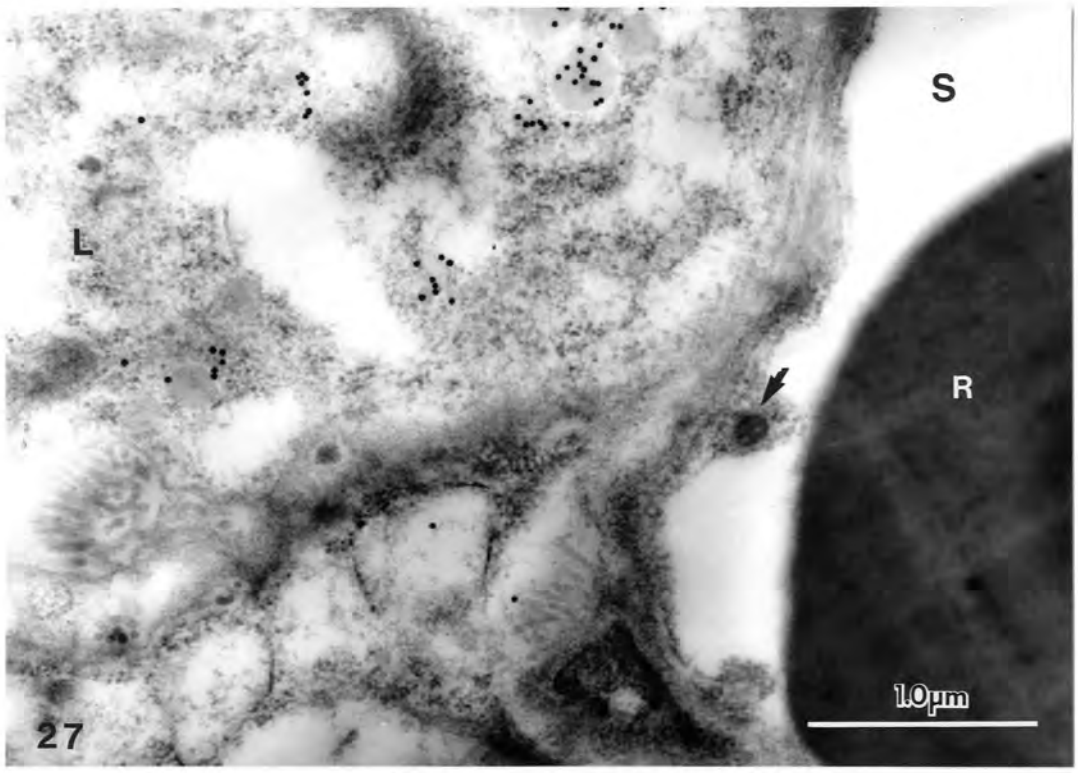


Figure 26: Sections of LH gonadotropes of male *M. schreibersii* from April, after 15 minutes (a) and 60 minutes (b) LHRH treatment. (Stain: IG labelling using 20 nm gold particles).



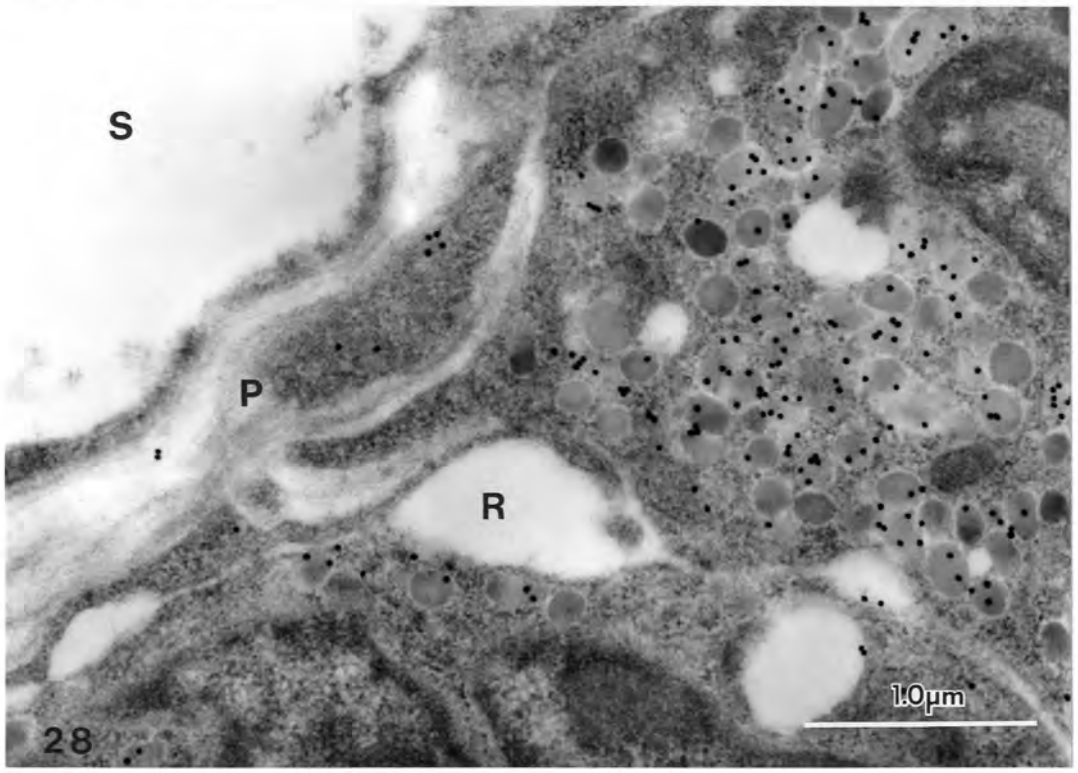


Figure 27: Section of an LH gonadotrope of female *M. schreibersii* from February, showing the secretion of a secretory granule (arrow) into the sinusoid (S). R - red blood cell; L - LH gonadotrope. (Stain: IG labelling using 20 nm gold particles).

Figure 28: Section of an LH gonadotrope of female *M. schreibersii* from March, showing the extensive dilations of the rough endoplasmic reticulum (R). S - sinusoid; P - perivascular space. (Stain: IG labelling using 20 nm gold particles).

the cisternae and within the perivascular space (Fig. 28).

Discussion

The gross morphology of the pituitary glands of *M. schreibersii* and *R. capensis* is the same as described for other bat species (Sawyer, 1936; Siegel, 1955; Patil, 1974; Anthony & Gustafson, 1984; Mikami *et al.*, 1988) and mammals in general (Nakane, 1970; Herbert, 1975; Baker & Gross, 1978) in that they comprise the anterior pituitary (pars distalis) which is separated from the pars nervosa by the pars intermedia.

In the past, identification of the different cell types using tinctorial methods proved difficult, and as a result, different distributions for basophils and acidophils were reported. Basophils are located at the anterior, and acidophils at the posterior end of the anterior pituitary of *Myotis lucifugus* (Siegel, 1955), while in the squirrel (Bhiwgade & Gulhane, 1980), rhesus monkey (Herbert, 1975) and *M. schreibersii* (present study) the basophils occur mainly in the middle region and the acidophils in the lateral regions of the anterior pituitary. LH gonadotropes are typically found scattered throughout the anterior pituitary, whereas the thyrotropes were present only in the middle region, resulting in the concentration of basophils in this region of the gland (Bhiwgade & Gulhane, 1980).

ICC studies show LH gonadotropes scattered either singly or in small groups throughout the anterior pituitary (rat: Nakane, 1970; mouse: Baker & Gross, 1978; dog: El Etreby *et al.*, 1977;

rhesus monkey: Herbert, 1978; *Myotis lucifugus*: Anthony & Gustafson, 1984; *Miniopterus schreibersii*: Mikami *et al.*, 1988 and present study). LH gonadotropes are often aggregated around or close to the infundibular stalk ("sex zone"), where the cells are closest to the incoming portal vessels (which transport the gonadotropic-releasing hormone to the pituitary) (Nakane, 1970; Baker & Gross, 1978; Anthony & Gustafson, 1984; present study).

The greater relative abundance of LH gonadotropes in male compared to female *M. schreibersii*, is similar to the pattern found in the little brown bat, although the relative abundance of LH gonadotropes were lower (Anthony & Gustafson, 1984). In contrast the relative abundance of LH gonadotropes of female *R. capensis* is higher than that obtained for the male.

The ultrastructure of the gonadotropes of *M. schreibersii* and *R. capensis* is similar to that described for the rat (Nakane, 1970), mouse (Payette *et al.*, 1987) and pig (Batten & Hopkins, 1978). However, many workers have described two populations of gonadotropes, one with small granules (about 200 nm) and a second with large granules (300 - 700 nm) (Tougard, 1980; Kurosumi & Inoue, 1986). These two cell types were not observed in *M. schreibersii* and *R. capensis* and the largest granules in the gonadotropes had a diameter of 350 - 400 nm. Since both the LH and the FSH antibodies stained the same cell type, it is assumed that the gonadotropes produce both hormones. This is in agreement with findings on pituitary studies of several mammals (Moriarty, 1975; Richardson, 1979; Childs, 1986) where FSH and LH are produced by the same cell and may even occur in the granules (Payette *et al.*, 1987). The low immunoreactivity between the human FSH antibody and the bat FSH

antigen is possibly due to the fact that few amino acids are shared in the β-subunit.

The mammotropes and somatotropes of *M. schreibersii* contain large secretory granules ranging in size from 200 - 500 nm and 170 - 440 nm respectively. Reports from the literature indicate that prolactin secreting cells vary greatly, the size of the secretory granule being 170 - 200 nm in the mole and up to 600 nm in rats (Herlant, 1964a). Growth hormone cells in the rat possess secretory granules of 320 - 390 nm (Howell & Whitfield, 1973), an average of 350 nm in the mouse (Barnes, 1962) and 350 - 400 nm in bats (Azzali, 1971). Studies on the pituitaries of the cow and the Japanese house bat revealed the presence of prolactin and growth hormone in the same cell, termed mammosomatotrope, in addition to cells containing only prolactin or growth hormone (Fumagalli & Zanini, 1985; Ishibashi & Shiino, 1989). These cells contained a wide range of secretory granules from 200 - 900 nm, and in pregnant female Japanese house bats, *Pipistrellus abramus*, they were found to be more than 1000 nm in diameter. It is possible, that all anterior pituitary glands contain mammosomatotropes, but this can only be established with double immunogold labelling using two different sizes of gold particles.

The injection of LHRH into the bats induced changes in ultrastructure and pituitary LH content of the gonadotropes, and in the plasma LH concentration. The initial decrease in the number of secretory granules and the amount of LH present in the gonadotropes has been observed in rats treated with LHRH, where depletion of granules occurred after 10 - 15 minutes (Luborsky-Moore *et al.*, 1975; Roemmler *et al.*, 1978). With the depletion of

secretory granules during the first 15 minutes there should be an increase in the plasma LH concentration. This apparently did not occur in the LHRH treated bat, where the plasma LH concentration showed a decrease after 15 minutes. It is possible that there was an initial and rapid increase in plasma LH levels followed by a decrease during the first 15 minutes. This was found in LHRH injected rats (Mendoza *et al.*, 1973), chicken (Clarke & Ottinger, 1987) and ewes (Newton & Edgerton, 1989). However other workers found the plasma LH level increasing only after 15 - 20 minutes post LHRH injection (Roemmler *et al.*, 1987; Fink & Pickering, 1980; Smith & Bartke, 1987). The experimental control bat (injection of PBS for 15 minutes) showed a decrease in numbers of secretory granules, but this reduction was much less compared to the 15 minutes LHRH treated bat. This response could be due to stress caused by the injection of PBS into the bat. Furthermore, the amount of LH present in the secretory granules is higher compared to the control, indicating increased LH synthesis, possibly to counteract the stress related response in order to restore initial pituitary LH levels. Changes after 30 minutes indicate a period of LH synthesis and return to the typical condition of the control. All LHRH treated gonadotropes show extensive dilations of the Golgi bodies and the cisternae of the rough endoplasmic reticulum, indicating the cells synthetic and secretory activity (Shiino, 1982). The extensive dilation of Golgi body and cisternae of rough endoplasmic reticulum was also found in LH gonadotropes of rats (Tougard, 1980; Kurosumi & Inoue, 1986) and other bat species (Azzali, 1971).

Secretion of the hormones of the anterior pituitary by exocytosis

of the secretory granules is generally accepted (Herlant, 1967; Pelletier *et al.*, 1971; Howell & Whitfield, 1973). In the LH gonadotropes of *M. schreibersii* and *R. capensis*, secretory granules were observed being discharged from the basal membrane of the cell into the blood capillaries by apocrine secretion. This type of secretion involves the discharge of cytoplasm, granules and other cell organelles, and has been reported in pituitary cells of the rabbit (Salazar & Peterson, 1964). However, an alternative mechanism of intracisternal granule formation for rapid hormone release was suggested by Peters *et al.* (1989). During secretory activity of the gonadotropes, the cisternae of the rough endoplasmic reticulum become greatly dilated and form extensive channels, in which granules and cytoplasmic islands of granules were found. These channels were continuous with the perivascular space and increased the surface area for the release of hormones. In the LH gonadotropes of *M. schreibersii* and *R. capensis* secretory granules were observed being expelled by apocrine secretion, and were also found in the dilated cisternae of the rough endoplasmic reticulum and in the perivascular space. It is therefore assumed that LH secretion occurs by exocytosis and by intracisternal granule formation as an additional mechanism for rapid hormone release. Intracisternal granule formation was observed in LH gonadotropes of bat anterior pituitaries treated with LHRH for 15 and 30 minutes, thus probably contributing to the rapid release of LH following the LHRH injection.

Summary

While it is not possible to identify the various cells of the anterior pituitary based on ultrastructural and morphological features alone, the use of ICC allows positive identification. LH gonadotropes occur throughout the anterior pituitary of *M. schreibersii* and *R. capensis*, and the relative abundance varies between sexes and species. The cells possess generally small secretory granules in which LH is stored. FSH, is found in the same cell type and it is therefore assumed that both hormones are secreted by the same gonadotrope although this can only be confirmed using double immunogold labelling. Stimulation of the gonadotropes with LHRH, brought about a change in gonadotrope ultrastructure and a decreased pituitary LH content. The findings were in accordance with results obtained from pituitary studies on other mammals.

CHAPTER 5: SEASONAL CHANGES IN ACTIVITY OF GONADOTROPE AND PLASMA LH LEVELS IN RELATION TO THE REPRODUCTIVE CYCLE OF *Miniopterus schreibersii* AND *Rhinolophus capensis*

Introduction

Miniopterus schreibersii and *Rhinolophus capensis* employ different reproductive strategies (delayed implantation and sperm storage and delayed ovulation respectively) and since LH is essential in regulating mammalian reproduction (Austin & Short, 1984), differences in seasonal activity of LH gonadotropes and plasma LH levels in these two bat species are expected. Seasonal differences in the ultrastructure of LH gonadotropes have been described in *Macrotus californicus* (Richardson, 1979), *Myotis lucifugus* (Anthony & Gustafson, 1984), and other mammals (Clarke & Forsyth, 1964; Foresman & Mead, 1974; Canivenc & Bonnin, 1981; Licht *et al.*, 1982), and periods of gonadotrope activity coincide with reproductive activity. This chapter deals with the changes in numbers and ultrastructure of LH gonadotropes, and plasma LH levels of *M. schreibersii* and *R. capensis*, and relates such changes to their reproductive cycles.

Results

Seasonal changes in pituitary weight

The weight of the pituitary of female *M. schreibersii* is more or less constant during the year (c. 1 mg), except for November,

January and February, when the weight increases up to 1.6 mg (Fig. 29a). The weight of the pituitary of male *M. schreibersii* fluctuates greatly, ranging from 0.8 mg in December to 1.25 mg in May (Fig 29b). In male *R. capensis*, pituitary weight changes little during the year, with peaks in June (1.4 mg), October and November (1.5 mg), and January (1.35 mg) (Fig. 30a). Pituitary weight in female *R. capensis* shows the greatest fluctuation from 0.8 mg in May to 1.8 mg in October (Fig. 30b), but data for December, January and February were not available. These statistical significant fluctuations in pituitary weight ($p < 0.01$) for both species are not a consequence of a change in body size, since forearm length stays more or less constant during the year.

Seasonal changes in the abundance of LH gonadotropes

Seasonal changes in abundance of LH gonadotropes were estimated from immunogold/silver stained light microscope sections. This technique was used from October 1988 to May 1989 and consequently data were only available from this period.

The abundance of LH gonadotropes in the anterior pituitary changes seasonally. During follicular development in female *M. schreibersii* the abundance of LH gonadotropes is high, reaching a peak in April (13.3%) (Fig. 31a). The abundance of LH gonadotropes is low prior to and after follicular development (8.2% in November, Fig. 31b; 6.8% in January; 6.2% in May). A similar pattern is seen male *M. schreibersii*, and abundance of LH gonadotropes is higher than in the female. During reproductive inactivity the abundance of LH gonadotropes is low (10.4% in October). Just prior to spermatogenesis LH gonadotropes increase in

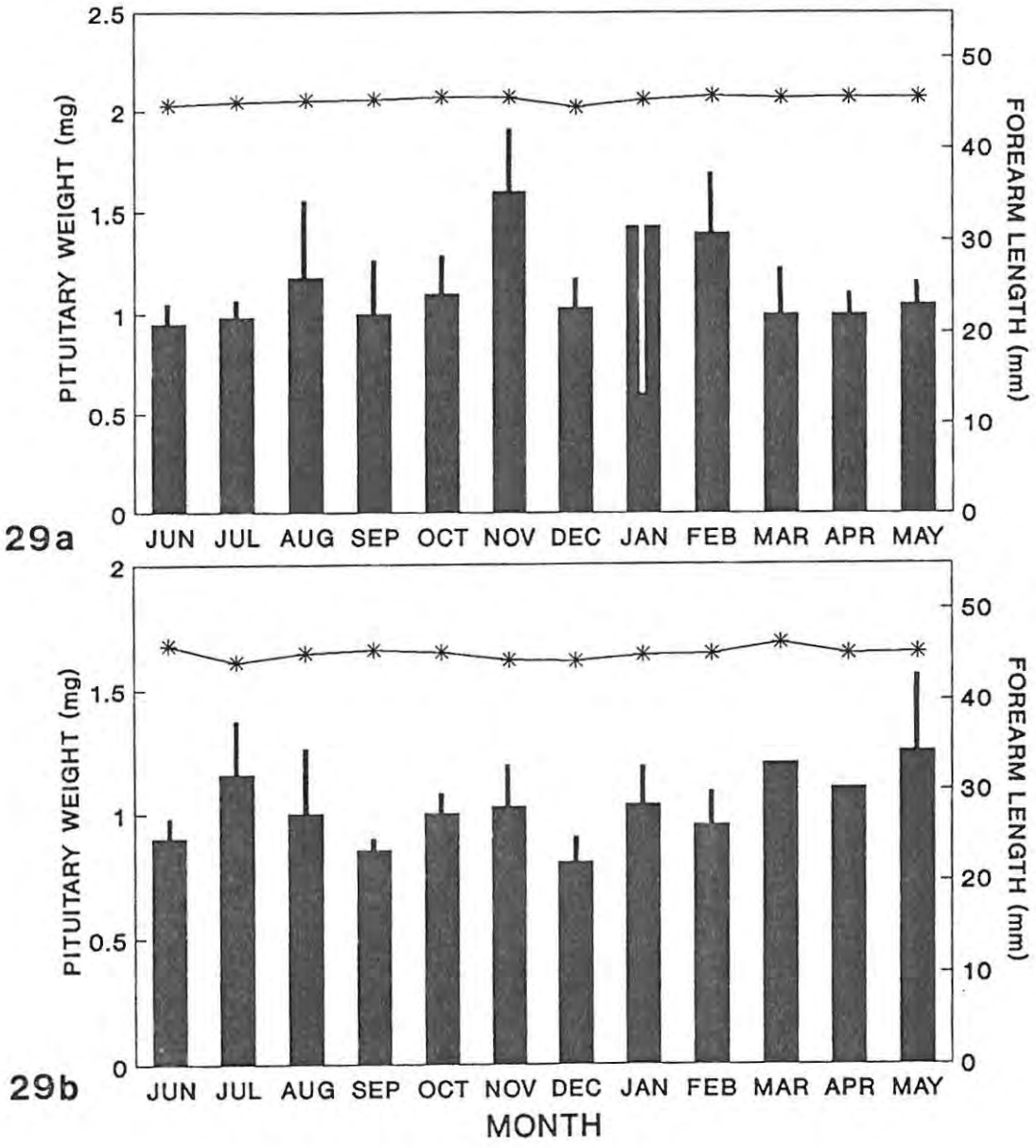


Figure 29: Seasonal changes in pituitary weight (bars) and forearm length (line) in female (a) and male (b) *M. schreibersii* from June 1988 to May 1989. (Lines on/in the bars indicate standard deviations).

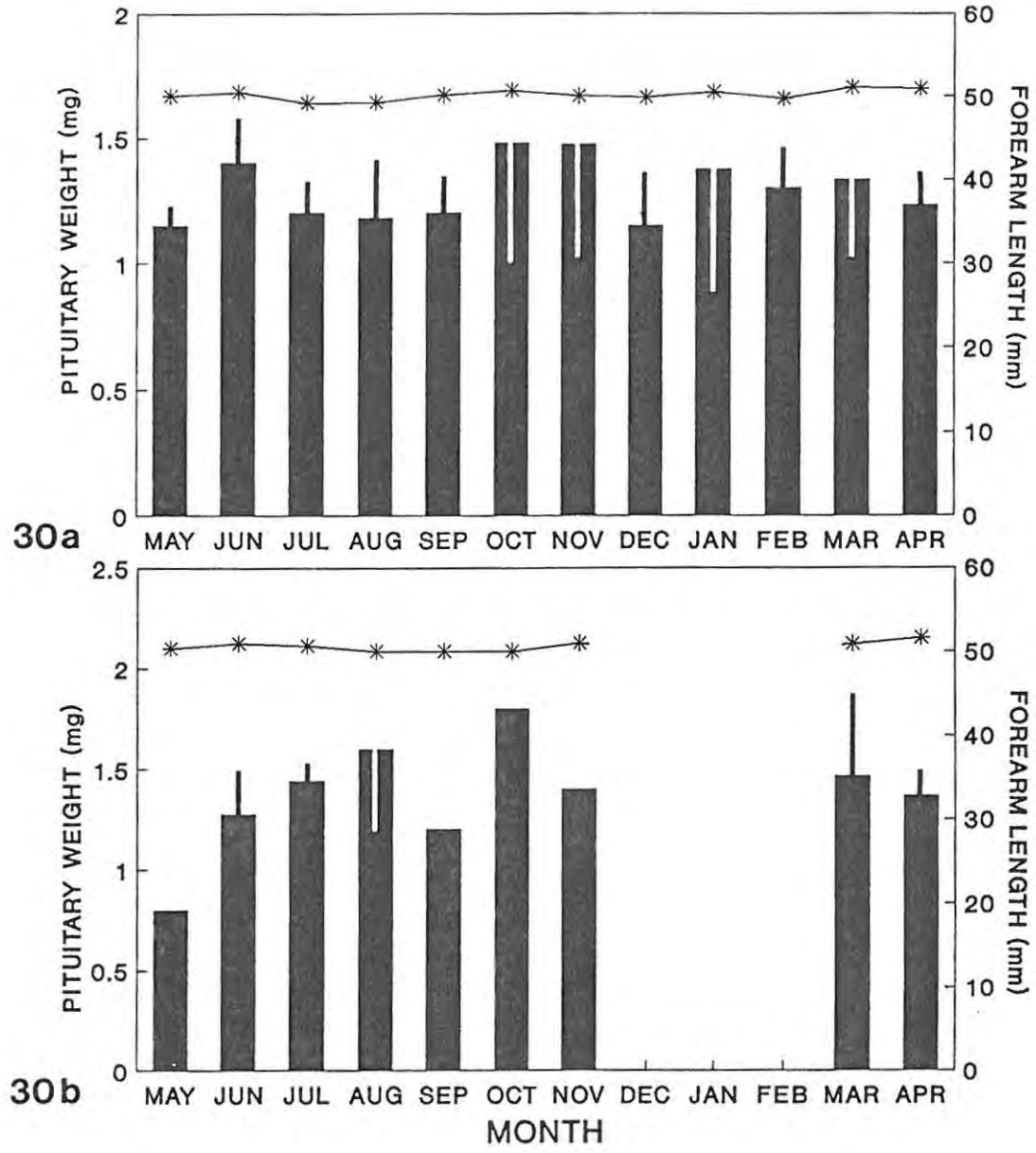


Figure 30: Seasonal changes in pituitary weight (bars) and forearm length (line) in male (a) and female (b) *R. capensis* from May 1988 to April 1989. (Lines on/in the bars indicate standard deviations).

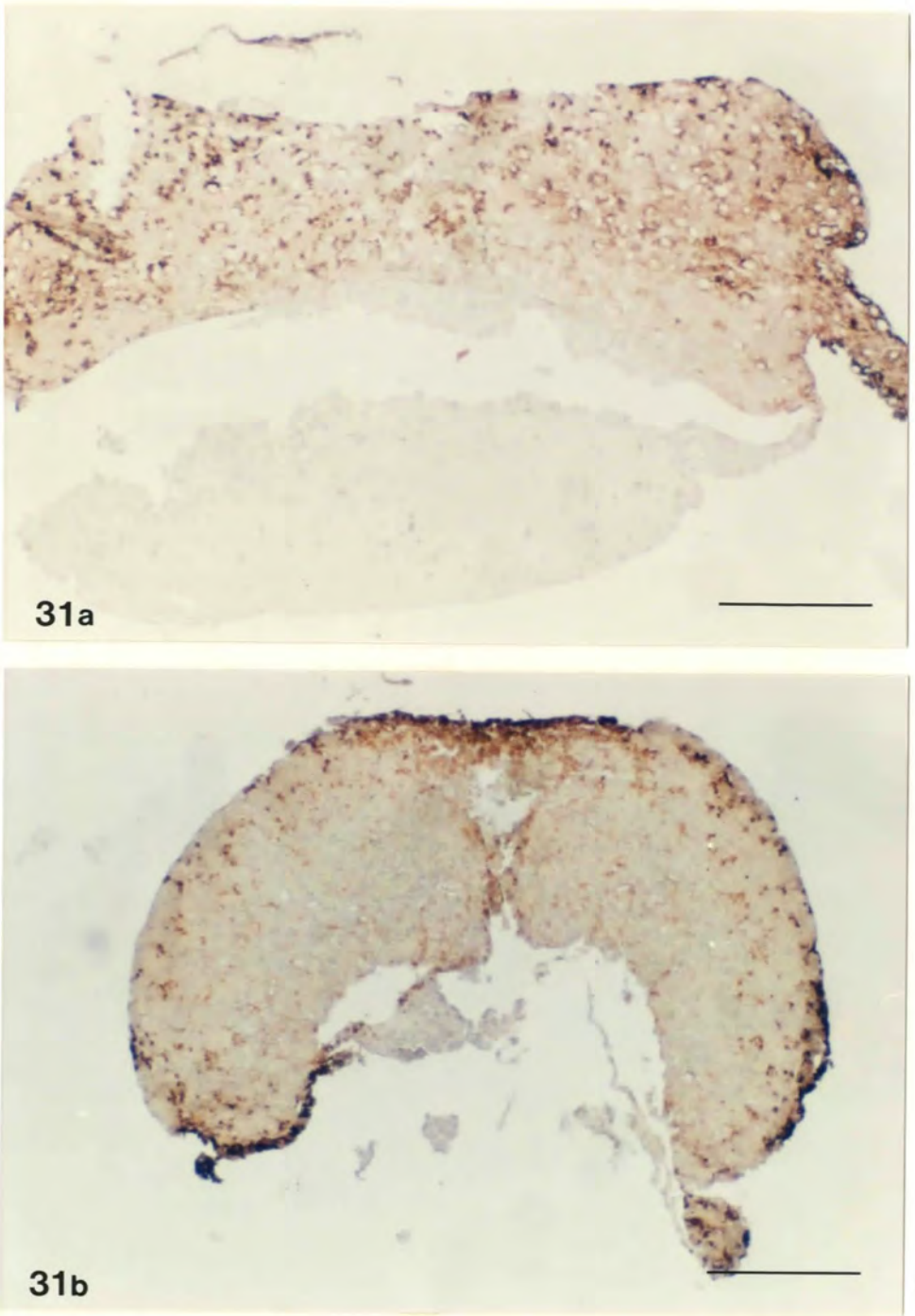


Figure 31: Horizontal sections of the pituitary gland, showing the abundance of LH gonadotropes in female *M. schreibersii* in April (a) and November (b). (Stain: IGSS; scale bars represent 200 μm).

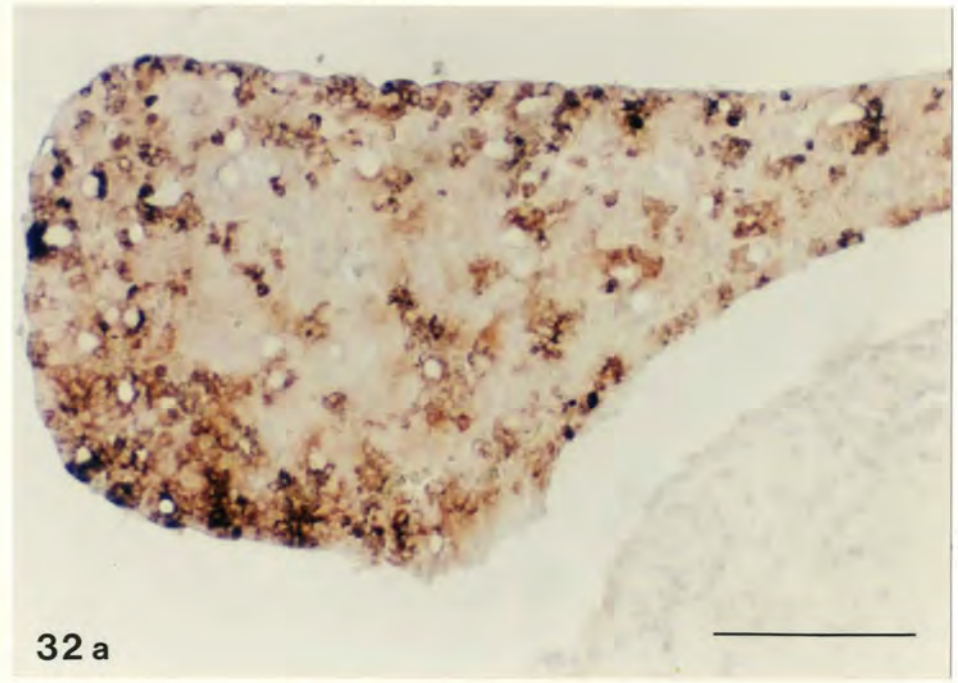
abundance to 13.4% (December), and during spermatogenesis the abundance of LH gonadotropes reaches a peak in March (19.4%) (Fig. 32a). Post spermatogenesis the numbers of LH gonadotropes drop to 10.7% in May (Fig. 15b). The abundance of LH gonadotropes in male *R. capensis* shows little variation, being slightly higher at the beginning of spermatogenesis (10.4% in October, Fig. 32b; 10.8% in December), than towards the middle and the end of spermatogenesis (7.9% in February; 9.7% in March; 7.7% in April, Fig. 16a).

No significant correlation (i.e. all r-values obtained are not significantly different from zero) occurs between the pituitary weight and the percentage of LH gonadotropes present in the anterior pituitary of *M. schreibersii* (Pearson's correlation coefficient r = 0.25 for males, r = -0.40 for females) and *R. capensis* (r = 0.20 for males) during the study period. For female *R. capensis* not enough data are available for comparative purposes.

However, in male *M. schreibersii* there is an increase in pituitary weight, which coincides with an increase in abundance of LH gonadotropes in the anterior pituitary, between February and March (the beginning of spermatogenesis). A similar phenomenon is also observed in male *R. capensis*, where in October (beginning of spermatogenesis) pituitary weight increases and the percentage of LH gonadotropes is high.

Seasonal changes in the ultrastructure of LH gonadotropes and plasma LH levels

LH gonadotropes display marked seasonal changes in the abundance



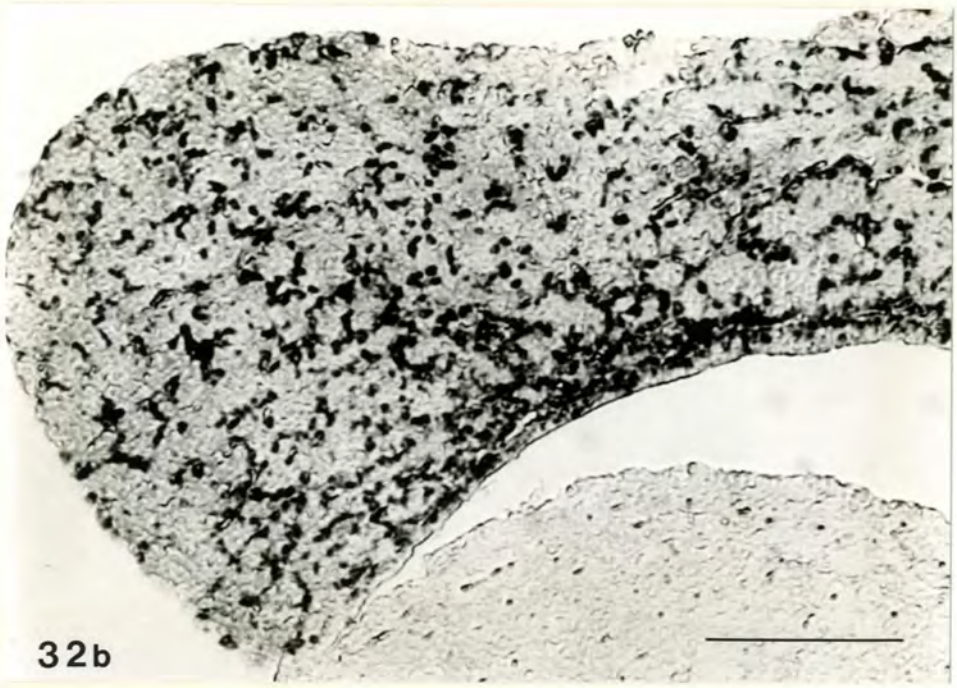


Figure 32: Section of the anterior pituitary, showing the abundance of LH gonadotropes in male *M. schreibersii* (a) in March, and in male *R. capensis* (b) in October. (Stain: IGSS; scale bars represent 100 μm).

of labelled secretory granules present, in the degree of dilation of the cisternae of rough endoplasmic reticulum and Golgi body, in the size of the secretory granule and in the immunoreactivity of the LH to the LH antibody.

In both species, during reproductive activity LH gonadotropes show extensive dilation of the cisternae of rough endoplasmic reticulum and Golgi body (Fig. 22), which are reduced during reproductive inactivity. During reproductive activity there is a higher proportion of larger secretory granules (250 - 400nm) (Fig. 20a), compared to reproductive inactivity, where the majority of secretory granules range in size from 100 - 250 nm (Fig. 20b). The abundance of secretory granules present in gonadotropes, and the pituitary LH content is higher during reproductive activity. In male *M. schreibersii* (Figs 33a & b) pituitary LH content (measured by counting the number of gold particles per secretory granule) is low during reproductive inactivity (June to December); there is a marked increase (about 25%) from December to January, and pituitary LH content peaks in March. The numbers of secretory granules present in the LH gonadotropes more or less follow the same pattern, except for October, when there is a marked increase in the number of secretory granules. Plasma LH levels (Figs 33a & c) are elevated during reproductive inactivity (August and October) and during spermatogenesis, at which time plasma testosterone levels are elevated. In female *M. schreibersii* (Figs 34a & b) the highest numbers of secretory granules in the cells occur during follicular development, and this coincides with a high pituitary LH content. During April/May, when copulation, ovulation and fertilisation

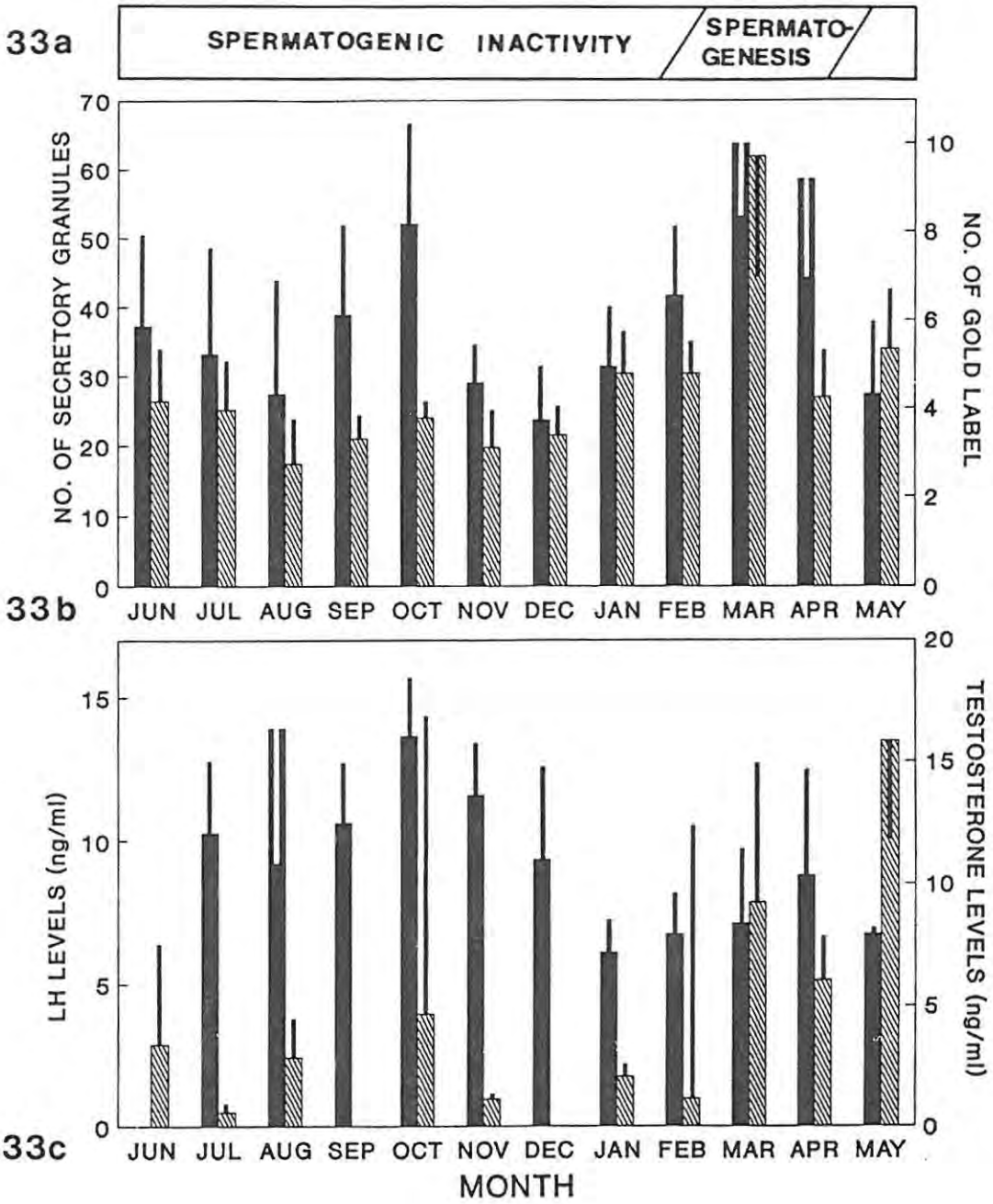


Figure 33: Seasonal changes in abundance of secretory granules (b), pituitary LH content (number of gold particles per granule; b), plasma LH levels (c) and plasma testosterone levels (c) during the reproductive cycle (a) of male *M. schreibersii*. (Key to fig. 33b: ■ - no. of sec. gran., ▨ - no. of gold particles; key to fig. 33c: ■ - LH levels, ▨ - testosterone levles; lines on/in the bars indcate standard deviations).

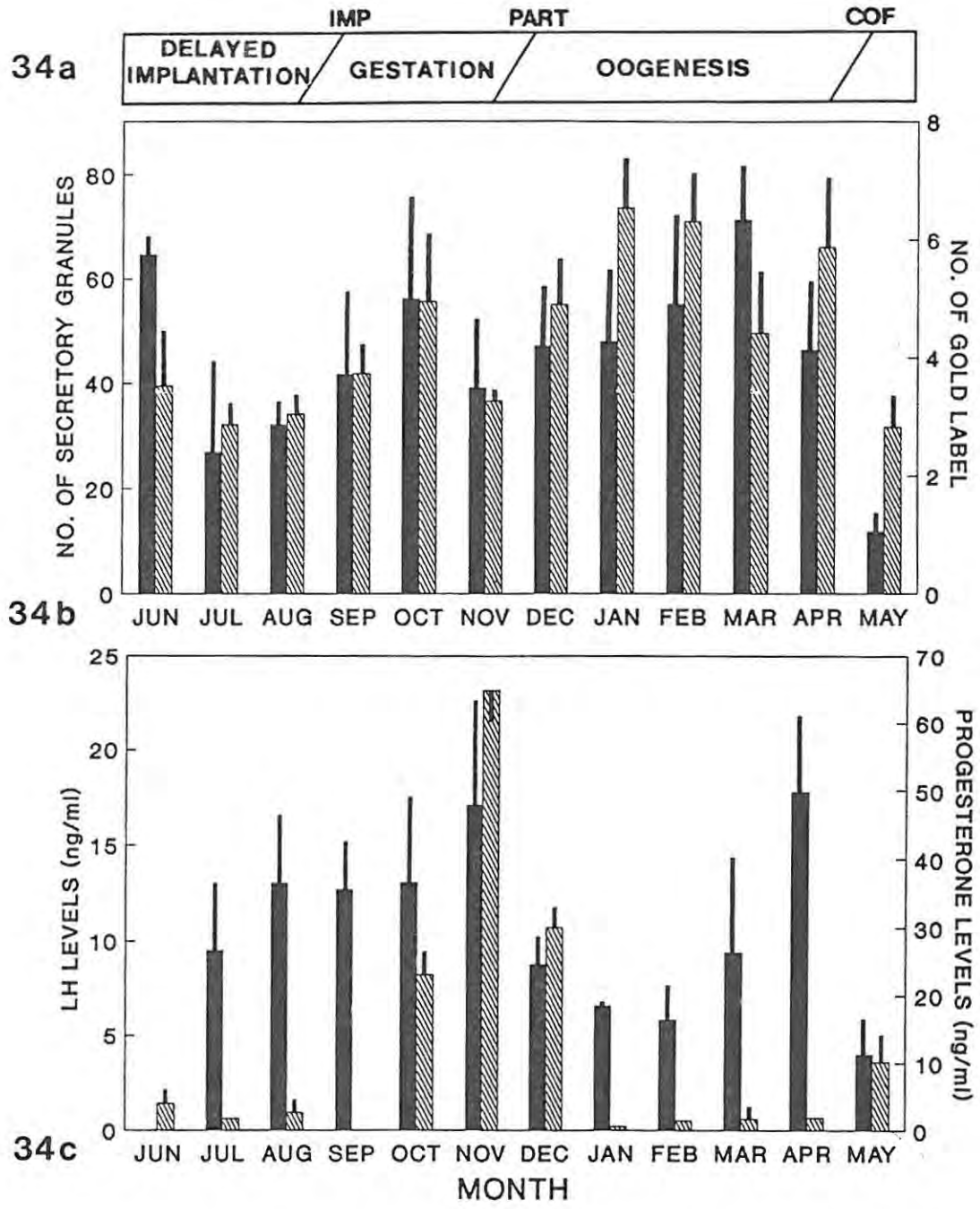


Figure 34: Seasonal changes in abundance of secretory granules (b), pituitary LH content (number of gold particles per granule; b), plasma LH levels (c) and plasma progesterone levels (c) during the reproductive cycle (a) of female *M. schreibersii*. (Key to fig. 34a: COF - copulation, ovulation & fertilisation, IMP - implantation, PART - parturition; key to fig. 34b: ■ - no. of sec. gran., ▨ - no. of gold particles; key to fig. 34c: ■ - LH levels, ▨ - progesterone levels; lines on/in the bars indicate standard deviations).

take place, there is a marked decrease in both the numbers of secretory granules and pituitary LH content, which then remain low during winter hibernation. In August/September, when implantation takes place, numbers of secretory granules and pituitary LH content increase, reaching a peak in October. LH content and the abundance of secretory granules decrease in November and increase again in December. Plasma LH levels (Figs 34a & c) are high during pregnancy and follicular development, corresponding to elevated plasma progesterone levels from October to December and in May. Plasma LH levels are low between December and February and during winter, increasing towards the end of hibernation. The low plasma LH levels coincide with low plasma progesterone levels.

In male *R. capensis* (Figs 35a & b) both the number of secretory granules present in LH gonadotropes and the pituitary LH content are high during spermatogenesis reaching a peak in March, after which secretory granule numbers and pituitary LH content drop. The lowest levels of pituitary LH content and secretory granule numbers are recorded at the end of hibernation in July. Plasma LH levels (Figs 35a & c) are elevated from December to April, showing a peak in March. During hibernation LH levels decrease and remain low until November. In female *R. capensis* (Figs 36a & b), the limited data indicate high numbers of secretory granules and pituitary LH content in March and April during follicular development. Both levels drop during winter and pituitary LH content and secretory granule numbers are lowest at the end of

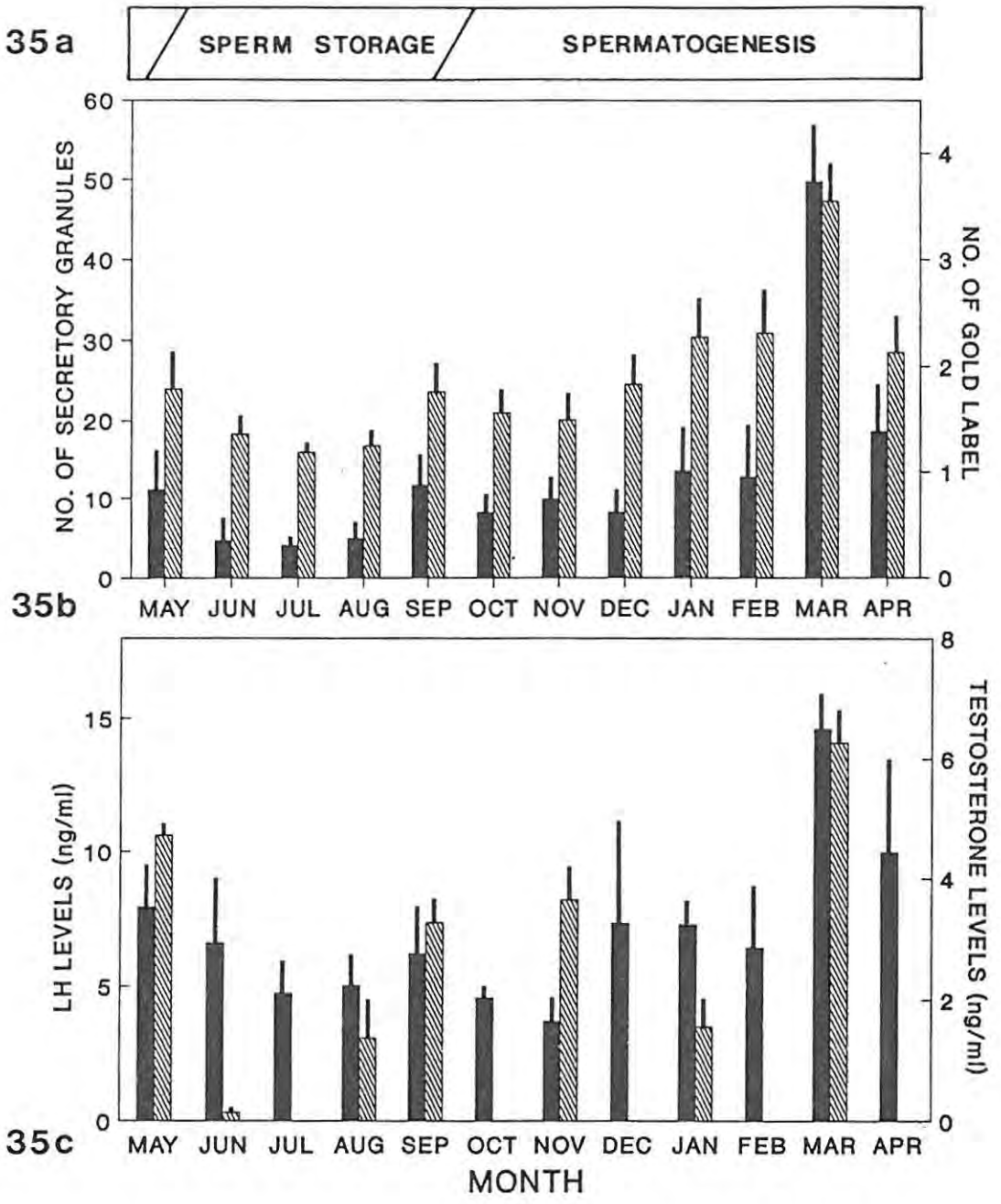


Figure 35: Seasonal changes in abundance of secretory granules (b), pituitary LH content (number of gold particles per granule; b), plasma LH levels (c) and plasma testosterone levels (c; after Bernard, 1986) during the reproductive cycle (a) of male *R. capensis*. (Key to fig. 35b: ■ - no. of sec. gran., ▨ - no. of gold particles; key to fig. 35c: ■ - LH levels, ▨ - testosterone levels; lines on the bars indicate standard deviations).

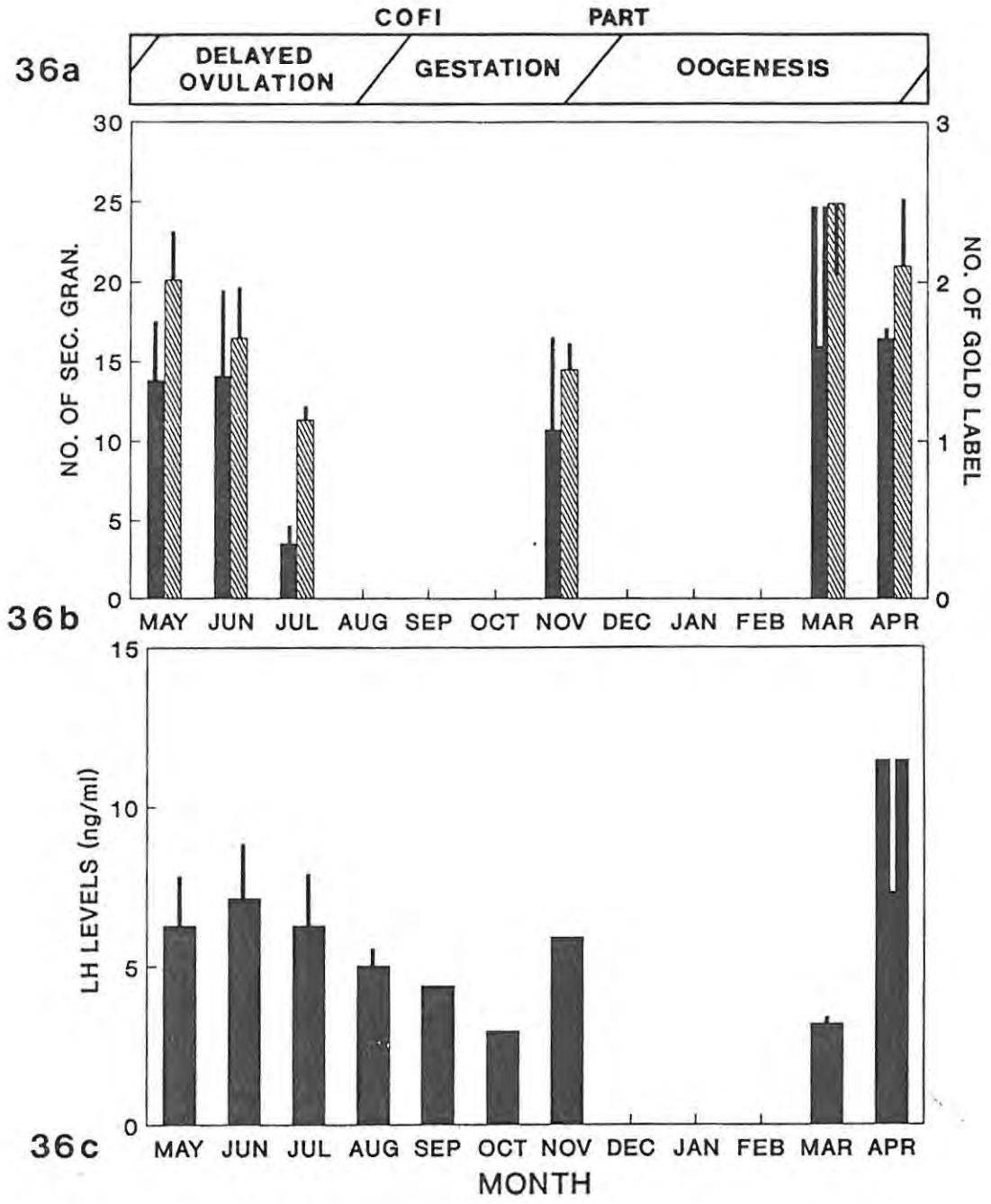


Figure 36: Seasonal changes in abundance of secretory granules (b), pituitary LH content (number of gold particles per granule; b) and plasma LH levels (c) during the reproductive cycle (a) of female *R. capensis*. (Key to fig. 36a: COFI - copulation, ovulation, fertilisation & implantation, PART - parturition; key to fig. 35b: ■ - no. of sec. gran., ▨ - no. of gold particles; lines on/in the bars indicate standard deviations).

hibernation. Plasma LH levels (Figs 36a & c) are elevated in April (late follicular development); remain high during winter hibernation; and decrease towards the end of hibernation and during early pregnancy. High levels of LH are recorded during November. Data for December, January and February were not available. In March plasma LH levels are low.

Discussion

The anterior pituitary in both bat species displays marked seasonal changes in ultrastructure and pituitary LH content. In addition to these cytological changes, plasma LH levels and the pituitary weight show seasonal variation.

The fact that pituitary weight in both species and the abundance of LH gonadotropes are not significantly correlated is probably due to the presence of other cell types, which may also show seasonal changes in abundance, hence contributing to the change in pituitary weight. However, Richardson (1979) found that a weight increase in the anterior pituitary of female *Macrotus californicus*, prior to parturition and mid-lactation, coincides with increases in numbers and size of immunoreactive prolactin cells. In female *M. schreibersii*, the increase in pituitary weight prior to parturition (December) and during lactation (January and February) could be due to an increase in numbers of prolactin cells. Furthermore Racey (1974) found the weight of the pituitary gland of *Nyctalus noctula* to be highest at the beginning of spermatogenesis, which could be due to an increase in the abundance

of LH gonadotropes. This is also observed in *R. capensis*, but in *M. schreibersii* the pituitary weight is high only one month after the beginning of spermatogenesis.

In male *M. schreibersii*, LH gonadotropes are active just prior to and during spermatogenesis. From December to January the twenty-five percent increase in pituitary LH content and in the numbers of secretory granules present indicates an increase in LH synthesis. This probably follows a cue, possibly in late December, when external factors ultimately initiate the spermatogenic cycle. During spermatogenesis the increased abundance of secretory granules and the high pituitary LH content coincide with increasing plasma LH and testosterone levels. During reproductive inactivity, LH gonadotropes appear to be inactive, except for October, when there is an increase in the number of secretory granules per cell. The elevated plasma LH levels between July and November can not be explained since the males are reproductively inactive and pituitary LH content is low. The peak in plasma LH levels in October coincides with elevated plasma testosterone levels and an increase in numbers of secretory granules in the LH gonadotropes, but neither the seminiferous epithelium nor the accessory glands respond to the high plasma testosterone concentration (Bernard, unpublished data). There have been no studies of seasonal changes in plasma LH levels in bats, but elevated plasma testosterone levels, unrelated to spermatogenesis, have been reported in other bat species (Racey, 1974; Bernard, 1986), and in none of these cases is the biological significance understood. In other mammals (ram: Sanford *et al.*, 1974 & Pelletier *et al.*, 1982; fallow deer: Asher *et al.*, 1989; hedgehog: El Omari *et al.*,

1989) LH levels are high during the breeding season and low during reproductive inactivity. Plasma LH levels in *M. schreibersii* are the opposite and the reason for this is not known.

In male *R. capensis*, LH gonadotropes are active prior to and during spermatogenesis, reaching a maximum in synthesis and secretion during spermiogenesis and spermiation. The peak in LH gonadotrope activity coincides with elevated plasma LH and testosterone levels (Bernard, 1986). During winter, male *R. capensis* store spermatozoa in the cauda epididymides, and the accessory gland complex remains active (Bernard, 1986). The gradual decrease in pituitary and plasma LH levels during hibernation and sperm storage may indicate secretion of LH and consequently the maintenance of the accessory glands. The minor increases in pituitary and plasma LH levels at the end of winter are possibly due to external factors initiating a new spermatogenic cycle. These results, indicating activity of LH gonadotropes during spermatogenesis and inactivity during hibernation (period of sperm storage and spermatogenic inactivity) are similar to results obtained from gonadotrope and plasma LH studies on other mammals (vole: Clarke & Forsyth, 1964; ground squirrel: Licht *et al.*, 1982; hedgehog: El Omari *et al.*, 1989).

During follicular development in *M. schreibersii* LH gonadotropes are active and plasma LH levels increase. Increased activity of gonadotropes during follicular development has been reported in a number of bat species (*Macrotus californicus*: Richardson, 1979; *Myotis lucifugus*: Anthony & Gustafson, 1984; *R. capensis*: present study), and LH is known to be required for the activation of the thecal cells to produce oestrogen precursors; initiation of ovulation, and

development of the corpus luteum (Gore-Langton & Armstrong, 1988). A pre-ovulatory peak in plasma LH levels, reported in other bat species also (Anthony & Gustafson, 1984; Richardson, 1979; Canney & Butler, 1987) and in many other mammals (European badger: Canivenc & Bonnin, 1981; blue fox: Mondain-Monval *et al.*, 1985; brown hare: Caillol *et al.*, 1986), is followed by the formation of the corpus luteum in May (Bernard *et al.*, unpublished data), and slightly elevated plasma progesterone levels. A preovulatory surge in female *R. capensis* was not detected, possibly due to the small sample size collected in August.

Gonadotrope activity and plasma LH levels during delayed implantation and pregnancy apparently vary greatly. Gonadotrope inactivity during delayed implantation, as described in the present study, has been described for *M. schreibersii* (Peyre & Herlant, 1963), and plasma LH levels are low in the spotted skunk (Foresman & Mead, 1974) and the European badger during the delay (Canivenc & Bonnin, 1981). By contrast, Mikami *et al.* (1988) report that the LH gonadotropes in female *M. schreibersii* show the highest immunoreactivity during delayed implantation. This would suggest an increased LH synthesis during a period when follicular development has terminated, the corpus luteum is inactive and plasma progesterone levels are low, hence this activity can not be explained.

Studies on small carnivores (Murphy, 1979; Papke *et al.*, 1980; Berria *et al.*, 1989) have indicated that delayed implantation is terminated by increasing levels of prolactin which reactivates and maintains the corpus luteum during early stages of pregnancy. LH plays only a minor role, if any, in maintaining the activity of

the corpus luteum during pregnancy, which is in accordance with low gonadotrope activity or inactivity in *Macrotus californicus* (Richardson, 1979), *Myotis lucifugus* (Anthony & Gustafson, 1984) and *Miniopterus schreibersii* (Mikami *et al.*, 1988). Furthermore, plasma LH levels during pregnancy are found to be low in red deer (Kelly *et al.*, 1982) and blue fox (Mondain-Monval *et al.*, 1985) and *R. capensis* (present study). However, studies on the ferret (Agu *et al.*, 1986), sheep (Denamur, 1973) and spotted skunk (Foresman & Mead, 1974), show that both LH and prolactin are necessary for progesterone synthesis in the corpus luteum. Although prolactin levels were not measured in the present study, the apparent activation of the LH gonadotropes and the elevated LH levels from August to October suggest that LH might be required to maintain the corpus luteum of *M. schreibersii*. The importance of LH in corpus luteum maintenance has also been emphasised in studies on the monkey (Moudgal *et al.*, 1973), hamster (Rao *et al.*, 1973) and rat (Maneckjee *et al.*, 1973). These results explain reports of increasing plasma LH levels and gonadotrope activity during pregnancy in rat (Merchant, 1974), spotted skunk (Foresman & Mead, 1974) and several bat species (*M. schreibersii*: Peyre & Herlant, 1963 & present study; *Myotis myotis*: Herlant, 1964b; *Hipposideros fulvus*: Patil, 1974).

Summary

Seasonal changes in activity of the gonadotropes and plasma LH levels in male and female *M. schreibersii* and male *R. capensis* can be related to the reproductive strategies of delayed implantation and

sperm storage respectively. Not enough data are available for female *R. capensis* to relate seasonal changes in gonadotropes to reproductive activity. Such differences in seasonal gonadotropic functions can be expected, since mammals, especially bats, exhibit a variety of different reproductive cycles (Anthony & Gustafson, 1984).

CHAPTER 6: GENERAL DISCUSSION & CONCLUSION

Seasonal changes in the ultrastructure of gonadotropes, and pituitary and plasma LH contents were successfully related to the reproductive strategies of delayed implantation in *Miniopterus schreibersii* and sperm storage and delayed ovulation in *Rhinolophus capensis*. The activation of LH gonadotropes shortly before spermatogenesis, ovulation and implantation clearly demonstrates the role of this pituitary hormone in initiating reproductive processes.

The application of immunocytochemistry is essential since it allows the specific identification of a particular cell type in the anterior pituitary. Furthermore, it is important to monitor seasonal plasma LH concentrations in relation to gonadotrope activity, as high immunoreactivity (pituitary LH content) of LH gonadotropes could be due to an increased rate of hormone synthesis and little or no secretion, whereas low immunoreactivity could be caused by a very high rate of secretion, or a low rate of hormone synthesis. In this study high plasma LH levels generally coincided with reproductive and gonadotropic activity in both bat species, except for male *M. schreibersii* in spring, when plasma LH levels were highest. At this time of the year the males were reproductively inactive and the reason for the activity of the gonadotropes is not known.

Knowing the activity pattern of LH gonadotropes in relation to the reproductive cycle, it is possible to examine the factors which initiate gonadotrope activity and hence reproduction. In many mammals seasonal reproductive periodicity is conditioned by the

external environment, the responses being mediated through a neural mechanism involving the activation of the hypothalamus, by incoming sensory impulses (Wimsatt, 1960). The hypothalamus in turn activates the pituitary to release gonadotropic hormones, which activate reproductive processes (Austin & Short, 1984). Reduced gonadotrope activity or inactivity and hence the suppressed rate of hormone secretion during winter, is probably a reflection of the low metabolic rate during the hibernation period (Wimsatt, 1960). Factors controlling seasonal breeding of mammals are food availability, ambient temperature, rainfall and photoperiod (Bronson, 1985). Temperature was found to be the controlling factor for spermatogenesis in the rat-tailed bat (*Rhinopoma kinneari*: Anand Kumar, 1965), pallid bat (*Antrozous pallidus*: Beasley & Zucker, 1984) and little brown bat (*Myotis lucifugus*: Gustafson, 1979), and maximal food availability coincided with spermatogenesis in species of *Pipistrellus*, *Myotis* and *Miniopterus* (Gustafson, 1979). Furthermore, Beasley & Zucker (1984) found that photoperiod and possibly the pineal gland influence the reproductive cycle of the male pallid bat, *Antrozous pallidus*. However Pevet & Racey (1981) found no evidence of the pineal controlling spermatogenesis in the male pipistrelle bat, *Pipistrellus pipistrellus*. In male *M. schreibersii* and *R. capensis* the gonadotropes appear to be active about 1 month before the beginning of spermatogenesis, which in *M. schreibersii* is during summer (December/January) and in *R. capensis* in early spring (August/September), shortly after arousal from hibernation. Cues that might initiate gonadotropic activity in male *M. schreibersii* can not include ambient temperature and food availability, as both factors do not change much during summer.

However photoperiod might play a role, as the summer solstice occurs at the end of December, and the change to a decrease in daylength might initiate gonadotropic activity and ultimately the spermatogenic cycle. In *R. capensis* the winter solstice probably does not play a role, because the switch to increasing daylength occurs about 2 months before apparent gonadotrope activity. It is more likely, that a change in ambient temperature as well as an increase in food abundance in spring, at the time of arousal from hibernation initiate gonadotrope activity.

Wimsatt (1960) has suggested that the low metabolic rate during hibernation may be responsible for reduced secretory activity of the anterior pituitary and consequently reproductive delays. However, more recent studies of delayed implantation in small carnivores have indicated that the high levels of melatonin during winter may be responsible for preventing implantation (Murphy & James, 1974; Sundqvist *et al.*, 1989). During hibernation, gonadotropes of *M. schreibersii* and *R. capensis* were inactive and it is possible that, as in the small carnivores, this reduced activity is associated with elevated plasma melatonin concentrations.

Initiation of implantation in the equatorial fruit bat, *Eidolon helvum*, is probably controlled by rainfall (which is high at the time of implantation), as photoperiod varies very little at the equator (Mutere, 1967). However, photoperiod appears to play a role in initiating implantation in *Miniopterus* species, as implantation coincides with increasing daylength, suggesting that the winter solstice marks the onset of the environmental stimulus for implantation (Racey, 1981).

Another interesting aspect in the hormonal control of

reproductive processes is the role of LH during pregnancy. Gonadotropes appear to be active and plasma LH levels are high during pregnancy in female *M. schreibersii*, suggesting LH is responsible for reactivation and maintenance of the corpus luteum, and thus for progesterone production during pregnancy. This was also reported in *Myotis myotis* (Herlant, 1964b) and *Miniopterus schreibersii* (Peyre & Herlant, 1963). However, in *Myotis lucifugus* (Anthony & Gustafson, 1984) and *Macrotus californicus* (Richardson, 1979), there is no reactivation of the corpus luteum, and the LH gonadotropes were found to be inactive during implantation and pregnancy. This difference in seasonal gonadotropic function is difficult to explain, but the variety of different reproductive cycles exhibited by bats, especially the different forms of delay phenomena (delayed ovulation, delayed implantation and delayed embryonic development) might account for this difference (Anthony & Gustafson, 1984).

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APPENDICES

APPENDIX 1: ICC staining protocol for light microscopy using the PAP labelling method

SOLUTION	TIME (minutes)
Xylene I	2
Xylene II	2
Xylene III	2
100% ethanol I	2
100% ethanol II	2
95% ethanol I	2
95% ethanol II	2
PBS (pH 7.3)	3x3
6% hydrogen peroxide	15
PBS (pH 7.3)	3x3
1/10 normal goat serum	20
1/200 primary antibody	60
PBS (pH 7.3)	3x3
1/50 GAM IgG	20
PBS (pH 7.3)	3x3
1/100 PAP	20
PBS (pH 7.3)	3x3
0.05% 3,3'DAB & 0.01% hydrogen peroxide or 0.04% 3-amino-9-ethylcarbazole in N,N-dimethylformamide & 0.01% hydrogen peroxide diluted to 1/50 in 0.05M acetate buffer (pH 5.2)	10
distilled water	rinse
Mayr's hematoxylin	1
distilled water	rinse
ammonia water	10 sec.
distilled water	rinse
dehydrate and mount	

APPENDIX 2: Protocol for immunogold labelling with silver enhancement for light microscopy

SOLUTION	TIME (minutes)
Xylene I	2
Xylene II	2
Xylene III	2
100% ethanol I	2
100% ethanol II	2
95% ethanol	2
70% ethanol	2
PBS (pH 7.3)	2x5
blocking solution[1]	30
washing buffer[2]	5
1/200 primary antibody	60
washing buffer	3x10
1/40 GAM Ig coated colloidal gold (1nm)	180
washing buffer	3x10
PBS (pH 7.3)	3x5
2.5% glutaraldehyde in PBS (pH 7.3)	10
distilled water	2x5
initiator & enhancer solutions[3]	6-18
distilled water	2x5
mount using a water soluble mounting medium	

1. made up of PBS (pH 7.3), containing 0.8% w/v BSA, 0.1% v/v IGSS gelatin, 0.1% v/v Triton X, 5% v/v NGS and 0.01% w/v sodium azide.

2. made up of PBS (pH 7.3), containing 0.8% w/v BSA, 0.1% v/v IGGS gelatin, 0.1% v/v Triton X and 0.01% sodium azide.

3. Reagents A & B of the IntenSE M kit.

APPENDIX 3: ICC staining protocol for electron microscopy using the PAP labelling method

SOLUTION	TIME (minutes)
3% hydrogen peroxide	15
distilled water	4x1
1/10 normal goat serum	10
1/400 primary antibody	180
Tris buffer (pH 7.3) & 0.1% BSA	4x1
1/10 normal goat serum	10
1/100 GAM IgG	15
Tris buffer (pH 7.3) & 0.1% BSA	4x1
1/300 PAP	25
Tris buffer (pH 7.3) & 0.1% BSA	4x1
0.05% 3,3'DAB & 0.01% hydrogen peroxide	10
Tris buffer (pH 7.3)	4x1
distilled water	4x1
1/15 hydrogen gold chloride	15
distilled water	5x2

APPENDIX 4: Protocol for immunogold labelling for electron microscopy.

SOLUTION	TIME (minutes)
1/10 normal goat serum	10
1/400 primary antibody	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
1/40 GAM Ig G coated colloidal gold (20 nm), pH 8.2	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	4x1

ERRATA SHEET

Page	v	Talbles should read Tables.
Page	3	line 7 form should read from.
Page	9	line 18 recieved should read received.
Page	10	line 10 titrated should read tritiated.
Page	16	line 4 Brandzaeg should read Brandtzaeg.
Page	29	line 13 Parafin should read Paraffin.
Page	37	figure 9 caption should read Diagrams of the dorsal view of the pituitary gland of *Miniopterus schreibersii* and *Rhinolophus capensis*.
Page	59	line 10 Roemmler *et al.*,1987 should read Roemmler *et al.*, 1978.
Page	63	line 24 seen male should read seen in male.
Page	79	line 8 Denamur, 1973 should read Denamur *et al.*, 1973.
Page	99	line 13 Ovo-implantation differee et correlations hypophyso-genitales chez la femelle du Minioptere should read Ovo-implantation différée et corrélations hypophyso-génitales chez la femelle du Minioptère.