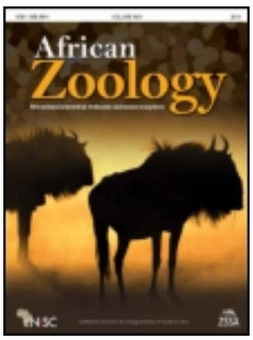

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Changes in the abundance of cells in the anterior pituitary gland and the possible roles of luteinizing hormone, prolactin and progesterone in the control of delayed implantation in the straw-coloured fruit bat (*Eidolon helvum*)

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Eidolon helvum **(Megachiroptera) is a large frugivorous bat found in equatorial and tropical Africa. The reproductive cycle is characterized by a three-month period of delayed implantation and the total length of pregnancy may be as much as 10 months. A histochemical study of the gonadotrophs and mammotrophs of the anterior pituitary, in conjunction with assays of LH-like, progesterone-like and prolactin-like immunoreactivity in the plasma suggest that during delayed implantation the gonadotrophs were inactive while the mammotrophs were active and plasma PRL-like immunoreactivity high. We interpret this as indicating that, in the straw-coloured fruit bat, implantation was inhibited by high levels of prolactin and, as such, the endocrine control of delayed implantation may be quite different from that described for other mammals.**

Key words: **Megachiroptera, reproduction, mammotroph, gonadotroph.**

INTRODUCTION

Eidolon helvum, the straw-coloured fruit bat, occurs in tropical Africa where it breeds seasonally (Mutere 1965, 1967; Fayenuwo & Halstead 1974; Kingdon 1974). Mating is not synchronized within populations and occurs between April and June, and implantation, which is synchronised (Kingdon 1974), is delayed until October. After implantation, normal embryonic development resumes, and births occur in February and March (Mutere 1967; Fayenuwo & Halstead 1974). The period of delayed implantation is about three months long and more or less coincides with one of two annual dry periods (Mutere 1967; Fayenuwo & Halstead 1974). Delayed implantation and the other forms of reproductive delay (delayed ovulation/sperm storage and retarded or delayed embryonic development) that occur in the Chiroptera and some other mammals, effectively separate reproductive processes that normally occur together, and thus species that have evolved these delays may be used as models for studying the control of events such as implantation (Bernard & Bojarski 1994).

In most mammals that use delayed implantation, the steroidogenic activity of the corpus luteum (CL) is suppressed and plasma progesterone concentrations are low during the delay (skunk: Sinha & Mead 1975; roe deer: Sempéré 1977; mink: Martinet *et al.* 1981; long-fingered bat: Peyre & Herlant 1963a,b; Kimura *et al.* 1987; Crichton *et al.* 1989; Bernard *et al.* 1991). Typically, implantation coincides with activation of the CL and increasing plasma progesterone concentrations (Mead & Eik-Nes 1969; Møller 1973; Canivenc & Bonnin 1981; Bernard *et al.* 1991). In the mustelid carnivores and the long-fingered bat, prolactin (PRL) is the pituitary hormone responsible for activating the CL and terminating delayed implantation, while progesterone and luteinizing hormone (LH) play little or no role (Martinet *et al.* 1981; Berria *et al.* 1989; Bernard & Bojarski 1994). For both the mustelid carnivores and the long-fingered bat it has been suggested that the termination of delayed implantation is triggered by changing daylength (Murphy *et al.* 1990; Bernard

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& Bojarski 1994). *E. helvum* is the only fruit bat (Megachiroptera) known to use delayed implantation, and it is tropical in distribution. At low latitudes the changes in daylength may not be sufficient to be used as a cue for reproduction (Bronson 1989), and if this is the case, the control of delayed implantation in *E. helvum* may be different from that proposed for other mammals. The aim of this study was therefore to examine the roles of LH, progesterone and prolactin in the control of delayed implantation in the straw-coloured fruit bat.

MATERIALS & METHODS

Animals

Monthly samples of adult female straw-coloured fruit bats (Table 1) weighing between 175 and 225 g were collected, using mist nets, from Mbale in Kakamega Forest Belt (Western Kenya, 0°20′N) between March 1994 and February 1995. Five stages of the reproductive cycle were recognized (pre-ovulatory, ovulatory, delayed implantation, post-implantation and lactation; Mutere, 1965, 1967; Fayenuwo and Halstead, 1974), and monthly samples were grouped based on the stage of the reproductive cycle that the specimens represented (Table 1).

Histochemistry

The pituitary gland was removed from five bats each month, fixed in Bouin's solution for 24 h and prepared for light-microscopy using standard techniques. Serial sections were cut at 7–8 μm and all sections were mounted. A number of staining procedures were used (Table 2) and the cells in the pituitary gland were identified based on the staining properties of their cytoplasmic granules and on their shape and size. The nomenclature used for the cells follows that recommended by the International Committee for Nomenclature for the Adenohypophysis (Van Oordt 1965). Ten sections through the pituitary gland of each specimen were stained with PAS/OG/methylene blue, which allowed us to identify each cell type, and these sections were used for the quantification of cell abundance and granulation. The abundance of each cell type, for each specimen, was calculated as the mean number of cells of each type in 10 randomly selected fields at ×1000 magnification in each of 10 sections through the pituitary gland of each bat. At ×1000 magnification, the field of view was 314.2 μm^2 and there were no more than 10 cells in a field of view. The degree of granulation was given a subjective score on the scale of + (cells with low granulation; poorly-stained cytoplasm with the stain concentrated in a few areas) to +++ (cells packed with granules and well-stained cytoplasm). The diameters of 10 cells of each type from five specimens per month were measured with an ocular vernier micrometer at ×1000 magnification.

Table 1. Monthly sample sizes and the reproductive condition of female straw-coloured fruit bats.

Month	Sample size	Reproductive condition
January	5	Lactation
February	5	
Early March	5	
Late March	5	Pre-ovulatory
April	5	
May	5	Ovulatory
June	5	
July	5	
August	5	Delayed implantation
September	5	
October	5	
November	10	Post-implantation embryonic development
December	5	

Hormone assays

Blood (5–7ml) was drawn from the vena humeri profunda of the wing of all specimens that were sacrificed for the histochemical study of the pituitary. The whole blood was transferred to a heparinized glass test-tube (62 I.U. heparin/tube) and centrifuged at 48 000 rpm at 4 °C for 20 minutes. Plasma was removed and stored at –20 °C until assayed. All hormone assays were undertaken at the Tata Cancer Research Institute, Endocrinology Division, Bombay, India, using routine radioimmunoassay protocols for human LH, progesterone and PRL. The antibodies used in the assays were all anti human and the assays were not validated for the bat hormones, and this must be taken into account when analysing the results. Each plasma sample was run in triplicate and the intra- and interassay coefficients of variation were <10% in all cases. Specimens were grouped according to their reproductive stage (Table 1 for sample sizes) and mean plasma hormone levels calculated.

Table 2. Staining reactions of the cells of the anterior pituitary gland of the straw-coloured fruit bat. The chromophobes stained weakly in all stains. Putative cell type is given in brackets. / = no clear reaction with the stain.

Staining technique[1]	Basophil I (thyrotroph)	Basophil II (FSH gonadotrophs)	Basophil III (LH gonadotrophs)	Acidophil I (mammotroph)	Acidophil II (somatotroph)
PAS	+ve	+ve	+ve	–ve	–ve
PAS/OG/methylene blue	Magenta-red	Purple	Brick-red	Orange	Yellow
AB/PAS/OG	Brick-red	Pink	Blue	Orange	Yellow
PAS/OG	Dark brick-red	Light brick-red	Light brick-red	light blue	/
Masson's trichrome	Red	Red	Red	/	/
BrAB/OG/F	Blue	Green	Green	Orange	Orange
Azan method	Reddish blue	Blue	Blue	Carmine	Red
CHP	Dark blue	Blue-black	Blue	Orange/red	Orange/red
Brooke's method	Green	Green	Green	Orange	Red
AT/PAS/OG	Deep blue-black	Red	Red	Deep blue-green	/
Herlant's tetrachrome technique	/	Blue	Purple	Red	/
Gomori's AF	Deep purple	Red	Red	/	/
AR/LG/OG	Green	Green	Green	Red	Pale yellow
AF/LG/OG	Dark blue	Green	Green	Pale yellow	Pale yellow

[1]PAS, periodic acid Schiff; OG, orange G; AB, alcian blue; BrAB, bromine alcian blue; CHP, chrome alum, haematoxylin, phloxine; AT, aldehyde thionin luxal fast blue; Gomori's AF, Gomori's aldehyde fuchsin technique; AR, acid red; f, fuchsin.

Statistical analyses

The mean values for the abundance of the pituitary cell types and the hormone levels in the different reproductive stages were compared using ANOVA followed by the Student-Newman-Keuls test, or Kruskal-Wallis ANOVA on ranks followed by Dunn's all pairwise multiple comparison when the data were not normally distributed. The mean granulation scores were compared using ANOVA on ranks followed by Dunn's all pairwise multiple comparison. Values are given as mean ±1 S.D. Statistical tests were carried out using SigmaStat (Jandel Scientific, San Rafael, CA).

RESULTS

Histochemistry of the anterior pituitary

The cells of the anterior pituitary were readily divided into basophils, which stained positively with periodic acid Schiff (PAS), acidophils, which stained negatively with PAS and positively with orange G (OG) (Table 2), and chromophobes, which lacked stainable granules and exhibited a poor reaction with all stains. Based on morphological characteristics and tinctorial affinities, three types of basophils (Types I, II and III), two types of acidophils (Types I and II) and the chromophobes were identified (Table 2). Based on their tinctorial affinities, we have given each of these cells putative identities as one of the functional cell types in the anterior pituitary (Table 2; Herlant 1953, 1956; Amin & Gilbert 1970; Patil 1974 and Richardson 1979 for review).

Type I basophils (putative thyrotrophs) were polygonal with a diameter of 9–10 μm. They stained magenta red with PAS/OG/methylene blue, deep blue-black with aldehyde thionin, and deep purple with aldehyde fuchsin.

Type II basophils (putative FSH gonadotrophs) were oval with a diameter of 12–14 μm and a centrally placed nucleus. These cells stained purple with PAS/OG/methylene blue, blue with Herlant's tetrachrome, and blue-black with chrome-alum/haematoxylin/phloxine (CHP).

Type III basophils (putative LH gonadotrophs) were the smallest of the three basophils and were round or oval with a diameter of 3.5–4 μm and an eccentrically positioned nucleus. They differed from the other basophils and stained brick-red with PAS/OG/methylene blue, and purple or violet with Herlant's tetrachrome technique.

Type I acidophils (putative mammotrophs) had a diameter of 9–10 μm, and were distinguished from Type II acidophils (putative somatotrophs; diameter 6–7 μm) using Brookes' (1964) method in which the Type I cells stained an orange colour while Type II cells stained a red hue (Table 2).

Chromophobes were generally devoid of stainable cytoplasmic granules and stained poorly

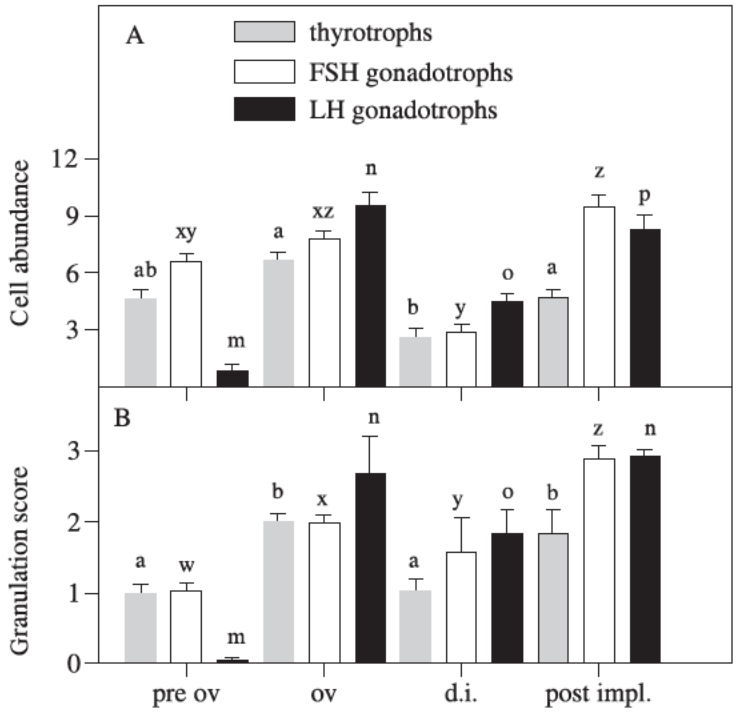

Fig. 1. Changes in abundance (**A**) and granulation (**B**) of the three basophils in the anterior pituitary of the straw-coloured fruit bat during the different stages of reproduction (pre ov, preovulatory; ov, ovulatory, d.i, delayed implantation; post impl., post implantation embryonic development stages). Significant differences are indicated by letters above the bars, and bars with a letter in common are not significantly different.

with most dyes.

The three types of basophil showed a similar trend of changes in abundance in relation to the stages of reproduction (Fig. 1A). Cell abundance increased from the pre-ovulatory period to the ovulatory period after which there was a significant decrease in abundance of all three basophils during delayed implantation ($P < 0.05$ for all). The abundance of these cells increased significantly during the post-implantation stage ($P < 0.01$ for all; Fig. 1A). A similar, statistically significant trend was seen in the granulation of the cytoplasm of the three basophils which was low in the pre-ovulatory and delayed implantation stages and significantly higher at ovulation and during post-implantation embryonic development ($P < 0.01$ for all; Fig. 1B).

The putative somatotrophs were significantly more abundant and the cytoplasm more granular during the preovulatory period than during the other reproductive stages ($P < 0.05$ and <0.001, respectively; Fig. 2A,B). The putative mammotrophs showed a significant increase in abundance from the pre-ovulatory period to reach a peak abundance during delayed implantation ($P < 0.001$; Fig. 2A). The cytoplasm of the putative mammotrophs had few granules during the pre-ovulatory period and was highly granular at ovulation and during the period of delayed implantation (Fig. 2B).

Plasma hormone levels

Since the hormone assays were not validated for the straw-coloured fruit bat, the results are presented as levels of antigen-like immunoreactivity rather than levels of the antigen.

The level of LH-like immunoreactivity was lowest during the pre-ovulatory stage, significantly elevated during the ovulatory stage and low during delayed implantation ($P < 0.001$; Table 3). After implantation there was a significant increase in plasma LH-like immunoreactivity ($P < 0.001$). Plasma progesterone-like immunoreactivity levels were low during the pre-ovulatory stage, when the females did not have CL in the ovaries, and did not change significantly during the ovulatory and delayed implantation stages ($P > 0.05$). Post-implantation plasma progesterone-like immuno-

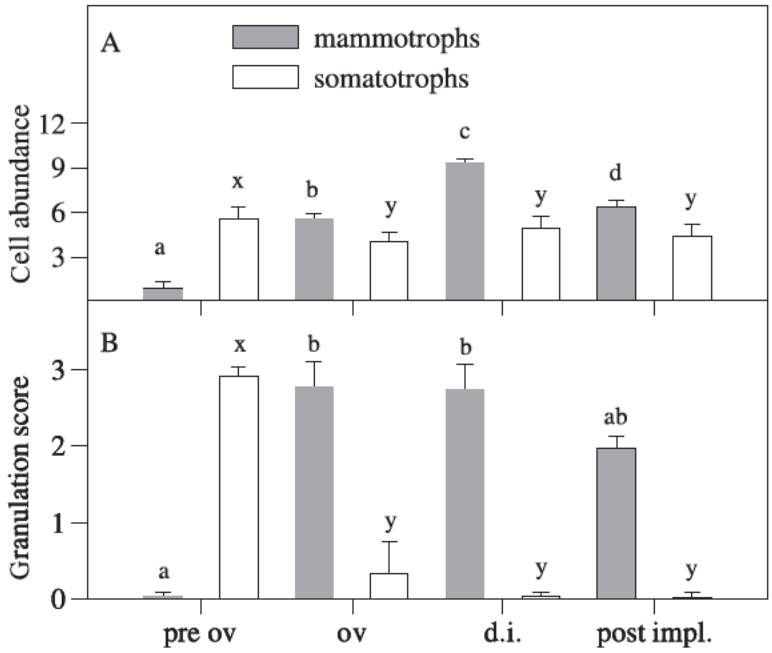

Fig. 2. Changes in abundance (**A**) and granulation (**B**) of acidophils in the anterior pituitary of the straw-coloured fruit bat during the different stages of reproduction (pre ov, preovulatory; ov, ovulatory, d.i, delayed implantation; post impl., post implantation embryonic development stages). Significant differences are indicated by letters above the bars, and bars with a letter in common are not significantly different.

reactivity increased significantly to about 60 ng/ml ($P < 0.001$; Table 3). The plasma PRL-like immunoreactivity levels were lowest during the pre-ovulatory period and significantly elevated at ovulation and further elevated during delayed implantation ($P < 0.001$; Table 3). Plasma PRL-like immunoreactivity levels decreased significantly after implantation, and were highest during lactation $P < 0.01$; Table 3).

DISCUSSION

The size and tinctorial affinities of the putative cell types identified in the anterior pituitary of *E. helvum* were similar to those reported for other mammals (Herlant 1964; Purves 1966; Patil 1974; Bhalchandra 1980, 1987; Bhiwgade *et al.* 1989). However, we did not confirm the identity of these cell types using immunocytochemistry as has been done in some studies of the endocrine control of reproductive delays in bats (Richardson 1979; Anthony 1987; Bernard *et al.* 1991; Singh & Krishna 1996). Although two types of gonadotrophs have been identified in the present and other histochemical studies (Herlant 1956, 1964), immunocytochemical studies have shown that in many mammals some gonadotrophs stain positively for both LH and FSH (Anthony 2000 for review).

Plasma hormones were assayed at a medical laboratory and it was not possible to have the assays validated for *E. helvum*. However, the high levels of PRL-like immunoreactivity during lactation, of progesterone-like immunoreactivity during post-implantation embryonic development, and of LH-like immunoreactivity at ovulation and during post-implantation development suggest that the assays were indeed measuring LH, progesterone and PRL.

Endocrine control of delayed implantation

The seasonal changes in abundance and appearance of mammotrophs and gonadotrophs, and the seasonal changes in plasma LH-like, progesterone-like and PRL-like immunoreactivity in *E. helvum* were complementary. Delayed implantation was characterized by a reduced abundance and granularity of the gonadotrophs and a reduction in plasma LH-like and progesterone-like immunoreactivity. By contrast, apparent secretory activity of the mammotrophs and plasma PRL-like immunoreactivity were significantly elevated during delayed implantation.

Table 3. Levels of progesterone-like, LH-like and prolactin-like immunoreactivity in the stages of reproduction of female straw-coloured fruit bats. Mean values are given ±1S.D. and in any row, values with a superscript in common are not significantly different (ANOVA, $P > 0.05$).

Hormone (ng/ml)	Pre-ovulatory stage	Ovulatory stage	Delayed implantation	Post-implantation	Lactation
LH	3.0 ± 0.2^{a}	5.7 ± 0.52^{b}	2.0 ± 0.1^{c}	8.1 ± 0.8^{d}	
Progesterone	1.5 ± 0.07^{a}	2.1 ± 0.6^{a}	1.6 ± 0.1^{a}	62.1 ± 3.2^{b}	
Prolactin	0.9 ± 0.05^{a}	1.1 ± 0.13^{b}	7.7 ± 2.7^{c}	4.5 ± 0^{d}	10.6 ± 0.8^{e}

A common feature of delayed implantation and delayed embryonic development is that the abundance and immunoreactivity of the gonadotrophs is reduced during the period of delay, and this is reflected in a reduction in progesterone production by the CL (Burns & Easley 1977; Richardson 1979; Crichton *et al.* 1990; Bernard *et al.* 1991; Anthony 2000; Martin & Bernard 2000 for review; present study). An unexplained exception to this is the report of an increased abundance of gonadotrophs during delayed implantation in *M. schreibersii fuliginosus* from Japan (Mikami *et al.* 1988). The coincidence of luteal inactivity and delayed implantation might suggest a causal role for LH and progesterone in the termination of the reproductive delay, but this is not the case as neither exogenous LH nor exogenous progesterone will trigger implantation in *Miniopterus schreibersii* from South Africa (Bernard & Bojarski 1994).

In *Macrotus californicus* (delayed development) and *Miniopterus schreibersii* (delayed implantation) there is a reduction in the abundance and PRL immunoreactivity of the mammotrophs during the reproductive delay (Richardson 1979; Bojarski 1993; Anthony 2000). In both species the abundance, size and immunoreactivity of the mammotrophs increase at the time of the transition from delay to normal embryonic development and are elevated during lactation (Richardson 1979; Bojarski 1993; Anthony 2000 for review). Furthermore, experimental treatment of *M. schreibersii* in early delayed implantation with exogenous PRL stimulates premature implantation (Bernard & Bojarski 1994). Together these results indicate that low levels of PRL inhibit implantation in these species and, conversely, that PRL stimulates implantation. Our results for *E. helvum* were quite different. The apparent secretory activity of the mammotrophs and plasma PRL-like immunoreactivity were high during delayed implantation suggesting that high levels of PRL may be responsible for delaying implantation rather than stimulating the process as seen in other mammals.

Many mammals from temperate latitudes have an annual cycle of PRL secretion, with secretion stimulated by increasing daylength (Curlewis 1992; Anthony 2000). In such species (e.g. *M. schreibersii*) PRL may have a stimulatory effect on reproduction, and consequently one or more reproductive events are triggered by increasing daylength. At more tropical latitudes, species may not show a seasonal pattern of PRL secretion and, in some instances, PRL may have an inhibitory effect on reproduction (Greenwald & Terranova 1988; Curlewis 1992; Anthony 2000). For example, in the male fruit bat *Pteropus poliocephalus,* plasma PRL levels are high in summer and decline as they enter breeding condition in autumn (O'Brien *et al.* 1990).

In conclusion, we suggest that the endocrine control of delayed implantation in *E. helvum* is different from that established for *M. schreibersii* and other mammals. In *E. helvum,* mammotrophs appeared to be active and PRL-like immunoreactivity was high during delayed implantation, and we suggest PRL has an inhibitory effect on implantation rather than a stimulatory effect as in *M. schreibersii* and other mammals.

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