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CHARACTERISATION OF UTERINE PROGESTERONE AND OESTROGEN RECEPTORS IN THE AFRICAN ELEPHANT, *LOXODONTA AFRICANA*

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Characterisation of uterine progesterone and oestrogen receptors in the African elephant, *Loxodonta africana.*

by

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Characterisation of uterine progesterone and oestrogen receptors in the African elephant, *Loxodonta africana.*

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ABSTRACT

The present study is directed at obtaining information on the interaction between uterine steroid receptors and circulating steroids. Furthermore the study aimed at quantifying the affinity of these receptors for possible contraceptive agents that could be applied to constrain individual reproductive output.

Scatchard analysis showed that both progesterone and oestrogen receptors had high binding affinities for their appropriate ligands. The binding affinity of the oestrogen receptor decreased with progressing pregnancy while that of the progesterone receptor showed no definite pattern. The concentrations of the two types of receptors were positively correlated and decreased with advancing pregnancy. The down-regulation of the progesterone receptor could possibly be attributed to an increase in the circulating levels of both 5α-pregnane-3,20-dione and progesterone. In the pregnant animals the uterus horn containing the foetus had a lower receptor concentration than the corresponding uterus horn that was not in contact with the developing foetus.

Both the oestrogen and progesterone receptors displayed high levels of specificity for their natural ligands. The relatively high binding affinity of 5α-pregnane-3,20-dione for its appropriate receptor, indicates that this hormone may be of biological importance to the maintenance of pregnancy in this species and in this regard needs further investigation. Competitive binding assays showed that mifepristone (RU 486) could not be used to terminate pregnancy in the African elephant as the progesterone receptor had a very low affinity for this steroid. Norethindrone and levonorgestrel may, however, be of potential use when considering their relatively high affinity to the uterine progesterone receptor.

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AELIA:	Amplified enzyme-linked immunoassay
B_{max}	Intercept of Scatchard line with the x-axis
BSA:	Bovine serum albumin
Ci:	Curie
CITES:	Convention on the International Trade in Endangered Species of fauna and flora
DCC:	Dextran coated charcoal
DTT:	Dithiothreitol
EDTA:	Ethylene-diamino-tetra-acetate
EIA:	Enzyme immunoassay
FSH:	Follicle-stimulating hormone
IgG:	Immunoglobulin (class A)
K_d:	Dissociation constant
kDA:	Kilodaltons
LH:	Luteinizing hormone
N:	Number of receptor binding sites/binding capacity
MCF-7:	Michigan Cancer Foundation breast cancer cell line (pleural metastatic cell line from human breast cancer)
NADPH:	Nicotinamide adinine dinucleotide phosphate linked to a hydrogen atom
PBS:	Phosphate buffered saline solution
RIA:	Radioimmunoassay
RBA:	Relative binding affinity
SEM:	Standard error of the mean
$TEDAG_{10}$:	Buffer containing Tris HCL, EDTA, Dithiotreitol, Sodium azide and glycerol (10%)
$TEDAG_{60}$:	Buffer containing Tris HCL, EDTA, Dithiotreitol, Sodium azide and glycerol (60%)
Tris:	Tris (hydroxymethyl) aminomethane
V_{max}	Represents the rate achieved when the total amount of enzyme is saturated with substrate

Trivial name	**Systematic name** (Moss 1989)
5α-DHP; 5α-Dihydroprogesterone	5α-pregnane-3,20-dione
Androstenedione	4-androstene-3,17-dione
Cortisone	17,21-dihydroxypregn-4-ene-3,11-20-trione
Danazol	17α-pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol
Dehydroepiandrosterone	3β-Hydroxyandrost-(5)-ene-17-one
Dehydrotestosterone	17β-Hydroxy-5α-androstan-3-one
Deoxycorticosterone	21-hydroxypregn-4-ene-3,20-dione
Diethylstilboestrol; DES	Stilbestrol
Equilenin	3-hydroxyestra-1,3,5,7,9-pentaen-17-one
Equilin	3-hydroxyestra-1,2,5(10),7-tetraen-17-one
Ethinyl oestradiol	17α-ethiylestra-1,3,5(10)-triene-3,17β-diol
Hydrocortisone	11β,17,21-trihydroxypregn-4-ene-3,11,20-dione-3-dione
Levonorgestrel	13-ethyl-17-hydroxy-18,19-dinorpregn-4-en-20-yn-3-one
Medroxyprogesterone	17-hydroxy-6α-methylpregn-4-ene-3,20-dione
Norethindrone	19-norethisterone-17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one
Oestradiol-17β	estra-1,3,5(10)-triene-3,17β-diol
Oestriol	estra-1,3,5(10)-triene-3,16α,17β-triol
Oestrone	3-hydroxyestra-1,3,5(10)-trien-17-one
ORG 2058	16α-ethyl-21-hydroy-19-norpregn-4-ene-3,20-dione
Pregnenolone	3β-hydroxy-5-pregnen-20-one
Progesterone	pregn-4-ene-3,20-dione
Promegestone; R 5020	17α,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione
RU 486; RU 38486; Mifepristone	17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(prop-1-unyl)-estra-4,9-dien-3-one
Tamoxifen	2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N-dimethylethanamine
Testosterone	17β-hydroxyandrost-4-ene

CHAPTER 1

INTRODUCTION

General

The African elephant, *Loxodonta africana*, usually lives in family units consisting of related adult cows and their immature offspring (Buss 1961; Douglas-Hamilton 1972; Moss 1981). Families are led by a matriarch (usually the oldest cow) and members of a family unit maintain a close association during the normal course of a day (Douglas-Hamilton 1972; Laws, Parker & Johnstone 1975; Moss & Poole 1983). Female calves stay in the family unit and reach sexual maturity from 10 to 15 years of age. Breeding usually starts when they are at about 11 years old, with the first calf being born at 13 years of age. Calves are generally born at four year intervals and the gestation period is approximately 22 months (Douglas-Hamilton 1972; Moss 1983; Moss & Poole 1983). Oestrus is not associated with a swollen vulva or a vaginal discharge but subtle behavioural changes during the two to six day period of sexual heat, such as an increased wariness, the characteristic 'oestrus walk', the 'chase' and 'consortship' occurs. Copulation typically lasts from 40 -60 seconds and may be repeated several times during consortship. Oestrus tends to occur in synchrony within families with cows coming into oestrus within a few weeks of one another (Moss 1983).

Bulls are physiologically capable to reproduce when 14-17 years old (Laws 1969). As bulls get older they spend more time in bachelor groups on the periphery of the family unit, in so-called 'bull-areas' (Moss & Poole 1983; Hall-Martin 1987; Poole 1987). Bulls do not compete for oestrus females until 26-35 years old, and start to experience musth when about 30 years old. Musth in bulls is an annual, unsynchronised event and the occurrence thereof, in a particular animal,

depends on whether another dominant bull in the group is in musth or not. It can be defined as a period of heightened sexual activity, and is characterised by high testosterone levels, temporal gland secretions, continuous dripping of urine and increased aggression. Cows in oestrus show a definite preference for musth bulls (Poole & Moss 1981; Poole 1987; Poole 1989a and b; Dickerman, Zachariah & McConathy 1995; Rasmussen, Hall-Martin & Hess 1996).

Conservation and management.

The conservation of African elephants involves the paradoxical situation of local over-abundant populations but concurrent continental declines. The general decline in elephant numbers over the past decade is ascribed to poaching and the illegal ivory trade (Lewis 1984; Douglas-Hamilton 1987; Barnes & Kapela 1991; Caughley, Dublin & Parker 1990; Dublin, Milliken & Barnes 1995). The ivory trade's annual demand of 825 tons during the 1980's, resulted in an estimated at a loss of 70 000 elephants each year. The African elephant is listed as an Appendix I species since the October 1989 CITES convention in Luasanne, Switzerland, and at present ivory trading is illegal throughout the world (Martin 1992). This has reduced the pressure of intensive poaching in certain areas such as Tsavo, where a 1000 elephants were poached in 1988 and a mere 15 poached during 1990 (Douglas-Hamilton 1992). Although the ivory ban has hampered poaching, increasing human populations are infringing on elephant habitat with resulting conflict for space (Parker & Graham 1989; Poole 1993; Dublin *et al.* 1995; Njumbi, Waithaka, Gachago, Sakwa, Mwathe, Mungai, Mulama, Mutinda, Omondi & Litoroh 1996).

While elephant numbers have been declining elsewhere in Africa, several elephant populations in southern Africa are reduced through costly culling operations in designated conservation areas. As elephants are a charismatic species, capable of arousing tremendous public sentiment, the apparent destruction of life, possible reduction in genetic diversity and the

disturbances to survivors caused by culling operations, are of immediate concern to many conservationists. The use of reproductive biotechnology to control reproductive output is viewed, by some, as an alternative management tool, the potential of which has not been investigated as yet (Short 1992, Van Aarde 1996). Although the development of techniques for the possible control of reproduction in African elephants will still include intensive management, it offers an alternative to culling, which may prove to be ethically more appealing to the general public. Reproductive technologies for the manipulation of elephant numbers depends on detailed information on fundamental reproductive processes. Controlling the fertility of selected bulls within a population will have little effect on the population as a whole, as bulls are able to cover large areas in search of oestrus females. Furthermore androgenic steroids aimed at inhibiting sperm production may influence aggression and thereby affect the social status of an individual (Short 1992). Thus the cows in which either inhibition of ovarian activity or implantation of the foetus can be addressed, is a more suitable candidate. The aim would be to decrease the reproductive output of a selected proportion of the population of the females, either by delaying the age of first reproduction and/or by increasing the intercalving interval (Short 1992; Poole 1993). However, such interference may only be acceptable once all the relevant information has been obtained and the physiological consequences thoroughly investigated. Present applications of immunocontraception (Bertschinger, Fyarer-Hosken, Kirkpatrick, Soley, Steffens & Ard 1996, Raath 1996) and slow releasing oestrogen implants (The Economist 1996; Meyer, Quandt, Raath, Göritz, Hildebrandt & Hofmann (*in litt.*)) in elephants in the Kruger National Park have thus far not taken these factors into consideration. Due to logistic and economic considerations, the use of a contraceptive agent may only be applicable in small, private game reserves with small elephant populations but appears to be impractical where large elephant populations need to be managed (Whyte, Van Aarde & Pimm 1997). Translocation of elephants to less populated reserves is a common practise, but often proves

financially costly, impacting considerably on the social behaviour of the herd, unless whole families can be translocated (Garai 1993). Furthermore, translocation can only be seen as a short-term solution as these satellite herds may become over-abundant and eventually be faced with land-shortage problems due to pressure from surrounding, burgeoning human populations. Therefore, the most feasible, although not always practical, alternative to culling would be the enlargement of present reserves to create a more natural system with migrations between subpopulations.

Physiological aspects of reproduction

Elephants are apparently monovular but polyestrous. Corpora lutea appear to accumulate in their ovaries until pregnancy (Austin & Short 1985). Indian elephants are spontaneous ovulators with oestrus occurring at 18 week intervals (with an average of 15.1 ± 0.3 weeks), coinciding with a decline in the small amounts of progesterone in the peripheral blood (Austin & Short 1984; Olsen, Chen, Boules, Morris & Coville 1994). In African elephants the cycle lengths have been estimated at 15.9 ± 0.6 weeks (Plotka, Seal, Zarembka, Simmons, Teare, Phillips, Hinshaw & Wildt 1988), 16 ± 2 and 13.3 ± 1.3 weeks (Brannian, Griffin, Papkoff & Terranova 1988; Plouzeau, Cunha & Shaw 1994).

Although previous studies have considered aspects of reproductive physiology, such as circulating oestrogen levels and concentrations of progesterone in the plasma and corpus lutea of African and Indian elephants (Short & Buss 1965; Laws 1969; Smith, Hanks & Short 1969; Hanks & Short 1972; Plotka, Seal, Schobert & Schmoller 1975; Smith & Buss 1975; Hodges, Henderson & McNeilly 1983; McNeilly, Martin, Hodges & Smuts 1983; Brannian et al. 1988; Plotka et al. 1988; De Villiers, Skinner & Hall-Martin 1989; Niemuller, Shaw & Hodges 1993; Heistermann, Beard, Van Aarde & Hodges 1994; Hodges, Van Aarde, Heistermann & Hoppen

1994; Schwarzenberger, Straub, Hoppen, Schaftenaar, Dieleman, Zenker & Pagan 1996; Trohorsch, Hodges & Heistermann 1996; Hodges, Heistermann, Beard & Van Aarde (*in prep.*); Van Aarde, Ford & Allen (*in prep.*)) there is a lack of information concerning the interaction between these steroids and their receptors in the reproductive tract.

Research on the interaction of the progestins and oestrogens with their uterine receptors, may be an important step in the development of an effective contraceptive for female African elephants. Until such knowledge is available, the application of alleged contraceptives for female elephants based on the induction of abortion through the use of RU 38486 (Short 1992) and the use of slow releasing oestrogen implants (Meyer *et al.*(*in litt.*)) are unacceptable.

Basic action and criteria for receptor functioning

The classic model of hormone-receptor interaction involves the entry of the steroid hormone into the cell, the activation of the cytoplasmic steroid-receptor complex, the translocation of the receptor from the cytoplasm to the nucleus, the interaction of the steroid receptor with nuclear components, dissociation of steroids from receptor sites and finally tissue responses to steroid hormones and their relationship to receptors (Gorski & Cannon 1976). According to this model, free receptor is found predominantly in the cytoplasm in the absence of steroids and the steroid-receptor complex undergoes a temperature-dependent alteration in its physical properties after translocation to the nucleus (McCarty & McCarty 1977). However, a more recent model was proposed with the discovery of unoccupied receptors loosely bound to the nucleus. According to this model, oestrogen and progesterone receptors with their nuclear location undergo a less complex conformational change than that suggested by the classic model (Cooke, King & Van der Molen 1988). Knowledge on the conformational changes that receptors undergo once steroids have

interacted with them, remains limited, but steroid hormone-receptor complexes are known to recognise specific hormone response elements (HREs) of the DNA near the promoter region of regulated genes (Baulieu 1989).

Various criteria have been set for the characterisation of steroid receptors (King 1982, McCarty & McCarty 1977, O'Malley & Birnhaumer 1977). These criteria used to be met to allow intracellular, non-specific binding proteins to be distinguished from cytoplasmic low capacity specific binding proteins (McCarty & McCarty 1977). They include (1) high affinities of receptors due to the low blood levels of circulating steroids (2) steroid specificity (3) tissue specificity in terms of the quantity of receptor being high in target as opposed to non-target tissues (4) finite binding capacities which demonstrate that the binding activity of interest can be saturated by its specific ligand, and (5) a quantitative correlation with an identifiable biological response.

As binding specificity of steroid hormone receptors may be very similar to that of plasma binding proteins, it becomes imperative that specific steroid binding plasma proteins be distinguished from cytoplasmic receptors by characteristic differences in physio-chemical properties which include the following methods of differentiation (Wagner 1978):

Thermolability: Thermolabile receptors are destroyed by heating cytosols to 40°C while steroid binding plasma proteins are thermostable at these temperatures.

Molecular weight: Most serum proteins sediment at approximately 4S with steroid receptors sedimenting in the 6-8S region (King 1982) in low-ionic strength density gradient centrifugation.

Electric charge: Cytoplasmic receptors migrate during electrophoresis as α-globulins and are separated from steroid binding ß-and-Y-globulins.

Selective modification of electric charge: Steroid binding plasma proteins are glycoproteins and are

desialicated by neuraminidase, a treatment which will decrease their electrophoretic mobility. Most receptors are left unaffected.

Ligand specificity: By using radioactive synthetic steroid hormones which selectively bind to receptors. Receptors are usually more specific with higher affinities for their ligands than plasma binding proteins. This method will be used extensively throughout the present thesis.

The aims of the study

The present study aims to contribute to our understanding of the reproductive physiology of the African elephant at the level of steroid - receptor interactions. This information may be relevant to the development of chemical contraception as an alternative to culling for the control this species' population numbers. The present study characterises the oestrogen and progesterone receptors in the uterine tissues of the African elephant by investigating the following aspects:

- the binding affinity of each of the receptor types for their natural ligands,

- the ligand specificity of the oestrogen and progesterone receptors, and

- The temporal chances in receptor concentrations with changes in reproductive status.

CHAPTER 2

BINDING AFFINITIES OF THE OESTROGEN AND PROGESTERONE RECEPTORS IN THE UTERINE TISSUE OF THE AFRICAN ELEPHANT

Introduction

Steroid receptors have been described as saturable, cytosolic binding proteins with high and specific affinity for their ligand and a particular ionic strength in sucrose or glycerol gradients (Savouret, Misrahi & Milgrom 1988). The biological potency of ligands depends on properties such as receptor affinity and persistence in target tissue (Katzenellenbogen 1980).

The primary force that promotes binding of the steroid to the receptor is a hydrophobic interaction between the receptor and both planar faces of the steroid. The receptor envelopes the steroid within a hydrophobic region (King 1982). Some steroids bind to more than one receptor and are known as secondary steroids. The selectivity index of a steroid receptor is then given as a ratio of the desired agonistic receptor binding to the undesired secondary agonistic receptor binding (Collins 1994). The cytosolic or non-activated forms of the receptors have a strong tendency to form complexes with a 90 kDa heat-shock protein (hsp) or with RNA (Cooke *et al.* 1988). Hsp 90 is a common receptor component with hsp 70 being a component of progesterone receptors. These protein components appear to be important as general mediators of both protein folding and assembly events (Smith, Baggenstoss, Marion & Rimerman 1993). The ligand binding domain in the progesterone receptor, the glucocorticosteriod receptor and the androgen receptor include two pockets capable of accommodating relatively large hydrophobic substituents in the 11ß- and 17α-positions, in addition to the hydrophobic site for the steroid framework, thereby allowing some

scope for the metabolism of the steroid without influencing binding affinity too markedly (Baulieu 1989).

The high affinity or acceptor sites in nuclear extracts are located in the non-histone protein fraction (Wagner 1978). The subcellular localisation of the progesterone receptor has already proved to be predominantly nuclear (King & Greene 1984). The oestrogen receptor has also been found to have a nuclear localisation (Jensen & DeSombre 1972).

Binding affinities of oestrogen and progesterone receptors differ across and within species. For instance, the progesterone receptor of the chick oviduct has a dissociation constant (K_d) of 8 x 10^{-10} M, the guinea pig uterus a K_d of 2 x 10^{-10} M (Jensen & DeSombre 1972) and the rat uterus a K_d of 1 x 10^{-10} M (McGuire & Bariso 1972). The half-life of the progesterone receptor in the uterus of the guinea pig is about five days with a predicted longer half life in rat uteri (Laio 1975). Heterogeneity of oestrogen nuclear binding sites has already been found in the cytosol of the rat uterus, where type I sites represent the classical oestrogen receptor with a K_d of 0.8nM and a sedimentation coefficient of 8 S on low salt sucrose density gradients. Type I sites also undergo translocation from the cytoplasm to the nucleus. Type II sites have a K_d of 30nM and are found predominantly in oestrogen sensitive tissues with low levels occurring in nontarget tissues. Type II sites do not translocate to the nucleus. The possible function of type II sites is that they act as concentrating agents in oestrogen sensitive cells or represent intermediates in the formation of type I sites (Savouret *et al.* 1988). Katzenellenbogen (1980) also reported on the heterogeneity of the oestrogen as well as the aldosterone nuclear binding sites. The purified progesterone receptor also consists of two separable subunits that both bind steroid but which serve different functions in the transduction of the steroid signal (Zaneveld & Chatterton 1982). Multiple receptor forms have been found by O'Malley & Birnbaumer (1978) where density gradient centrifugation has

distinguished two distinct types of progesterone binding substances. Component 1 is found in the cytosol from hamster vagina and uterus and sediments at 6-7 S in low ionic strength medium. It is thermolabile, insensitive to cortisol competition and has a high affinity for progesterone. The serum and cytosol from nontarget tissues (such as the liver and kidney) contains component 2 which sediments at 4-5 S, is heat stable, and sensitive to cortisol competition. Component 2 has a lower affinity for progesterone than Component 1. Heterogeneity is thought not to be due to variations in the coding regions, but rather due to variations in the noncoding regions (Misrahi, Loosfelt, Atger, Guiochonmantel, Applanat, Bailly, Thi, Lescop, Lorenzo, Bouchard & Milgrom 1980).

The binding affinity of a particular receptor type for its appropriate ligand is thus expected to be highly species and tissue specific. The binding affinities of the uterine oestrogen and progesterone receptors have not been determined in the African elephant and the present chapter describes the binding affinities of these receptor types. Equilibrium binding assays conducted on different compartments of the uterus as well as on animals of different in reproductive status, will specify whether this particular molecular characteristic (K_d) of the receptor remains constant, irrespective of the reproductive status or the uterine compartment of a particular animal. Determination of the binding affinity of radiolabelled oestrogen, promegestone and progesterone for the oestrogen and progesterone receptors respectively, contributes towards our understanding of the possible biological activity of these gestagens through the specificity of the hormone-receptor interaction.

Materials and methods

Animals and tissue collection

Endometrial and myometrial tissue was collected from female African elephants killed in the Kruger National Park, South Africa during May 1995. Samples were divided into those taken from the fundus and corpus regions of the uterus horn in both pregnant and nonpregnant animals. Similar tissue sections were removed from the myometrium. The tissues were placed on crushed dry ice within 30 min of death. They were then wrapped in aluminium foil and immediately snap frozen in liquid nitrogen. Subsequently, the samples were transported to the laboratory on dry ice and stored at -70°C until processed.

Gestational age was determined as $t = 106w^{1/3} + 138$, where t is the age of the foetus in days and w is the foetal mass in kg (Craig 1986). The animals selected were classified as early- (one to seven months), mid- (eight to 14 months) and late- (15 to 22 months) pregnant. Of the 22 animals sampled, four were subadult; six were nonpregnant-lactating; six were early-pregnant; two were mid-pregnant and four were late-pregnant.

Equilibrium binding assays

Oestrogen and progesterone receptor assays were carried out simultaneously and the tissue for each elephant was analysed separately. Cytosol fractions of a particular tissue type were prepared separately using the method described by Potgieter, Spies, Klein, Thierry & Van der Watt (1985). After thawing on ice the tissue was cut into thin slices on powdered dry ice. Leupeptin (Sigma, St. Louis, USA), at a final concentration of 10mM, was added to 10% (wt/vol) tissue in $TEDAG_{10}$ buffer, before homogenisation, to inhibit protease activity (Leake & Habib 1987). The tissue was homogenised in three bursts of 10 seconds duration, alternated with a 50 second cooling period on crushed ice. A TP Ultra Turrax (Janke and Kunkel, Staufen, Germany) was used during homogenisation. The homogenate was centrifuged using

a SW 50.1 rotor at 105000 × *g* (*g* = gravitational constant) for 30 min at 4°C, using a Beckman L8-80 centrifuge.

Duplicate aliquots (100µl) of cytosol were incubated with 50µl [2,4,6,7,16,17-^{3}H] oestradiol-17β (^{3}H-E_2 ; specific activity: 157Ci/mmole; Amersham International, Buckinghamshire, UK) for the oestrogen receptor and both [1,2,6,7-^{3}H] progesterone (^{3}H-P; specific activity: 95Ci/mmole; Amersham International, Buckinghamshire, UK) and [17α-Methyl-^{3}H] promegestone (^{3}H-R5020; specific activity: 86.7Ci/mmole; New England Nuclear, Massachusetts, USA) for the progesterone receptor. These radiolabelled compounds were made up in $TEDAG_{10}$ buffer at different concentration ranges according to the required number of Scatchard points (refer to the validation). The buffer ($TEDAG_{10}$) contained 10mM Tris HCL (Sigma, St. Louis, USA), 1.5mM EDTA (Sigma, St.Louis, MO), 1mM dithiotreitol (Sigma, St.Louis, MO), 1mM sodium azide (Sigma, St. Lious, USA) and 10% (m/v) glycerol with the pH adjusted to 7.4 at 4°C. Non-specific binding was determined by adding 1000-fold excess of unlabelled diethylstilbestrol (DES) (Sigma, St. Louis, MO) for the oestrogen receptor and progesterone (Sigma, St. Louis, MO) and promegestone (New England Nuclear, Massachusetts, USA) for the progesterone receptor assays. All volumes in the tubes were adjusted to a final volume of 400 µl by the addition of an appropriate volume of buffer. Total count tubes contained 50 µl of the radioligand and 350 µl buffer. Incubations for the oestrogen receptor lasted 30 min at 20°C. Progesterone receptor assays were incubated on crushed ice for 2 h, after which 100 µl of $TEDAG_{60}$ (60% glycerol (wt/vol)) was added, followed by an additional incubation on crushed ice for a further 2 h. All reactions were terminated by adding 500 µl cold dextran coated charcoal (DCC). Fresh DCC solutions were prepared every fortnight and consisted of 0.5% (wt/vol) pre-washed, activated charcoal (Merck, Darmstadt,

Germany), 0.1% (wt/vol) gelatine (Carraggenan Type 1; Sigma, St. Louis, MO) and 0.5% dextran T70) (Pharmacia, Uppsala, Sweden) in $TEDAG_{10}$ buffer. After the addition of DCC the suspension was vortexed, left on ice for 10 min and centrifuged at 2000 × *g* for 15 min. Supernatants (500 µl) were placed in scintillation vials (Packard Instrument company Inc., Johannesburg, South Africa) and counted for radioactivity after adding 4 ml scintillation cocktail (Emulsifier scintillator 299^{TM}, Packard Instument Company Inc., Johannesburg, South Africa) to a confidence limit of 95% and an uncertainty of 1% using a Packard 1500 Tri-carb liquid scintillation counter.

Validation

Initially, a 15-point Scatchard plot was used to analyse pooled endometrial tissue obtained from subadult elephant cows. These animals were selected because of their similarity in age and reproductive status. The objective was to exclude potential compounding effects of some hormones found during pregnancy and lactation. Since receptor concentrations are under multihormonal control, various hormones could modulate hormone receptor levels directly or alter the hormone response pathway at a later stage (Katzenellenbogen, 1980). McNeilly *et al.* (1983) found a dramatic increase in the prolactin concentrations during pregnancy, in elephants, but no apparent relationship between lactation and prolactin in nonpregnant elephants. Although prolactin has been shown to increase the oestrogen receptor levels in the mouse (Muldoon, 1978; Muldoon, 1981) and the rat mammary gland, there was no effect in the uterus (Lignon & Rocheford, 1976). Wathes & Hamon (1993) and Zollers, Garverick, Smith, Mofatt, Salfen & Youngquist (1993) showed a positive relationship between oxytocin and oestrogen receptors. Changes in receptor concentrations have also been known to occur during pregnancy in a variety of species (Jänne, Kontula, Vihko, Feil &

Bardin 1978). The use of subadult, non-reproductive females, therefore, minimised the possible interference of pregnancy or lactation on the observed characteristics of the receptors. A wide concentration range (0.5 nM - 64 nM) indicated that saturation of the receptors occurred at 12 nM for both receptors. The progesterone receptor reached saturation at a lower concentration of radiolabelled ligand than that of the oestrogen receptor (Figure 1a & b). Subsequent radiolabelled compounds were made up in buffer at seven different concentrations, ranging from 1 to 12 nM. Seven samples of endometrial and five samples of myometrial tissue were used in further analysis.

Since the binding parameters of the endometrial and myometrial oestrogen and progesterone receptors did not differ significantly from each other, only the endometrial tissue samples were processed in the other cows. In these cases a three-point method was used (with radiolabelled compounds made up in buffer to a final concentration of 2 nM, 4 nM and 8 nM). In order to test the feasibility of using three-point Scatchard plots, as opposed to seven-point Scatchard plots, to determine binding affinities, the values obtained for each of these methods were compared using a t - test for dependent variables (Sokal & Rohlf 1969). No significant difference in binding affinities were found between the two methods for either the oestrogen receptor ($t = -1.49$, $n = 12$, $p > 0.05$) or the progesterone receptor ($t = -1.03$, $n = 15$, $p > 0.05$). Further analyses therefore used three- point Scatchard plots which allowed a large number of samples to be analysed simultaneously.

K_d values from Scatchard plots and those obtained from saturation curves were similar for the oestrogen receptor ($t = -1.32$, $n = 12$, $p > 0.05$) and for the progesterone receptor ($t = -1.49$, $n = 12$, $p > 0.05$). The dissociation constant of a saturation curve is proportional to the slope of the curve at its origin on a plot of the bound steroid fraction as a function of the total

steroid concentration (Clark & Peck 1977). For a Scatchard plot, the slope of the plot (-1/ K_d), when the concentration of the bound steroid divided by the concentration of the free steroid versus the concentration of the bound steroid is plotted, gives the dissociation constant (Scatchard 1949). Since a Scatchard plot relies on the linear transformation of data from a saturation curve, there was no significant difference in the dissociation constants obtained by means of these two methods.

Based on frozen duplicates samples of the same tissue processed on different days, the inter-assay coefficients of variation for the binding affinities of the progesterone and oestrogen receptors were 17.9% and 18.4% respectively. The intra-assay coefficients of variation for both receptor types were < 10%.

Statistics

Scatchard and saturation curves with the Rosenthal-correction for non-specific binding were drawn by means of the COMBICEPT 2000CA software program (Packard Instrument Company, Illinois, USA). Dissociation constant (K_d) values are given in nM (mean ± SEM). The Kruskal-Wallis *H* and Mann-whitney *U* test was used to test the differences in binding affinities between the different tissue types and different reproductive stages. Correlations between the binding affinities of the oestrogen and progesterone receptor and that of foetal age, were determined by means of the Pearson product-moment correlation (*r*) (Sokal & Rohlf 1969). Significance is defined at the 95% level and values are presented as means ± SEM. Results form Scatchard plots were subjected to least square regression analyses and only data with correlation coefficients of ≥ 0.9 were considered reliable for further statistical analyses.

Results

The mean K_d values for each of the tissue and receptor types for the subadult elephant cows, using seven-point Scatchards, are presented in Table 1. The mean K_d value for the progesterone receptor assay using [^{3}H] progesterone as radioligand, was significantly higher than that calculated from the binding data where [^{3}H] promegestone was employed as radioligand (Z ($U = 2$) = -4.04, $p < 0.05$).

There was no significant difference between the binding affinities of the endometrial and myometrial tissue for either the oestrogen receptor (H = 1.44, n = 12, $p > 0.05$) or the progesterone receptor (H = 2.33, n = 12, $p > 0.05$). When the binding affinities of both receptor types were compared across tissue types within a particular stage of pregnancy, no significant difference in K_d values could be found for either of the receptors ($p > 0.05$).

The K_d values that were obtained from three-point Scatchard plots of the endometrial tissue from nonpregnant, lactating and early-, mid- and late-pregnant elephant cows, for the oestrogen and progesterone receptors are given in Tables 2 and 3 respectively. There was no significant difference in K_d values for the progesterone receptor across the various reproductive stages for a specific tissue type ($H = 4.65$, $n = 21$, $p > 0.05$). The K_d values of the oestrogen receptor was significantly different when the various reproductive stages were compared for the fundus region of the nonpregnant uterus horn ($H = 14.05$, $n = 21$, $p < 0.05$).

The binding affinities of the uterine oestrogen receptor in the nonpregnant, lactating and the early-pregnant cows are significantly higher (lower K_d values) than those of the mid- and late-pregnant cows. Subadult cows also had a significantly higher binding affinities for

TABLE 1. Binding affinities (mean K_d nM ± SEM for no. of individual animals in parentheses) of the oestrogen and progesterone receptors determined by means of seven-point Scatchard plots in subadult female African elephants.

Receptor type	Tissue type			
	Endometrial, corpus region (3)	Endometrial, fundus region (4)	Myometrial, corpus region (3)	Myometrial, fundus region (2)
Oestrogen	0.140 ± 0.025	0.185 ± 0.018	0.223 ± 0.038	0.190 ± 0.100
Progesterone (with ^{3}H promegestone)	0.250 ± 0.056	0.235 ± 0.033	0.233 ± 0.072	0.135 ± 0.025
Progesterone (with ^{3}H progesterone)	0.677 ± 0.223	1.058 ± 0.263	1.470 ± 0.105	0.830 ± 0.180

TABLE 2. The binding affinities (mean K_d nM ± SEM for no. of individual animals in parentheses) of the oestrogen receptor in the African elephant determined by means Scatchard plots for various tissue types obtained from different regions of the uterus.

Tissue type	Reproductive status			
	Nonpregnant, lactating	Early-pregnant	Mid-pregnant	Late-pregnant
Endometrial, corpus region in the nonpregnant uterus horn	0.118 ± 0.009 (4)	0.200 ± 0.055 (3)		
Endometrial corpus region in the pregnant uterus horn		0.135 ± 0.045 (2)		
Endometrial, fundus region in the nonpregnant uterus horn	0.122 ± 0.015 (6)	0.145 ± 0.017 (6)	0.300 ± 0.020 (2)	0.320 ± 0.038 (3)
Endometrial, fundus region in the pregnant uterus horn		0.257 ± 0.128 (5)	0.180 ± 0.020 (2)	0.293 ± 0.134 (4)

TABLE 3. The binding affinities (mean K_d nM ± SEM for no. of individual animals in parentheses) of the progesterone receptor in the African elephant determined by means Scatchard plots for various tissue types obtained from different regions of the uterus.

Tissue type	Reproductive status			
	Nonpregnant, lactating	Early-pregnant	Mid-pregnant	Late-pregnant
Endometrial, corpus region in the nonpregnant uterus horn	0.618 ± 0.300 (4)	0.313 ± 0.044 (3)		
Endometrial corpus region in the pregnant uterus horn		0.235 ± 0.055 (2)		
Endometrial, fundus region in the nonpregnant uterus horn	0.353 ± 0.047 (6)	0.267 ± 0.042 (6)	0.215 ± 0.025 (2)	0.220 ± 0.029 (3)
Endometrial, fundus region in the pregnant uterus horn		0.347 ± 0.126 (5)	0.285 ± 0.125 (2)	0.328 ± 0.144 (4)

the uterine oestrogen receptor in late-pregnant elephant cows. Similarly significantly lower oestrogen receptor binding affinities (higher K_d value) were observed for tissues from nonpregnant, lactating elephant cows (Table 4).

Figure 2 (a) depicts the positive relationship between the foetal age (gestational age) and K_d values of the oestrogen receptor in the fundus region of the nonpregnant uterus horn ($r = 0.87$, $n = 11$, $p < 0.05$). The progesterone receptor K_d values that were associated with gestational stage are depicted in Figure 2 (b).

Discussion

The oestrogen receptor had a higher binding affinity for its natural ligand than the progesterone receptor and our results therefore support those of Jänne *et al.* (1978) who found that the progesterone receptor has a lower affinity for progesterone than do the appropriate receptors for androgens and oestrogens. The lower affinity of the progesterone receptor for its appropriate ligand is probably due to a rapid rate of dissociation of the progestin receptor-progesterone complexes when compared to those of the oestrogen receptor (Jänne, Kontula & Vihko 1976). Geynet, Millet, Truong & Baulieu (1972) proposed that the differential affinity of the progesterone receptors for various ligands is determined by changes in the rate of association.

The fact that progesterone receptor assays using [^{3}H] promegestone had significantly lower K_d values than assays using [^{3}H] progesterone, indicates that this receptor has a higher affinity for promegestone than for progesterone. This may be due to the fact that promegestone has a high progestin selectivity (Ojasoo, 1995), a nanomolar K_d value for the

TABLE 4. *P* values of the Mann-Whitney *U* test for the binding affinity values of the oestrogen receptor with changes in the reproductive status of African elephant cows for endometrial tissue from the fundus region of the uterus ($n = 21$). Values marked with an asterisk denote significant differences at a 95% confidence level.

	Subadult	Nonpregnant, lactating	Early-pregnant	Mid-pregnant
Nonpregnant, lactating	$Z\ (U = 2) = -2.139$ $p = 0.032$*			
Early-pregnant	$Z\ (U = 4.5) = -1.604$ $p = 0.109$	$Z\ (U = 45) = -0.966$ $p = 0.334$		
Mid-pregnant	$Z\ (U = 0) = -1.852$ $p = 0.064$	$Z\ (U = 0) = -2.012$ $p = 0.044$*	$Z\ (U = 0) = -2.000$ $p = 0.005$*	
Late-pregnant	$Z\ (U = 0) = -2.121$ $p = 0.034$*	$Z\ (U = 0) = -2.334$ $p = 0.020$*	$Z\ (U = 0) = -2.324$ $p = 0.020$*	$Z\ (U = 3) = -0.000$ $p = 1.000$

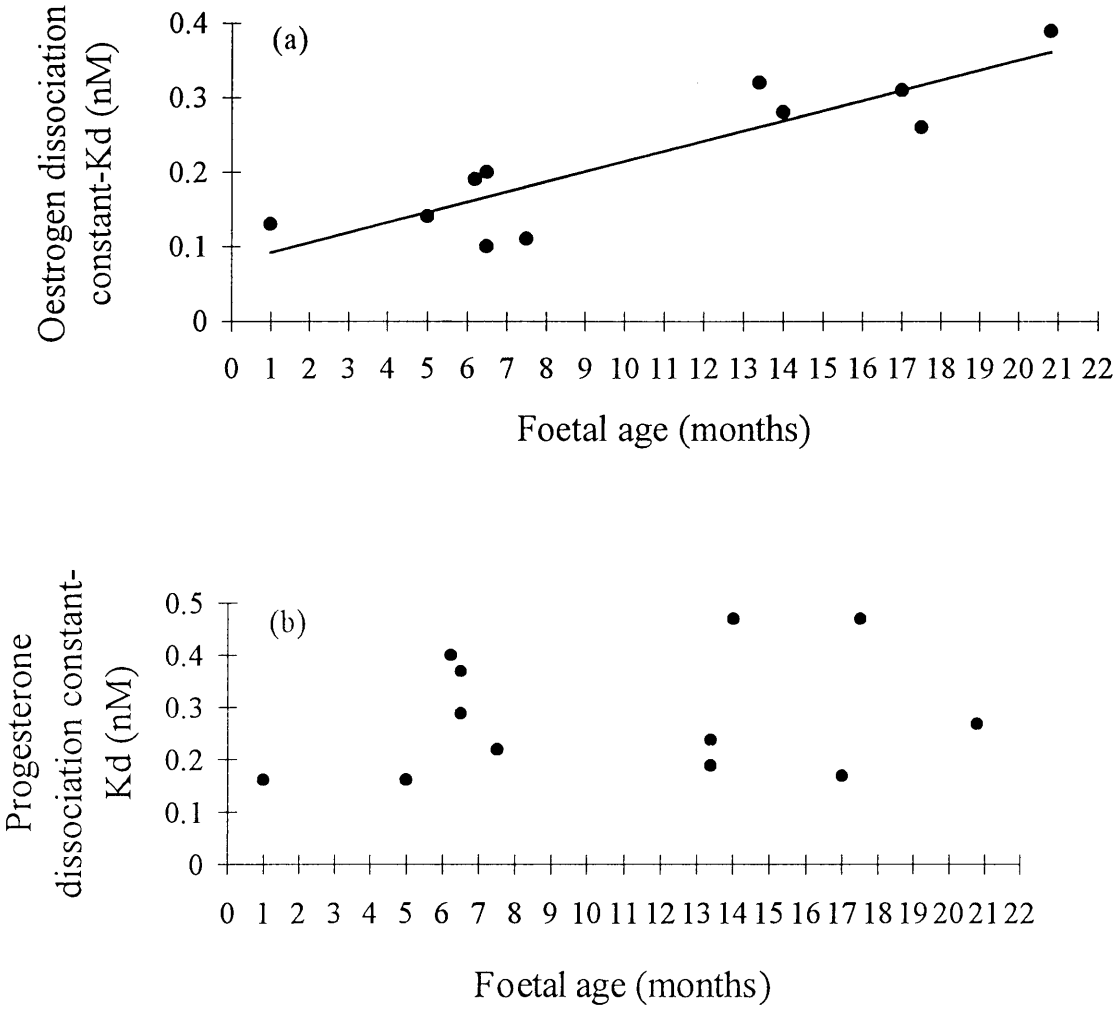

FIGURE 2. The effect of gestational stage on the binding affinity of the oestrogen (a) and progesterone (b) receptors in the endometrial, fundus region of the nonpregnant uterus horn of pregnant African elephant cows.

progesterone receptor and an inability to recognise corticosteroid binding globulin (Milgrom, Luu Thi & Baulieu 1973). Furthermore, progesterone has a faster dissociation rate than promegestone because of the hydrophobic substituents of promegestone which gives this steroid a long half-time of dissociation (Feil, Miljkovié & Bardin 1976).

The K_d values for the endometrial and myometrial oestrogen and progesterone receptors were similar, suggesting that the receptors of these tissues may have similar molecular characteristics. This finding is also in agreement with the observation of Grossman & Leavitt (1974) who showed that progesterone receptors in the endometrium of hamsters have the same physio-chemical properties as the receptors in the myometrium.

Muldoon (1981) found a difference in oestrogen receptor affinity between virgin and late lactating mouse mammary gland. Leavitt & Grossman (1974) and Milgrom, Perrot, Atger & Baulieu (1972) observed that with increased concentrations of endogenous ligands in the hamster, the slope of the Scatchard plot decreased, which implies a decrease in the binding affinity of the receptor for its ligand. It is therefore possible that an increase in oestradiol-17β concentrations (or related oestrogens) with pregnancy would cause the decreased affinity of the oestrogen receptor. This is supported by the positive correlation between the foetal age (gestational age) and the K_d values for the oestrogen receptor. With increasing foetal age there is probably an increase in plasma concentrations of oestradiol-17β sulphate (Hodges *et al.* 1983) but not in the concentrations of unconjugated oestradiol-17β. The higher concentrations of the conjugated oestrogen could result in the lower observed affinity of the oestrogen receptor for oestradiol-17β in pregnant animals. This is probably due to a ligand-induced phenomenon and not to changes in mRNA or protein levels of the oestrogen receptor, which would imply changes in the molecular characteristics of the receptor at gene level. As the

physio-chemical properties of a particular receptor (sedimentation rate, binding kinetics and thermal instability) have been reported to be indistinguishable between different tissues for the same receptor type (Atger, Baulieu & Milgrom 1974), it seems unlikely that these properties will change with changes in the reproductive status of the animal. These results are therefore in agreement with those Padayachi, Hofmeyr, Joubert, Norman & Pegorado (1987) who reported on a decreased affinity in the oestrogen receptor with progressing human pregnancy. The K_d values of the elephant's progesterone receptor did not vary in any particular pattern with changes in the reproductive status of the animals, thereby concurring with the findings of Wiehle (1983) where the K_d values for the progesterone receptor did not vary extensively with the stage of development in the rat.

Conclusion

In the African elephant both oestrogen and progesterone receptors have high binding affinities (nM K_d values) and finite binding capacities (saturation) for their natural ligands. Although the binding affinity of the progesterone receptor for progesterone was not as high as that of the oestrogen receptor for oestradiol-17β, promegestone did proved to have a higher affinity for the progesterone receptor ligand than progesterone. The molecular characteristics of both receptor types were not dependent on the distribution of the receptors within the uterus, as receptors in the endometrium had similar binding affinities to those in the myometrium. There also was no difference in the binding affinities of either of the receptor types when the various categorised, tissue-types were compared within a particular stage of gestation. However, progressing pregnancy did lead to a decrease in the binding affinity of the oestrogen receptor. This was not observed for the progesterone receptor. The positive

correlation between the oestrogen receptor's binding affinity and foetal age, suggested that an increase in oestrogens with progressing pregnancy could cause a ligand-induced phenomenon such as co-operative binding. It is unlikely that these changes in affinity were due to changes in the molecular characteristics of the oestrogen receptor at gene level.

CHAPTER 3

THE COMPETITIVE EFFICIENCY OF OTHER STEROIDS FOR BINDING WITH EACH OF THE RECEPTORS

Introduction

All steroids consist of a steroid nucleus, arranged in such a way that four carbon rings (A, B, C and D) are formed (Zaneveld & Chatterton 1982). The interaction of a steroid and its receptor are dependent on the types of functional groups that this steroid nucleus contains and their spatial arrangement. Binding specificity of receptors are determined by hydrophobic substituent groups (the cytochrome P-450 system in the pig has been implicated with the ring and side-chain oxidation of progesterone (Senciall & Rahal 1988)) on the steroid, the size of these groups and the conformation (planarity) of steroid ring A (Zaneveld & Chatterton 1982). The difference in conformation between the α and ß isomers of a steroid can evidently influence the biological properties of the molecule. The rate of metabolism of circulating steroids is related to the reproductive state of the animal (Makawiti, Osaso & Gombe 1991). Although McCarty & McCarty (1977) state that there is little to suggest the necessity of metabolic conversions for the specific binding and biologic activity of most steroids, the receptors are highly specific in the recognition of the stereochemical properties of the A ring .

As carbon five is contained within the A ring, 5α- reduced metabolites, such as those found in the African elephant which are metabolised from progesterone (Heistermann *et al.* 1994; Hodges *et al.* 1994), may have very different biologic activities. Although progesterone and 17α-hydroxyprogesterone have been found to be the main circulating progestin in most mammals

during pregnancy, 5α–reduced progestins, 5α–pregnan-3α-ol-20-one and 5α–dihydroprogesterone (5α-DHP, 5α-pregnane-3,20-dione), are the main progestins contained within and biosynthesised by the corpora lutea of the African elephant (Heistermann *et al.* 1994; Hodges *et al.* 1994). The mare also contains high plasma concentrations of circulating 5α–reduced progestins during pregnancy, of which the function is unknown (Hamon, Clarke, Houghton, Fowden, Silver, Rossdale, Ousey & Heap 1991; Holtan, Houghton, Silver, Fowden, Ousey & Rossdale 1991).

Clear inter-species differences exist in terms of progesterone uptake by steroid binding proteins, metabolism and the nature of the progesterone-receptor protein interaction (King & Mainwaring 1974). Therefore, a synthetic anti-progestin which may prevent pregnancy in one species, may prove ineffective in another species if the metabolised steroid is of greater importance than the parent compound. The following examples illustrate the inter-species differences that exist when it comes to the conversion and binding probabilities of progesterone and its metabolites:

- Metabolism of progesterone in the rat uterus has proved to be extensive, with a wide variety of progesterone metabolites being formed (Weist 1963a and b). 5α reduced metabolites may be important in the rat uterus as at least one effect of oestrogen priming is to elevate nuclear 5α-reductase which is responsible for metabolic conversions of progesterone to the 5α-reduced metabolites (King & Mainwaring 1974).
- In the guinea pig oestrogens stimulates the uptake of radio-labelled progesterone with little consequent metabolism (Armstrong & King 1971; Falk & Bardin 1970). However, 5α-pregnane-3,20-dione do show significant affinity for the progesterone receptor in the guinea pig uterus (Milgrom, Atger & Baulieu 1970).

- In the shell gland of the chick oviduct, metabolism of progesterone leads to the production of 5α–pregnane-3,20 dione. Progesterone and testosterone are the active substrates for an NADPH-dependent 5α–reductase which was associated with the nuclear chromatin (Morgan & Wilson 1970). 5α-pregnane-3,20 dione also shows significant affinity for progesterone receptors in the chick oviduct (Mueller, Gorski & Aizawa 1961). In the chick oviduct system cortisol, androsterone, aldosterone, androstenedione and 5α-pregnane-3ß,20α-diol are all inactive in inducing avidin protein synthesis, whereas progesterone activates the synthesis of this protein (King & Mainwaring 1974).
- 5α-pregnane-3,20 dione is less tightly bound to the progesterone receptors in rabbit uterus (Jensen & DeSombre 1972). In rabbit endometrium, 5α-pregnanedione will compete with radio-labelled progesterone for binding sites but remains inactive in both the endometrial proliferation and stromal cell chromatin structure assays (King & Mainwaring 1974).

Although metabolism of progesterone may be extensive in certain tissues, unchanged progesterone still constitutes the principal steroid in most of the species studied (Jensen & DeSombre 1972; Liao 1975; Higgins & Gehring 1978). 5α–pregnanedione has been found to have relatively low progestin activity (Hecht-lucari, Baldratti & Sala 1961). Steroid receptors often display considerable cross-reactivity, i.e. progesterone receptors might fail to differentiate between progesterone and its metabolites (Evans 1988; Baulieu 1989; Arevelo, Taussig & Wilson 1993). The metabolism of most active steroids is known to invariably decrease the affinity of the steroid for its receptor thereby diminishing its potency (Liao 1975; Zaneveld & Chatterton 1982).

Poole (1993) and Short (1992) have suggested that consideration should be given to the use of contraceptives or contragestins, such as an antiprogestin, like mifepristone (RU 486; Roussel Uclaf, Paris), which may block uterine receptor activity in the African elephant.

Such treatments do, however, require detailed knowledge of the reproductive endocrinology in the elephant. The potential for antiprogestins to interfere with uterine receptor activity in the elephant have not been investigated. The present chapter thus focuses on the relative binding affinities of the uterine progesterone and oestrogen receptors of the elephant for a variety of hormones and their analogues. The importance of the 5α–reduced metabolites of progesterone when it comes to the hormone-receptor interaction, were also assessed.

Materials and methods

Substances used

The radioligands [1,2,6,7-^{3}H]progesterone (^{3}H-P; specific activity: 95Ci/mmol) [2,4,6,7,16,17-^{3}H]oestradiol-17β (^{3}H-E_2; specific activity: 157Ci/mmol) were purchased from Amersham International, Buckinghamshire, UK and [17α-Methyl-^{3}H]promegestone (^{3}H-R5020; specific activity: 86.7Ci/mmol) as well a unlabelled promegestone from New England Nuclear, Massachusetts, USA. All other unlabelled steroids, leupeptin and gelatine (Carraggenan Type 1) were purchased from Sigma, St. Louis, MO, while SA-Druggists (Port Elizabeth, South Africa) kindly donated levonorgestrel, ethinyl oestradiol, tamoxifen and danazol. Roussel Uclaf (Paris, France) kindly supplied RU 486. Activated charcoal was purchased from Merck Chemicals (Darmstadt. Germany) and dextran T 70 from Pharmacia Fine Chemicals (Uppsala, Sweden).

Animals and tissue collection

Tissue samples were collected as described in Chapter 2 (p. 10-11). During all dissections, the endometrium was removed first as this tissue was considered to be the most important to this study.

Competitive binding assays

Endometrial and myometrial tissues from the four subadult elephant cows were kept separately and finely sliced with a sharp scalpel while frozen in a petri dish filled with powdered dry ice. A homogenous mixture was made of the pieces of tissue and this mixture was subsequently homogenised and assayed for oestrogen and progesterone receptors according to the method of Potgieter *et al.* (1985). Cytosol aliquots (100µl) were incubated in triplicate for 30 min at 20°C with 50µl of either [^{3}H]oestradiol (made up in $TEDAG_{10}$ buffer to a final concentration of 8 nM radiolabelled compound), or [^{3}H]progesterone (made up in $TEDAG_{10}$ buffer to a final concentration of 8 nM radiolabelled compound) in the presence of 50µl of increasing concentrations (0.3125 nM - 0.1 mM) of each of the following competitors: ORG 2058, norethindrone, promegestone, levonorgestrel, RU 486, medroxyprogesterone, 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol-20-one, 5α-pregnane-3β-ol-20-one, 5α-pregnane-3β,20β-diol, 5β-pregnane-3,20-dione, 5β-pregnane-3α,20β-diol, 5β-pregnane-3α,20α-diol, 17α-hydroxyprogesterone, 11α-hydroxyprogesterone, oestradiol-17β, diethylstilbestrol (DES), ethinyl oestradiol, oestrone, oestriol, tamoxifen, pregnenolone, 4-pregnen-20α-ol-3-one, hydrocortisone, cortisone, testosterone, dehydrotestosterone and danazol. All the volumes in the tubes were adjusted to 400µl with the addition of the appropriate volume of buffer. The tubes that were used to determine total binding contained radiolabelled compound and cytosol while those that were used to measure total counts, only contained radiolabelled compound. The reactions were terminated with the addition of dextran coated charcoal and counted for radioactivity as set out in the previous chapter (p. 12-13).

Validation

Each assay included a set of tubes containing a 1000 molar excess of unlabelled DES or progesterone for the oestrogen and progesterone competitive binding assays respectively. This was used to correct for non-specific binding (Wakeling 1987). If the specific binding did not account for > 60% of the total binding, the tissue was regarded as degraded and the results were not used. Calculation of the relative binding affinity of each compound was determined at the 50% competition level from a displacement curve for each ligand, constructed by plotting the percent specific bound radiolabelled compound versus log of the concentration of the competing steroid. The displacement curves were subjected to least square regression analyses and only data with correlation coefficients of ≥ 0.9 were considered reliable for the application of Rodbard's equation (1973).

The time dependent, temperature lability of receptors (King 1982) implies a loss in the binding capacity of tissue with time (Leclercq 1987). In this regard it would be germane to determine the influence of storage on the degradation of the receptors. The homogenate for each competitive binding assay was, therefore divided into cytosol fractions (10 ml) which were then stored at -70°C. After storage competitive binding assays were conducted for nine consecutive days. To test the influence of storage on the degradation of each of the receptor types, controls were included in each assay. The control for the progesterone competitive binding assay consisted of a triplicate set of tubes that contained 100µl of cytosol, 50µl of radiolabelled progesterone (made up in $TEDAG_{10}$ buffer to a final concentration of 8 nM radiolabelled compound), 50µl of norethindrone (made up in $TEDAG_{10}$ buffer to a final concentration of 10 nM) and 200µl of $TEDAG_{10}$ buffer. The control for the oestrogen competitive binding assay was similar except for the use of 50µl of radiolabelled oestradiol-17β (made up in $TEDAG_{10}$ buffer to a final concentration of 8 nM radiolabelled compound)

and 50µl of DES (made up in $TEDAG_{10}$ buffer to a final concentration of 10 nM). Norethindrone and DES were used as competitors as these steroids had relatively high binding affinities for their respective receptors. Any decrease in the percentage specific binding of these controls with time of storage, would indicate that tissue degradation was taking place in accordance to the increase in the non-specific binding.

Figure 3 illustrates that there was no significant change in the binding capacity of the endometrial, progesterone and oestrogen receptors (r = -0.20, n = 23, $p > 0.05$ for the progesterone receptor and $r = 0.15$, $n = 11$, $p > 0.05$ for the oestrogen receptor) with the time of storage of the frozen cytosols. Wiehle (1983) could find no significant difference between the level of oestrogen receptors in unfrozen versus frozen (for a five to six month period) rat mammary gland at 14 or 20 days of pregnancy or at 1 or 10 days of lactation. In the present study the myometrial tissue appeared to be more sensitive to degradation over time (r = -0.75, $n = 15$, $p < 0.05$). This was reflected in a smaller difference between the specific and the non-specific binding components of such material and therefore the significant decrease in percentage specific binding over time. Luu Thi, Baulieu & Milgrom (1975) found that the myometrial, cytoplasmic progesterone receptor of the guinea pig was more sensitive to down-regulation by progesterone than the endometrial receptors. The lability of the myometrial tissue probably resulted from the time delay in preservation during field collection as this tissue was only removed after the endometrial tissue had been removed.

Results

The relative binding affinity and apparent K_d values for the various competitors for endometrial progesterone receptors are presented in Table 5. Degradation of the tissue, as

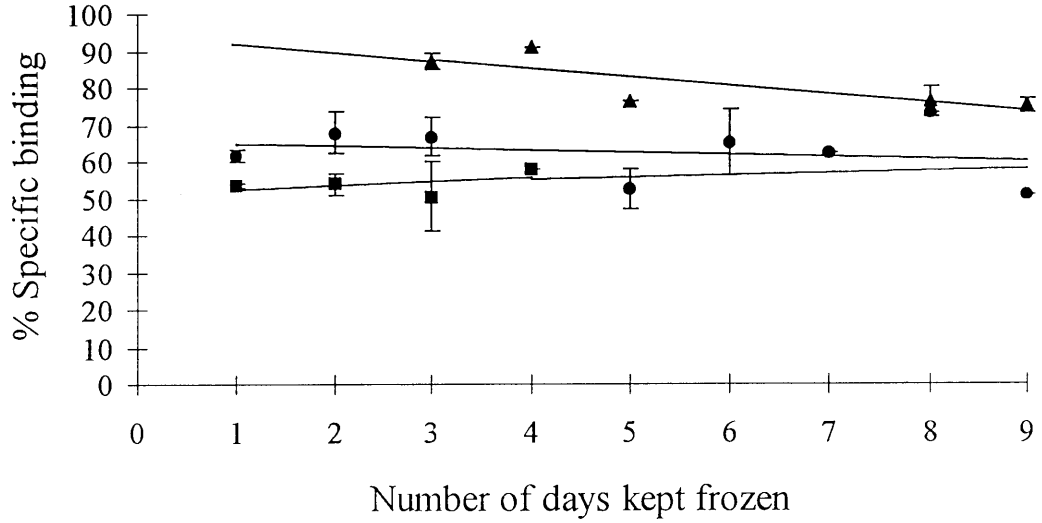

FIGURE 3. The influence of storage on the degradation of myometrial tissue for the progesterone receptor (▲), and endometrial tissue for the progesterone (●) and oestrogen receptors (■) respectively.

TABLE 5. Relative binding affinity and apparent K_d values of selected potential competitors for the endometrial progesterone receptor in four subadult female African elephants.

Competitor	Apparent K_d (nM)	Relative binding affinity (%)
ORG 2058	0.24	380
Norethindrone	0.30	293
Promegestone	0.65	138
Progesterone	0.89	100
5α-pregnane-3,20-dione	2.1	43
Levonorgestrel	3.6	24
5α-pregnane-3α-ol-20-one	4.5	20
5α-pregnane-3β-ol-20-one	27	3
5β-pregnane-3,20-dione	29	3

RBA < 0.1% for 5α-pregnane-3β,20β-diol, 5β-pregnane-3α,20β-diol, 5β-pregnane-3α,20α-diol, 17α-hydroxyprogesterone, 11α-hydroxyprogesterone, Medroxyprogesterone, Mifepristone (RU 486), Oestradiol-17β, Oestrone, Oestriol, DES, Tamoxifen, Pregnenolone, 4-pregnen-20α-ol-3-one, Hydrocortisone, Cortisone, Testosterone, Dehydrotestosterone and Danazol.

discussed previously, precluded estimation of these values for the myometrium, except for the binding of promegestone (relative binding affinity = 199%, K_d = 0.61 nM), levonorgestrel (relative binding affinity = 65%, K_d = 1.9 nM), 5∝-pregnane-3,20-dione (relative binding affinity = 26%, K_d = 4.8 nM), RU 486 (relative binding affinity < 0.1%) and oestrone (relative binding affinity < 0.1%). These relative binding affinity values were similar to those obtained for the same competitors with endometrial tissue. The progesterone receptor had a high relative binding affinity for both 5∝-pregnane-3,20-dione (43%) and 5∝-pregnane-3∝-ol-20-one (20%), known to be produced in the corpus luteum of the pregnant elephant (Heistermann *et al.* 1994, Hodges *et al.* 1994). The two synthetic progestins norethindrone and levonorgestrel, used as oral contraceptives in women, both exhibited a relatively high affinity for the elephant's progesterone receptor (norethindrone: relative binding affinity = 293%, K_d = 0.30 nM and levonorgestrel: relative binding affinity = 24%, K_d = 3.6 nM) . However, RU 486, as well as oestrogens, androgens and corticosteroids, had relative binding affinity values < 0.1%. The competitive binding plots for selected ligands are presented in Figure 4.

Table 6 summarises the relative binding affinity and K_d values of the competitors that were tested on the endometrial oestrogen receptor and their displacement curves are given in Figure 5. These assays were also conducted on the myometrium but, due to tissue degradation, estimates could be obtained only for DES (relative binding affinity = 252%, K_d = 0.08 nM), ethinyl oestradiol (relative binding affinity = 102%, K_d = 0.20 nM), oestrone (relative binding affinity = 24%, K_d = 0.88 nM) and oestriol

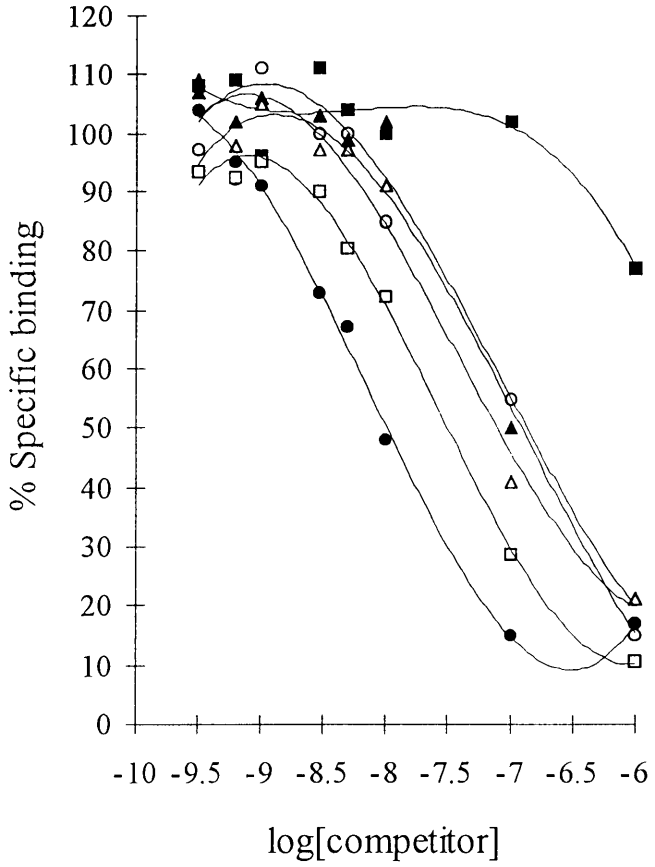

FIGURE 4. Competition of various ligands for binding to the African elephant endometrial progesterone receptor: norethindrone (●), progesterone (□), 5α-pregnane-3,20-dione (Δ), levonorgestrel (▲), 5α-pregnane-3α-ol-20-one (o). RU 486 (■).

TABLE 6. Relative binding affinity and apparent K_d values of selected potential competitors for the endometrial, oestrogen receptor in four subadult female African elephants.

Competitor	Apparent K_d (nM)	Relative binding affinity (%)
Diethylstilbestrol	0.14	119
Ethinyl oestradiol	0.15	112
Oestradiol-17β	0.17	100
Oestrone	1.4	12
Oestriol	1.7	10

RBA of < 0.1% for Tamoxifen, Progesterone, Promegestone, Norethindrone, Mifepristone (RU 486), 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol-20-one, Hydrocortisone, Cortisone, Testosterone and Dehydrotestosterone.

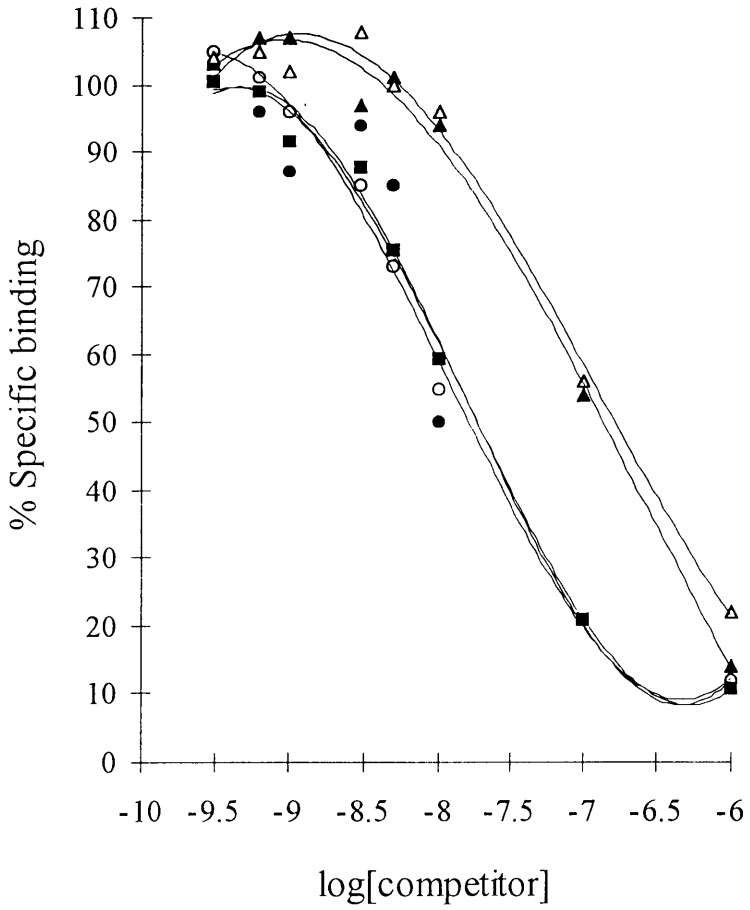

FIGURE 5. Competition of various ligands for binding to the African elephant endometrial oestrogen receptor: ethinyl oestradiol (●), diethylstilbestrol (o), oestrone (▲), oestriol (Δ), oestradiol-17β (■).

(relative binding affinity = 12%, K_d = 1.8 nM). These values were similar to those obtained with the endometrium.

Discussion

Antiprogestins could prevent conception by creating a uterine environment hostile to implantation (Cooke *et al.* 1988) and this makes the progesterone hormone receptor the most specific and most accessible target for intervening in hormone action (Baulieu 1989). However, differences between species in the uptake, binding and transport of steroids and also in the nature of the progesterone receptor interaction (King & Mainwaring 1974) caution against the use of synthetic steroids without first examining each compound within the particular species of interest and correlating binding affinity with biological activity (Gray & Leavitt 1987). Prior to this study no information was available on uterine steroid hormone receptors in the African elephant. Until the relative binding affinities of the oestrogen and progesterone receptors have been screened for a range of antigestagenic steroids, the potential use of antigestagens such as mifepristone (RU 486) to induce abortion, as suggested by Short (1992), cannot be considered seriously as a means of birth control in elephants. Competitive binding studies, such as those used in the present study should indicate which antigestagens may be of potential use in the development of techniques to control conception rate in elephant populations. However, a high affinity of a specific ligand for the receptor would still have to be correlated with biological response *in vivo*.

The relative binding affinities of the competitors tested for both receptors in the endometrium and myometrium compared favourably, suggesting that the receptors in these uterine compartments are homogenous.

The only progestin that competed successfully for the progesterone receptor in the present ligand binding studies was norethindrone, which bound to the receptor nearly three times as strongly as progesterone. Norethindrone and norethindrone acetate are used widely to prevent pregnancy in women (Taitel & Kafrissen 1995). Low doses of norethindrone and of levonorgestrel (relative binding affinity = 24% in the present study), are considered to have an antifertility effect, while high doses can have an anti-ovulatory effect (Lobo, & Stanczyk 1994). On the other hand, RU 486, which is known to act strongly as an antiprogestin in humans (Gravanis, Schaison, George, De Brux, Satyaswaroop, Baulieu & Robel 1985), rabbits and rats (Philibert 1984), showed a very low affinity for both the progesterone and oestrogen receptors of the uterus of the African elephant. A low relative binding affinity for RU 486 has also been reported in the hamster (Gray & Leavitt 1987), the chicken (Groyer 1985) and the Tammar wallaby (Fletcher & Blandon 1988).

The low binding affinity of RU 486 could be attributed to the presence of glycerol in the buffer. In the first incubation for the progesterone receptor assays, 10% glycerol was used, while in the second incubation step 60% glycerol was used. Gravanis *et al.* (1985) reported that in the absence of glycerol, the human endometrial progesterone receptor had a higher affinity for RU 486 than progesterone. This was not the case when a higher proportion of glycerol was present. Bramley & Menzies (1994) stated that modifications to the 3,4,5 and 11 positions of progesterone could cause phenomenal decreases in binding potency. RU 486 has a phenyl group attached to carbon 11, which could possibly explain its low affinity for the receptor. Gray & Leavitt (1987) found in the hamster that the low binding affinity of the

progesterone receptor for RU 486 did not prevent decidualisation and normal implantation in the uterus. Should this be the same for the elephant, RU 486 may no longer be considered to be of potential use to control reproductive output in the elephant (Short 1992; Poole 1993). Although comparative strong binding to the receptor does predict a possible potency as a contraceptive agent, as suggested for norethindrone and levonorgestrel in this present study, a discrepancy in the observed potency may still arise once an agent is applied.

Neither of the two 5α-reduced metabolites of progesterone (5α-pregnane-3,20-dione and 5α-pregnane-3α-ol-20-one) known to be produced in the corpus luteum of the elephant (Heistermann *et al.* 1994; Hodges *et al.* 1994) had a stronger binding affinity for the progesterone receptor than progesterone. For a metabolite to be of significant biological importance, the following conditions must be met: it must account for at least 80% of the recovered radiolabelled primary steroid; the receptor must preferentially bind the metabolite; and the conversion enzyme must occur only in the target tissue (Higgins & Gehring 1978). Despite a low affinity, a low metabolic clearance rate could ensure sustained concentrations in the blood and thereby increase efficiency dramatically (Sutherland & Jordan 1981). Biological potency therefore depends on a combination of receptor affinity and persistence in target tissues (Katzenellenbogen 1980). If a binding protein in the blood or uterine fluid of the elephant has a stronger affinity for the metabolites of progesterone, the progesterone that is not converted can interact freely with the receptor at low concentrations. Uteroglobin, found in the rabbit uterus binds poorly to synthetic progestins but binds to 5α-pregnane-3,20-dione with a three fold higher affinity than the progesterone receptor (Fridlansky & Milgrom 1976). In binding to its regulator it modulates progesterone activity and transport

(Savouret *et al.* 1988). Likewise, serum albumin has a higher affinity for oestradiol-17β than oestriol, this ensures that oestriol is freely available and results in a greater tissue interaction than that of oestradiol-17β (Anderson, Peck & Clark 1974).

The relatively high binding affinity of the progesterone receptor in the elephant for 5α-dihydroprogesterone (43%) which occurs at a 10-20 fold higher concentration than progesterone in the plasma of pregnant animals (Heistermann *et al.* 1994), suggests that this metabolite may have some biological activity in this species. The metabolic clearance rate of the 5α-reduced metabolites of progesterone, and the binding affinities of carrier proteins for these substances in the plasma, have yet to be determined for the African elephant. Until these studies are undertaken it remains inappropriate to speculate further on the biological significance of 5α-dihydroprogesterone in this species. However, it is worth noting that progesterone metabolites can act as intracellular mediators of progesterone action in the endometrium (Armstrong & King 1971).

In the present study, 5β-reduced-isomers of progesterone displayed a very low binding affinity for the progesterone receptor when compared to 5α-reduced metabolites. This is in agreement with King & Mainwaring (1974), who found that 5α-reduced compounds usually retain some of the biological properties of the parent compound, whereas 5β-isomers lose or change the properties completely.

In the African elephant, both the oestrogen and progesterone receptors proved to be highly specific. The progesterone receptor interacted poorly with the three other classes of steroids, namely the oestrogens, androgens and the adrenocortical hormones. The oestrogen receptor had no affinity for any of the four major steroid classes, other than the oestrogens. Oestradiol-17β had nearly the same affinity for the oestrogen receptor as ethinyl oestradiol

and DES, while tamoxifen had a very low affinity for the receptor. This may have been caused by the hydrophobic nature of tamoxifen resulting in the adsorption to the glass or plastic apparatus, thereby leading to significant errors at low concentrations (Wakeling 1987). It is more likely, however, that the 4-hydroxytamoxifen metabolite of tamoxifen would have had a stronger affinity for the oestrogen receptor than tamoxifen since Rochefort, Garcia & Borgna (1979) found that this metabolite is 10 times more potent than its parent compound.

Conclusion

Both the oestrogen and the progesterone receptors displayed a high level of ligand specificity. The 5α-reduced metabolites of progesterone exhibited a high relative binding affinity for the progesterone receptor. This indicates that these metabolites may be of some biological importance in the African elephant. The synthetic antiprogestin, RU 486 did not compete successfully with progesterone in competitive binding studies. However, norethindrone displayed an extremely high affinity for the progesterone receptor, and may have some potential in the future development of a technique to control reproductive output in the African elephant. In this regard the high relative binding affinity of norethindrone for the progesterone receptor deserves further investigation. Both oestrogen and progesterone receptors proved to be highly specific, which necessitates the screening of all anti-gestagenic substances before they can be applied to reduce the reproductive output in elephants.

CHAPTER 4

CHANGES IN RECEPTOR CONCENTRATION WITH CHANGES IN THE REPRODUCTIVE STATUS

Introduction

Hormones display an ability to modulate the levels of their own receptors or of receptors for other hormones giving rise to tissue sensitivity to multiple hormones. The oestrogen: progesterone ratio is very important in determining the synergistic and/or antagonistic effects of these hormones on different tissues of the reproductive tract. Therefore, regulation of receptor levels is frequently under multihormonal control with regulation of a particular receptor involving different hormones in different tissues (Katzenellenbogen 1980). Hormones may also mutually antagonise each others activities through negative regulation of receptor levels (Zaneveld & Chatterton 1982). There are two central concepts concerning steroid receptor proteins (1) the concentration of circulating steroid represents a gradation steroid-hormone-response and (2) the activation or repression of the synthesis of a second hormone receptor due to the response of a given steroid hormone, occurs as a marker of cellular differentiation (McCarty & McCarty 1977). Thus, the formation of hormone-receptor complexes and receptor initiation of responses have proved to be complex and multifaceted, with distinct temporal components (Katzenellenbogen 1980).

Depending on the physiological state, progesterone may antagonise oestrogen action. One effect of oestradiol is to increase the levels of progesterone receptor. Binding of progesterone to its

receptors leads not only to progestational effects, but also to anti-oestrogenic effects (a reduction in oestrogen secretion into the systemic circulation by stimulation of the enzyme, 17ß-hydroxysteroid dehydrogenase, which converts oestradiol to the less active oestrogen, oestrone) (Cooke *et al.* 1988). Thus progesterone can suppress oestrogen receptor levels in the presence of continuously elevated serum oestradiol. The morphological response is tissue specific even though progesterone results in equally diminished levels of cytoplasmic and nuclear oestrogen receptor in the tissues of the reproductive tract. This has been illustrated in cats and monkeys where the endometrium hypertrophies and undergoes marked secretory development while the oviduct atrophies, loses its ciliation and de-differentiates (Katzenellenbogen 1980). Progesterone has anti-oestrogen effects on a variety of target tissues. Progesterone does not appear to interfere with oestrogen uptake, oestrogen receptor activation, translocation or the amount of oestrogen receptor that can bind to nuclei, but does interfere with the replenishment of cytoplasmic oestrogen receptor in the rat uterus, causing impaired oestrogen sensitivity (Zaneveld & Chatterton 1982). The repression of the activity of the oestradiol receptor by progesterone is an example of a negative steroid effect (McCarty & McCarty 1977).

Oestrogen priming has led to an increase of progesterone receptor in the uterus, oviduct, vagina, anterior pituitary and hypothalamus of a variety of species (Katzenellenbogen 1980). Oestrogen exposure can be described as a prerequisite for the initiation of the LH surge and which is responsible for several signal production and signal response events, one of which is the increase in the progesterone receptor in the gonadotrope (Turgeon & Waring 1992).

The only rate-limiting factor in the steroid response has been described as the amount of total steroid receptor (McCarty & McCarty 1977). Receptor concentrations are, therefore, of paramount importance when it comes to steroid - receptor interactions. Although oestradiol and

progesterone show negligible binding to each other's receptors (King 1982), progesterone exerts an influence on the action of oestrogen by controlling nuclear retention of the oestrogen-receptor complex (Okulicz, Evans & Leavitt 1981). Elucidation of the relationship between these two receptors would begin with their simultaneous characterisation.

Very little is known about the interaction of progestins, oestrogens and their respective receptors in the African elephant. No information is as yet available on the cyclic changes in receptor concentrations as a function of the reproductive status of this species. Understanding the influence of gestation on receptor concentrations and more specifically, the influence of circulating progesterone and 5α-pregnane-3,20-dione (5α-DHP) on progesterone receptor levels, would provide valuable information on their biological importance. The present chapter deals with the changes in oestrogen and progesterone receptor concentrations with changes in reproductive status in the African elephant and also investigates the homogeneity in the distribution of each receptor type within the uterus. The possible influence that circulating hormones, such as oestradiol-17β, progesterone and 5α-DHP might have on receptor concentrations was also examined.

Materials and methods

Collection of blood samples

Blood was collected into heparinised glass tubes from the jugular vein of 19 of the elephant cows from which uterine tissue samples were also taken. Blood was stored on ice for transport to the laboratory where it was centrifuged and the plasma removed. All plasma samples were frozen at -20°C until the assays were done.

Equilibrium binding assays

Cytosols were prepared and assays were conducted according to the method of Potgieter *et al.* (1985) and as described in Chapter 2 (p. 11-13).

Determination of the protein concentration

The Bradford method (1976) was used to determine protein concentrations employing the Bio-Rad protein assay kit with bovine serum albumin as a standard (Chemlab, Johannesburg, South Africa).

Hormone assays

To determine the levels of progesterone in the blood, duplicate aliquots of 100 µl plasma were extracted with 4 ml of analytical grade petroleum ether (distillation range: 40-60°C from Saarchem, Krugersdorp, South Africa) as described by Van Aarde (1985). Dried residues from ether extracts were reconstituted in 100 µl phosphate buffered saline (PBS, 0.1% gelatin, pH 6.8-7) and the concentrations of circulating progesterone determined by RIA (following the procedure of Van Aarde (1985). Hormone concentrations were determined using a personal computer software programme (SecuRia 2 000) purchased from Packard Instruments (Packard, Downers Grove, Illinois).

To determine the plasma concentrations of oestradiol-17ß, duplicate aliquots of 200 µl plasma were extracted and assayed in the same manner as described for the progesterone extraction, except that 3 ml of analytical grade diethyl ether (Saarchem, Krugersdorp, South Africa) was used instead of petroleum ether, and there was no incubation period after the addition of antibody. The labelled steroid that was used in this case was [2,4,6,7-^{3}H] oestradiol with a specific activity of 75 Ci/mmol from Amersham International, Buckinghamshire, UK.

Levels of 5α-DHP were determined by first extracting plasma samples (50 µl, 100 µl and 200 µl, depending on reproductive status) with diethyl ether (peroxide-free from Rectapur, Prolabo, Fontenay S/Bois) as described by Van Aarde (1985). The dried residue was reconstituted in phosphate buffered saline (PBS, pH 7-7.5). The levels of 5α-DHP in these plasma extracts were determined using an amplified enzyme-linked immunoassay (AELIA) similar to the assay described in Hamon *et al.* (1991). Assay plates (Nunc-Immuno Plate, Nun, Denmark) were coated with 100 µl of antigen per well, sealed and kept at 4°C overnight. The plates were washed four times with 0.05% Tween-20 (Polyoxyethylene-sorbitan monolaurate, Sigma Chemical Co., St. Louis, MO), after which 50 µl of standard or plasma extracts were added to the wells. Antibody was reconstituted to 100 mg/ml, diluted in PBS (1: 400) and added (50 µl) to each well. The antibody was raised in rat and supplied by M. Hamon of the Babraham Institute, Cambridge University, UK. The plates were incubated at 4°C for 1-2 h, after which they were washed and 100 µl of conjugate (anti-rat IgG alkaline phosphatase, developed in goat, Sigma Immuno Chemicals, St. Louis, USA), diluted 1:10 000, were added to the wells. After an incubation of 20 min at 4°C the plated were washed again. NADPH, which served as a substrate for the alkaline phosphatase, was added (100 µl per well) and incubated for 10 min at room temperature. Amplifier (diaphorase and alcohol dehydrogenase) for the reaction was added to each well and colour development was terminated by the addition of 100 µl of 0.3 M H_2SO_4 after about seven min. The optical density was measured at 490 nm in a V_{max} kinetic microplate reader (Molecular Devices, Novo BioLabs, Cambridge, UK) against an assay buffer blank. The steroid concentrations were determined using software from Novoclone (Microplate Data, Version 14-06-008, Novo BioLabs, Cambridge, UK).

Validation of equilibrium binding assays

As indicated earlier (chapter 2, p. 14), three-point titration assays were performed on endometrial uterine tissue of nonpregnant, lactating, early-, mid- and late-pregnant elephant cows. Receptor concentration values (B_{max}) were calculated from the binding data by means of Scatchard plots. A comparison of data, by means of a *t* -test for dependant variables (Sokal & Rohlf 1969), between the three-point assay and the seven-point titration assay, revealed no significant differences in receptor concentrations obtained with the two methods for either the oestrogen receptor (($t = 0.51$, $n = 12$, $p > 0.05$) or the progesterone receptor (($t = -0.14$, $n = 15$, $p > 0.05$).

The receptor concentration values from Scatchard plots and those obtained from saturation curves were compared by means of a *t* -test for dependant variables (Sokal & Rohlf 1969). The receptor concentrations that were determined by using these different methods did not differ significantly for both the oestrogen ($t = 0.05$, $n = 12$, $p > 0.05$) and progesterone receptors ($t = 1.67$, $n = 12$, $p > 0.05$). The number of receptor binding sites (N or binding capacity) of a saturation curve can graphically be represented as the horizontal asymptote to the curve on a plot of the bound steroid fraction as a function of the total steroid concentration (Clark & Peck 1977). For a Scatchard plot, where the concentration of the bound divided by the concentration of the free steroid versus the concentration of the bound steroid is plotted, the intercept on the x-axis will denote the receptor binding site concentration (Scatchard 1949). The values that were obtained by the use of these two methods were similar.

The processing of duplicates of the same tissue on different days was used to determine the variability in receptor concentrations within and between assays. The inter-assay coefficients of variation for the progesterone receptor and oestrogen receptor was 16.8

% and 11.5 % respectively. The intra-assay coefficients of variation for both receptor types was < 10 %.

Validation of hormone assays

Progesterone antibody (AS 1529), supplied by R.P. .Millar of the Department of Chemical Pathology at the University of Cape Town was raised in a goat to progesterone-11-succinyll-bovine serum albumin. It was, South Africa. Cross-reactions with other steroids were: 11α-hydroxyprogesterone: 85%, 17α-hydroxyprogesterone: 12.5%, 5β-pregnane-3,20-dione: 12.5%, 5α-pregnane-3,20-dione: 3%, 5β-pregnane-3β-ol-20-one: 1.73%, 11-deoxycorticosterone: 1.1%, 5α-pregnane-3β-ol-20-one: 1%, 20α-hydroxypregn-4-ene-3-one, 20β-hydroxypregn-4-ene-3-one, 11-deoxycortisol, testosterone, androstenedione, pregnenolone, 5β-pregnane-3α,20α-diol and oestradiol-17β: < 0.7%. Intra- and inter-assay coefficients of variation were 5.8% (n = 6) and 13.5% (n = 6) respectively. The mean recovery of hormone added to stripped plasma ranged from 83% to 107% (mean ± SEM., 95 ± 6.9%, n = 3). The slope of the line relating percentage binding and serial volumes of plasma was parallel to that of the slope of the line of the standard curve ($F_{1,8}$ 0.423, $p > 0.05$). Values recorded for buffer blanks included in each assay ranged from 0 to 0.007 ng/ml and that for ether blanks from 0 to 0.006 ng/ml. The detection limit, defined as two standard deviations of the buffer blank, of the assays ranged from 0.005 to 0.03 ng/ml (mean ± SEM, 0.023 ± 0.01 ng/ml, n = 6).

Oestrogen antibody (E29BI) antibody, which was also provided by R.P.Millar form the Department of Chemical Pathology at the University of Cape Town, South Africa, was raised in a rabbit against a conjugate of oestradiol-6-(O-carboxymethyl)oxime:BSA. Cross-reactivity with other steroids is as follows: 17β-oestradiol: 100%, oestrone: 0.01%, cortisol: 0.005%, deoxycorticosterone: 0.002%, corticosterone: 0.001%, 17α-OH-pregnanolone,

androstenedione, progesterone and testosterone < 0.001%. Inter- and intra-assay coefficients of variation were 5.2% (n = 4) and 5.3% (n = 4), respectively. Serially diluted plasma yielded a curve parallel to that of the standard ($F_{1,8}$ 3.44, $p > 0.05$). The recovery of known amounts of oestradiol-17β from stripped plasma was 95.6% (n = 5). The detection limit of the assays ranged from 0.51 to 3.90 pg/ml (mean ± SEM, 3.52 ± 0.38 pg/ml, n = 9).

The cross-reactivity of the antibody of 5α-DHP was described by Hamon *et al.* (1991) and was as follows: 5α-dihydroprogesterone: 100%, progesterone: 56%, 5α-pregnane-3β-hydroxy-20-one: 21%, 5β-dihydroprogesterone: 17%, pregnenolone: 5%, 20α-dihydroprogesterone: 1.2%, 20β-dihydroprogesterone, 5α-pregnane-20α-hydroxy-3-one, 5α-pregnane-3β,20α-diol, Δ^5-pregnene-3β,20α-diol, Δ^5-pregnane-3β,20β-diol, equilin, equilenin: < 0.01%. Intra- and inter-assay coefficients of variation were 9.4% (n = 4) and 16.8% (n = 4) respectively. The mean recovery of hormone added to stripped plasma ranged from 68% to 88% (mean ± SEM, 76 ± 6.1%, n = 3). The slope of the line relating percentage binding and serial volumes of plasma was parallel to that of the slope of the line of the standard curve ($F_{1,7}$ 2.91, $p > 0.05$). Values recorded for buffer blanks included in each assay ranged from 0 to 0.07 ng/ml and that for ether blanks from 0 to 0.03 ng/ml. The detection limit of the assays ranged from 0.05 to 0.09 ng/ml (mean ± SEM, 0.3 ± 0.07 ng/ml, n = 5).

Statistics

Scatchard and saturation curves with the Rosenthal-correction for non-specific binding were drawn by means of the COMBICEPT 2000CA software program (Packard Instrument Company, Illinois, USA). Receptor concentration values are given in fmol/mg protein (mean ± SEM). The Kruskal-Wallis H and Mann-whitney U test was used to determine statistical differences in receptor concentrations between (a) the different tissue types and (b) different

reproductive stages. Correlations between oestrogen and progesterone receptor levels and the circulating levels of their natural ligands were determined by means of the Pearson product moment correlation (r) and multiple regression analysis (Sokal & Rohlf 1969). Non-linear regressions were obtained by determining correlation matrices for the power transformations in the ladder of powers (Fry 1993). Significance was taken at a 95% level and mean values are followed by one standard error of the mean. Results form Scatchard plots were subjected to least square regression analyses and only data with correlation coefficients of ≥ 0.9 were incorporated into the present chapter.

Results

The mean receptor concentrations for the different tissue and receptor types for subadult elephant cows, using seven-point Scatchards, are presented in Table 7. Values for the endometrial and myometrial tissue for both receptor types were similar ($p > 0.05$). Figure 6 illustrates receptor concentrations for all the tissues types that were assayed. Receptor concentrations for the different reproductive stages differed significantly for both the oestrogen (H (4, n = 49) = 34.75, $p < 0.05$) and the progesterone receptors (H (4, n = 49) = 32.52, $p < 0.05$). However for both receptors, tissues obtained from subadults and from nonpregnant, lactating elephant cows did not differ significantly. Values for both categories of nonpregnant animals were significantly higher than those for the nonpregnant uterus horn of pregnant animals (Tables 8 and 9). These differences are reflected in the tissue obtained from the endometrial, fundus region of the nonpregnant uterus horn. Receptor concentrations in tissue taken from the pregnant uterine horns were significantly lower than those taken from the nonpregnant uterus horn during early pregnancy for both the oestrogen (Z (U = 2) = -2.34,

TABLE 7. Receptor concentrations (mean fmol/mg protein ± SEM for no. of individual animals in parentheses) of the oestrogen and progesterone receptors determined by means of seven-point Scatchard plots in subadult female African elephants.

Receptor type	Tissue type			
	Endometrial, corpus region (3)	Endometrial, fundus region (4)	Myometrial, corpus region (3)	Myometrial, fundus region (2)
Oestrogen	585 ± 333	472 ± 153	433 ± 128	478 ± 176
Progesterone (with ^{3}H promegestone)	925 ± 602	654 ± 213	518 ± 245	339 ± 76
Progesterone (with ^{3}H progesterone)	463 ± 124	828 ± 291	755 ± 250	422 ± 43

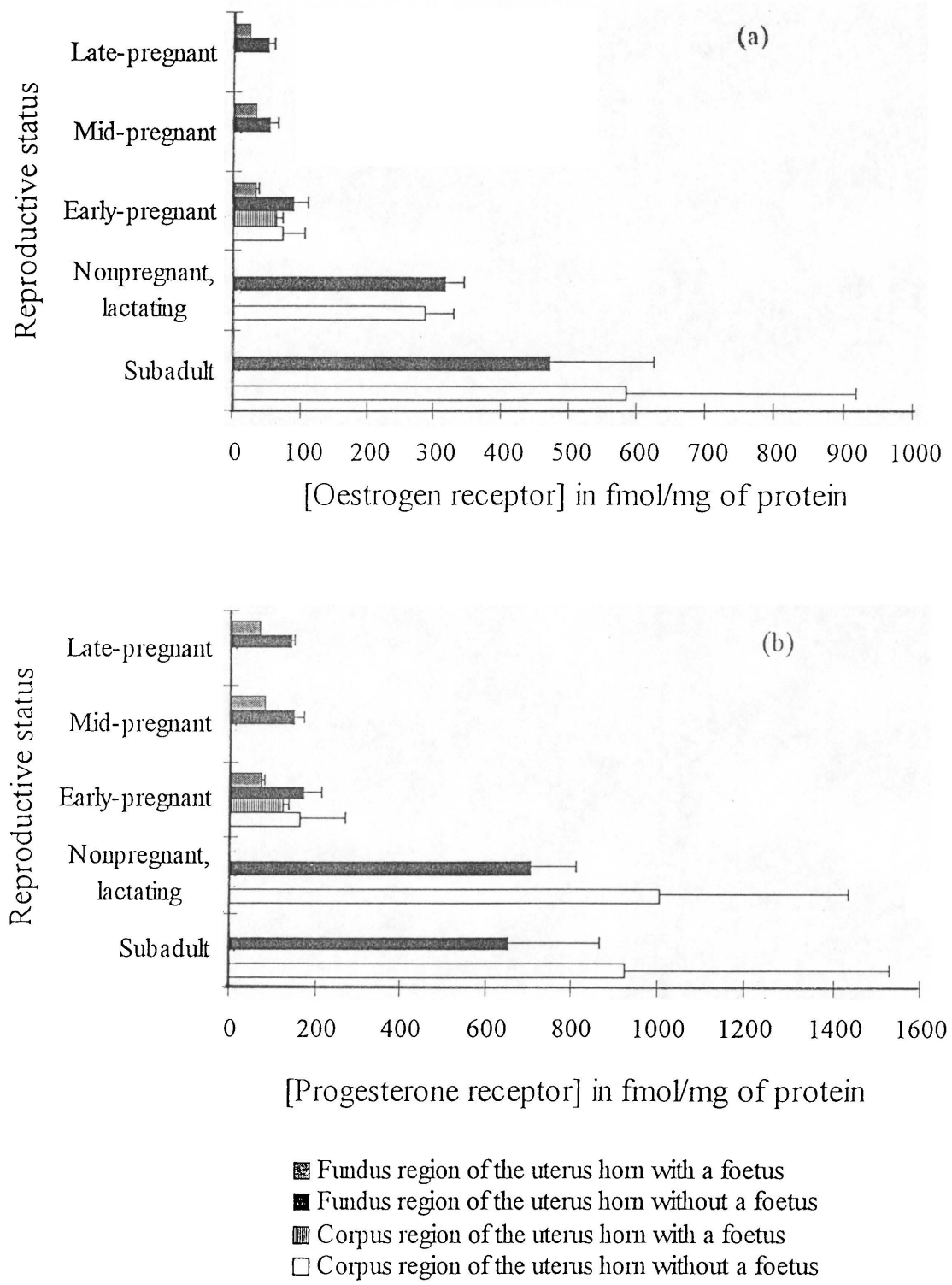

FIGURE 6. Oestrogen (a) and progesterone (b) receptor concentrations (fmol/mg protein ± SEM) for the various endometrial tissue types as a function of reproductive status.

TABLE 8. *P* values of the Mann-Whitney U test for the oestrogen receptor concentration levels with changes in the reproductive status of African elephant cows across all tissue types. Values marked with an asterisk are significant at a 95% confidence level.

	Subadult	Nonpregnant, lactating	Early-pregnant	Mid-pregnant
Nonpregnant, lactating	$Z\ (U=41) = -1.286$			
	$P = 0.198$			
Early-pregnant	$Z\ (U=6.5) = -4.157$	$Z\ (U = 0) = -4.218$		
	$P < 0.001$*	$P < 0.001$*		
Mid-pregnant	$Z\ (U = 0) = -2.913$	$Z\ (U = 0) = -2.832$	$Z\ (U = 29) = -0.284$	
	$P = 0.004$*	$P = 0.005$*	$P = 0.777$	
Late-pregnant	$Z\ (U = 0) = -3.551$	$Z\ (U = 0) = -3.418$	$Z\ (U = 42) = -0.938$	$Z\ (U = 12) = -0.382$
	$P < 0.001$*	$P = 0.001$*	$P = 0.348$	$P = 0.702$

TABLE 9. *P* values of the Mann-Whitney U test for the progesterone receptor concentration levels with changes in the reproductive status of African elephant cows across all tissue types. Values marked with an asterisk are significant at a 95% confidence level.

	Subadult	Nonpregnant, lactating	Early-pregnant	Mid-pregnant
Nonpregnant, lactating	$Z\ (U = 35) = -1.648$ $P = 0.993$			
Early-pregnant	$Z\ (U = 14) = -3.807$ $P < 0.001$*	$Z\ (U = 0) = -4.216$ $P < 0.001$*		
Mid-pregnant	$Z\ (U = 1) = -2.789$ $P = 0.005$*	$Z\ (U = 0) = -2.828$ $P = 0.005$*	$Z\ (U = 28) = -0.378$ $P = 0.705$	
Late-pregnant	$Z\ (U = 3) = -3.296$ $P = 0.001$*	$Z\ (U = 0) = -3.416$ $P = 0.001$*	$Z\ (U = 52) = -0.301$ $P = 0.764$	$Z\ (U = 12) = -0.378$ $P = 0.705$

$p < 0.05$) and the progesterone receptors (Z ($U = 3$) = -2.19, $p < 0.05$). This was also the case during late pregnancy for the oestrogen (Z ($U = 0$) = -2.14, $p < 0.05$) and progesterone receptors (Z ($U = 0$) = -2.12, $p < 0.05$)). Although both oestrogen and progesterone receptor concentrations were lower in the pregnant uterine horn of the mid-pregnant animals, the difference was not significant when compared to the nonpregnant uterine horn's receptor concentrations.

Oestrogen and progesterone receptor concentrations were significantly linearly related ($r = 0.75$, $n = 49$, $p < 0.05$; Fig. 7). Foetal age correlated positively with circulating concentrations of 5α-DHP ($r = 0.70$, $n = 13$, $p < 0.05$) but were not significantly correlated to the concentrations of circulating progesterone. Bivariate scatterplots (Fig. 8a and b) illustrate the negative relationship that exists between the progesterone receptor concentration with circulating 5α-DHP ($r = -0.93$, $n = 12$, $p < 0.05$; Fig. 8a) and progesterone ($r = -0.45$, $n = 12$, $p < 0.05$; Fig. 8b) respectively. Although the slope of the regression lines (b) of progesterone receptor concentrations versus progesterone concentrations (-329.28) in Fig. 8b differs from that of progesterone receptor concentrations versus 5α-DHP concentrations (-342.43) in Fig. 8a, the difference can be attributed to sampling error ($t_{20} = 0.051$, $p > 0.05$).

Multiple regression analysis ($F_{2,9} = 30.76$, $R = 0.93$, $n = 12$, $p < 0.05$) support the strong relationships between the variables. The magnitude of the standardised regression coefficient (*beta*) for circulating 5α-DHP ($t_9 = -6.86$, *beta* = -1.004, $p < 0.05$) compared with that of progesterone ($t_9 = 0.89$, *beta* = 0.131, $p > 0.05$) illustrated the strong relative contribution that circulating 5α-DHP made to the changes of the progesterone receptor concentrations. In this analysis the relationship between circulating 5α-DHP and progesterone receptor concentrations was also significantly negative. The contribution of circulating 5α-

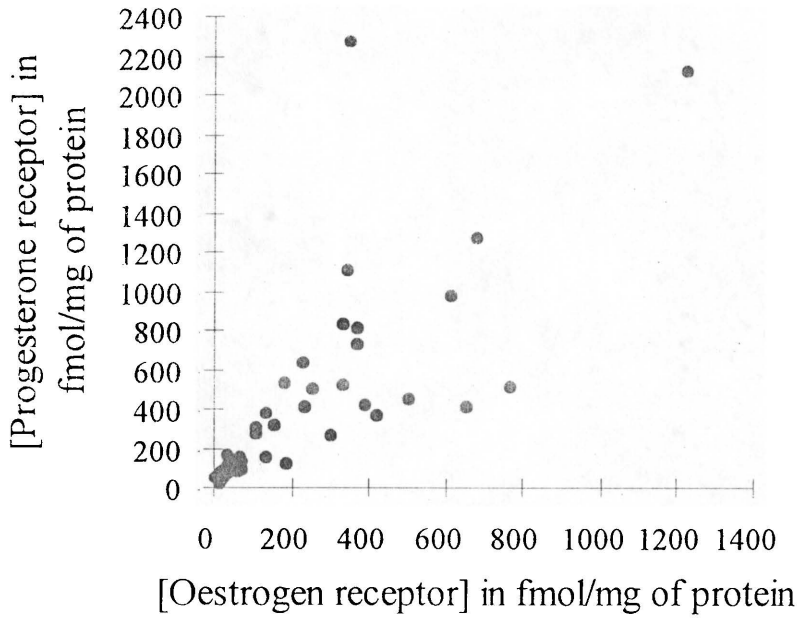

FIGURE 7. The relationship between progesterone and oestrogen receptor concentrations of African elephant cows ($n = 49$, $r = 0.75$, $p < 0.05$).

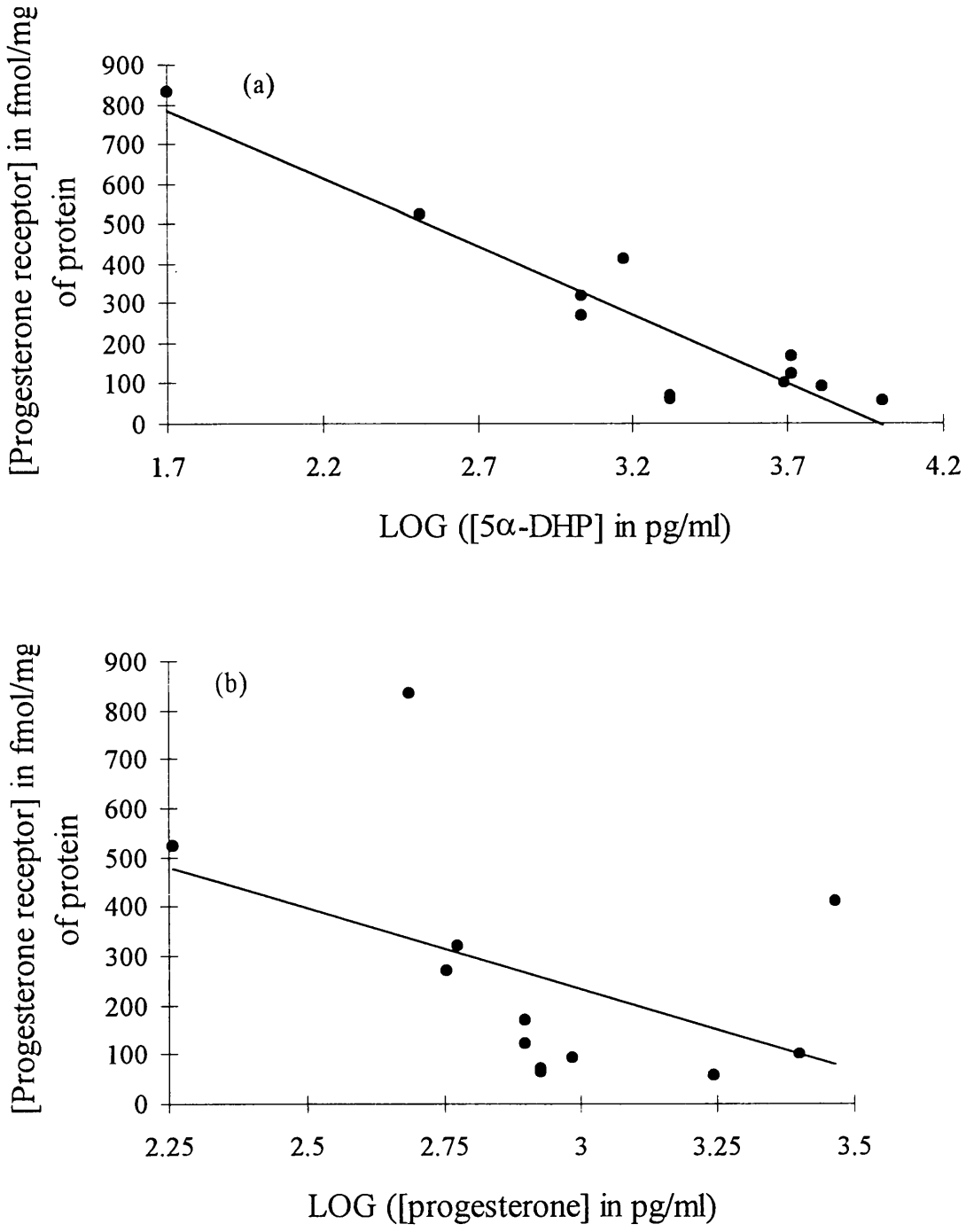

FIGURE 8. The relationship between uterine progesterone receptor concentrations (in fmol/mg of protein) and plasma concentrations (pg/ml) of (a) 5α-DHP and (b) progesterone.

DHP was also expressed in the partial correlation values (r) of 5α-DHP (-0.92) compared to those of circulating progesterone (0.29). There was no relationship between circulating oestradiol-17β and the oestrogen or progesterone receptor.

Discussion

Receptor concentrations may be the key factor in the modulation of biological responses to steroid hormones (Jänne *et al.* 1978). Due to the different functions of progesterone and oestradiol-17β during the oestrous cycles and pregnancy, receptor concentrations often correspond to different reproductive states. The receptor concentrations of both receptor types were significantly higher in nonpregnant than in pregnant animals. The variation in receptor concentrations were also greatest in the nonpregnant animals as they could be in any stage of their 15 week oestrus cycle (Plotka *et al.* 1988). Receptor concentrations are known to change in a dynamic manner during the oestrus cycle (Wathes & Hamon 1993) which would account for large variation between individuals at different stages of their oestrus cycle.

The uterine tissues from non-pregnant, lactating elephant cows had lower concentrations of the oestrogen receptor than tissues from the subadult animals. Although the differences were not significant, the results differ from the findings of previous workers such as Leung (1978) who found that prolactin was a positive up-regulatory factor of the oestrogen receptor in mammary tissues. Muldoon (1978; 1981) came to similar conclusions. It is not known whether prolactin displays the same influence on uterine oestrogen receptors. The higher levels of the progesterone receptors in the nonpregnant, lactating

elephants as opposed to the subadult elephants may not be of biological importance, but merely a reflection of the large variation within these groups of animals.

Padayachi *et al.* (1987) reported that in humans the concentrations of receptors decreased as a function of gestational stage. At full term pregnancy human uterine tissues had no detectable receptors, however, endogenous hormones were extremely elevated. Perrotapplanant, Deng, Fernandez, Lelaidier, Meduri & Bouchard (1994) found that in the human uterus, significant progesterone down regulation of the progesterone receptor occurred during pregnancy in the epithelial cells of the endometrium. In the rat uterus the progesterone-binding sites in myometrial cytosol increased at the beginning of pregnancy but decreased to a minimum during the last week of gestation (Liao 1975). This pattern of decreasing progesterone receptor concentrations with advancing pregnancy also occurs in the guinea pig, where receptor concentrations in the uteri of early pregnant animals where similar to those of non-pregnant animals, but then decreased to a low concentration at the time of implantation (Milgrom *et al.* 1972; Vu Hai, Logeat, Warembourg & Milgrom 1977). The lack or low concentration of oestrogen receptor staining in the various cell types of the endometrium during gestation also resulted from the down-regulation of the oestrogen receptor mRNA or protein concentrations by steroid hormones (Perrotapplanant *et al.* 1994).

The present analyses of tissue from both pregnant and nonpregnant uterine horns of elephants in early-, mid- and late stages of pregnancy, provides an opportunity to compare between tissue in close contact with the conceptus and tissue not in direct contact with the progesterone-secreting-placenta. Similarly Padayachi *et al.* (1987) could collect decidua from ectopic pregnancies as an ideal model for tissue 'uncontaminated' by conceptus. A significant difference in receptor concentrations existed between the pregnant and nonpregnant horns of early and late pregnant elephant cows for both the oestrogen and progesterone receptors.

Although the pregnant uterine horns of the mid-pregnant animals had lower receptor concentrations than tissue collected from the nonpregnant uterine horns, the small sample size (n = 2) probably accounts for the lack of statistical significance between the receptor concentrations in the different uterine compartments of these animals. The high concentration of endogenous hormones circulating in the pregnant horn of the uterus, which is produced by the placenta probably accounts for the low concentrations of receptors. These high concentrations of hormones can thus either lead to the down regulation of their appropriate receptors or saturate the receptors and make detection thereof impossible with equilibrium binding assays. High serum progesterone concentrations could result in the saturation of available cytosolic receptors leading to complete nuclear accumulation (Wiehle 1983). Padayachi *et al.* (1987) also found that full term pregnancy tissue had no detectable receptor concentrations when using binding assays similar to those used in the present study and the removal of endogenous steroids by pre-assay incubation of the cytosol with dextran-coated charcoal did not improve the detection of the receptors in these tissues. MacLaughlin & Richardson (1976) also reported a failure to detect progesterone receptors in the dicidua of early pregnancy.

When comparing the receptor concentrations in the nonpregnant uterus horn with those of the pregnant uterus horn of the elephant, it appears as though approximately 50% of the pregnant horn's receptors are being down-regulated by the steroids produced by the conceptus. The use of alternative methods, such as enzyme immunoassay (EIA), could clarify whether the observed results are a biological reality or merely a reflection of methodological artefacts. The main advantage of an EIA method resides in the fact that monoclonal antibodies bind to immunological sites on the receptors allowing one to quantify the receptors regardless

of whether the receptors are occupied with endogenous hormones or not (Jarque, Lluch, Vizcarra, Munoz, Alberola & Garcia-conde 1994).

Horwitz & McGuire (1978) suggested that, in the human mammary carcinoma tissue cell line MCF-7, the oestrogen induced the synthesis of the progesterone receptor via the oestrogen receptor. Leavitt, Chen & Allen (1977) came to a similar conclusion in ovariectomised hamsters. The oestrogen receptor concentrations measured in the elephant in this study were, on average, lower than the progesterone receptor concentrations and were positively correlated with the concentrations of progesterone receptors. These results therefore support those of Syrjäla, Kontula, Jänne, Kauppila & Vidko (1975) who also found a significant positive correlation between the concentrations of oestrogen and progesterone receptors in the same endometrial cytosol.

Oestrogen priming leads to an increase of progesterone receptors in the uterus, oviduct, vagina, anterior pituitary and hypothalamus of a variety of mammals (Katzenellenbogen 1980). Oestrogen exposure is responsible for several signal transduction events, one of which is the increase in progesterone receptors in the gonadotrope (Turgeon & Waring 1992). The effective biological activity of progestational compounds are often dependent on prior exposure to oestrogen. Nyholm, Nielsen, Lyndrup & Thorpe (1993) found that the tumour biochemical progesterone receptor content correlated positively with free oestradiol serum concentrations. Kontula (1975) also reported a significant positive relationship between circulating oestradiol and cytosol progestin receptor concentrations in human myometrium. The endometrial progesterone receptor concentrations in the elephant however, did not significantly correlate with serum concentrations of oestradiol-17β. This suggests that oestradiol-17β is not responsible for the up-regulation of progestin receptors. Hodges *et al.* (1983) however, reported that oestradiol-17β sulphate was

probably the most abundant circulating oestrogen during pregnancy in the African elephant and that there was no distinction between the non-pregnant and pregnant animals when the unconjugated hormone, oestradiol-17β, was assayed. As the concentrations of oestradiol-17β that were measured were very low and exhibited considerable individual variation, the expected pattern may have emerged if the conjugated form of this hormone was measured.

The progesterone receptor concentrations were negatively correlated to the concentrations of circulating 5α-DHP. The foetal age was positively correlated to the plasma concentrations of 5α-DHP, while the circulating concentrations of progesterone could not be significantly correlated to any of the above. This may indicate that the 5α-reduced progesterone metabolite probably exerts the greatest influence on the receptor concentrations in the African elephant. The importance of this circulating steroid in the African elephant has been discussed previously (Hodges *et al.* 1994; Heistermann *et al.* 1994). The small sample size and the considerable cross-reactivity of the antibody of 5α-DHP for progesterone (56%) could be responsible for the lack of significant differences between the slopes of the regression lines in Figure 8. The importance of the influence of both progesterone and 5α-DHP on the down-regulation of receptor concentrations can therefore not be disregarded.

The inverse relationship between progestins and progesterone receptor concentrations has already been reported by Padayachi *et al.* (1987) who found that the total steroid receptor concentration (cytosolic and nuclear) decreased with advancing gestation and was inversely related to the increasing endogenous progesterone concentrations. Perrotapplanant *et al.* (1994) observed significant progesterone receptor down-regulation by progesterone during pregnancy, only in epithelial cells of the human endometrium. Kontula (1975) also found an inverse correlation between the progesterone concentration in the human myometrium and the

concentration of progesterone receptors. Lin, Billiar & Little (1972) found that the loss of cytosol receptor after progestin administration exceeded the amount that could be accounted for by nuclear translocation. Progestin receptor depletion is known to last for days in contrast to the oestrogen receptor which is replenished within hours (Freifeld, Feil & Bardin 1974; Milgrom *et al.* 1973). Progesterone, and in this case probably 5α-DHP, is known to promote the movement of the cytosol receptor into the nuclei of the target cell and also to regulate the degradation of the cytoplasmic receptor (Jänne *et al.* 1978).

Conclusion

Progesterone and oestrogen receptors were positively correlated. Down-regulation of progesterone receptors occurs with progressing gestation in the African elephant. This down-regulation appears to be linked to increasing concentrations of 5α-DHP in the plasma of pregnant animals. Progesterone and oestradiol-17β do not seem to be related to receptor concentrations in the uterus. However, the low concentrations of receptors that were detected in the pregnant uterine horns could be the result of a negative steroid effect that the conceptus exerts on the receptors or may reflect a weakness in the applied methodology.

CHAPTER 5

SYNTHESIS

The conservation of the African elephant, *Loxodonta africana,* presents a challenge, not only due to the ethical issues that are involved, but also because of the dearth of information on which management decisions could be based. An example involves the possible used of contraceptives as an alternative to the culling of local over-abundant populations. The application of any contraceptive agent would necessitate a sound knowledge of the basic reproductive physiology of the African elephant.

In the present study high affinity, saturable oestrogen and progesterone receptors were found in the uterine tissue of the African elephant. Both of these receptor types had nanomolar dissociation constants for their appropriate ligands and were highly specific. In an attempt to adhere to some of the requirements proposed by Lincoln (1992) for the ideal contraceptive in humans, an effort needs to be made to address the paucity of information before the application of any contraceptive agent in the African elephant. Lincoln (1992) suggested that:

- the contraceptive must express an absolute specificity of action, preferably intercepting a discrete mechanism expressed before fertilisation.

- an efficiency of 100% must be provided with the potential to commence use at any time in the cycle.

- the use of a contraceptive must be appropriate for continuous use over many years.

- there must be immediate reversibility without the need of expensive therapeutic measures and without compromising reproductive potential as a necessary requirement.

- the contraceptive must preferentially have a very low annual cost in terms of manufacture, application and the duration of action.

- endogenous levels of sex steroids should be maintained within a safe prophylactic window.

Exogenous progesterone causes the hypothalamic-pituitary gonadotrophin axis to be disrupted and blunts the midcycle FSH and LH surge, resulting in the inhibition of ovulation. The application of anti-progestins could thus prevent conception by creating a uterine environment hostile to implantation (Cooke *et al.* 1988). Anti-progesterones are described as compounds which inhibit the synthesis of progesterone or antagonise its biological action. Alternatives to agent which induce abortion would be the use of compounds which inhibit 3-ß-ol steroid dehydrogenase (3ß SDH) which converts pregnenolone to progesterone, and which will inhibit the production of steroids that are in excess (Baird 1993). The anti-progestin RU 486 (which has a high affinity for progesterone receptor without having agonistic actions and competitively inhibits the binding of progesterone to receptors (Horwitz 1985)) has recently been hailed as a contraceptive for female African elephants (Short 1992). If administered early in pregnancy, RU 486, increases oestrogen receptors in human decidua as the anti-oestrogenic effects of progesterone are blocked (Perrotapplanat *et al.* 1994). RU 486 action is exerted predominantly at a post-DNA-binding step (Beck, Estes, Bona, Murocacho, Nordeen & Edwards 1993).

In the African elephant RU 486 has a very low affinity for the progesterone receptor, suggesting that the use of this agent to curtail individual reproductive output would not be feasible. Norethindrone did, however, bind nearly three times as strongly as progesterone and may therefore have some potential as a contraceptive agent in the African elephant. Norethindrone and norethindrone acetate are used widely to prevent pregnancy in women (Taitel *et al.* 1995). Levonorgestrel also had a relatively high binding affinity for the progesterone receptor. Although

the comparative high binding affinity of these steroids offers potential as contraceptive agents, this would have to be correlated to a biological response *in vivo,* as affinity is not necessarily a fundamental criteria in assessing the efficiency of anti-hormonal compounds. Hormones present at physiological levels of interaction are generally receptor specific, but pharmacological doses can have unexpected biological results (Katzenellenbogen 1980).

Recent research on endocrine correlates of reproduction in the African elephant centred on the production of 5α-reduced metabolites and the concentration at which they circulate in the African elephant. As these 5α-reduced metabolites and not progesterone, as in most other mammalian species, are the primary luteal steroids formed during pregnancy (Heistermann *et al.* 1994; Hodges *et al.* 1994), the importance of investigating the interaction of these and related hormones with their appropriate receptors becomes clear. 5α-Pregnane-3,20-dione and 5α-pregnane-3α-ol-20-one, were found to have comparatively high binding affinities for the progesterone receptor. Given the high concentration at which these metabolites circulate in relation to progesterone, this high affinity suggests biological relevance (Hodges *et al.* (*in prep*)). Circulating levels of 5α-DHP were correlated to a decrease in the uterine progesterone receptor concentrations with progressing pregnancy. In contrast, no relationship could be found between circulating progesterone levels and this receptor type, and thus 5α-DHP is responsible for the down regulation of the progesterone receptor. This metabolite may, therefore, be responsible for maintenance of pregnancy in the African elephant. In this regard, the interaction of 5α-DHP and the plasma binding proteins needs further investigation. If blood-binding-proteins have a stronger affinity for 5α-DHP than for progesterone, unconverted progesterone could interact freely with the receptor and despite its low concentrations, still be responsible for the maintenance of pregnancy in

the African elephant. It would also be of importance to determine the metabolic clearance rate of this steroid since low metabolic clearance rates could ensure sustained concentrations in the blood and thereby increase efficiency dramatically (Sutherland & Jordan 1981, Balieu 1989). Although more information is needed to develop a contraceptive agent for the African elephant, the present study has illustrated the importance of first screening potential contraceptives to determine their binding affinity for the uterine progesterone receptor.

SUMMARY

The characterisation of the uterine oestrogen and progesterone receptors in the African elephant provides information on the binding affinity, binding capacity and specificity of these receptor types. This information contributes towards our understanding of the reproductive physiology of this species. This has proved to be important in view of the possible application of steroidal contraceptives for the African elephant.

The uterine progesterone and oestrogen receptors of the African elephant both had high binding affinities for their specific ligands. Scatchard analysis suggested nanomolar dissociation constants for both progesterone and oestrogen receptors. The endometrial and myometrial receptors appeared to be similar since the receptors in both tissue types displayed the same physio-chemical properties in terms of binding affinity and specificity. The myometrial receptors appeared to be more susceptible to degradation than the endometrial receptors. This was probably due to the time delay during the removal and preservation of this tissue type.

Pregnancy in the African elephant is characterised by a decrease in the progesterone and oestrogen receptor concentrations with advancing pregnancy. Both receptor types were positively correlated to each other. The decrease in the progesterone receptor concentrations could be linked to the increase in the concentration of 5α-DHP in the plasma. As circulating levels of progesterone and oestradiol-17β did not correlate to the concentrations of the progesterone and oestrogen receptors respectively, these hormones may not be responsible for the down-regulation of their specific receptors. The pregnant uterus horn of a particular animal had a lower concentration of receptors when compared to the nonpregnant uterus horn. This could either be due to the saturation of the

receptors by hormones secreted by the developing conceptus and the consequent difficulty in detecting saturated receptors with equilibrium analysis, or due to the down-regulation of receptors by these secreted hormones. The oestrogen receptor had a decreased affinity for its ligand with advancing gestation while the progesterone receptor maintained the same binding affinity for its ligand throughout pregnancy.

Both the oestrogen and progesterone receptors exhibited a high level of specificity. Although 5α-DHP did not displace progesterone completely from the receptor, this 5α-reduced metabolite of progesterone did bind relatively strongly to the receptor (43%) when compared to the other progestins. The only synthetic progestins that could show possible potential as contraceptive agents, were norethindrone and levonorgestrel. RU 486, had a very low affinity for the progesterone receptor. Only oestrogens interacted with the oestrogen receptor.

The possible down-regulation of the progesterone receptor by circulating 5α-DHP and the affinity of this metabolite for the uterine progesterone receptor, substantiates the possible biological importance of this steroid in the maintenance of pregnancy in the African elephant. The importance of this steroid and its binding to the plasma binding proteins, needs further investigation. The progesterone receptor in the target tissue of the African elephant will have to be screened for more contraceptive agents with higher relative binding affinities, before the application thereof *in vivo*.

OPSOMMING

Die karakterisering van die estrogeen en progesteroon reseptore in die uterus van die Afrika olifant verskaf inligting oor die bindingsaffiniteit, bindingskapasiteit en spesifisiteit van dié reseptor tipes. Hierdie inligting dra by tot ons begrip oor die voortplantingsfisiologie van die olifant en is belangrik vir die moontlike aanwending van geboortebeperkingsmiddels.

Beide progesteroon en estrogeen reseptore in die uterus van die Afrika olifant het `n hoë bindingsaffiniteit vir van hul onderskeie ligande gehad. Duur die gebruik van Scatchard analiese kon aangetoon word dat beide reseptors dissosiasie konstantes in die nanomolare gebied gehad het. Beide reseptor tipes het nanomolare dissosiasie konstantes, wat deur middel van Scatchard analiese vasgestel is, geopenbaar. Die endometriële en miometriële reseptore is blykbaar homogeen en het dieselfde fisies-chemiese eienskappe (bindingsaffiniteit en spesifisiteit). Die miometriële reseptore was egter meer sensitief vir degradasie wat waarskynlik aan die vertraging in die versameling en bewaring van die weefsel toegeskryf kan word.

Dragtigheid in die Afrika olifant word gekenmerk deur `n afname in die progesteroon en oestrogeen reseptor konsentrasies soos dragtigheid vorder. Daar was `n positiewe korrelasie tussen biede reseptore. Die afname in die progesteroon reseptor konsentrasies hou verband met die toename in die konsentrasie van sikulerende 5α-DHP in die bloed. Aangesien die plasma konsentrasies van estradiol-17β en progesteroon nie hierdie inverse verwantsakp met hul onderskeie reseptore geopenbaar het nie, kan hierdie hormone nie verantwoordelik wees vir die afnemende konsentrasies van hul onderskeie reseptore nie. Die uterus horing wat die fetus bevat het, het `n baie laer konsentrasie reseptore gehad as die uterus horing daar sonder. Dit kan moontlik toegeskryf word

aan die fetus wat hormone sekreteer wat die omliggende reseptore versadig en tot gevolg het dat reseptor konsentrasies nie met behulp van titrasie analise bepaal kon word nie. Verder kan hierdie verskynsel ook moontlik toegeskryf word aan die hormone wat wel reseptor konsentrasies afreguleer. Die oestrogeen reseptor se bindingsaffiniteit het afgeneem met toenemende dragtigheid terwyl die bindingsaffiniteit van die progesteroon reseptor nie verander het met dragtigheid nie.

Beide reseptore was baie spesifiek teen opsigte van hul onderskeie ligande. Alhoewel 5α-DHP nie sterker as progesteroon aan die progesteroon reseptor gebind het nie, het hierdie hormoon wel met geweegsame affiniteit aan die progesteroon reseptor gebind in vergelyking met ander progestiene. Die enigste sintetiese progestien wat moontlik as geboortebeperkingsmiddels aangewend kan word, blyk norethindrone en levonorgestrel te wees. RU 486, wat algemeen gebruik word om aborsie in ander soogdiere te induseer, het `n baie lae affiniteitt vir die progesteroon reseptor gehad. Die oestrogeen reseptor het slegs estrogene gebind.

Na aanleiding van 5α-DHP se affiniteit vir die progesteroon reseptor en die inverse verwantskap tussen die sirkulerende hormoon en reseptor konsentrasies, speel hierdie hormoon mootlik `n belangrike biologiese rol in die handhawing van dragtigheid in die Afrika olifant. Die belangrikheid van die interaksie tussen plasmabindingsproteiene en 5α-DHP, moet nog ondersoek word. Die progesteroon reseptor se bindingsaffiniteit vir ander geboortebeperkingsmiddels in die teikenweefsel van die Afrika olifant, sal verder ondersoek moet word voor enige middels *in vivo* toegepas kan word.

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