EXPRESSION OF ESTROGEN RECEPTORS ALPHA AND BETA, AROMATASE, STEROID SULFATASE AND ESTROGEN SULFOTRANSFERASE IN TESTES OF IMMATURE AND MATURE BOARS

HENRY M'IKIUGU MUTEMBEI

INAUGURAL DISSERTATION
submitted to the Faculty of
Veterinary Medicine in partial
fulfillment of the requirements
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by

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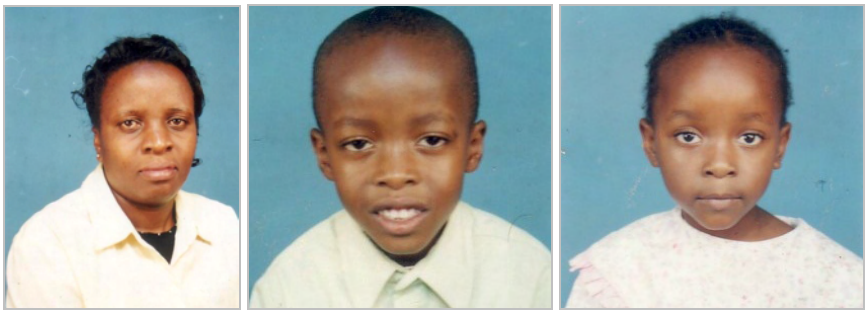
Mutembei Henry M'Ikiugu

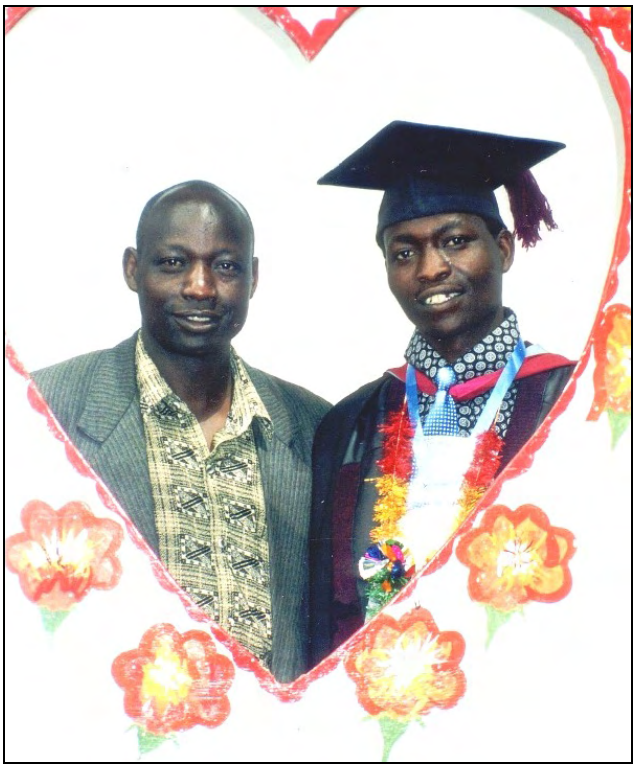
TO MY WIFE MARY, AND MY KIDS, RON AND SHEILA



You all continuously showed me great love, patience, tolerance and understanding during the entire period of our separation for this study. While each of these will be cherished separately, they will be carried in my heart forever.

&

TO MY LATE BROTHER FRANCIS



Francis tirelessly stood by us since our daddy's death in January 1984. He continuously showed us kindness and compassion until his untimely demise in July 2005. May his soul rest in peace and since history reminds us how little we know of those who came before us, I will try to gather and preserve his legacy.

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LIST OF IMPORTANT ABBREVIATIONS

3βHSD	3β-hydroxysteroid dehydrogenase
ABP	Androgen binding protein
AMH	Antimüllerian hormone
AP1	Activated protein-1
ArKO	Aromatase knockout
BCIP	5-bromo-4-chloro-3-indolyl phosphate
cAMP	Cyclic adenine monophosphate
cDNA	Complementary DNA
CRE	cAMP responsive elements
CREB	cAMP response element binding protein
CTE	COOH-terminal extension
CYP	Cytochrome
dATP	2’-deoxyadenosine 5’-triphosphate
DBD	DNA binding domain
dCTP	2’-deoxycytidine 5’-triphosphate
DEPEC-water	Deionised double distilled water
dGTP	2'-deoxyguanosine 5'-triphosphate
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dTTP	2’-deoxythymidine 5’-triphosphate
dUTP	2'-deoxyuridine-5'-triphosphate
E1	Estrone
E2	Estradiol
EDS	Ethane dimethansulfonate
EM	Electron microscope
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERαKO	Estrogen receptor alpha knockout
ERβKO	Estrogen receptor β knockout
ERαERβKO	Estrogen alpha and beta knockout male
ERE	Estrogen response element
ERM	Ets related molecule
ERs	Estrogen receptor proteins
ES	Elongated spermatids
ES1	Estrone sulfate
EST	Estrogen sulfotransferase
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
HAT	Histone acetyltransferase
HDAC	Histone deacylase complex
HMG	High mobility group
hpg	Hypogonadal
IHC	Immunohistochemistry

ISH	*In situ* hybridisation
L	Leptotenes
LBD	Ligand binding domain
LDL	Low density lipoprotein
LH	Luteinizing hormone
MAP	Mitogen activated protein
MIS	Müllerian inhibiting substance
mRNA	Messenger ribonucleic acid
NADPH	Nicotamide adenine dinucleotide phosphate hydrogen
NBT	Nitroblue tetrazolium
NCad	Cell adhesion molecule neural cadherin
P	Pachytenes
PBS	Phosphate buffered saline
PBSM	Phosphate buffered saline with magnesium
P450 arom	Cytochrome P450 aromatase
PGs	Primodial germ cells
PK	Protein kinase
PL	Preleptotenes
RB	Residue body
RER	Rough endoplasmic reticulum
RS	Round spermatids
RT-PCR	Reverse transcription polymerase chain reaction
SBP	Sex steroid binding protein
SCC	Side chain cleavage
SER	Smooth endoplasmic reticulum
SERMs	Selective estrogen receptor modulators
SF-1	Steroidogenic factor 1
SG	Spermatogonia
SH	Steroid hormone
SHBG	Sex steroid hormone binding globulin
Sox 9	SRY containing HMG box family of transcription factors
SRE	Steroid response element
SRY	Sex determining region on Y chromosome
SSC	Standard saline citrate
ST	Seminiferous tubule
St1	Spermatid 1
StS	Steroid sulfatase
T	Testosterone
TAF	Transcription associated factor
TATA	Transcription initiation DNA sequence
TESAP	3-triethoxysilyl-propylamine
TGF	Transforming growth factor
USP	Upstream promoter
WT-1	Wilm’s tumour suppressor gene

1. INTRODUCTION

In recent years ample evidence has been accumulated that estrogens are essential for normal male fertility and that they likewise may also interfere with fertility. Thus a series of male reproductive disturbances, such as cryptorchidism, epididymal defects, impaired fertility and an increased incidence of testicular cancer (McLachlan et al., 1975a, b, Gill et al., 1979, Jensen et al., 1995; Raman-Wilms et al., 1995, Saunders et al., 1997, Cooper and Kavlock 1997, Toppari and Skakkebaek 1998, Toppari et al., 1996) has been reported to occur after administration of estrogens and xenoestrogens during fetal and neonatal development.

The opinion that estrogens are essential for male fertility, however, was delayed until a knockout mouse (KO) (Lubahn et al., 1993, Korach 1994, Eddy et al., 1996) and rat (Hess et al., 1997) lacking a functional estrogen receptor α (ERα) gene (ERαKO) was developed. While the infertility observed in the ERαKO mouse for the first time demonstrated that estrogens are required for male fertility (Lubahn et al., 1993), it was the observations in the ERαKO rat that revealed that the infertility is primarily due to a defect in efferent ductule development and function (Hess et al., 1997, Lee et al., 2000). Mice lacking a functional aromatase gene (ArKO) are also infertile, probably due to a specific defect in germ cell development (Robertson et al., 1999). Thus although estrogen receptor β (ERβ) knockout mice (ERβKO) seem to have a normal phenotype, observations in ERαKO, in ERαβKO (Couse and Korach 1999, Dupont et al., 2000), in ArKO mice (Honda et al., 1998, Robertson et al., 1999) and in aromatase-inhibited Leydig cells (Kmicikiewicz et al., 1997), provide compelling evidence for a likelihood of estrogens being involved in spermatogenesis.

Apart from being important regulators of epididymal development and function (Toney and Danzo 1989; Dhar et al., 1998) estrogens also synergize with 5α-reduced androgens to induce prostate gland growth as observed in the dog (Walsh and Wilson 1976, Markham and Coffey 1994, Suzuki et al., 1994).

A particular situation seems to exist in the boar. The boar is remarkable for its high levels of testicular estrogen secretion (Velle 1958, Reaside 1965, Claus and Hoffmann 1980, Reaside et al., 1993, Rostalski et al., 2000, Rostalski 2005) as well as for its exceedingly large volume of semen (200–500 ml) and output of spermatozoa (30-60 $x10^9$/ejaculate) (McKenzie et al., 1938, Frankenhuis et al., 1982). In respect to biological function of estrogens in the boar, earlier work in castrated animals revealed that estrogens

act synergistically with testosterone on the accessory sex glands and libido development (Joshi and Reaside 1973, Booth 1980). Claus et al. (1987) developed the hypothesis that estrogens in the seminal fluid of the boars might play a role in myometrial contraction and in synchronising induction of ovulation in females during copulation. However these provided no further hints on a likely functional role in the boar himself.

Metabolism of estrogen in the body occurs mainly in the liver. However, evidence has accumulated that local metabolism in target organs may contribute to the overall response to the hormone (Zhu and Conney 1998). It was hypothesised that the biological effects of an estrogen depends on the profile of multiple metabolites formed, and the biological activities of each of the metabolites in the target cells (Reaside et al., 1999). In the boar concentrations of estrone sulfate (ES1) exceeds by far those of free estrone (E1) (Reaside 1966, Reaside et al., 1998, Rostalski et al., 2000, Rostalski 2005) in testicular venous blood and high activities of steroid sulfatase (StS) and a lower activity of estrogen sulfotransferase (EST) was observed in testicular homogenates (Rostalski et al., 2000, Rostalski 2005). Although their localization was not known, it may be postulated from these data that both StS and EST seem important factors that regulate local availability of biologically active estrogens in testis of the boar.

Based on this background information, the hypothesis of the present study was that estrogens may have functional roles in the testis of the boar like in other species. In order to provide evidence, the expression of ERα and ERβ was tested in conjunction with the identification of those cells expressing aromatase, StS and EST.

2. LITERATURE REVIEW

2.1 Anatomy and histology of the boar testis

2.1.1 Location and gross morphology of the boar testis

Mature boar testis measure approximately 10 cm in length and 5 to 6.5 cm in width, and weigh about 400g (Amman, 1970, Dym and Cavicchia 1978). They descend during foetal life from the abdominal cavity to lie extra-abdominally in the scrotum that is located in the sub anal region of perineum.

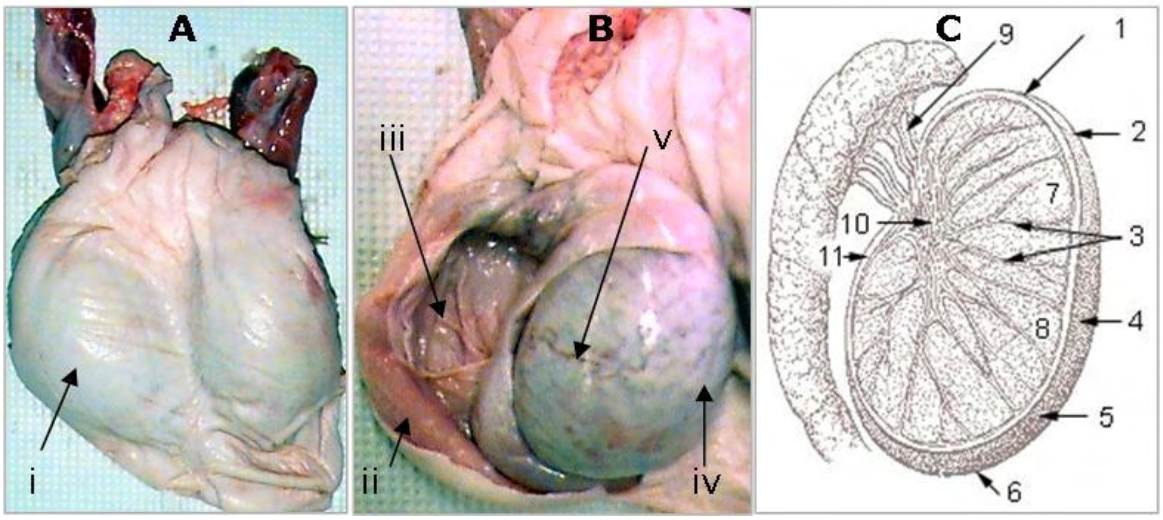

Fig. 1: Morphology of the boar testis. A - intact testes covered by the scrotum, B - intact testis with opened scrotum, C - sketched cross section of a testis; i - scrotum, ii – tunica dartos, iii - tunica vaginalis (parietal layer), iv - tunica vaginalis (visceral layer), v - blood vessel, 1 - head or upper pole of testis, 2 - tunica albuginea, 3 - testicular septa, 4 - free margin, 5 - lateral margin, 6 - tail or lower pole of testis, 7 - testicular lobules, 8 - parenchyma of testis, 9 - efferent ductules, 10 - mediastinum testis, 11 -posterior margin (Animal Science; http://www.ansi.okstate.edu)

The scrotum (Fig.1A, i) is composed of an outer layer of thick skin with numerous large sweat and sebaceous glands. Its inner layer is lined by the tunica dartos (Fig.1B; ii) consisting of fibroelastic tissue and smooth muscle interspersed with connective tissue. The tunica dartos divides the scrotum into two pouches and is attached to the tunica vaginalis at the bottom of each pouch. The external spermatic fascia from the external oblique muscle, the cremaster muscle from the internal oblique muscle and the internal spermatic fascia extend between the tunica dartos and vaginalis. The tunica vaginalis lines the process vaginalis with an outer parietal layer (Fig.1B; iii) and a visceral layer contacting the surface of each testis (Fig.1B; iv).

The tunica albuginea (Fig.1C; 2) is a tough capsule that encloses each testis and consists mostly of dense irregular fibrous connective tissue, predominantly of collagen- and a few elastic fibres. The capsule is probably involved in maintaining fluid pressure inside the testis or in moving fluid and sperm into the epididymis. The tunica albuginea has blood vessels (Fig.1B; v) from the numerous branches of the testicular artery and vein.

The tissue trabeculae or the septula testis (Fig.1C; 3) originate from the tunica albuginea and converge centrally to form the loose connective tissue of the mediastinum testis (Fig.1C; 10). The trabeculae or septula testis, composed predominantly of collagen fibres, contain blood vessels and nerves and divide the testis into a varying number of testicular lobules (Fig.1C; 7), each containing one to four convoluted seminiferous tubules. In the boar, like in most other domestic mammals, the mediastinum testis occupies a central position of the testis. Within the mediastinum testis are the channels of the rete testis by which the spermatozoa and the fluid, in which they are suspended, leave the testis. The rete testis is lined by a simple squamous-, cuboidal-, or columnar epithelium and it is connected to the cephalic portion of the epididymis by efferent ductules (Fig.1C; 9) (for review see Wrobel 1998).

Thus the parenchyma of the testis (Fig.1C; 8) consists of seminiferous tubules separated by interstitial tissue. The bulk of the testicular tissue is the seminiferous tubules and in the boar, they comprise about 60-70 % of the testis. The outer wall of each seminiferous tubule is a single layer of boundary tissue cells, the myoid- or peritubular contractile cells that are similar in some ways to smooth muscle cells (Allrich et al., 1983, Wrobel 1998). Between the seminiferous tubules in the interstitial region, there are the peritubular capillaries, the venous and lymphatic vessel networks, nerves and the Leydig cells.

A supporting system consisting mainly of various suspensory ligaments maintains the testis in its scrotal position. The ligamentum proprium testis that originates from the head or the upper pole of the testis connects and anchors the testis to the tail of the epididymis and the ligamentum cauda epididymis suspends and anchors the tail of the epididymis onto the fundus of the vaginal process. These ligaments are part of the degenerated gubernaculum testis that acted to direct and guide the testis into the scrotum during its descent from the abdominal cavity (for review Schummer et al., 1987).

The spermatic cord also partially contributes to the support system of the testis. It runs from the abdomen and is attached to the testes in the scrotum. It is a band of tissue

that carries the ductus deferens, blood vessels, nerves and muscles associated with the testis. In the boar it is 8 to 10 inches or more in length (Wrobel 1998).

2.1.2 Vascular supply and innervations

The testis is supplied by two arteries, the internal spermatic artery originating directly from the aorta and the ductus deferens artery coming from the urogenital artery. The two arteries finally branch into unevenly distributed small non-fenestrated blood capillaries, which are associated with the lamina propria of the seminiferous tubules but also are interspersed among interstitial cells. The capillaries of scrotal testes are about 10µm in diameter and contain an endothelial layer formed by two cells, a basal lamina constituted by collagen fibres and glycoconjugates, and a pericyte layer formed by a single cell (for review see Pinart et al., 2001a).

The testis drains its blood through the testicular veins, which then form a pampiniform plexus around the internal spermatic artery and finally drain into the caudal vena cava. The testicular veins in the boar can be subdivided into 4 types according to their perivascular elements and their location to the testicular artery. A type I vein is a large vein which gives rise to type II and III veins. Type II vein is composed of a single layer of veins that run along the testicular artery, while type III vein is composed of several layers of veins that are located between type II veins. The Type IV vein is regarded as a venous portal system and is composed of small muscular and pericytic venules located in the tunica adventitia of type II veins and the testicular artery. Occasionally type IV veins penetrate deep into the tunica media of the testicular artery to provide a fenestrated endothelium in its thin portion. A direct arterio-venous anastomosis between the pampiniform plexus and the testicular artery is not found in the boar and Type IV veins are considered the most important vessel in reducing the capacity of the barrier between the testicular artery and the type II and III veins. It is therefore suggested that type IV venous network may play a role in transferring the substances between the artery and the veins (for review see Rerkamnuaychoke et al., 1991). Between the seminiferous tubules in the interstitial region there are lymphatic vessel networks through which the testis drains its lymphatic fluid via the lumbar lymph nodes (Pinart et al., 2001b).

Nearly all the testicular nerves represent postganglionic sympathetic axons from the lumbar sympathetic trunk that reaches the gonad by three different routes - the funicular, caudal and mesorchial route. These axons innervate the vascular structures of the spermatic cord, the tunica albuginea, nearly all the septula testis and the mediastinum. Only in

exceptional cases are axons in contact with Leydig cells. From their association with blood vessels, the majority of nerves are vasomotoric in function.

Innervations of the porcine testes remarkably change during the period from birth to adulthood, with the most intense innervation found in testes of piglets aged 3 to 5 weeks. In 7- to 10-week old pigs, testicular innervation shows varying degrees of withdrawal and the testes of adult boars are completely devoid of intrinsic nerves. Only the funicular nerves supplying the testicular artery and pampiniform plexus are preserved in the adult age group. So, the vasomotoric control of intratunical, septal and mediastinal vessels and of the complete microcirculation within the testicular parenchyma is accomplished without any direct nerve participation in the sexually mature boar (for review see Wrobel and Brandl 1998).

2.1.3 Temperature regulating system

The testis requires cooler (2-8°C) temperature below that of the body for normal spermatogenesis. An important purpose of the scrotum is to act as a temperature-controlling device. In the boar the scrotum is less pendulous and sweating is less efficient, which may explain the observed small difference between its scrotal and rectal temperatures of 2.3°C (Stone 1981). Another mechanism in temperature regulation is through the cremaster reflex; thus the external cremaster muscle within the spermatic cord draws the testes closer to the abdomen when cold and relaxes them away from the abdomen when warm. The tunica dartos muscle at the bottom of the scrotum also responds to temperature changes. It has also been shown that this muscle is sensitive to temperature changes only in the presence of testosterone (Stone 1981). Blood flowing to the testis is cooled by the adjacent venous return in the convoluted complex of the pampiniform plexus located just dorsal to the testes.

2.1.4 Brief histology of the boar testis

2.1.4.1 General histology

The histological appearances of the seminiferous tubules (ST) in mature- and in immature testes is different (Russell et al., 1990, Livera et al., 2000). Testicular histology of immature testis is characterised by ST dominated by Sertoli cells with few prespermatogonia (gonocytes) and a not yet developed lumen. The mature testis shows ST

characterised by the presence of germ cells from all stages of a fully developed spermatogenesis and lumina that are fully developed. In most mammals the ST is tortuous with a diameter between 150 and 300 µm and is lined by a stratified germinal epithelium consisting of two different basic cell types - sustentacular (supporting, Sertoli) cells and spermatogenic cells (Russell et al., 1990, Wrobel et al., 1988). Although immunohistological staining might not allow true revelation of the 8 stages of spermatogenesis that occur in the boar (Swierstra 1968a and b), tubules in different sparmatogenic phases can be easily recognised, and, as described by Kohler (2004), spermatogenesis can grossly be divided into three arbitrary phases, the first one immediately after spermiation without elongated spermatids, the second one during elongation of the spermatids and the third one just prior to spermiation with fully elongated spermatids.

2.1.4.2 Sertoli cells

The Sertoli cells or sustentacular cells (named after the Italian histologist Enrico Sertoli, 1842-1910) are easily identifiable by their nucleus. They are not part of the sperm cell line, but "nurse" cells that sustain the proper environment for spermatozoa to develop (Griswold 1998). Although a Sertoli cell has a very extensive and branching cytoplasmic structure, little of the Sertoli cell's true extent can be seen in the light microscope (Russell 1993a, b).

The extensive branching nature of the Sertoli cell's cytoplasm and its role in isolating the haploid cells of spermatogenesis from the rest of the body (Fig.2) were understood with the advent of the electron microscope (EM). Their shape is columnar but highly irregular; they extend from the basement membrane to the luminal surface of the seminiferous epithelium and their processes extend in between the spermatogenic cells. The nucleus of Sertoli cell is ovoid or angular, large and lightly stained, slightly separated from the tube border and often contains one or two prominent nucleoli. The long axis of the nucleus is oriented perpendicular to wall of the tubule. A fold in the nuclear membrane is characteristic for Sertoli cell but not always visible in the light microscope (Chevalier 1978, Schulze 1984, Russell 1993a, b, de Kretser and Kerr 1994).

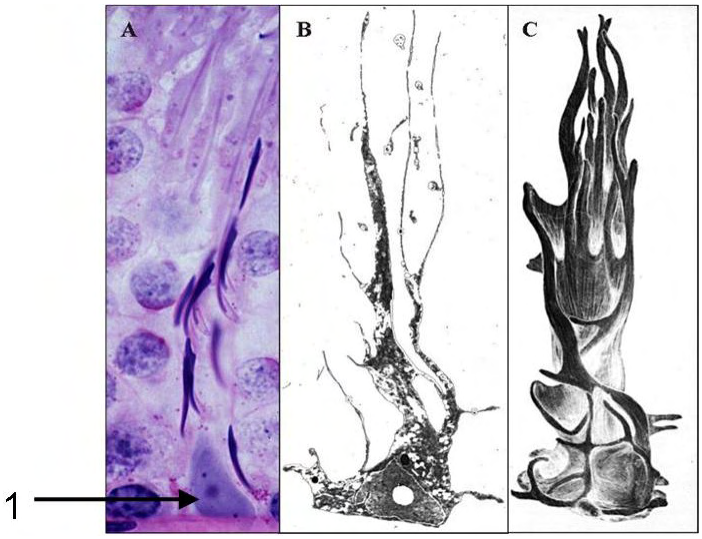

Fig. 2: Structure of Sertoli cell (1). A - histological section, B - electron micrograph, C - plastic model (Russell 1993a, Courtesy of Prof. Rex Hess, Illinois).

Sertoli cells hold germ cells in crypts in their luminal surface, thus maintaining the architecture of the seminiferous epithelium (Chevalier 1978, Dym and Cavicchia 1978, Russell 1993a, b, de Krestser and Kerr 1994). The base of the Sertoli cell sits on the basal membrane of the seminiferous tubule. The cell's lowest "branches" reach out to those of other Sertoli cells and when these processes make contact, they fuse to form long occluding (tight) junctions that effectively separate the tubule into two compartments - one is "below" (or outside of) the fused lower processes (the adluminal or basal compartment), and the other is "above" them, on the side open to the tube (the luminal compartment) (Yazama et al., 1988, Russell 1993a, b). These occluding junctions form the blood testis barrier. As a result blood borne components of the immune system are denied access to the luminal compartment containing the haploid stages of sperm development. Simultaneously the blood-testis barrier also permits the maintenance of different environments for different stages of sperm development and acts to conserve certain products of Sertoli cells within the seminiferous tubule, such as Androgen Binding Protein (ABP) (Yazama et al., 1988). As spermatocytes develop from spermatogonia, they are moved into the luminal compartment by the appropriate Sertoli cells which grow new processes, which then "undermine" the germ cells that are ready to move up. After this has happened, the junctions "above" them are broken and these germ cells are now (without having passed through any junctions) no longer in the compartment accessible to the blood - they leave the adluminal- and enter the luminal compartment (Russell 1977; Pelletier and Byers 1992; Lui et al. 2003).

Sertoli cells are involved in anchoring and nourishing germ cells, forming blood testis barrier, phagocytosing residual bodies, releasing sperms during spermiation, and participating in secretion and endocytosis of various substances, including ions and water. They transport water from the interstitial space into the lumen, serving as the vehicle for moving sperms from the testis to the epididymis. Sertoli cells are also responsible for secretion of numerous proteins into the seminiferous tubular lumen, such as glycoprotein androgen binding protein (ABP), which binds androgens with high affinity and transports them to the epididymis. Additionally they reduce testosterone into dihydrotestosterone and secrete inhibin and activin, hormones regulating FSH-release (Clermont 1972, Schulze 1984, de Kretser and Kerr 1988, Russell 1993c, Dadoune 1994, Grover et al., 2004).

2.1.4.3 Leydig cells

Interstitial cells (or Leydig cells named after a German anatomist Franz von Leydig, 1821-1908) appear as small groups of cells in the interstitial regions between the seminiferous tubules. Leydig cells are 15-20 μm in size, cuboidal in shape and appear in clusters of 2-20 cells. Their cytoplasm is strongly acidophilic, finely granular and their nucleus is large, round and often located eccentric in the cell (Christensen 1975, Desjardins and Ewing 1993). They are extremely abundant in the boar making the lymphatic vessels quite inconspicuous (Belt and Cavazos 1967). Their characteristics in the testis of the boar were revealed by Belt and Cavazos (1967) with the aid of an EM: The bulk of the cytoplasm to one side of an eccentrically placed nucleus consists predominately of a tightly packed agranular endoplasmic reticulum in a system of anastomosing tubules. Free ribosomes and a few short strands of granular endoplasmic reticulum are present only in a perinuclear or subplasmalemmal location. Mitochondria are abundant as a cluster in the centre of the cytosome. Surrounding them are dense bodies. Between contiguous cells, irregularly disposed microvilli protrude into an intercellular space of variable dimensions. Some sites of membrane fusion, resulting in maculae occludentes, are observed between adjacent cells, while occasionally small desmosomes are observed. Coated vesicles are frequent near to and continuous with the plasmalemma as well as deeper within the cell, especially in association with elements of the Golgi apparatus.

In the boar these cells secrete not only androgens but also abundant amounts of sulfo-conjugated estrone (Claus and Hoffmann 1980, Ford 1983, Reaside and Renaud 1983, Setchell et al., 1983, Claus et al., 1987, Rostalski et al., 2000). In addition,

expression of aromatase in the boar Leydig cell (Conley et al., 1996, Biliniska et al., 2000, Fraczek et al., 2000, Mutembei et al., 2005b), and activity of sulfatase and of sulfotransferase (Rostalski 2005) within its testis has been observed.

2.1.4.4 Spermatogonia

Spermatogonia are diploid and the first cells along the route of spermatogenesis. They are always situated in contact with the basal membrane of the seminiferous tubule. Subsets of type A spermatogonia (A0), which are the true stem cells of the germ cell population (Clermont 1972), multiply mitotically to form new generations of type A0-stem cells and type A1 spermatogonia. While the A0 spermatogonia enter a phase of arrest, the A1-spermatogonia divide further into A2, A3, A4, intermediate and B spermatogonia (Swierstra 1968a, b; Frankenhuis et al., 1982).

Type A spermatogonia have a rounded to oval nucleus with fine chromatin grains and one or two nucleoli, the type B spermatogonia have a more rounded nucleus with chromatin granules of variable sizes that are often attached to the nuclear membrane and contains one nucleolus. Division of type B spermatogonia is the final mitosis resulting in the formation of primary spermatocytes (Browder et al., 1991).

2.1.4.5 Primary spermatocytes

Primary spermatocytes and the following stages lie in the cell layer luminal to the spermatogonia. They immediately enter the prophase of the first meiotic division. Primary spermatocytes appear larger than spermatogonia and a large number of them are always visible in a seminiferous tubule cross-section. A primary spermatocyte has a round nucleus with fine chromatin granules and one nucleolus. Cell divisions, from the formation of primary spermatocytes and onwards to the production of elongated spermatids, are incomplete and the cellular associations remain connected by bridges of cytoplasm that appear as clonal units (Dym and Fawcett 1971, Weber and Russell 1987).

2.1.4.6 Secondary spermatocytes

Each primary spermatocyte divides meiotically to form two haploid secondary spermatocytes with 2N, which then divide to form four haploid spermatids with 1N. Secondary spermatocytes are smaller than primary spermatocytes. They rapidly enter and

complete the second meiotic division and are therefore rarely seen in histological preparations. Two important events with major genetic consequences occur during the two meiotic divisions - there is the random separation of homologous chromosomes and crossing over of genetic material, a process that maintains individual genetic specificity (Dym and Fawcett 1971, Weber and Russell 1987, Russell 1993c).

2.1.4.7 Spermatids

The initial forms are small (about 10 µm in diameter) and contain a very light-staining (often eccentric) nucleus (Dadoune 1994). The chromatin condenses highly during maturation of the spermatids into spermatozoa, a process that results in a smaller and darker staining nucleus. The development of spermatids from meiosis to detachment of the spermatozoa is known as spermiogenesis (Fawcett et al., 1970). During this process the cell changes from a simple rounded cell into a highly differentiated spermatozoon by undergoing complex morphological, physiological and biochemical changes (Dooher and Bennett 1973, Gilbert 2000).

2.1.4.8 Spermatozoon

The spermatozoon is the final product of spermatogenesis. The structure of the mammalian spermatozoon, including that of the boar, has been broadly studied and documented (Clermont 1972, Peterson et al., 1987, Browder et al., 1991, Shostak 1991, Curry and Watson 1995, Kalthoff 1996, Gilbert 1997, Wolpert et al., 1998). Briefly, it is divided into head, neck and tail. In the boar it is about 50 µ long, the head is about 5 µ long, 4 µ wide and 0.5 µ thick and chiefly consists of the flattened nucleus. The anterior 2/3 of the nucleus is covered by the acrosome which originates from the Golgi apparatus and contains enzymes important in the process of fertilisation. The neck (about 1 µ) is formed by the posterior part of the nuclear membrane and attached to the basal plate. A transversely oriented centriole is located immediately behind the basal plate. The neck also contains segmented columns of fibrous material that continue as the outer dense fibres into the tail. The tail is further divided into middle-, principal- and end piece. The axonema (the generic name for the arrangement of microtubules in all cilia) originates in the neck and extends through the end piece; it is surrounded by the outer dense fibres through the principal piece. The middle piece is about 8-10 µ long and terminated by a dense ring, the annulus; a sheath of mitochondria surrounds its axonema and dense fibres. The principal

piece is about 35-40 µ long. It contains a fibrous sheath, which consists of dorsal and ventral longitudinal columns interconnected by regularly spaced circumferential hoops; it does not extend to the tip of the tail. The end-piece is about 3-5µ. Motility of the spermatozoon is achieved after epididymal maturation (Wrobel 1998).

2.1.4.9 Lamina propria

The peritubular sheath of cells and the basal lamina form the two main components of the tubular lamina propria. Pinart et al. (2001a) reported the constitution of lamina propria in the boar to consist of an innermost non-cellular layer, referred to as the basal lamina, surrounded by 2 layers of peritubular cells that are separated by a fibrous layer. The non-cellular layer consists of collagen fibres and glycoconjugates with abundant N-acetylgalactosamine, galactose, fructose, N-acetylglucosamine and neuraminic acid residues. The inner peritubular cell layer is composed of myoid cells while the outer layer is composed of fibroblasts. The myoid or peritubular contractile cells have a spherical to rounded nucleus and are similar in some ways to cells of the smooth muscle. The basal lamina is the inner component of the lamina propria that covers the entire circumference of the seminiferous tubules and it is bound to the outer peritubular myoid cells by collagen and elastic fibres. The lamina propria is implicated in tubular contractility, in complementing the blood-testis barrier and in mediating the communication and the substrate diffusion between seminiferous tubules and interstitial tissue (Wrobel 1998).

2.2 Testicular and ductal system development and function

2.2.1 Fetal period

In male as well as in female embryos the gonads develop from an identical indifferent bipotential genital ridge (Klaus, 1996), a sub-region of the urogenital ridge. The epithelium of the coelomic cavity lines the urogenital ridge and serves as the source of multiple gonadal cell lineages (Schmahl et al. 2000).

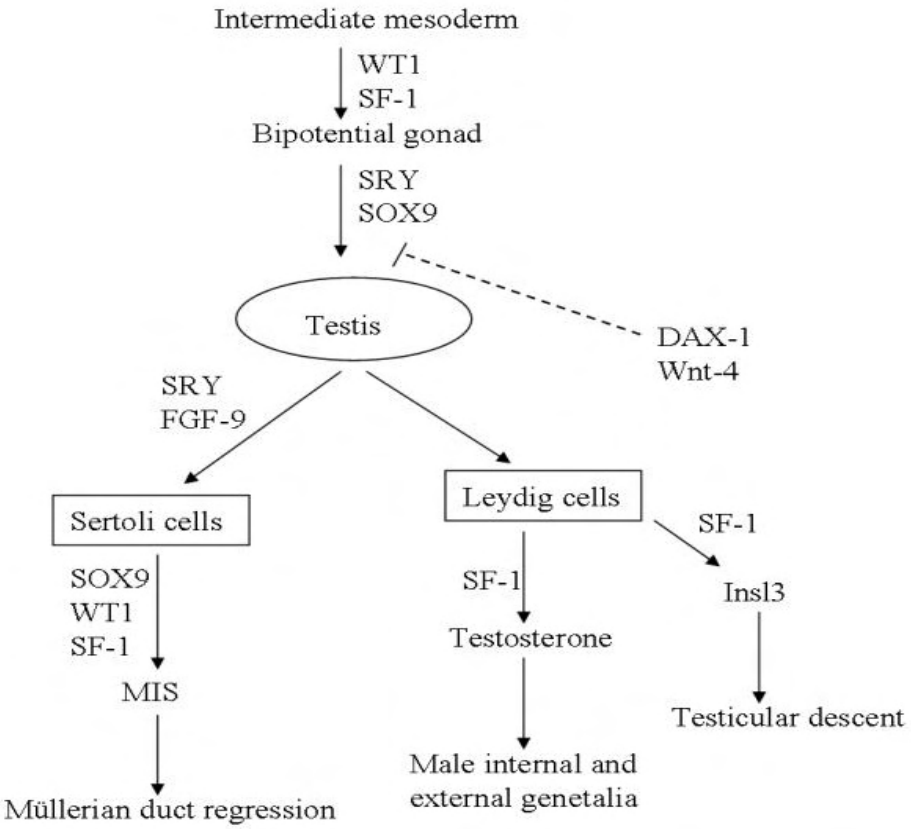

Fig. 3: Summary of genes essential for development of the testis. Solid lines indicate activating effects while dotted lines indicate inhibition effects.

Various genes play vital roles in the development of the testis (Fig. 3). Establishment of the bipotential genital ridge requires expression of Wilms' tumour suppressor gene (WT-1) and SF-1 (steroidogenic factor-1) (Luo et al., 1994, Achermann et al., 1999, Bakke et al., 2001) in both sexes during early embryonic development. According to our present understanding further differentiation into an ovary requires no special stimuli (principle of basic femaleness) while differentiation into a testis and the development of the male phenotype require specific signals. Differentiation is initiated when primodial germ cells, which might carry either the XX or XY combination, reach the indifferent bipotential gonadal ridge; male (XY) primodial germ cells migrate and reach into the male-signalling mark (Martineau et al., 1997) while the XX primodial cells get stuck in the cortex (Adams and McLaren 2002). The exact cellular origin of primordial germ cells (PGCs) is unknown (Ginsburg et al. 1990) but their ancestors are thought to arise from a pool of epiblast cells (cells of the heterogeneous, undifferentiated ectodermal tissue) (Kierszenbaum and Tres 2001).

Further development of the testis is triggered by SRY-factor (Sex determining Region on Y chromosome), a Y chromosome-linked gene. Deletions or mutations of the

SRY gene lead to XY female development (Gubbay et al., 1990, Lovell-Badge and Robertson 1990, Sinclair et al., 1990, Schmahl et al., 2000, Hawkins et al., 1992, Ikeda et al., 1994) and even reduced SRY expression causes XY sex reversal or ovotestis (Nagamine et al., 1999, Hammes et al., 2001, Washburn et al. 2001). A close relative of SRY is the SOX-9 gene - a gene containing the high mobility group (HMG) box domain of transcription factors and expressed in testes of all vertebrates (Kent et al., 1996, Morais da Silva et al., 1996, Bowles et al., 2000, Nagai 2001). Its mutations result in XY sex-reversal whereas ovarian development is normal, demonstrating that it is necessary for testis determination (Foster et al., 1994, Wagner et al., 1994, Koopman 1999). Furthermore SOX-9 expression in ovaries results in female-to-male sex reversal (Huang et al., 1999, Bishop et al., 2000, Vidal et al., 2001), which means, it may represent an ancestral sex-determining gene and mammals have evolved SRY as a Y-linked switching mechanism (Nagai 2001). Based on its expression pattern, SOX-9 serves as a putative target gene for SRY, although definitive proof for this is still lacking.

Although to date no target genes for SRY have been identified, its expression pattern in the mouse indicates that it initiates the differentiation of gonadal supporting-cell precursors, which are derived from the coelomic epithelium, to develop as testicular Sertoli cells (Hacker et al., 1995, Albrecht and Eicher 2001). Sertoli cells, which surround germ cells to form testicular cords in prenatal gonads (Karl and Capel 1998), proliferate and reach maximum numbers before birth (Orth 1982). The Fibroblast growth factor-9 (FGF-9) gene regulates the SRY-dependent processes, such as cell proliferation and migration of coelomic cells into the gonad, and further Sertoli cell differentiation. Male mice lacking this gene exhibit phenotypes ranging from hypoplasia to complete sex reversal (Colvin et al., 1999, 2001).

SRY may be counteracted by a nuclear receptor gene referred to as DAX-1 (Yu et al., 1998). DAX-1 gene duplication results in male-to-female sex-reversal (Zanaria et al., 1994) while its mutation leads to hypogonadotrophic hypogonadism (Tabarin et al., 2000). DAX-1 gene is co-expressed with SF-1 along the developing hypothalamic-pituitary-gonadal axis in the gonads of both sexes (Ikeda et al. 1996) where it may repress and/or modulate SF-1-mediated activity in the gonads for normal development (Wang et al. 2001).

Sertoli cell signalling is essential for further testicular development (Magre and Jost 1991); it enables the primordial germ cells to proliferate mitotically (McLaren 2000) and to

differentiate into gonocytes (prespermatogonia) that undergo mitotic arrest until birth when they resume proliferation (Sutton 2000, de Rooij 2001).

Leydig cell precursors originate either from the adjacent mesonephros or from the cells of the coelomic epithelium and migrate into the genital ridge (Buehr et al., 1993, Merchant-Larios and Moreno-Mendoza 1998; Karl and Capel 1998, Yao and Capel 2002). The fetal Leydig cells present at birth are not progenitors of the adult Leydig cell population; rather they remain present in low numbers in the mature testis, presumably in a quiescent state (Kerr and Knell 1988).

The ductal system arises from a structure adjacent to the genital ridge called mesonephros that regresses during embryonic development. In the male, both testosterone of Leydig cell origin and Müllerian inhibiting substance (MIS, also known as anti-Müllerian hormone, AMH), secreted by embryonic/fetal Sertoli cells, are needed for development of the male phenotype. Notably the steroidogenic factor-1 (SF-1) (Lala et al., 1992, Morohashi et al., 1992, Parker and Schimmer 1997) regulates this endocrine-dependent step of sex determination. MIS causes the regression of Müllerian ducts and testosterone and its derivative, dihydrotestosterone, stimulate the development of male-type genitalia (Behringer et al., 1994, Rey and Picard 1998). Male duct development can be inhibited if steroid production is down regulated by WNT-4 gene or if antiestrogens are applied (Vainio et al., 1999).

2.2.2 Post natal and mature period

The testis of a newborn animal is functionally an immature organ. For normal spermatogenesis, the correct gonadal position is essential and testicular descent from the fetal retroperitoneal position into the scrotum is initiated during the prenatal period. This activity is regulated by the INSL-3 gene (also known as relaxin-like factor) - disruption of this gene causes bilateral cryptorchidism (Zimmermann et al., 1999, Adham et al., 2000).

In most species the number of Leydig cells remains fairly constant from the late fetal period throughout the postnatal period. However in the boar, Herrera et al. (1983) reported abundant hyperplastic Leydig cells from 14 to 40 days postpartum, which thereafter decreased not only in number but also in size. Their numbers remained constantly low until the prepubertal period, when a second rapid increase was observed, yielding the final population of adult Leydig cells (Nistal et al., 1986, Hardy et al., 1991, Habert et al., 2001). When compared to juvenile Leydig cells, the adult Leydig cells

(Christensen 1975, de Kretser and Kerr 1988, Hardy et al., 1991) have a marked increase in volume of smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), Golgi vesicles and mitochondria. In addition, there is a rise in the total number of mitochondria per cell. In the boar these changes occurs between 100 and 130 days of age (Lunstra et al., 1986, Franca et al., 2000, Tripepi et al., 2000). These ultra structural characteristics are consistent with an increased capacity for the secretion of steroids by individual Leydig cells (de Kretser 1967, Kerr et al., 1979, Zirkin et al. 1980). The sharp increase in number of LH receptors per Leydig cell between 100 and 130 days in boars may be a causative factor in the subsequent hypertrophy of the Leydig cell at 130-160 days of age (Lunstra et al., 1986).

Differentiation of Sertoli cells commences during puberty (de Kretser and Kerr 1988), with the number of the Sertoli cells in the testis remaining stable throughout adulthood (Wang et al., 1989).

After birth prespermatogonia differentiate into spermatogonia and at puberty, spermatogenesis begins. In the boar onset of spermatogenesis is around day 80 and from 90 days on, pachytene primary spermatocytes could be found (Allrich et al., 1983, Herrera et al., 1983).

2.2.3 Spermatogenesis in the boar

During spermatogenesis spermatogonia (A1) undergo successive mitotic and meiotic divisions and metamorphic changes (spermiogenesis) to produce spermatozoa (Swierstra 1968a, b, Clermont 1972, Frankenhuis et al., 1982, Hess 1990, Browder et al., 1991). Duration of the spermatogenic process in man and farm animals is about two months, in the boar it is 39 days.

Spermatogenesis is inherently linked to the process of apoptosis (Jacobson et al., 1997, Raff 1998), a process that serves a role in regulating the development and survival of germ cells (Dunkel et al., 1997, Matsui 1998, Sinha Hikim and Swerdloff 1999, Kierszenbaum and Tres 2001). A balance between germ cell survival and death is a prerequisite for normal spermatogenesis and testis development. Before reaching maturity, a number of germ cells undergo physiological apoptotic death, which has been showed to be under genetic and hormonal control (Matsui et al., 1998, Braun 1998, Sinha and Swerdloff, 1999). FSH (Dunkel et al., 1997a), Testosterone, (Erkkilä et al., 1997) and estradiol (Pentikainen et al., 2000) are part of factors that promote germ cell survival.

Some of the genes known to support cell-survival include those for the Bcl-2 family of proteins and Bax (Rodriguez et al., 1997, Russell et al., 2002), the spermatogenic stage-specific transcription factors GATA-1 and GATA-4 and their cofactors FOG-1 and FOG-2, all of which largely act in anti-apoptotic process (Ketola et al., 1999, 2002). On the other hand the caspases, which are cell death-promoting enzymes, inactivate GATA-1 and -4 transcription factors (De Maria et al., 1999), thereby promoting cell death.

2.2.3.1 Spermatogoniogenesis, stem cell renewal and spermatocytogenesis

Spermatogonia stem cells proliferate by mitosis through several different types of spermatogonial population to provide a source for 30-60 x10^9 of spermatozoa that are daily produced in the boar testis (McKenzie et al., 1938, Frankenhuis et al., 1982). In this species, four classes of spermatogonia are observed; undifferentiated A spermatogonia (A1), differentiating A spermatogonia (A2, A3), intermediate and B spermatogonia. Mitosis ends when B type spermatogonia yield two preleptotene primary spermatocytes (Russell et al., 1990), a process that yields 16 primary spermatocytes from each of the A2 spermatogonia (Fig. 4) and takes about 7-10 days in the boar (Swierstra 1968a, b, Frankenhuis et al., 1982).

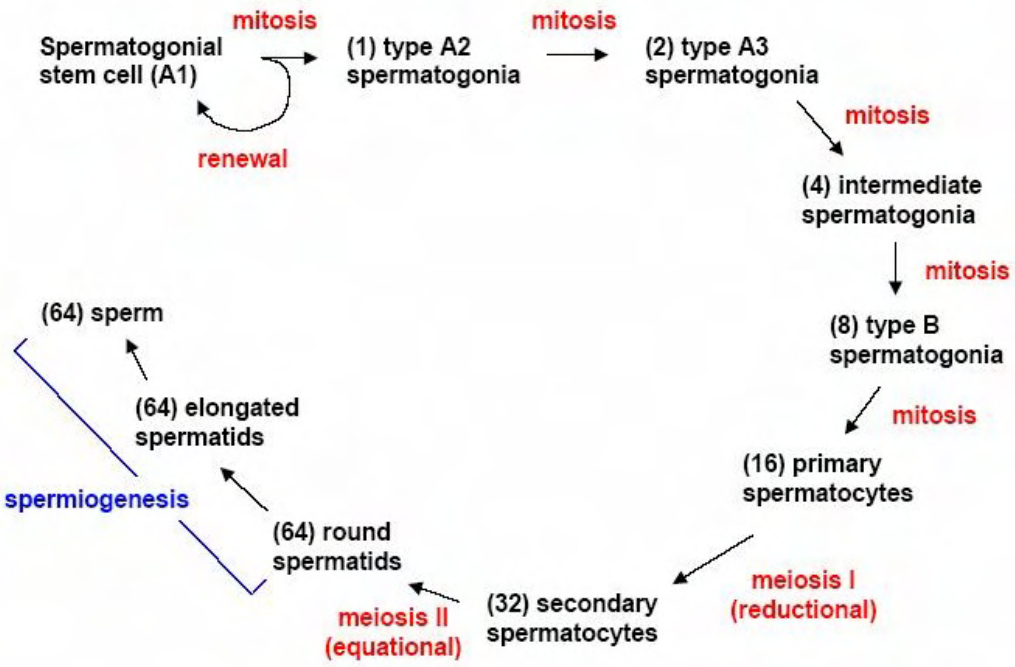

Fig. 4: A Schematic representation of spermatogenesis

Spermatogenesis is dependent on the replenishment of spermatogonial stem cell (A1). The mechanisms of germ cell renewal are not totally understood and there are presently two schools of thought. It has been a long standing opinion that a stem cell (A1)

probably divides into an A2 spermatogonia and an operative copy of itself as indicated in figure 4 (Clermont 1972). The more recent thought is that A1 divides only into A2 types and that certain Sertoli cell-derived factors influence the conversion of some of the A2 spermatogonia into A1 stem cells to be kept in reserve for future divisions while the rest of A2 cells become dedicated to spermatogenesis (Chen et al., 2005). These Sertoli cell-derived factors are secreted under the regulation of ERM, an Ets (epithelial specific protein) Related Molecule and a Pea3 subfamily (Ets transcription factor). ERM is expressed exclusively in Sertoli cells and it is essential for the regulation of spermatogonial stem cell self-renewal because it has been shown that ERM-/- males undergo only a first wave of spermatogenesis that is normal, but subsequent waves are lost through maturation depletion of germ cells, resulting in sterility. Testes from ERM-/- mice aged 3 months or older exhibit complete seminiferous tubular atrophy, characterized by depletion of all germ cells, but retention of Sertoli cells (Chen et al., 2005).

Meiosis 1 is initiated in preleptotene spermatocytes. In interim phase and prior to reducing haploidity with 2N, DNA-content in primary spermatocytes is 4N. This doubling of chromosomes is associated with doubling in cell size. These synthetic processes take time and this is the most prolonged period in spermatogenesis (Swierstra 1968a, b, Clermont 1972, Russell et al., 1990). During meiosis 1, primary spermatocytes are transformed into two secondary spermatocytes (2N), which then in turn are converted into four round haploid (1N) spermatids during meiosis II. The second meiotic division is rapid and therefore very few secondary spermatocytes can be identified in histological sections (Clermont 1972).

Whereas meiosis II is essentially like a mitotic division, meiosis I is different in that during this process, homologous chromosomes pair along their length and come in contact in discrete areas of synapsis (chiasmata), through which chromatids exchange base pairs by crossing-over. This allows for generation of genetic variability and also provides a mechanism for correcting damage in the DNA helix; the repair mechanisms ensure that precision in the DNA of the gametes is sustained (Creasv et al., 1985).

2.2.3.2 Spermiogenesis

Spermiogenesis defines the phase where round spermatids turns into the mature elongated spermatids ready for spermiation (Leblond and Clermont 1952b; Russell et al.,

1990). This process is divided into Golgi phase, cap phase, acrosomal phase and maturation phase.

In brief, during the Golgi phase, the centrioles, which serve as points of organization of spindle fibres, migrate to a postnuclear region. The acrosome is derived from the Golgi apparatus (Susi et al., 1971); proacrosomal granules fuse to form a single acrosomal vesicle.

In the cap phase the acrosomal vesicle move towards the anterior pole of the nucleus. The acrosomal vesicle grows and forms the head cap that covers approximately the anterior two thirds of the nucleus. The two centrioles assemble at the caudal pole of the nucleus, and the distal centriole gives rise to the outgrowing flagellum within the tail.

In the acrosomal phase the nucleus and cell body start to elongate in a craniocaudal direction. At the same time, the nucleus starts chromatid condensation and moves toward the cranial end, leaving the other organelles behind. In the mean time, the spermatids rotate so that the nucleus is directed toward the tubular periphery and the developing tail toward the lumen.

In the maturation phase nuclear condensation is completed and most of the mitochondria gather around the axoneme in a helicoidal manner within the region corresponding to the future middle piece of the spermatozoon. Outer fibers and the fibrous sheath of the future principal piece develop. The volume of a spermatid in late maturation phase amounts to only 20 to 30% of that of cap phase spermatid. After completion of the development of the tail, the residual body is separated from the spermatozoon and the intercellular bridges (IB) between the germ cells are now interrupted (Leblond and Clermont 1952b; Russell et al., 1990).

2.2.3.3 Spermatogenic cycle, stage and wave

Examination of serial cross-sections of a seminiferous tubule revealed that sperm cells differentiate in distinctive associations (Leblond and Clermont 1952a, Swierstra 1968a, b, Clermont 1972, Parvinen et al., 1986, Hess 1990, Russell 1993b). It is this unique association of germ cells with Sertoli cells that constitutes the cycle of the seminiferous epithelium, and each particular association of germ cells is referred to as a stage (Leblond and Clermont 1952a, Hess et al., 1990, Parvinen 1993). A spermatogenic cycle is defined as the time it takes for the reappearance of the same stage within a given segment of the tubule (Clermont 1972, Parvinen et al., 1986). Each stage of the cycle

follows in an orderly sequence along the length of the tubule and the distance between the same stages is called the spermatogenic wave (Perey et al., 1961). One tubule can contain numerous complete waves. Adjacent segments of the tubule communicate in some unknown manner. The number of stages in a particular species is thus defined as the number of morphologically recognizable germ cell associations within the testis. The number of stages within a spermatogenic cycle and the number of cycles required for the completion of spermatogenesis varies between species (Table 1) (Clermont 1972; Hess 1990; Onyango et al., 2000).

Table 1: Length (days) of cycle, duration of spermatogenesis and of stages of seminiferous cycle epithelium in various species

Stage	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*Cycle*	*Spermatogenesis*
Boar	1.1	1.4	0.4	1.2	0.8	1.6	1.0	0.8	8.3	39
Bull	4.2	1.2	2.7	1.7	0.2	0.8	1.1	1.6	13.5	61
Dog	2.6	1.3	0.8	1.8	0.7	1.2	0.7	1.0	10.4	48
Goat	1.7	1.4	2.2	1.1	1.2	1.0	0.8	1.2	10.6	47.7
Rabbit	3.1	1.5	0.8	1.2	0.5	1.7	1.3	0.9	11.0	48
Ram	2.2	1.1	1.9	1.1	0.4	1.3	1.1	1.0	10.1	47
Stallion	2.0	1.8	0.4	1.9	0.9	1.7	1.6	1.9	12.2	55

2.2.3.4 Stages of spermatogenesis in the boar

In the boar a seminiferous cycle of 8.3 days is comprised of 8 developmental stages (Fig. 7) (Swierstra 1968a). In this species approximately 4.5 cycles occur before a type A1 spermatogonium is transformed into a spermatozoon and thus, the entire process of spermatogenesis takes about 39 days. The seminiferous cycle begins with the accomplishment of spermiation (stage 1) and ends with apical migration and close attachment of late maturation phase spermatids at the Sertoli cell apex, separated from their residual bodies ready for spermiation (stage 8) (Fig. 5I-VIII) (Swierstra 1968 a, b). The 8 stages can grossly be divided into three phases (Hess 1990, Kohler 2004, Mutembei 2005b), Phase I seen immediately after spermiation and characterised by absence of elongated spermatids; corresponds to stage 1, Phase II seen during spermatid elongation

and characterised by presence of elongating spermatids at various stages of maturation; corresponds to any of stages 2-6 and Phase III seen prior to spermiation and characterised by presence of fully elongated spermatids; corresponds to any of stages 7-8.

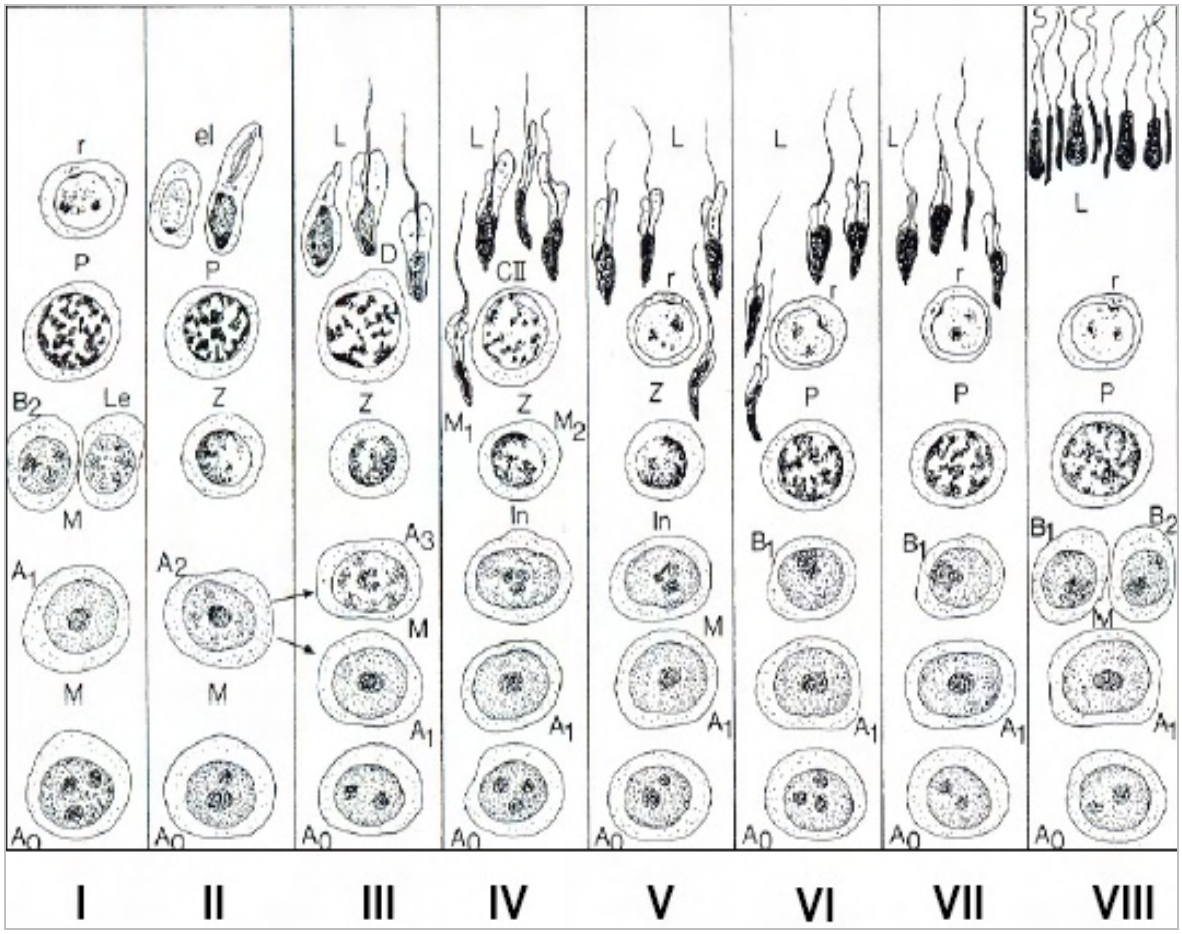

Fig. 5: Stages of spermatogenesis in the boar. A0-3 = Type A spermatogonia, In = Intermediate spermatogonia, B = Type B spermatogonia, Le = Leptotenes, Z = Zygotenes, P = Pachytenes, R = Round spermatids, el = elongated spermatids, M = mitosis, M1, M2 = meiosis 1 and meiosis II (Swierstra 1968 a).

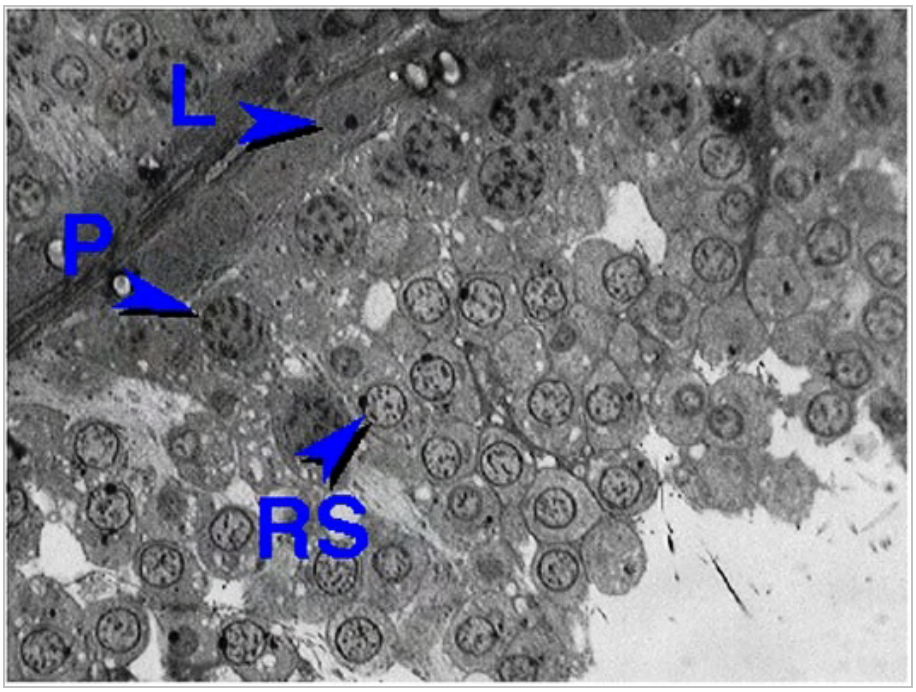

Fig. 5I: Stage 1 of spermatogenesis. Following spermiation, round spermatids (RS) lie nearest the lumen, followed basally by two generations of primary spermatocytes, i.e., old pachytenes (P) and young leptotenes (L). This stage is characterised by lack of any elongating/ed spermatids (Swierstra 1968 a).

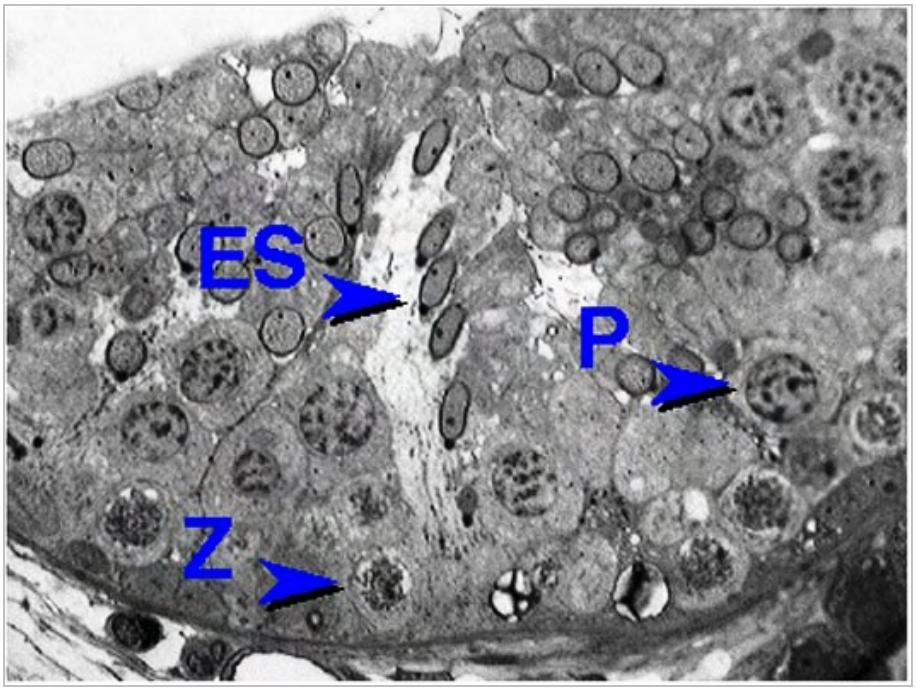

Fig. 5II: Stage 2 of spermatogenesis. This stage is characterised by spermatids whose dark staining nuclei are beginning elongation (ES). The two generations of primary spermatocytes are old pachytenes (P) and young zygotenes (Z), which were the leptotenes in the previous stage (Swierstra 1968 a).

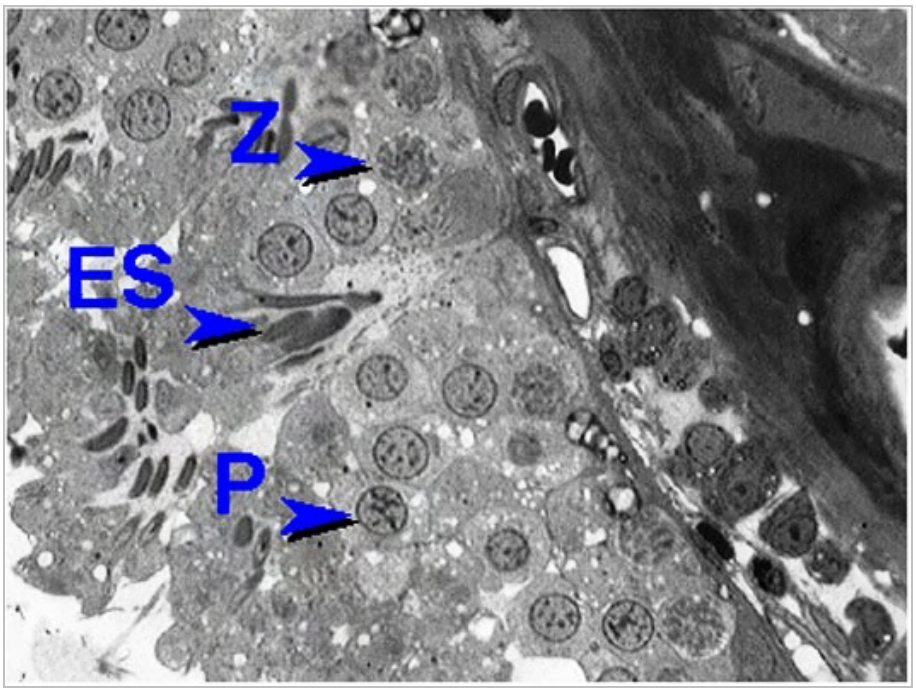

Fig. 5III: Stage 3 of spermatogenesis. This stage is characterised by elongating spermatids (ES) that are arranged in bundles and lying in deep apical recesses of the Sertoli cell close to its nucleus. The zygotene (Z) and pachytene (P) primary spermatocytes are arranged near the basal membrane (Swierstra 1968 a).

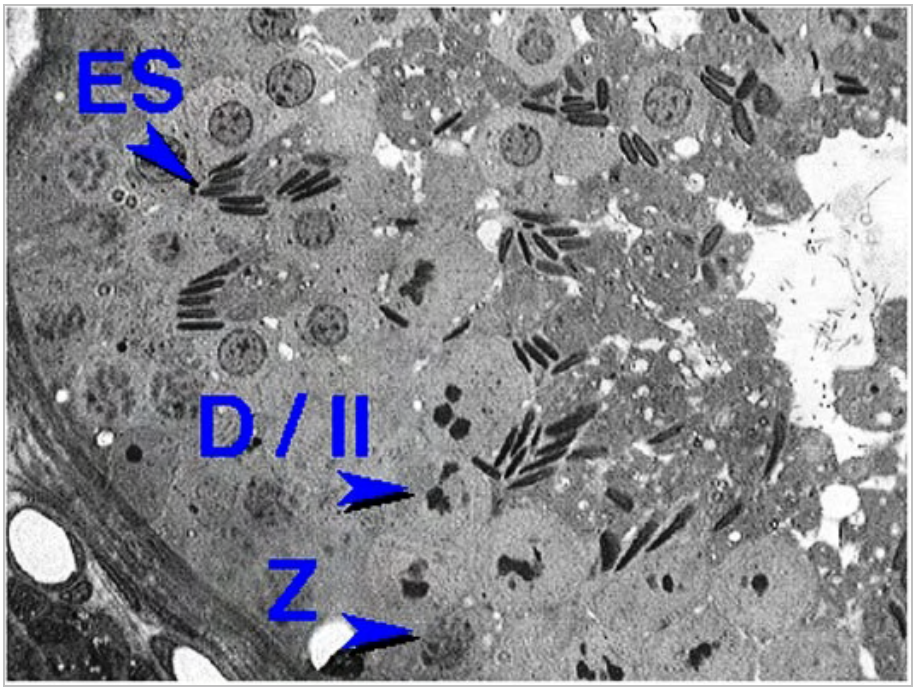

Fig. 5IV: Stage 4 of spermatogenesis. This stage is characterised by presence of the two meiotic divisions of spermatogenesis - the first and/or second meiotic divisions take place in this stage. In addition to bundles of maturing elongating spermatids (ES) and zygotene primary spermatocytes (Z) either diplotene secondary spermatocytes (D / II) or round spermatids are seen (Swierstra 1968 a).

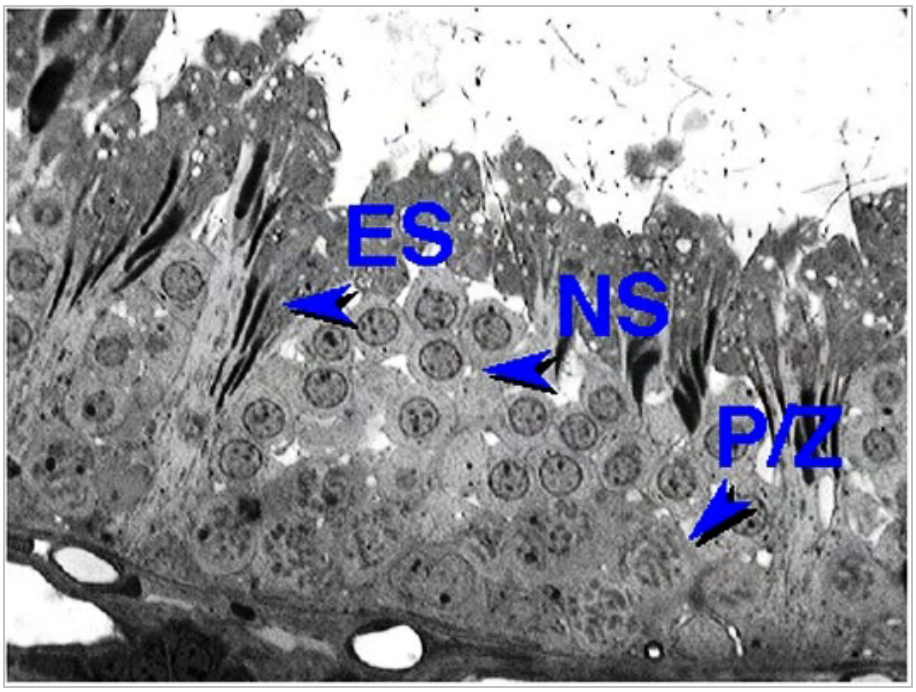

Fig. 5V: Stage 5 of spermatogenesis. This stage is characterised by presence of two generations of spermatids, older elongating spermatids (ES) and newly formed round spermatids (NS). The zygotene primary spermatocytes of stage 4 enter the pachytene (P/Z) stage and leave their basal position (Swierstra 1968 a).

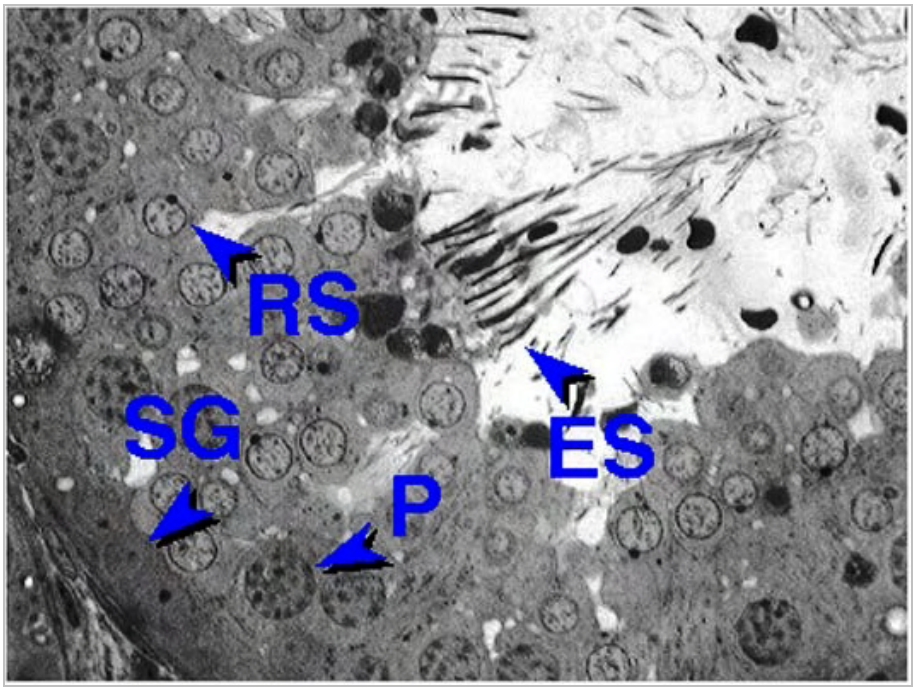

Fig. 5VI: Stage 6 of spermatogenesis. This stage is characterised by presence of the bundles of older elongated spermatids (ES) having moved away from the vicinity of the Sertoli cell nuclei with their tails projecting into the lumen of the tubules. In addition to round spermatids (RS) pachytene primary spermatocytes (P) and spermatogonia (SG) lie near the basal lamina (Swierstra 1968 a).

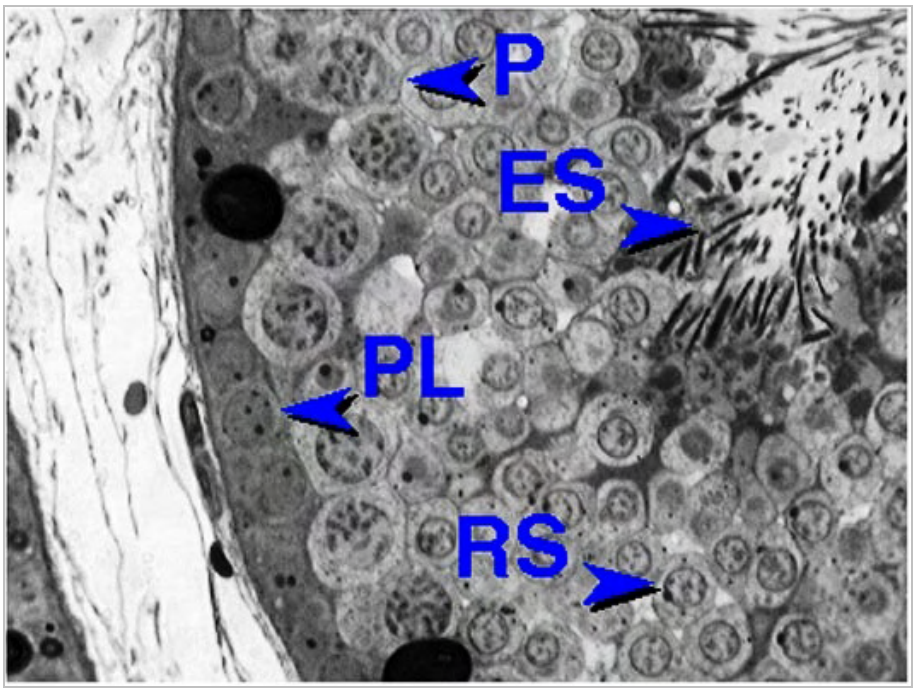

Fig. 5VII: Stage 7 of spermatogenesis. This stage is characterised by presence of elongated spermatids (ES) at the maturation phase – here spermatids continue to migrate centrally but are still not separated from their residue body. The spermatogonia divide to form preleptotene primary spermatocytes (PL). Also observed are pachytene primary spermatocytes (P) and round spermatids (RS) (Swierstra 1968 a).

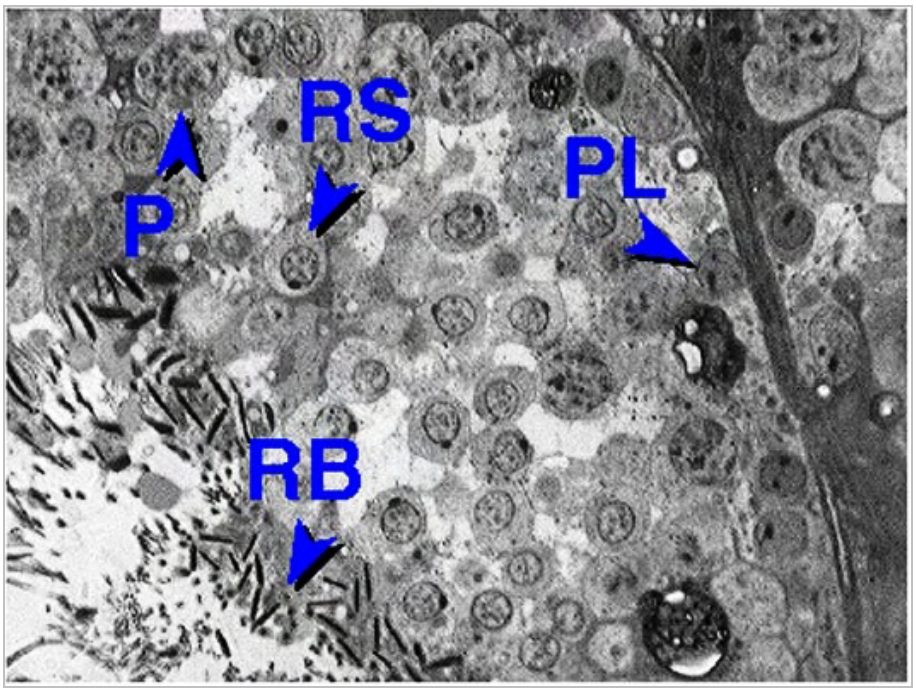

Fig. 5VIII: Stage 8 of spermatogenesis. In this stage the spermatozoa leave the tubular epithelium following separation from their residual bodies (RB). Remaining in the epithelium are round spermatids (RS) and two generations of primary spermatocytes (older pachytenes (P) and young preleptotene (PL) (Swierstra 1968 a).

2.2.3.5 Sperm release (spermiation) and maturation

Spermiation is a process by which spermatozoa are released from the seminiferous epithelium into the lumen of the tubule (Russel 1993b), leaving behind residual bodies that they discarded within the seminiferous epithelium (Russell 1993c). A small amount of cytoplasmic material (cytoplasmic droplet) remains attached within the neck region or around the middle piece as the spermatozoon makes its way into the epididymis via the excurrent duct system, the rete testis and the efferent ducts. Seminiferous spermatozoa lack motility and hence fertilizing capacity. During transit through the epididymis, which takes approximately two weeks (about 9 to 14 days in boar) (Swierstra 1968b; Frankenhuis et al., 1982), the spermatozoa undergo a series of biochemical changes to become motile and capable of fertilization (Clermont 1972, Hess 1990, Hess et al., 1997). The cytoplasmic droplet migrates distally along the tail of the spermatozoon and falls off. This event is correlated with an increase in motility, up to 60-70%.

2.2.3.6 Rate of sperm production in the boar

Daily sperm production per gram of testis in the boar is 20 to 30 $\times 10^6$ (Amann 1970, Frankenhuis et al., 1982, Trudeau and Sanford 1986). Sperm production increases with age in the post-pubertal period and it is not markedly affected by season of the year (Trudeau and Sanford 1986, Cameron 1997).

2.3 Neuroendocrine regulation of spermatogenesis

2.3.1 Sertoli-Germ cell interaction

Germ cell development relies on a highly coordinated interaction with the Sertoli cell; communication is via ligand/receptor-mediated interactions or paracrine factors. The production and secretion of many Sertoli cell proteins involved in germ cell development occur in a stage-dependent manner (Parvinen 1982). Although Sertoli cell signalling is the major controlling factor in the timing of germ cell development, also germ cells have an immediate input as was shown in experiments when rat germ cells being in contact with mouse Sertoli cells developed according to the kinetics of rat and not of the mouse spermatogenesis (Franca et al., 1998). Important factors that control Sertoli cell function are testis derived and anterior pituitary gland hormones.

2.3.2 LH, FSH, Testosterone

LH targets the Leydig cell to stimulate the secretion of testosterone, which in turn acts on androgen receptors in the Sertoli cells and peritubular myoid cells of the seminiferous epithelium to control spermatogenesis (Weinbauer and Nieschlag 1993, Sharpe 1994, McLachlan et al., 1996). Androgen-binding protein sequesters testosterone within the seminiferous tubule to ensure it is maintained at adequate effective levels. FSH targets receptors within the Sertoli cell, possibly inducing a multitude of responses. Well established is the secretion of inhibin and activin, which respectively inhibit or promote the release of FSH (Weinbauer and Nieschlag 1993, Sharpe 1994, McLachlan et al., 1996).

The roles of testosterone and FSH in spermatogenesis have been studied extensively. FSH (Dunkel et al., 1997b) as well as androgens (Erkkilä et al., 1997) act as germ cell survival factors but androgens alone have been shown to stimulate all phases of germ cell development in the hypogonadal (hpg) mouse, which is congenitally deficient in GnRH and hence LH and FSH (Singh et al., 1995).

FSH is essential for quantitatively normal spermatogenesis and fertility. Thus in transgenic male mice with a targeted disruption of the FSH receptor gene (Dierich et al., 1998) or of the FSH b-subunit gene (Kumar et al., 1997), though being fertile and displaying all stages of germ cell development (Singh et al., 1995), the testes are smaller and produced less spermatozoa (Singh et al., 1995, Dierich et al., 1998, Kumar et al., 1997). FSH receptor knockout mice also demonstrate defects in sperm development that lead to the production of low quality ejaculates (Krishnamurthy et al., 2000). These findings in the mouse are almost identical with those in human males exhibiting a mutation related to inactivation of the FSH receptor (Tapanainen et al., 1998). These observations underline the importance of FSH during the neonatal and postnatal period for testicular development and maintenance of quantitative normal spermatogenesis (Singh and Handelsman 1996a, b). It is also involved in fluid absorption/secretion (Jeguo et al., 1982).

2.3.3 Estrogens

In many papers estrogens and compounds exhibiting a partial estrogenic activity are described as negatively interfering with spermatogenesis and testicular function in general. Thus estrogen administration to experimental animals during the neonatal period or adulthood can impair sperm production and maturation (Steinberger and Duckett 1965, Meistrich et al., 1975) and there are various reports that exposure to estrogens in the

environment might have a detrimental effect on male reproductive development and health, and may be related to the reported decreases in sperm counts in men over the past 50 years (Auger et al., 1995, Sharpe and Skakkebaek 1993, Toppari et al., 1996, Sharpe 1997). Along this line Abney (1999) could show that neonatal estrogen exposure can interfere with Leydig cell development and proliferation during puberty. Ethane dimethansulfonate (EDS) has been shown to cause destruction of Leydig cells by blocking their regeneration (Abney and Myers 1991). However, a physiological role of estrogens in the male and hence also in testicular function is suggested by their production in the Leydig cell as was demonstrated for the boar (Claus and Hoffmann 1980; Conley et al., 1996, Fraczek et al., 2000, Rostalski et al., 2000; Mutembei et al., 2005b). Estrogen appears to inhibit proliferation of precursor and adult-type Leydig cells during pubertal development, a process important for the establishment of the adult complement of Leydig cells (Nistal et al., 1986, Hardy et al., 1991, Habert et al., 2001); they may therefore be important in controlling the steroidogenic capacity of the adult testis.

In granulosa cells of the ovary, estrogens induced the formation of transforming growth factor β (TGFβ), which in turn stimulated DNA synthesis; from these observations, Dorrington et al. (1993) concluded that in somatic cells of the gonads, FSH may induce aromatase activity and hence estrogen production, which in turn stimulates TGFβ, which then, along with FSH, promotes granulosa cell division. The fact that similar control mechanisms exist in Sertoli cells led to the hypothesis that prior to puberty estrogen may also participate in the FSH-mediated mitogenic activity on Sertoli cells via induction of TGFβ (Dorrington and Khan 1993, Dorrington et al., 1993).

Estrogens have also been suggested to stimulate postnatal spermatogonial division in the mouse (Li et al., 1997, Miura et al., 1999), they influence Sertoli cell maturation during the post proliferative period (Sharpe et al., 1998), where they have been shown to have a role in regulating the expression of the cell adhesion molecule neural cadherin (NCad) (MacCalman and Blaschuk 1994a, b) in cultured mouse Sertoli cells (MacCalman et al., 1997). Given that NCad is important for cell-cell interactions in the testis, particularly between germ cells and Sertoli cells (Perryman et al., 1996, Newton et al., 1993), this may be one way in which estrogens are involved in establishing and maintaining the seminiferous epithelium. There are also recent evidences for a direct role for estrogen in promoting survival and mitosis of germ cells. Estradiol implants in hypogonadal (*hpg*) mouse stimulated a 4-5-fold increase in seminiferous tubular volume in

the absence of measurable levels of androgens (Ebling et al., 2000) and treatment of boars actively immunised against GnRH with estrogen restarted up to 60% of the arrested spermatogenesis (Wagner and Claus 2004). Furthermore low concentrations of 17β-estradiol effectively inhibited male germ apoptosis, which was induced *in vitro* by incubating segments of human seminiferous tubules without survival factors (i.e., serum and hormones) (Pentikainen et al., 2000). Also round spermatid apoptosis was increased in the seminiferous tubules of older aromatase (Ar) KO mice (Robertson et al., 1999, 2002), also pointing to a role for estrogen in acting as a spermatid survival factor. Moreover adult monkeys treated with an aromatase inhibitor showed a decrease in the conversion of round to elongated spermatids and a decrease in sperm output from the testis, which suggests that estrogens are also important for spermatid differentiation (Shetty et al., 1997, 1998). Additionally the observation made in ArKO mice that round spermatids, that did not undergo apoptosis early in spermiogenesis, had acrosomal dysgenesis (Robertson et al., 1999) suggests that acrosome biogenesis could be an estrogen-dependent process.

The stimulatory effect of estrogens on spermatogenesis was also investigated by the administration of estradiol to *hpg* mice (Ebling et al., 2000), which lack FSH and LH due to a congenital deficiency of GnRH. The testes of these males were underdeveloped with spermatogenesis arrested at the early stages of germ cell development; however after administration of estradiol (SILASTIC implants) for a period of 70 days, their testes showed all stages of spermatogenic cell development (Ebling et al., 2000). This together with the fact that treating actively GnRH immunized boars with estrogen leads to a 60% restoration of the arrested spermatogenesis (Claus and Wagner 2004) strongly suggest that estrogens are capable of inducing spermatogenesis.

Although the above studies suggest a role for estrogen in germ cell development, it has been pointed out that the administration of a dual ERα/ERβ antagonist ICI 182,780 to wild-type mice for 35 days did not produce observable changes in the morphology of the seminiferous epithelium apart from the expected distension of the rete testis (Lee et al., 2000). However, the ability of the ICI antagonist to cross the blood-testis barrier and enter the adluminal compartment of the seminiferous epithelium to locally block estrogen action has not been demonstrated, nor has the ability of the antagonist to fully block the high levels of estrogen that may be present in the testis. Again, the possibility of other variant forms of ER taking over the roles of the classical ERs in the knockout models have not been eliminated (Scobie et al., 2002), which warrants further studies.

2.4 Steroid hormones and their mechanisms of actions

2.4.1 Overview on steroid hormones

Steroid hormones are a class of signalling molecules that are synthesized in response to a variety of neuroendocrine activities for a wide range of functions. All steroid hormones are derivatives of cholesterol and hence share the same basic ring structure, the cyclopentanoperhydrophenanthrene nucleus. According to the number of carbon-atoms and function, steroid hormones are divided into various classes (Table 2) (Schulster et al., 1976; Duke et al., 1995).

Table 2: Sources and functions of the main steroids

Steroid	Source	Main function
Estrogens (C18-steroids)	Adrenal cortex and gonads	Primarily involved in female sexual determination
Androgens (C19-steroids)	Adrenal cortex and gonads	Male sexual determination
Progestins (C21-steroids)	Ovaries and placenta	Maintains pregnancy
Glucocorticoids (C21)	Adrenal cortex	Gluconeogeneis, Decrease inflammation and increase resistance to stress
Mineralocorticiods (C21)	Adrenal cortex	Maintain salt and water balance

About 95% of the steroid hormone (SH) in blood is bound to plasma transport proteins and a sex steroid-binding protein (SBP), also known as sex hormone binding globulin (SHBG). SHBG specifically binds dihydrotestosterone (DHT), testosterone (T), and 17β-estradiol (E2) in plasma and hence contributes to the control of their metabolic clearance rates (Duke et al., 1995).

Until recently a conventional dogma held that protein–bound steroid hormones are inactive and that only free forms traverse cell membranes passively, owing to their lipophilic nature. However it has been demonstrated that megalin, a low density lipoprotein (LDL), functions as an endocytotic receptor in reproductive tissues (Petersen et al., 2002). Megalin provides a pathway for cellular uptake across the plasma membrane of androgens and estrogens bound to SHBG by forming a ternary complex with the steroid, the SHBG-complex (Hammes et al., 2005). The complex is thought either to endocytose the hormone into the cell, or to accelerate its diffusion by concentrating it onto the cell surface. In addition the ternary E2/SBP/SBP-receptor complex may function in signal transduction. In megalin receptor knockout mice, males had impaired descent of the testes and females had

blockade of vaginal opening, both of which critically dependent on sex-steroid signalling; similar defects are seen in animals treated with androgen- or estrogen-receptor antagonists.

Regardless of the type of steroid hormone, all act via membrane-bound and/ or nuclear receptors through a similar mechanism, with both genomic and non-genomic effects that interrelate. Their non-genomic functions are rapidly exhibited and are achieved via membrane-bound receptors through activation of second messengers, notably the mitogen-activated kinase signalling pathway (Aronica et al., 1994, Wehling 1997, Boldyreff and Wehling 2003). A steroid effect that occurs between a few seconds and 1–2 min after steroid application is likely due to a non-genomic rather than a genomic effect (Wehling 1997). Non-genomic responses e.g. glucose uptake, monovalent and divalent cation transport, water imbibitions, increased discharging activities of the secretory vesicles and rapid changes in electrical activity of neurones can not be blocked by inhibitors of DNA transcription or protein synthesis (e.g. actinomycin D or cycloheximide).

However the principal functions of steroids are through genomic activities achieved through nuclear receptors. Although genomic actions of steroids e.g. cell division (proliferation), cell growth and tissue development are much slower than the non-genomic responses, they are responsible for the prolonged synthetic effects. Though even more rapid effects of steroids on transcriptional processes are known, most actinomycin D– or cycloheximide-sensitive actions occur after a lag phase of 10 min or more. The additional delay at the effector level is probably the result of the time required for the processing of newly synthesized protein molecules (Wehling 1997). Hence a typical steroid hormone response has been described as the modulation of nuclear transcription, thus triggering genomic events that are responsible for physiological effects (Hendry et al., 1977, Hendry 1988, Rollerova and Urbancikova 2000).

Steroid-bound nuclear receptors act either at target genes that contain classical steroid responsive elements within their promoter regions or regulate transcription at promoter elements that directly bind heterologous transcription factors (Kushner et al., 2003), such as the AP1 (activated protein-1) sites and the variant cyclic-AMP responsive elements (CRE) which bind Jun/Fos- and c-Jun/ATF-2 transcription factors respectively. Nuclear steroid receptors are usually intranuclear but the unbound receptors shuttle between the nucleus and the cytoplasm (Schwabe et al., 1993). A cell's ability to respond to a particular steroid hormone is based on the presence or absence of the specific steroid receptors.

2.4.2 Steroid hormone receptors and induction of transcription

Steroid receptors are grouped into a large nuclear receptor superfamily, which evolutionally, might be derived from one ancestor. The three-dimensional structures of many nuclear receptor ligand binding domains (LBD) and of various ligand complexes have been determined and have provided an understanding of the structural basis of selectivity and mode of action of receptor agonists and antagonists (Gronemeyer et al., 2004). It is frequently the case that several paralogous genes that originated by gene duplications characteristic of the vertebrate lineage encode the receptor for a given ligand. This multiplicity of nuclear receptors is an important factor that contributes to both signal diversification and specification. However, whereas paralogue-selective ligands have been synthesized, it is not known whether endogenous ligands with such selectivities are operative *in vivo* (Gustafsson 2003).

Steroid receptors are a family of similar proteins with a six functional domain structure (Fig.6a) (O'Malley et al., 1991).

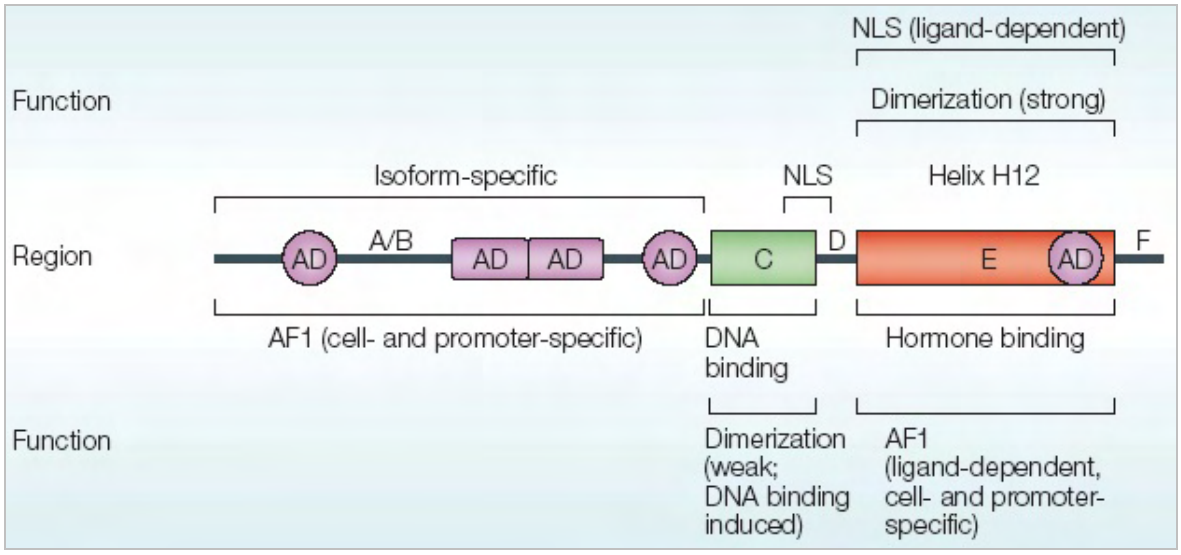

Fig. 6a: Schematic illustration of the structural and functional organisation of nuclear receptors. The evolutionarily conserved regions C and E are indicated as boxes (green and orange, respectively), and a black bar represents the divergent regions A/B on the N-teminal, D and Fon the C-terminal. Domain functions are depicted above and below the scheme. AD, activation domain; AF1, activation function 1; NLS, nuclear localization signal (Gronemeyer et al., 2004).

The N-terminal A/B domain contains transactivation function AF-1, which activates target genes by interacting with components of the core transcription machinery (Horwitz et al., 1996, Katzenellenbogen and Katzenellenbogen 2000).

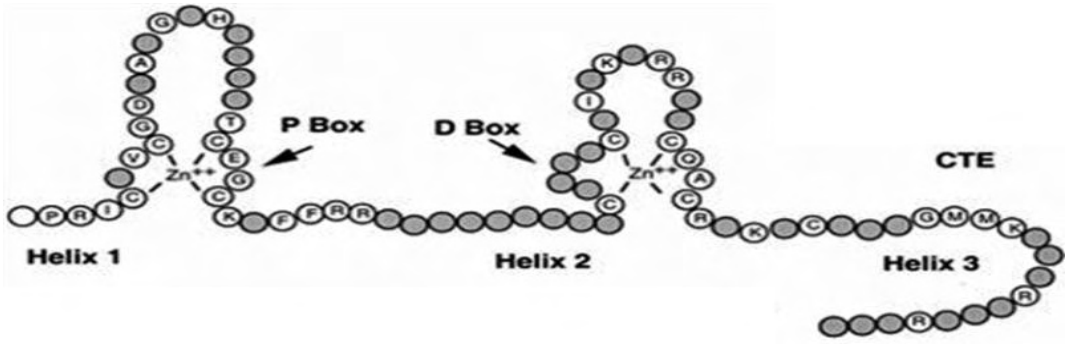

Fig. 6b: Structure of ER DNA binding domain (DBD) P box - transcription control region, D box - dimer specificity control region, CTE – COOH-terminal extension (Dechering et al., 2000).

The hydrophilic C domain is the DNA binding domain (DBD), which is highly conserved and contains two type II zinc-binding motifs that span 60-70 amino acids with a COOH-terminal extension (CTE). Each zinc ion is tetrahedrally liganded by four conserved cystein ligands forming a finger-like shape within the steroid receptor, hence the so-called first- and the second fingers of the receptor. The first finger (P box) controls the specificity of DNA binding while the second (D box) controls specificity of dimerization (Fig.6b) (Schwabe et al., 1993). The hinge or hyper variable D region allows the receptor protein to bend or alter its conformation (Tsai and O'Malley 1994).

The relatively large C-terminal E domain contains the ligand-binding domain (LBD) that determines ligand specificity (Mueller-Fahrnow and Egner 1999). Additionally, this domain harbours regions that allow the receptor to dimerize. The LBD contains 12 α-helices and two β-sheets, which are secondary structural elements, arranged in a so-called the α-helical sandwich-protein fold (Rollerova and Urbancikova 2000). The F domain in ER contains 42 amino acids, is not well conserved among different species and is concerned with transcription (Dechering et al., 2000).

Binding of a ligand to a nuclear receptor causes a series of downstream events, including receptor dimerization, receptor-DNA interactions mediated by SREs (steroid responsive elements) present in the promoter regions of the target genes, recruitment of and interactions with transcription factors, and formation of a preinitiation complex that ultimately causes changes in target gene expression (Fig. 6c). Unbound receptors are normally associated with chaperone proteins such as heat shock protein 70 and 90, which get dissociated after the receptor binds to its ligand. Transcription itself is affected by a number of factors, some receptor and cell-specific, and others harboured within the receptor. The receptor C-terminal domain contains AF-2 interacting surface that mediates ligand binding and dimerization to stimulate transcription activity and the N-terminal

domain encodes an activation function called AF-1 that mediates protein-protein interaction to induce transcription (Dechering et al., 2000, Gronemeyer et al., 2004).

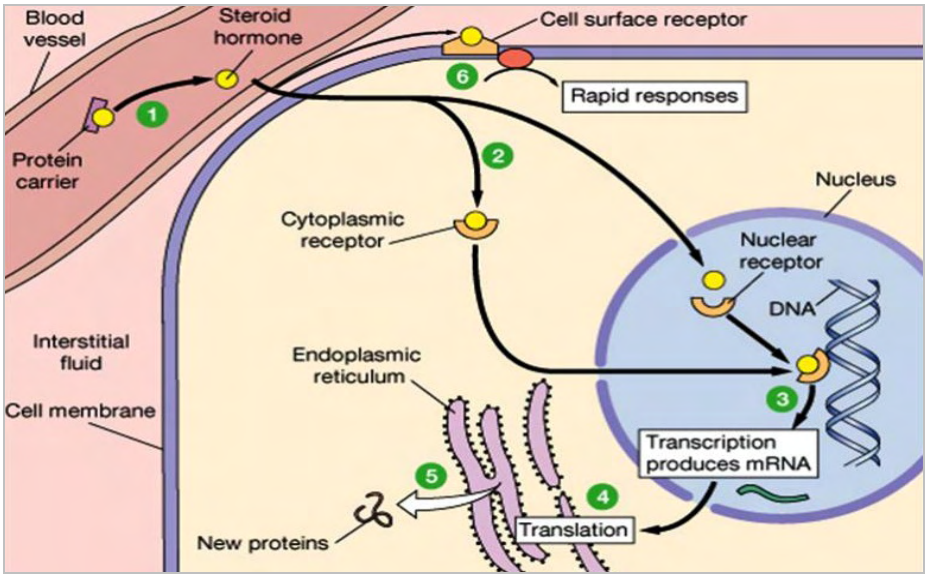

Fig. 6c: Steroid hormone-receptor interaction (Gronemeyer et al., 2004)

In the absence of the ligand, several of the nuclear receptors are believed either to be bound to the regulatory regions of target genes as a corepressor or associated with a histone deacetylase (HDAC) complex as apo receptors. Histone deacetylation causes chromatin condensation that accounts for the gene-silencing effect of apo receptors. Ligand binding releases the HDAC complex from the apo receptors and results in the recruitment of histone acetyltransferase (HAT) and chromatin-remodelling complex, a process that occurs in a receptor-, target-gene- and cell-specific manner. This process allows the polymerase II holoenzyme complex, comprising of the polymerase II enzyme, TATA-binding protein-associated factor (TAF) and other mediators to be recruited to initiate transcription. Then the activated genes create new mRNA that moves back into the cytoplasm and gets translated into new proteins for the required cellular processes. Also, the ligand can also interact with its cognate receptor within the cytoplasm where it can exert a 'non-genomic effect' by interacting either directly, for example, with membrane receptors that activate kinases or use second messengers to effect rapid non synthetic responses (Robyr et al., 2000, Dechering et a., 2000, Kushner et al., 2003, Gronemeyer et al., 2004).

2.4.3 Testicular steroid hormone synthesis

Cholesterol is the common precursor during steroidogenesis (Fig. 7) and it is initially converted to pregnenolone by CYP 450-SCC side chain cleavage enzyme.

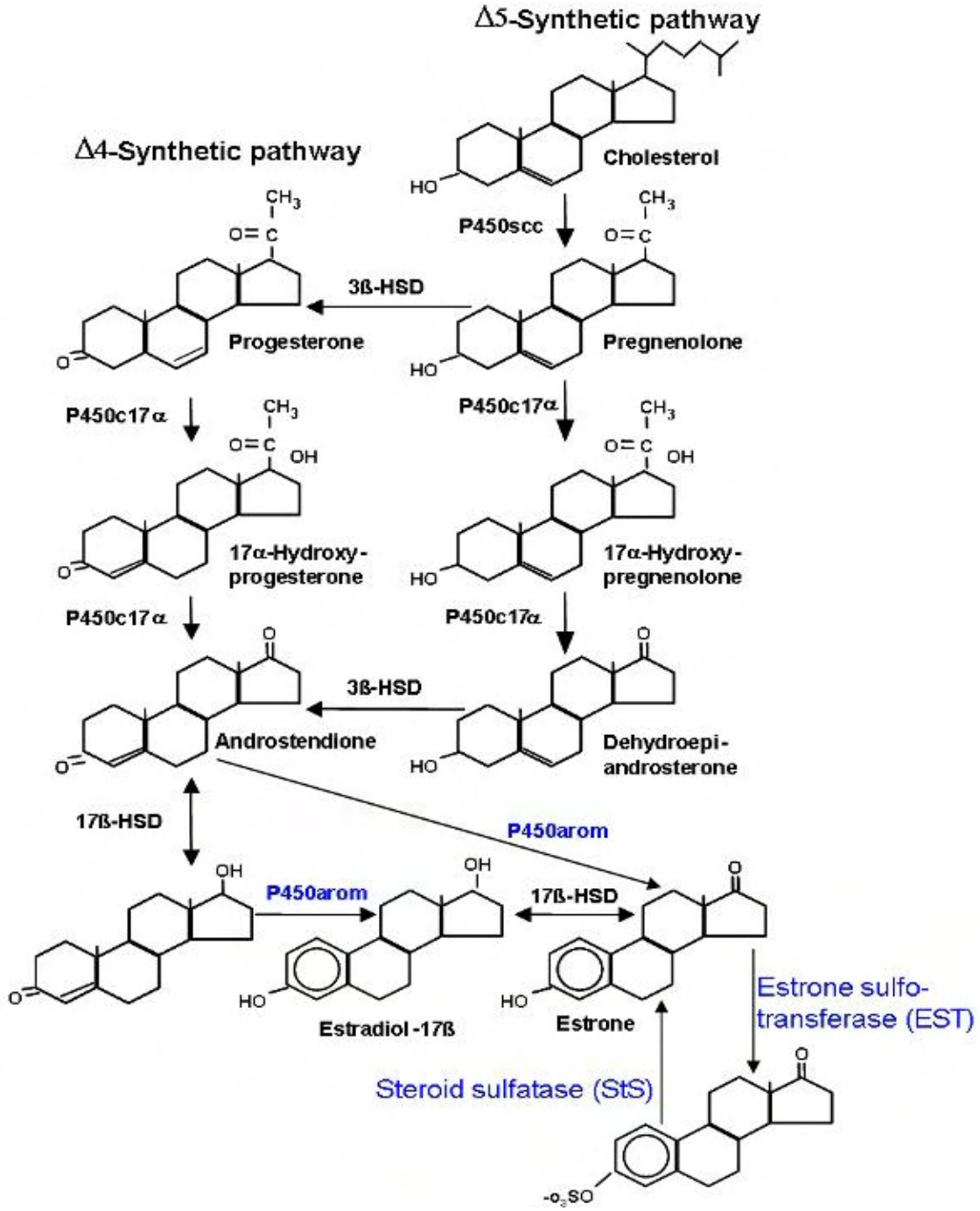

Fig. 7 Synthetic pathway of testicular steroid hormones P450 Scc –cytochrome P450 side chain cleave, P50c 17 α - Cytochrome P450c 17α hydroxylase, 3β-HSD - 3β-hydroxy steroid dehydrogenase, 17α-HSD - 17α-hydroxysteroid dehydrogenase, P450arom – Cytochrome P450 aromatase.

The conversion of cholesterol to pregnenolone by the CYP 450-SCC enzyme, which is present on the inner mitochondrial membrane, is the rate-limiting step in steroid production (Gill 1995). Following the Δ 4-pathway pregnenolone may be converted to

progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD) or via the Δ 5-pathway into 17α-OH-pregnenolone and dehydroepiandrosterone (DHEA) by the activity of 17α-hydroxysteroid dehydrogenase and C17,20-lyase. Both pathways lead to the formation of the C19-steroids androstenedione and testosterone.

Synthesis of estrogen occurs through conversion of the C19 steroids like androstenedione and testosterone to estrone (E1) and estradiol (E2), respectively. A microsomal member of the cytochrome P450 super family, the cytochrome P450 aromatase (P450 arom), which is a product of the CYP19 gene, catalyse this conversion. P450 arom is widely expressed in the testis across species (Oh and Robinson 1993, Simpson et al., 1997). The particular steroid hormone that a given tissue produces depends upon the nature of the tissue and the activity of the enzymes present in that tissue.

A special pathway involved in the regulation of estrogen bioavailability involves two enzymes, StS and EST. Sulfatase (StS) is an enzyme that is a product of the StS-gene located on the short arm of the X-chromosome (Yen et al., 1987). This enzyme is primarily localised to the endoplasmic reticulum (Chang et al., 1986). It catalyses removal of the sulfate group from 3- hydroxysteroid sulfates, such as cholesterol-3-sulfate, dehydroepiandrosterone-3-sulfate (DHEAS) and estrone-3-sulfate (Purohit et al., 1994). StS is expressed in various tissues, including in the testis (Munroe and Chang 1987) and placenta of the mouse (Shankaran et al., 1991), bovine (Greven et al., 2006) and human breast cells (Chandra 2003). Also in vitro studies with boar testicular tissue have demonstrated activity of this enzyme (Rostalski 2005). The presence of StS in the testis of various species may be suggestive of its involvement in the provision of free steroids.

Steroid sulfotransferases, which are cytosolic enzymes, reduce the bioavailability of free estrogens by catalysing the sulfonation of steroids (Hobkirk 1993, Strott 1997). Although their expression in the liver of animals and man has been assumed to play a role in maintaining steroid hormone homeostasis (Hobkirk 1985, Chatterjee et al., 1994), little was known about the structural and catalytic properties of the individual enzymes involved until molecular cloning studies and characterization of expressed enzymes yielded insights regarding their evolution and potential physiological roles (Hobkirk 1993, Weinshilboum et al., 1997). This led to identification of an estrogen-specific sulfotransferase (EST), which was shown to be evolutionarily distinct from steroid sulfotransferases that preferentially metabolize non phenolic hydroxysteroids (Weinshilboum et al., 1997, Nash et al., 1988, Oeda et al., 1992, Song et al., 1995).

The sequence of EST was found to have a higher degree of homology with the family of phenol sulfotransferases than with the hydroxysteroid sulfotransferases (Hobkirk 1993, Weinshilboum et al., 1997). In addition, functional characterization of heterologously expressed EST protein demonstrated that it catalyzes the specific sulfonation of estrogen (estradiol, estrone, and estriol) with K_m values in the low nanomolar range (Song et al. 1995, Falany et al., 1995). These observations provided good evidence for a specific role for EST in modulating the activities of estrogens in vivo. Thus, EST can be defined as a cytosolic enzyme that catalyzes the specific sulfonation of estrogens at the 3-hydroxyl position using 3'-phosphoadenosine-5'-phosphosulfate as an activated sulfate donor. Sulfated estrogens are hormonally inactive and no longer bind to the estrogen receptor.

Although the liver has been considered a primary site for steroid sulfotransferase activities, significant activities have also been found in other organs, e.g. the bovine placenta (Moore et al., 1988; Hoffman et al., 2001). EST is also expressed abundantly in the testis of rat and man (Song et al., 1995), suggesting that testicular expression of EST may be a common phenomenon among different species. In the boar, Reaside (1983) reported conjugation of estrogens within cultured Leydig cells and Rostalski et al. (2000) reported some activities of EST in testicular tissue homogenates. In the mouse testis, EST was localized selectively to the Leydig cells, with its expression being under the control of LH and regulated differentially during development (Song et al., 1997). Together, this information seem to suggest that testicular expression of EST may play a role in male reproduction, conceivably by modulating the activity of locally synthesized estrogens in the testis.

Steroids can also be conjugated with glucuronic acid by the enzyme glucuronyl transferase. This reaction takes place in the liver and uridine diphosphoglucuronic acid is the glucuronide donor. Like sulfonation glucuronidation makes the steroid more water-soluble and is also a process of their inactivation for excretion. Another important factor regulating steroid hormone activity is peripheral conversion, for example reduction of testosterone via 5α-reductase to dihydrotestosterone, producing a more potent form of testosterone (for review see O'Malley et al. 1991).

2.5 Estrogen receptors (ERs) and their expression in the testis and epididymis

Estrogen receptors exist in two isoforms, the ERα and ERβ, which originate from two separate genes on different chromosomes.

As indicated in Fig. 8 the two isoforms are highly homologous in their DNA binding domain (over 90%) while homogeneity in their ligand-binding domain is less than 60% (Cowley et al., 1997). They exhibit a similar affinity for estradiol-17β but not for other ligands and form homo- and heterodimers (Muramatsu and Inoue 2000), allowing them to act as selective estrogen receptor modulators (SERMs) (Riggs and Hartmann 2003).

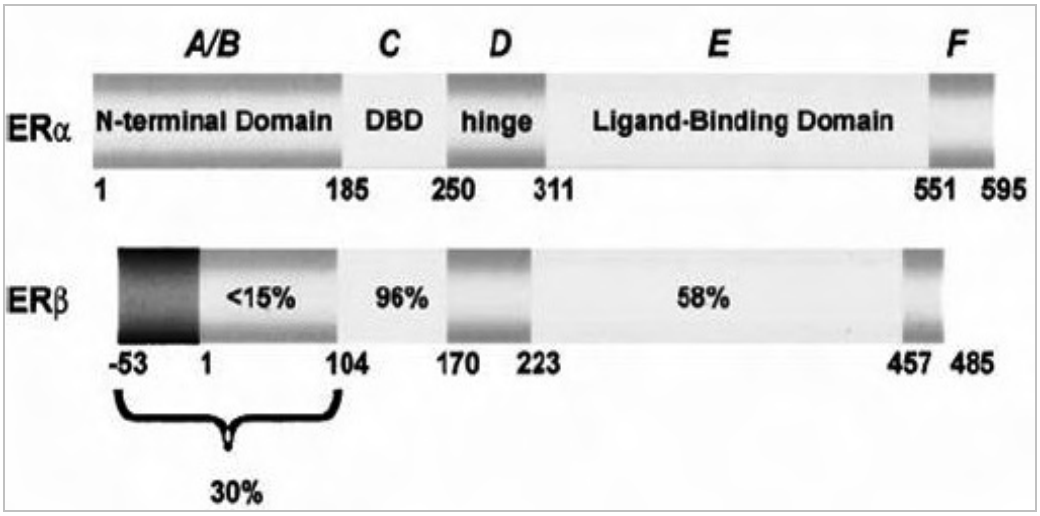

Fig. 8: Comparison of the structure of ERα and ERβ proteins. The functional domains A–F and the percentage homology of ERβ compared with ERα are shown. Indicated are the N-terminal domain (A/B domain), the DNA binding domain (DBD) (C domain), the hinge region (D domain), the ligand-binding domain (E domain), and the C-terminal region (F domain). The two potential start sites on ERβ are designated -53 and +1 (O'Donnell et al., 1991).

More recently it has become clear that estrogen signalling can be viewed as a balancing act between ERα and ERβ (Weihua et al., 2000, Lindberg et al., 2003, Gronemeyer et al., 2004); ERα is often an activating factor, whereas ERβ suppresses and/or modulates the effects of ERα. For instance, estradiol-17β-activated ERα stimulates the proliferation of MCF-7 breast cancer cells, whereas ligand-activated ERβ acts as an antiproliferative factor and prevents the effects of ERα. The antiproliferative effects of ERβ are also seen in colon and prostate cancer cells (Gustafsson 2003).

Both, the ERα and ERβ, are widely distributed in the epididymis of the male reproductive tract (Echeverria et al., 1994, Hess et al., 1997, 2001, Carreau et al., 1999, 2003, O` Donnell et al., 2001, Hess 2003, Carpino et al., 2004, Hess and Carnes 2004).

They are also expressed in the testis as was shown for the human (Enmark et al., 1997, Durkee et al., 1998, Pelletier and El-Alfy 2000, Pentakainen et al., 2000, Taylor and Al-Azzawi 2000, Takeyama et al., 2001, Saunders et al., 2002), monkey (West and Brenner 1990, Fisher et al., 1997, Saunders et al., 2001), goat (Goyal et al., 1997), dog and cat (Nie et al., 2002), rat and mouse (Lubahn et al., 1993, Saunders et al., 1998, Zhou et al., 2002, Oliveira et al., 2004).

In the testis, ERβ is the more abundant receptor and is typically found in nearly every cell type of the interstitium and the seminiferous tubule, except for the elongated spermatids (Table 3) (Saunders et al., 1997, 1998, Rosenfeld et al., 1998, van Pelt et al., 1999, Bilinska et al., 2000, 2001, Jefferson et al., 2000, Pelletier 2000, Pentikainen et al., 2000, Taylor and Al-Azzawi 2000, Makinen et al., 2001, McKinnell et al., 2001, Saunders et al., 2001, Takeyama et al., 2001, Nie et al., 2002, Zhou et al., 2002).

Table 3: Localization of ERα and ERβ in the testis and epididymis in various species

Cell type	**Rat**	**Mouse**	**Dog**	**Cat**	**Man**
Leydig	αβ	αβ	α	αβ	-/+α
Sertoli	β	β, -/+α	-	β	-
Germ cells	β, -/+α	β, -/+α	-	β	αβ
Peritubular	αβ	-/+β, -/+α	αβ	β	-
Rete testis	β	αβ	αβ	αβ	-
Efferent ductus	αβ	αβ	αβ	αβ	α
epididymis	αβ	αβ	β	β	-

-/+ means some authors detected presence of the receptors while others reported otherwise

The ERα has been identified in the interstitium of the testis of the rat, mouse dog, cat and human (see in Table 3) (Iguchi et al., 1991, Echeverria et al. 1994, Fisher et al., 1997, Pelletier and El-Alfy 2000, Pentikainen et al., 2000, Nie et al., 2002, Zhou et al. 2002) but it is not apparently expressed in the Leydig and peritubular myoid cells of the testis of the goat, monkey and human (West and Brenner 1990, Goyal et al., 1997, Saunders et al., 2001). ERα has also been localised in germ cells of the rat (Lubahn et al., 1993, Saunders et al., 1998, Oliveira et al., 2004), mouse (Bilinska et al., 2001, Zhou et al., 2002, Sipila et al., 2004), and vole (Bilinska et al., 2001), but not of the dog and cat (Nie et al., 2002, Zhou et al., 2002).

2.6 Functions of estrogen in the epididymis and testis

Estrogens are involved directly in regulating epididymal fluid reabsorption (Hess 1990), a process not only indirectly affecting testicular function but also essential for maintenance of normal fertility (Lubahn et al., 1993, Korach 1994, Hess et al., 1997, 2000). However, the direct roles for estrogens in testicular cells still need further clarification.

It is well established that administration of estrogens during the neonatal period or adulthood impairs sperm production and maturation (Steinberger and Duckett 1965, McLachlan et al., 1975a, b, Stillman 1982, Raman-Wilms et al., 1995, Cheek and McLachlan 1998, Toppari et al., 1996, Toppari and Skakkebaek 1998, Vom Saal et al., 1998, Akingbemi and Hardy 2001, Norgil Damgaard et al., 2002), indicating that estrogens have the ability to perturb spermatogenesis. More so, loss of estrogen synthesis in the aromatase knockout mouse (O'Donnell et al., 2001, Robertson et al. 2002) resulted in decreased fertility with age due to lesions associated with round spermatids and decreased volume and length of the seminiferous tubule. These effects were accelerated and enhanced when the ArKO male was maintained on a soy-free diet, suggesting soy based phytoestrogens likely protected the testis somewhat in the ArKO mouse. Testosterone concentrations are elevated in the ERαKO male (Eddy et al., 1996) and estrogens seem to have a subtle effect on Leydig cell function because pure ER inhibitor ICI 182,780 on them showed increased steroidogenesis (Akingbemi et al. 2003). ERα has been shown to support somatic cells and spermatogenesis (Mahato et al., 2002).

Other studies with mice evidence in developing testis have revealed that estradiol has a significant activity in initiating gonocyte mitosis, establishing Sertoli cell function

(O'Donnell et al., 2001), inhibiting germ cell apoptosis (Pentikainen et al., 2000) and establishing Sertoli-germ cell adhesion (MacCalman and Blaschuk 1994b, MacCalman et al., 1997). *In vitro* models have also provided evidence that 17β-estradiol can induce neonatal testicular gonocyte and spermatogonia proliferation (Li et al., 1997, Miura et al., 1999). A stimulatory effect of a 4 to 5-fold increase in seminiferous tubular volume in estradiol-implanted *hpg* mice (Ebling et al., 2000) and a restoration of up to 60% of previously arrested spermatogenesis in estrogen-treated boars actively immunised against GnRH (Wagner and Claus 2004) proved that estrogens influence spermatogenesis. In the boar this effect could be directly on germ cells given the fact that estrogen does not appear to play a regulatory role on gonadotropin secretion (At-Tarus et al., 2006a). Estrogens have been shown to activate genes with AP1 and CRE promoters elements, which respectively binds Jun/Fos and c-Jun/ATF-2 transcription factors to mediate breast and uterine cellular proliferation (Peach et al., 1997) and probably a similar ER-mediated mechanism of action occurs in testicular germ cells to initiate and maintain their proliferative activities.

Another direct testicular function for estradiol is inhibition of germ cell apoptosis (Pentikainen et al., 2000), a process that is most likely mediated via the novel non-genomic signalling pathway through ERs located on plasma membrane (Collins and Webb 1999). 17β-estradiol bound to membrane receptors results in rapid (within minutes) activation of mitogen-activated kinase signalling cascade, which in endothelial cells, has been associated with activation of endothelial nitric oxide synthetase and production of nitric oxide, which, at relevant physiological levels, has been shown to suppress apoptotic pathways in a variety of cell types (Kim et al., 1999).

Although ERβKO mice seemed surprisingly fertile (Krege et al., 1998), the recently discovered variant forms of ERβ (Scobie et al., 2002), including forms expressed specifically in normal fetal germ cells (Gaskell et al., 2003) and in testicular germ cell tumors (Pais et al., 2003) may have indeed supported functions originally attributed to the classical ERβ. Moreover, its fertility was assessed between 6 and 12 weeks of age, at which age, also no change in testicular morphology in ArKO mice was observed (Robertson et al., 1999). Therefore evaluation of fertility in older ERβKO animals could be interesting. Thus overall, understanding the role that estrogen plays in the testis is certainly complicated but it does appear to have subtle functions, not only at the Leydig cell but also possibly targeting the seminiferous epithelium, too. These roles need to be studied further and preferably from integrated approaches, certainly also involving animal models.

2.7 Aims and objectives of the study

2.7.1 Aim

The aim of the present study was to test if in the boar testis there are sources and targets of estrogens like in other species. Thus, the study aimed at tracking for the presence of estrogen receptors α and β, aromatase, steroid sulfatase and estrogen sulfotransferase proteins and their mRNA with age of development and spermatogenesis (Immature and mature boars).

2.7.2 Objectives

The following were the objectives of the present study.

1. To study expression patterns of estrogen receptor α and β (targets).
2. To confirm expression of P450 aromatase (source)
3. To study expression patterns of steroid sulfatase and estrogen sulfotransferase, the enzymes likely to influence bioavailability of free estrogens in the testis.

3. MATERIALS AND METHODS

3.1 Tissue Collection and Sample Preparation

3.1.1 Experimental animals and design

Testicular samples for this study were collected from 25 healthy Large White boars kept at the Liebig-University Hardthof veterinary farm in Giessen. The animals were randomly obtained from 5 litters, allotted to five age groups (5 animals per group) and castrated at ages of 50, 100, 150, 200 and 250 days. Tissue collection and conservation was aimed at preserving protein and mRNA in order to study their expression patterns. Porcine uterine control samples for ER trials were obtained from previously conserved blocks that were made using tissue collected from a local slaughterhouse in Giessen.

3.1.2 Sample collection and tissue preservation

The boars were castrated following routine clinical procedures. For anaesthesia the 50 day-old animals were injected intramuscularly, and the older ones infused intravenously via the vena auricularis with a drug combination comprising of azaperone (20mg/kgbwt), levomethadone (0.5mg/kgbwt) and ketamine (20mg/kgbwt).

Following removal, the testes were trimmed off the epididymides and excess connective tissue, cleaned off excess blood using phosphate buffered saline (PBS, pH 7.2) and cut longitudinally into two halves using a scalpel blade. From each half, five to six approximately 1 cm^3 parenchyma samples were taken from the area between the tunica albguinea and the mediastinium testis. Samples for immunohistochemistry (IHC) and *in situ* reverse transcription polymerase chain reaction (RT-PCR) were fixed for 24h at 4°C in neutral phosphate buffered 4% formalin with gentle agitation. After washing in PBS and dehydration through a graded ethanol series (30%, 50% and 70%), the samples were infiltrated with paraffin (60°C) overnight, embedded in paraffin wax (Histo-Comp-Vogel, D-35392, Giessen) and mounted onto blocks, which were then stored at 4°C until use.

For *in vitro* RT-PCR testicular parenchyma samples were frozen immediately in liquid nitrogen and stored at –80°C until RNA extraction.

3.2 Immunohistochemistry (IHC)

3.2.1 Slide preparation

5µm thick sections were cut from the tissue blocks using a microtome (Reichard Jung AG, Heidelberg), and mounted onto Super Frost-plus glass slides (Menzel Glaeser, D-38116 Braunschweig). The slides were mostly dried at room temperature overnight prior to immunostaining but in the cases when the procedure was done the same day, drying was achieved by letting the slides stay at 60^{o}C for one hour.

3.2.2 Staining procedure

Indirect immunoperoxidase staining methods were applied using the strepavidin-biotin technique for signal enhancement following standard procedures (Schuler et al. 2002). Sections were deparaffinised for 2x4 min in xylene, rehydrated for 2x2 min through graded ethanol (99%, 95%, 70%, and 30%) and washed under running tap water (5 min).

Antigen retrieval was achieved by pre-incubation of the rehydrated sections in 10mM citrate buffer, pH 6.0 for 5 min prior to 3x5 min microwave irradiation in pre-heated citrate buffer in an oven run at 560W. The slides were allowed to cool for 20min at room temperature, then washed for 5 min under running tap water and incubated in 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity.

Sections were then washed with IHC buffer (phosphate buffered saline with 0.3% tritonX) and covered for 20 minutes with appropriate serum to block non-specific binding sites. Following draining of excess blocking serum, the respective primary antibodies, diluted in IHC buffer at appropriate dilutions (Table 4) were applied and the sections were incubated for 20 h (over night) at 4°C.

In the following day, excess antibody solution was drained and the sections were washed 2x10 min in IHC buffer and incubated with biotinylated secondary antibody diluted in IHC buffer for 30 min at room temperature. Immunolocalisation of epitope antibodies was achieved using the streptavidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlingame, CA 94010, USA). After washing with IHC-buffer for 3x5 min immunoreactivity was visualized using Novared substrate kit (Vector Laboratories, Burlingame, CA94010, USA) as the chromogen.

Sections were then thoroughly rinsed in running tap water, lightly counterstained with haematoxylin, dehydrated, cleared in xylene and mounted using Histokit (Assistant, D-35520 Osterode).

Negative controls were set up using the irrelevant isotype-specific antibodies (see table 4) at an equal concentration of the respective primary antibody. For the estrogen receptor isoforms, porcine uterus was used as a positive control.

For ease of application and control, the IHC procedure was summarized in a tabulated format (Annex 1).

Table 4: Details of antibodies used for immunohistochemistry

Antigen	*Clone*	*Source*	*Immunogen*	*Dilution*	*Species*	*Description*
ERα	HT227	Dr. H.H Thole 1*	c-terminus of the porcine ERα	1:100	Mouse	Mouse antipig ERα monoclonal
ERβ	PPG5/10	Serotec, D-40210 Düsseldolf	conserved c-terminus of the human ERβ1	1:20	Mouse	Mouse antihuman ERβ1, monoclonal
Aromatase	SM1671P	Acris, D-32120 Hidden-hausen	conserved region in human P450 arom	1:500	Mouse	P450 aromatase primary monoclonal
Steroid sulfatase		Dr. Ugele 2*	Epitope within human sulfatase	1:2500	Rabbit	Rabbit antihuman polychronal
Estrogen sulfotransferase		Dr. Wenchao Song 3*	Mouse EST	1:7000	Rabbit	Rabbit antimouse EST, polychronal 4*
Negative	IgG1	Dianova GmbH, D-20148 Hamburg	Non-specific	1:100	Mouse	mouse isotype control antibody for ERα
negative	IgG2a	Vector Laboratorie, Burlingame, CA94010	Non-specific	1:200 1:500	Mouse	mouse isotype control antibody for ERβ and P450 aromatase
Secondary antibody	BA 2000	Beckman Coulter Miami, CA 33196-2500	Mouse IgG	1:200	Horse	Biotinylated horse anti-mouse IgG

1* Dr. H. H. Thole formerly at the Max-Planck Institute for Experimental Endocrinology, Hanover; 2* Dr. B. Ugele in Frauenklinik Innenstadt, Universität München; 3* University of Pennsylvania, Philadelphia, USA; 4* negative was set up using rabbit serum.

3.2.3 Quantitative Evaluation of Positive Cells

For quantitative assessment of ER positive cells, a person blinded for group and animal information, evaluated three immunostained sections from each animal for each receptor isoform. The types of positive staining cells were identified and in each of the three sections for each cell type, the total number and the numbers of ER positive cells were counted at a 200-fold magnification.

In the case of sexually mature boars, from each slide, at least three different arbitrarily views corresponding to different stages of germ cell development (see below) were chosen. In immature boars three arbitrarily chosen views of approximately 200 cells each were evaluated. Based on the results obtained, expression of ER in the testis was assessed in relation to the sexual development (immature and mature), location of the cells (seminiferous epithelium and interstitium) and stage of spermatogenesis. The histological appearance of the seminiferous tubules was used to define animals as immature (prespermatogonia and Sertoli cells only) or mature (fully developed spermatogenesis).

In respect to the stage of spermatogenesis, staining by IHC did not allow a complete application of Swierstra's criteria to divide spermatogenesis into the eight stages (Swierstra 1968a, b). Hence and as described by Kohler (2004), spermatogenesis was grossly divided into three arbitrary phases, the first immediately after spermiation without elongated spermatids, the second during elongation of the spermatids and the third just prior to spermiation with fully elongated spermatids.

For statistical evaluation, a three-factorial partial hierarchical analysis of variance (age group [G], animal within age group [A (G)], section within animal and age group [S (AG)] using BMDP statistical software, program BMDP8V (Dixon 1993) was performed.

3.3 *In vitro* reverse transcription polymerase chain reaction (RT-PCR)

3.3.1 RNA isolation

3.3.1.1 Extraction procedure

Coarse pieces of deep-frozen (-85 °C) testicular parenchyma tissue were enveloped in sterile aluminium foil and grounded into smaller particles by hammering against a solid surface. The resulting particles were then powdered under liquid nitrogen using a pestle in a mortar, which were placed into ice for one hour prior to isolation and thereafter pre-chilled to about -80°C using liquid nitrogen. Approximately 200mg of the ground tissue

were then immersed in 3ml lysis buffer containing 30µl β-mercaptoethanol and homogenized by three times 30 sec bursts using an Ultra Turrax T25 (IKA-Werke GmbH & Co KG, D-79219 Staufen i.Br.). Subsequently, a silica gel based RNA extraction was performed according to the instructions of the kit supplier (RNeasy Mini Kit, Qiagen, D-40724 Hilden) (Annex 2).

3.3.1.2 Measuring concentration

After extraction the RNA concentration in microgram per millilitre (µg/ml) was measured using a UV-Eppendorf BioPhotometer 6131 (BioPhotometer, Eppendorf® AG, Hamburg) applying wavelengths 230nm, 260nm and 280nm. A dilution of 1:20 (5µl of the sample in 95µl of double distilled water) in a plastic cuvette tube (Eppendorf Uvette® 220-1600 Nm, MAGV laboratory need & laboratory instruments, Rabenau Londorf) was utilised.

3.3.1.3 Standardisation of concentration and RNA storage

In order to ensure that a uniform amount of RNA was used during RT reactions, a standard concentration of 200ng/µl in the working solution aliquots was prepared using double deionised water (DEPC-Water) from the stock solution. The following formula was used to achieve this concentration in 30µl of the standardised RNA solutions.

200 ng x 30 µl = measured RNA conc. (ng/µl) in stock solution x Y µl (required volume).

This means the volume (Yµl) of RNA to be drawn from the stock solution would be equivalent to 6000/the measured RNA conc.

Then this volume (Yµl) from the stock RNA solution is toped up to 30µl using DEPC-Water.

The standardised RNA (200ng/µl) was then stored at –20°C as the working solution and the rest of the RNA was stored at –85°C as stock solution.

3.3.2 RT Step of the in vitro RT-PCR

GeneAmp RNA PCR Kit (Perkin Elmer, Foster City, CA) was used for the RT-PCR and the first strand cDNA was synthesized from 300ng of total RNA (1.5µl of

200ng/µl) using 8.5µl of the RT-mix (Table 5) prepared according to the instructions of the kit supplier (Perkin Elmer, Foster City, CA). This was pipetted in multiples according to the number of mRNA samples.

Table 5: Summarized formula for the RT-mix protocol

Components	*Simple calculation (in µl)*	*Stock solution*	*Used solution*
$MgCl_2$	2	25mM	5mM
10xPCR-buffer*	1	10x	1x
dGTP	1	10mM	1 mM
dTTP	1	10mM	1 mM
dATP	1	10mM	1 mM
dCTP	1	10mM	1 mM
Random Hexamers	0.5	50µM	2.5µM
RNAse-Inhibitor	0.5	20U/µl	1U/µl
Reverse Transcriptase	0.5	50U/µl	2.5U/µl
Total volume	8.5		

*10 times concentrate of 500 mM KCl, 100mM Tris-HCl

A one step reverse transcription was carried out in plastic tubes (Biozym Diagnostik GmbH, Hessisch Oldendorf) in an automated program in a T1 Thermocycler 48 (Whatman Biometra, Göttingen) using the settings of 8 min. at 21 ºC, 15 min. at 42 ºC, 5 min. at 99 ºC, 5 min. at 5 ºC, and then 4 ºC until withdrawal.

3.3.3 PCR step of the RT-PCR

The primer pairs for the PCR step were deduced from published porcine sequences using a computer Software Oligo Analyser 1.0.2/Oligo Explorer 1.1.0. (Free Internet software; © Teemu Kuulasmaa, University of Kuopio, 70211 Kuopio, Finnland), ordered from MWG-Biotech (D-85560 Ebensburg, Germany) and used in PCR reactions to amplify products of expected base pair cDNA fragments in a T1-Thermo Cycler (Biometra GmbH, D-37005 Göttingen) (Table 6).

Table 6: Primers used in RTPCR for detection of mRNA specific for ER, aromatase, StS and EST, their nucleotide positions (nt) in published porcine mRNA sequences and the length of resulting DNA fragments

Primer	*Sequence utilized*	*Position (nt)*	*Amplicons length (nt)*	*Accession No.*
ERα-forward	5'-AGG GAG AGG AGT TTG TGT G-3'	170–189	305	AF035775
ERα-reverse	5'-TCT CCA GCA GCA GGT CAT AG-3'	474 455		
ERβ-forward	5'-ACA CCT CTC TCC TTT AGC C-3'	243–262	239	AF267736
ERβ-reverse	5'-CCT GAC GCA TAA TCA CTG-3'	481 464		
P450arom-forward	5'-TTA GCA AGT CCT CAA GTG TG-3'	421–440	324	U37311
P450arom-reverse	5'-CCA GGA AGA GGT TGT TAG AG-3'	744 724		
EST-forward	5'-GAG AAC GAA TCC ACA AGT	819 828	303	AF389855
EST-reverse	5'-TGC TGC TCA TAG TGA ATG TC	1121–1101		
StS-forward	GAA GAC AGG ATC ATT GAC G	137 155	172	DQ139314
StS-reverse	AGA ACT TGG GTG TGA AGA AG	308–298		

The PCR reaction mixture per tube consisted of 10µl of cDNA sample added to 40µl of a premixed solution containing 1µl primer-mix (forward and reverse; each 15pmol/µl), 0.25µl Amplitaq Gold DNA polymerase (5U/µl), 2µl $MgCl_2$ (25mM), 4µl 10x PCR-buffer (500mM KCl, 100mM Tris-HCl) and 32.75µl double distilled autoclaved water; this solution was prepared for a set of 15 samples. At first, $MgCl_2$, PCR-buffer and DEPC water were prepared (Table 7) and thereafter the pre-mixed primer (reverse and forward) added (Table 8). Porcine-specific β-actin primer pairs (Gen. Acc.No. SSU07786) were used as an internal control while negative controls were set up using autoclaved water instead of RNA in the RT reaction mix.

Table 7: Summarized PCR-premix preparation protocol

Components	*Simple calculation (in µl)*	*Stock solution*	*Used solution*
$MgCl_2$	2	25mM	1mM
10xPCR-buffer*	4	10x	1x
DEPC-H_2O	32.75		
Total volume	38.75		

Table 8: Final PCR mix preparation protocol

Components	*Simpler calculation (in µl)*	*Stock solution* *Used solution*	
PCR-Premix	38.75		
Primer-mix*	1.00	10pmol/µl	0.2pmol/µl
GOLD Amplitaq	0.25	5U/µl	0.025U/µl
Total volume	40		

* 1µl primer-mix contains both forward and reverse primers, each at 15pmol/µl.

The hot-start PCR reaction was run for 10min at 95°C to activate Amplitaq Gold DNA polymerase followed by the program comprising of 35 cycles (Table 9).

Table 9: PCR Program settings

Program step	*Temperature*	*Duration*	
Initial Denaturing	94°C	1 min	
Amplification steps (Begin of cycles)			
Denaturing	94°C	1 min	Repeated for 35 cycles
Anealing	At 58.5°C (ERα), 55°C (ERβ)	2.15min	
Extension	72°C	1.30 min	
End of cycles			
Final extension	72 °C	6 min	
Until withdrawal	4°C		

3.3.4 Analysis of the PCR-amplicons by Gel electrophoresis

The amplicons were separated and visualized on a 2% ethidium bromide stained agarose gel and visualized under UV transillumination. 1.82g of agarose in 91 ml of Tris-Borate-EDTA (TBE) buffer was first heated in the microwave to dissolve completely and allowed to cool to about 60-65°C. Then 1.9 µl of the ethidium bromide (Table 10) (Carl Roth Gmbh&Co., 2218, Karlsruhe) was added, carefully mixed and poured into a horizontally set gel container (GibcoBRL, Life Technologies, Karlsruhe). The gel was then allowed to set for about 25-30 min at room temperature.

Table 10: Protocol for preparing 2% ethidium bromide stained agarose gel

Gel	*TBE-buffer (ml)*	*Agarose (g)*	*Ethidium bromide (µl)*
Small	20	0.4	0.5
Medium	34	0.68	0.8
Large	91	1.82	1.9

Meanwhile, the probe and the marker were prepared as follows: -

Probe 1.5 µl loading dye + 15 µl PCR product (cDNA)

100 bp Marker 1.5 µl loading dye (6x Loading Dye Solution; MBI Fermentas, St. Leon-Rot) + 1.5 µl Marker (100 ng/µl) (MBI Fermentas, St. Leon-Rot) + 7 µl DEPC-Water

Subsequently, the gel was immersed in TBE buffer contained in an electrophoresis machine (LKB Bromma, Golden, USA), the probe and the marker were loaded into the, wells and let to run for 35-40min under a voltage of 125 V and a current of 300 mA for the amplicons to separate. The separated ampilcons were then visualized under UV transillumination using a UV-Transluminator fitted with camera linked to Photoshop Software; Phoretix Grabber 3.01 (Biostep GmbH, Jahnsdorf) and photographs taken.

3.4 One Step *in situ* RT-PCR for ERα and ERβ

3.4.1 Introduction and slide preparation

This method detects incorporated digoxigenin (DIG)-labelled uridine triphosphate (11-dUTP) in the DNA amplified *in situ* (Sander et al., 1996; Schiller et al., 1998). Sections of 5µm cut from formalin-fixed paraffin-embedded tissue were mounted onto the cavities of TESAP (3-triethoxysilyl-propylamine) coated Superior glass slides (Paul Marienfeld GmbH and Co. KG, Lauda-Koenigshofen, Germany) and dried for 1h at 37°C. They were then deparaffinized in xylene (15min) and rehydrated in descending isopropanol (100%, 70%; 5min each) series. Then the slides were air-dried for 1hr and subsequently washed twice in DEPC water. Permeability was improved with protease K (Sigma, Deisenhofen, Germany) at a concentration of 2µg/ml 0.1N HCl at 25°C for 5 min, which was then stopped by washing three times in DEPC water. To digest genomic DNA, the slides were treated overnight at 37°C in an RNase-free DNase solution (Roche Molecular Biochemicals, Mannheim, Germany) at a concentration of 1U/µl in DNase buffer (100 mM NaAc, pH 5.0; 5mM $MgSO_4$), omitting this step constituted the positive controls.

3.4.2 In situ RT-PCR step

In the following day the slides were washed twice in DEPC water, then in 100% isopropanol and finally air-dried for 1hr. The *in situ* RT-PCR step was performed with the GeneAmp *in situ* PCR thermal cycler (System 1000; Perkin Elmer). Slides were placed on an assembly tool (Perkin Elmer) and overlaid with 50µl of the reaction mix containing 29µl DEPC-H_2O, 1µl each of nucleotides, the dATP, dCTP, dGTP, dTTP (each 10 mM stock), 1µl Dig-UTP 10mM (Roche Diagnostics GmbH, Steinheim, Germany), 6µl Mn (OAc) 225mM, 1µl RNAse inhibitor (Roche Diagnostics GmbH), 10µl 5'-EZ buffer (EZ rTth RNA PCR kit; Perkin Elmer), 3.0 µl of primers forward and reverse (20 µM stock) and 2µl recombinant thermus thermophilus (rTth)-DNA polymerase (2.5U/µl). They were then covered with mineral oil and incubated at 62C for 45min to allow for the reverse transcription. Cycling conditions (Table 6) and the primers (Table 3) were the same as described for *in vitro* PCR.

3.4.3 Washing and Immunostaining steps

To visualize the amplified PCR products, the DIG-labelled cDNA segments were detected by alkaline phosphatase-conjugated sheep anti-DIG Fab fragments (Roche Molecular Biochemicals) diluted at 1:3000 in TRIS *in situ* detection buffer I (1M Tris, pH 7.5 with NaCl) for 1hr. Initial 15 min incubation with 0.3% ovine serum prepared in 0.1x standard saline citrate (SSC) buffer was used to block any non-specific signals. Then the sections were washed for 2x15 min using SSC buffer to remove the any excess Dig-labelled dUTP that could have been bound to the excess antibody. Subsequently, the sections were equilibrated in TRIS *in situ* detection buffer II (1M Tris-HCl, pH 9.5; containing NaCl and $MgCl_2$) for 10min and reacted with the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the presence of nitroblue tetrazolium (NBT) (Roche Molecular Biochemicals) (i.e., 2480µl TRIS *in situ* detection buffer II+11.25µl NBT+8.75µl BCIP) to form a dark blue precipitate at the site of the label. To stop the reaction, slides were washed in distilled water and embedded in glycerol gelatine.

3.5 *In situ* hybridization for StS and EST

3.5.1 Introduction

In situ hybridization (ISH) is an effective approach for the localization of gene expression at the cellular level. The method is based on the specific annealing of a labelled probe to complementary sequences of target nucleic acids in a fixed specimen, followed by detection and visualization of nucleic acid hybrids with cytological methods (Baumgart et al., 2001). Although there are many descriptions of ISH protocols (Baumgart et al., 2001), none of them is universally sensitive and efficient due to the huge scope of target nucleic acids and wide variance of tissue types.

3.5.2 Generation of the probes

Total RNA from the porcine testis was reverse-transcribed using the same procedures as described for *in vitro* RT (section 3.3.2 above) and the cDNA was amplified using the same primers and conditions as in PCR step (section 3.3.3 above). The PCR products were then cut out and extracted from the gel according to the Qiagen extraction kit (QIEX II, gel extraction kit, Qiagen) and subsequently ligated to the pGEM-T system I Promega vector (Promega, WI, USA) (Fig. 12) according to the manufacturer's instructions

(Annex 3). The vector contained a 3` terminal thymidine to both ends which improved the efficiency of ligation of the PCR product and prevents recirculization of the vector. It also contained T7 and Sp6 RNA polymerase promoter flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allowed recombinant clones to be directly identified by colour screening on indicator plates. Then the vector with the PCR insert was used for transformation of competent *E.coli* bacteria using Promega transformation protocol (Annex 4) to yield white recombinant colonies (with PCR inserts) against blue non-transformed colonies after culturing overnight in agar gel plate coated with ampicillin and tetracycline antibiotics.

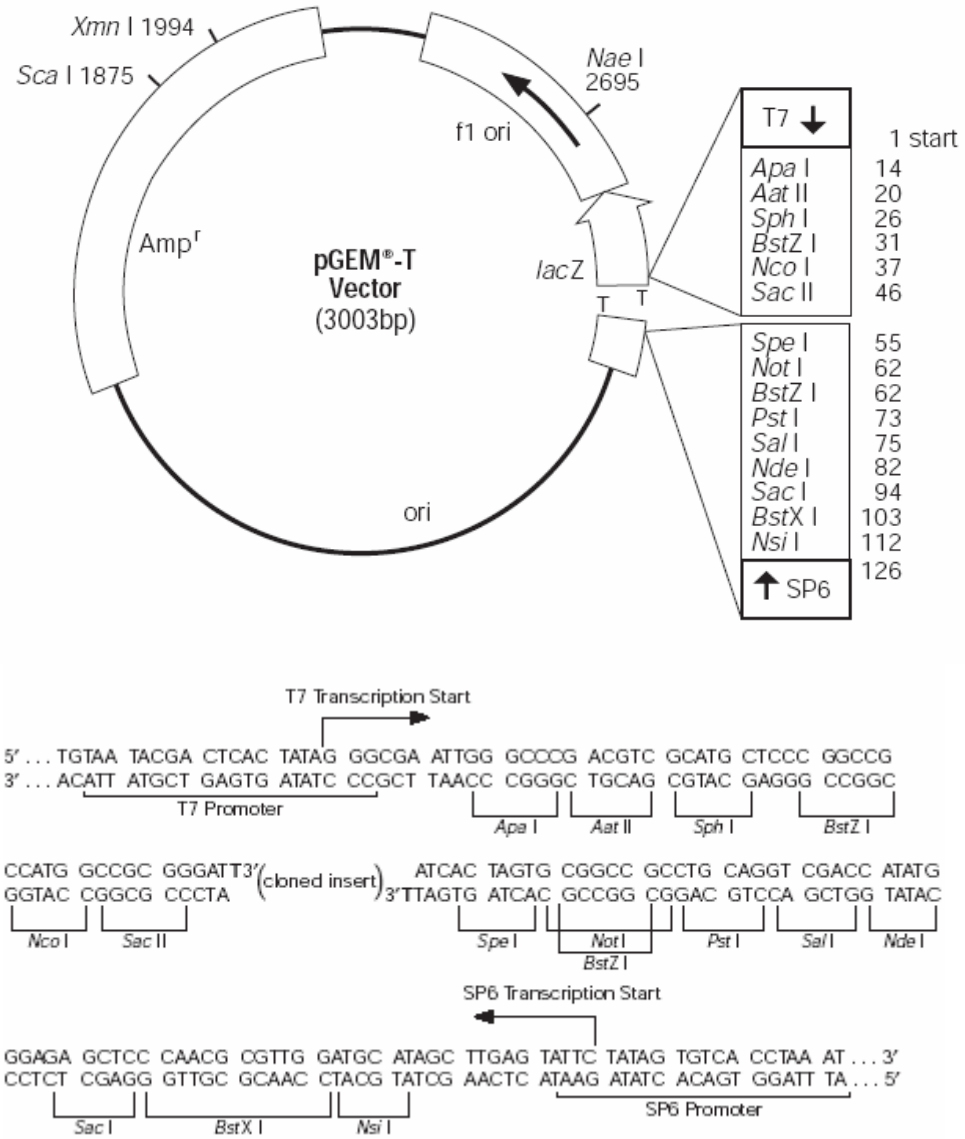

Fig. 9: pGEM®-T Vector and its multiple cloning sequences; the top strand of the sequence shown corresponds to RNA synthesized by T7 and the bottom by Sp6 (Promega manual, WI, USA).

A single white colony was then picked and cultured in fluid medium overnight to obtain multiple copies of the transformed bacteria. Subsequently the recombinant plasmids were isolated from the bacteria using the Promega Standard Plasmid Miniprep Procedure. This was followed by single digestion using either NCO1 or NOT1 restriction enzyme to linearize and release the T7 and/or Sp6 template DNA inserts respectively, which were sent for sequencing for identification. The linearized template DNA was extracted with phenol/chloroform (1:1), then with chloroform/isoamyl alcohol (1:1), precipitate with ethanol and finally dissolved in DEPC water. Following this transcription and labelling of the riboprobes was done through a procedure that incorporates one modified nucleotide (DIG-UTP) at approximately every 20 – 25th positions in the transcripts. Since the nucleotide concentration does not become limiting in the following reaction, 1 µg linear plasmid DNA (with a 1 kb insert) can produce approximately 10 µg of full-length labelled RNA transcript in a 2 h incubation. The reaction mixture for seven vials was prepared following Roche labelling kit (Roche Diagnostics GmbH, Mannheim), containing the Dig-RNA labelling mixture of 10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP; in Tris-HCl, pH 7.5 (+20°C). 4µl of the purified template DNA was added to 2µl of 10x transcription buffer, 2µl of 10x Dig-RNA labelling mixture, 1 µl of Rnase inhibitor and RNA Polymerase SP6 or RNA Ploymerase T7. The mixture were gently mixed, centrifuged briefly and incubated for 2 h at 37^{o}C. The reaction was stopped by adding 2 µl 0.2 µ EDTA (pH 8.0). The probes were then stored at -20 ^{o}C until usage.

3.5.3 In situ Hybridisation step

5µm thick sections were deparaffinized with xylene (5 min at 60^{o}C, 2x5 min at room temperature (RT), rehydrated through descending ethanol concentrations (2x5 min 100%, shortly in 96%, 5min 70%) and shortly in DPEC water. They were then equilibrated (20 min 0.2N HCL, 2x15 min SSC (standard saline citrate) at 70^{o}C and shortly in phosphate-buffered saline containing magnesium chloride (PBSM) and treated with proteinase K (40 µg/ml in PBSM at 37^{o}C) for 25 min to enhance permeability. The slides were then immersed in 0.2% (w/v) glycine in PBSM (5 min) to stop protease K reaction, acetylated for 15 sec with 20% (v/v) acetic acid in DEPC water to denature proteins and then shortly put in PBSM and in 4% paraformaldehyde (10 min) for architectural conservation. After a short immersion in PBSM, the sections were put in pre-hybridisation

buffer (20% glycerol in DEPC water) for 1 hr. Meanwhile the probe and the buffer were prepared as follows

Probe:

Salmon sperm DNA		2µl
Yeast-t-RNA		4µl
Dig-c-RNA (probe*)	1:25 =	8µl in 200µl

Hybridisation buffer

20x SSC	20µl	
Denhardt`s solution	2µl	
Deionised formamide (50%)	100µl	
Dextran sulfate (50%)	40µl	(pre-heated to 70°C in water bath and added lastly)
DEPC water	24µl	

Note: * prepared for both sense and antisense

Subsequently, after a short wash in 2xSCC, the sections and the probe (both sense and antisense) were simultaneously pre-heated for 12 min at 70°C by placing the sections on a hot plate surface (70°C) and the probe (14µl mixture) in a water bath (70°C) to denature the DNA and straighten the mRNA. During heating, the sections were fitted with sterile slide frames to provide cavities (wells) over them. Thereafter, the sections were placed onto a ice-cooled surface and the probe into ice-cooled water. The probe (14µl) was then added into the buffer (146µl) and mixed, then the pre-heated dextran sulfate (40µl) was added and finally 100µl of this mixture (hybridization solution) was applied over each section. This was covered with sterile cover sterile slips and incubated in a formamide-humidified chamber at 37°C overnight.

3.5.4 Washing and Immunostaining

After hybridization the sections were sequentially washed with 4x10 min 4xSSC at 37°C, 15 min 2xSSC at 60°C, 15 min 0.2xSSC at 42°C, 5 min 0.1xSSC at RT and 5 min 2xSSC at RT. Then they were incubated with 3 % (w/v) ovine serum *in situ* detection buffer I (30µl ovine serum in 970µl buffer) at RT for 1 hr to block non-specific signals.

The sections were then incubated in a humidified chamber overnight at 4°C with sheep anti-DIG antibody conjugated with horseradish peroxidase (Boehringer Mannheim

GmbH, Germany), diluted to 1:5000 in buffer 1 containing 1% blocking reagent (2.485ml buffer I, 15µl ovine serum and 1µl anti-Dig antibody).

On the third day, the sections were first washed with *in situ* detection buffer 1 (2x10 min), buffer II (5 min) and buffer II+1M levamisol (200µl in 50ml buffer) (5 min) at RT. Then the immunoreactions were visualized by reacting the sections with the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the presence of nitroblue tetrazolium (NBT) (Roche Molecular Biochemicals) (i.e., 2480µl TRIS *in situ* detection buffer II+11.25µl NBT+8.75µl BCIP) to form a dark blue precipitate at the site of the label. To stop the reaction, slides were incubated in buffer II+levamisol (5 min), then washed in distilled water and embedded in glycerol gelatine.

Negative controls were the anti-sense probes and the results were observed under a light microscope with a colour camera linked to a computer system with Photoshop software (Leica, Wetzlar, Germany).

3.6 Buffers and Solutions

Ethanol (Alcohol)-Series

Ethanol (95 %)		Ethanol (70 %)	
Pure ethanol (100%)	95 ml	Pure ethanol (100 %)	70 ml
Deionised water	5 ml	Distilled water	30 ml

Neutral (pH 7.0) phosphate buffered 4 % Formalin tissue fixative (Lillie 1951)

Formalin (app. 40 % aqueous Formaldehyde solution)	500 ml
NaH_2PO_4 x H_2O	20 g
Na_2HPO_4	32.50 g
Deionised water	500 ml

0.3 % H_2O_2 solution

30% H_2O_2	2 ml
Deionised water	200 ml

IHC-buffer (pH 7.2-7.4)

Na_2HPO_4	1.2 g
KH_2PO_4	0.2 g
KCl	0.2 g
NaCl	8.0 g
Double deionised water	ad 1 Liter
After pH-control (7.2-7.4)	3 ml Triton X-100

Paraformaldehyde tissue fixative (pH 7.4)

Paraformaldehyde	4 g
Distilled water	95 ml
20 x PBS	5 ml
NaOH (1,025 N)	150 µl

Heated at 70 °C, until all solute dissolves and thereafter stored at -20 °C; thawed just before use.

PBS (Phosphate Buffered Saline) pH 7 .2

5 x Stock solution:

NaCl	41 g
Na_2HPO_4 x $2H_2O$	11 g
KH_2PO_4	2.75 g
Double deionised water	1 Litre

1x PBS working solution

5 x PBS	200ml
Double deionised water	ad 1000ml

PBS-$MgCl_2$ buffer (PBSM buffer)

PBS-Tablet	1
Double deionised water	200 ml
1 M $MgCl_2$ solution	1 ml

PBST-buffer

PBS-Puffer (pH 7.5)	1000 ml
Tween 20	1 ml

Citrate buffer (pH 6.0)

Stock solution A: 0.1 M citronic acid

$C_6H_8O_7$ x H_2O	21.0 g
Deionised water	ad 1 Litre

Stock solution B: 0.1 M sodium citrate

$C_6H_5O_7Na_3$ x $2H_2O$	29.41 g
Distilled water	ad 1 Litre

Working solution

Stock solution A:	9 ml
Stock solution B:	41 ml
Deionised water	450 ml

Phosphate buffer for tissue stabilisation during Formalin fixation

Solution 1:

NaH_2PO_4 x H_2O	13.8 g
Deionised water	ad 1000ml

Solution 2:

Na_2HPO_4 x $2H_2O$	17.8 g
Deionised water	ad 1000 ml

Used buffer

Solution 1: 14.15 ml
Solution 2: 35.85 ml

0.5 M EDTA Na_2 x $2H_2O$ (Ethylenediaminetetraacetic acid disodium dihydrate)

EDTA Na_2 x $2H_2O$	37.22 g
Distilled water	ad ca. 160 ml
pH-control by adding ca. 4 g NaOH to pH 8.0	
Double deionised water	ad 200ml

In situ detections buffer I (1M Tris with Nacl pH 7.5)

TRIS-HCL	12.70 g
TRIZMA Base	2.36 g
NaCl	8.76 g
Double deionised water	ad 1000 ml

In situ detections buffer II (1M Tris with Nacl and $MgCl_2$, pH 9.6)

TRIS-HCL	1.52 g
TRIZMA Base	10.94 g
NaCl	5.84 g
$MgCl_2$	4.76 g
Deionised water	ad 1000 ml

10x TBE Buffer (Tris-Borate-EDTA)

Tris base	108 g
Borate	55 g
0.5 M Na4EDTA (pH 8.3)	40 ml
Deionised water	ad 1000 ml

1x TBE Buffer (Tris-Borate-EDTA)

10 x TBE	100 ml
Deionised water	ad 1000 ml

20 x SCC, pH 7.0 (Standard saline citrate)

$C_6H_5O_7Na_3$ x $2H_2O$	88,23 g
NaCl	175,29 g
Deionised water	ad 1000 ml

Paraformaldehyde solution, pH 7.0 used during in situ-hybridisation

Paraformaldehyde	25 g
1 x PBS-$MgCl_2$-buffer	500 ml

Add 4 N NaOH-solution until it clears (colourless)

Proteinase K for in situ RT-PCR (2 µg/ml)

Proteinase K (10mg/ml)	2µl
PBSM buffer	100µl

Proteinase K for in situ hybridisation (20 µg/ml)

Proteinase K (10mg/ml)	20µl
PBSM buffer	80µl

50 % Dextran sulfate solution

Dextran sulfate	5 g
Double deionised water	10 ml

Stored at –20 °C

Denhard reagent

BSA	200 mg
Ficoll 400	200 mg
Polyvinylpyrrolidone	200 mg
Double deionised water	10 ml

Levamisol solution for quenching endogenous AP-activity

Levamisol	2.4 g
1 x sodium chloride Tris buffer	10 ml

20 % acetic acid

Acetic acid	40 ml
Double deionised water	160 ml

Stored at 4 °C

1 M $MgCl_2$ solution

$MgCl_2$	81.4 g
Double deionised water	ad 400 ml

0.2 % glycine solution

Glycine	200 mg
PBS-$MgCl_2$ buffer	100 ml
Stored at RT	

3.7 Reagents and Sources

- β-Mercaptoethanol: M-3148 (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- 100 bp DNA marker (MBI Fermentas, St. Leon-Rot)
- ABC-reagent: Vector® - VECTASTAIN® Elite ABC Kit, PK-6101, Rabbit IgG (Vector Laboratories, Burlingame, CA 94010, USA via Linaris Biologische Produkte GmbH, D97877 Wertheim)
- Anti-DIG-Fab-Antibody: Nr.: 1093274 (Roche Diagnostic GmbH, Mannheim)
- BA2000; Horse antimouse IgG biotinylated antibody (Vector Labolatories. Inc., Burlingane, USA)
- BCIT: 5-bromo-4-chloro-3-indolyl-phosphate**,** toluidine salt; Stock Solution: Art. Nr.: 1383221 (Roche Diagnostic GmbH, Mannheim)
- Boric acid, Pufferan® (H_3BO_3): Art. Nr.: 6943.2 (Carl Roth GmbH + Co., Karlsruhe)
- Bovine Serum Albumin (BSA): Art. Nr.: 05482 (Fluka, Neu-Ulm)
- Citronic acid monohydrate (Merck KGA, Darmstadt)
- Denhard´s Solution: (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- Dextran sulfate (Sigma-Aldrich Chemicals GmbH, Deisenhofen)
- Diethylpyrocarbonate (DePC; Roth GmbH & Co., Karsruhe)
- DIG RNA Labelling Kit (SP6 / T7) : (Roche Diagnosic GmbH, Mannheim)
- Di-sodium hydrogen phosphate -dihydrate: Art. Nr: 6580 (Merck KGaA, Darmstadt)
- Di-sodiumydrogen phosphate solution: Art. Nr.: 6585 (Merck KGaA, Darmstadt)
- DNase 1, RNase-free, 10 U/µl (Roche Molecular Biochemicals, Mannheim)
- E. coli (XL-1 Blue) (Stratagene Europe, Holland)
- EDTA Sodium (Roth GmbH & Co., Karsruhe)
- Ethanol: 99.6%, DAB 10, Art.Nr.: 5054.5 (Carl Roth GmbH + Co., Karlsruhe)
- Ethidium bromide 1% solution: Art. Nr.: 2218.1 (Carl Roth GmbH + Co., Karlsruhe)
- Formaldehyde: Art. Nr.: 3999 (Merck KGaA, Darmstadt)
- Formamide: Art Nr.: 47671 (Fluka, Neu-Ulm)
- Gene Amp RNA Core Kit®: (Perkin Elmer Applied Biosystems GmbH, Weiterstadt)

- Glycerine Rotipuran®: 99.5 % Wasserfrei Art. Nr.: 3783.1 (Carl Roth GmbH + Co., Karlsruhe)
- Glycine: G-7126 (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- Gold PCR Core kit (Perkin Elmer Applied Biosystems GmbH & Co., Karsruhe)
- HCL: 37 %, Art. Nr.: 906 K11409117 (Merck KGaA, Darmstadt)
- Hefe-tRNA (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- Histokitt No: 1025/100 (Assistent, D-37520, Osterode).
- Hydrogen peroxide 30 %, Art. Nr.: 64271 (Merck KGaA, Darmstadt)
- Isopropanol (Roth GmbH & Co., Karsruhe)
- Levamisol: L-9756 (Sigma-Aldrich Chemie GmbH, Steinheim)
- Magnesium Chloride: M-0250 (Sigma-Aldrich Chemie GmbH, Steinheim)
- Marker buffer for Electrophoresis: Roti®-Load 1 (4x-Konz) Art. Nr.: K929.1 (Carl Roth GmbH + Co., Karlsruhe)
- Methanol: Art. Nr.: 1.06008.2500 (Merck KGaA, Darmstadt)
- $MsIgG_{2a}$: CA 33196-2500 (Beckman Coulter Miami, USA)
- NTB: 4-Nitroblue Tetrazolium Chloride, Art. Nr.:1383213 (Roche Diagnostics GmbH, Mannheim)
- Pap Pen: (G.Kisker Biotech, Steinfurt)
- Paraformaldehyde: Art. Nr.: 76240 (Fluka, Neu-Ulm)
- Peroxidase Kit: Vector® (Nova-RED), SK-4800: (Linearis Biologische Produkte GmbH, Wertheim)
- pGEM-T-Vector (pGEM-T Vector System 1; Promega GmbH, Manheim)
- Phosphate Buffered Saline Tablets: P-4417 (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- Potasium chloride reinst: Art. Nr.: 4936 (Merck KGaA, Darmstadt)
- Potasium dihydrogen phosphate: (Fluka Chemie GmbH, Buchs)
- Potasium phosphate monobase: Art. Nr.: 60220 (Fluka, Neu-Ulm)
- Proteinase K: P-2308 (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- QIAquick® PCR Purification Kit: (Qiagen Gmbh, Hilden)
- RNase inhibitor, 40 U/µl (MBI Fermentas, St. Leon-Rot)
- RNase-Away: (Molecular Bioproducts, San Diego, USA)
- rTth PCR Kit (Pelkin Elmer Applied Biosystems GmbH, Weitstadt)

- Salmon Testes DNA: D-7656 (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- Sodium chloride: Art. Nr.: 71367 (Fluka, Neu-Ulm)
- Sodium hydrogen phosphate-monohydrate: Art. Nr.: 6346 (Merck KGaA, Darmstadt)
- Sodium hydroxide: Art. Nr.: 60164531 (Merck KGaA, Darmstadt)
- T7-RNA-Polymerase, Sp6-RNA-polymerase, 5x Transcriptions buffer (Promega, Manhein)
- Tris hydroxymethylaminomethane (Trizma® Base): T-4661 (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- Tris-sodium citrate-dihydrate: Art. Nr.: 6448, 302 K 10020548 (Merck KGaA, Darmstadt)
- Triton 100: Serva Feinbiochemica Art. Nr.: 37238 (Serva, Heidelberg)
- Tween 20: Polyoxyethylenesorbitan Monolaurate, P-1379 (Sigma-Aldrich Chemie GmbH, Deisenhofen)
- Vectastain Mouse IgG Elite kit (Vector Laboratories. Inc., Burlingame, USA)
- X-gal
- Xylol: Art. Nr.: 8.18754.2500 (Merck KGaA, Darmstadt)

3.8 Materials, Equipments and their Sources

- ACS: 180®; Automated Chemiluminescence System, Bayer (Ciba-Geigy GmbH, D-79662 Wehr)
- Slides, Menzel-Gläser: SuperFrost®, 24x40 mm, 24x50 mm und 24x60 mm, Art. Nr.:31800 AMNZ – (MAGV Laborbedarf, Rabenau-Londorf)
- Eppendorf Biophotometer: (Eppendorf AG, Hamburg)
- Embedding automatic machine: (Microm Laborgeräte GmbH, Heidelberg)
- Embedding medium: Histokitt (Assistent, D-37520, Osterode)
- Mikrotome blade: Leica DB 80L, Order No: 0358 37040, Leica Microsystems (Nußloch GmbH, Nußloch)
- Elektrophoresis machine-Agarosegel: (GibcoBRL, Life Technologies, Karlsruhe)
- Hand gloves UniGloves® : (MAGV Laborbedarf + Laborgeräte Rabenau-Londorf)
- Lab water deminiralisation system: Aqua demineralisata Seral-Anlage mit angeschlosener Millipore-Reinigungsanlage: Milli Q, Water Purification System Firma Millipore, Typ MQ 4-fach UF (Millipore Gmbh, Eschborn)

- Microtome: (Reichard Jung AG, Heidelberg)
- Paraffin machine: Histoembedder EG 1160 (Leica Instruments GmbH, Nußloch)
- PapPen: (G. Kisker - Biotech, Steinfurt)
- PCR-Tubes: 0,5 ml, ultradünn, RNase-/DNase-Pyrogenfrei (Biozym Diagnostik GmbH, Hessisch Oldendorf)
- pH-Meter: (MAGV Laborbedarf + Laborgeräte Rabenau-Londorf)
- Vacuum Pump: KNF Neuber, Typ: No 035.1.2 AN. 18, Nr: 472678; VDE 0530, Nr: 83424, Motortyp: MW 63/4, IP 44, Volt:220, 1,8 A, 0,12kW, 50 Hz, 1400 U/min (Freiburg)
- Powersupply (PCR): 2301 Macrodrive 1 Powersupply (LKB Bromma, Golden, USA)
- Reactions tubes: Biozym Scientific GmbH, Art. Nr: D-31833 (D-31833, Oldendorf)
- Pipettes: 50 µl Rheodyne Cat. Nr.: 7205 ((Eppendorf AG, Hamburg)
- T1 Thermocycler 49: (Whatman Boimetra, Göttingen)
- Ultra-Turrax ®: T-8 mit Dispergierwerkzeug S8 N-5g (IKA-Werke GmbH, Staufen)
- UV-Transluminator with Photoshop software Phoretix Grabber 3.01: (Biostep GmbH, Jahnsdorf)
- UV-Spectrometer (BioPhotometer, Eppendorf® AG, Hamburg) mit Eppendorf Uvette® 220-1600 nm (MAGV Laborbedarf + Laborgeräte Rabenau-Londorf)
- Vortexer: Heidolph REAX control (MAGV, Rabenau-Londorf)
- Water bath: Typ WB-24; V220; W 550; Fabrik-Nr.;8810, max. 90C; (Medax Nagel KG Kiel)
- Warm chamber: Memmert, Typ: 3 26, F-Nr: 340 073, 220 Volt, 380 Watt, 50/60 Hz, Din 12880-Kl. 0, Nenntemp.: 70C, Schutzart Din 40050-IP20 (Schwabach)

4. RESULTS

4.1 Expression of estrogen receptors

4.1.1 Immunolocalisation of ER by IHC

4.1.1.1 Positive control experiments

In the porcine uterus used as positive control, a strong immunostaining for the ERα and ERβ was found in the glandular epithelium (arrow 1), weak signals were detected in the luminal epithelium (arrow 2) (Fig. 10a, b).

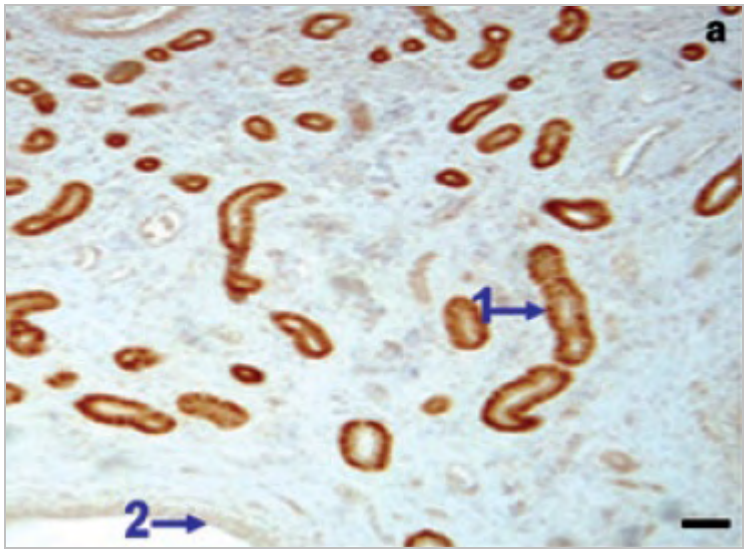

Fig. 10a: ERα immunostaining in porcine uterus used as positive control. A strong immunoreactivity was observed for ERα in the glandular epithelium (arrow 1) and weak immunoreactivity in luminal epithelium (arrow 2). Magnification X200, bar = 50µm.

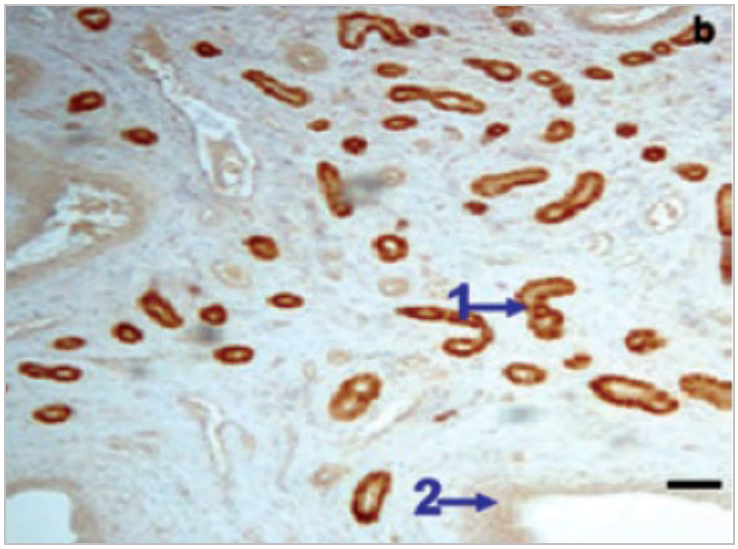

Fig. 10b: ERβ immunostaining in porcine uterus used as positive control. A strong immunoreactivity was observed for ERβ in the glandular epithelium (arrow 1) and weak immunoreactivity in luminal epithelium (arrow 2). Magnification X200, bar = 50µm.

4.1.1.2 Negative control for ER in porcine uterus

In the negative controls in the porcine uterus, apart from the occasional traces of a weak non-specific staining in the lumen of blood vessels (black arrow), obviously associated with residual serum components, there were no other non-specific signals (Fig. 11a).

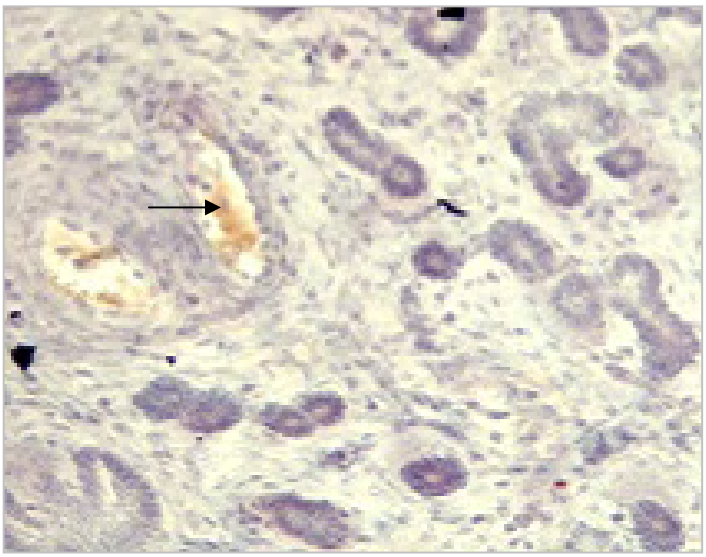

Fig. 11a: Negative control slide in the porcine uterus using non-specific mouse IgG isotypes. Only traces of weak non-specific staining were observed in the lumen of the blood vessels (arrow). Magnification X200

4.1.1.3 Negative control for ER in 50-day old boars

In the negative controls in the juvenile boar testis, which were conducted using the respective mouse non-specific IgG isotypes, there were no traces of any signals (Fig. 11b)

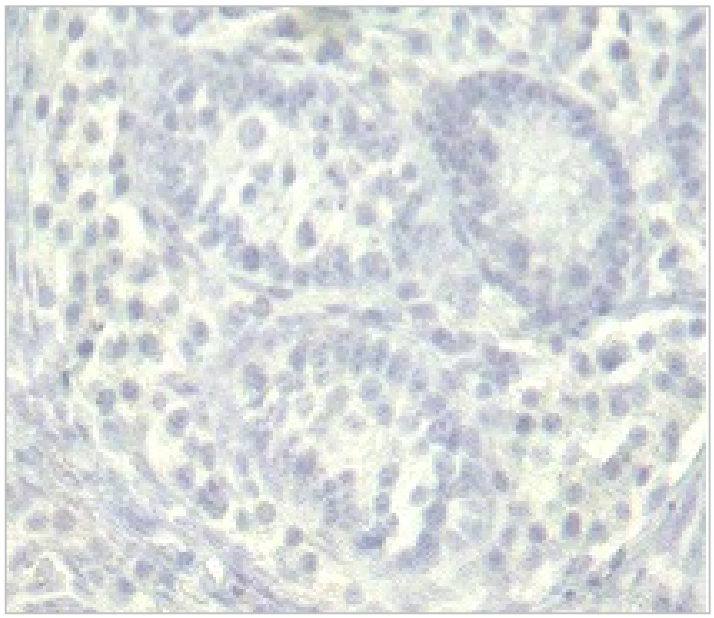

Fig. 11b: Negative control slide in the 50 day old boar testis using non-specific mouse IgG isotypes. Magnification X200

4.1.1.4 Negative control for ER in mature boars

In the negative controls in the mature boar testis (day 150-250), which were also conducted using the respective mouse non-specific IgG isotypes, there were no traces of any signals (Fig. 11c)

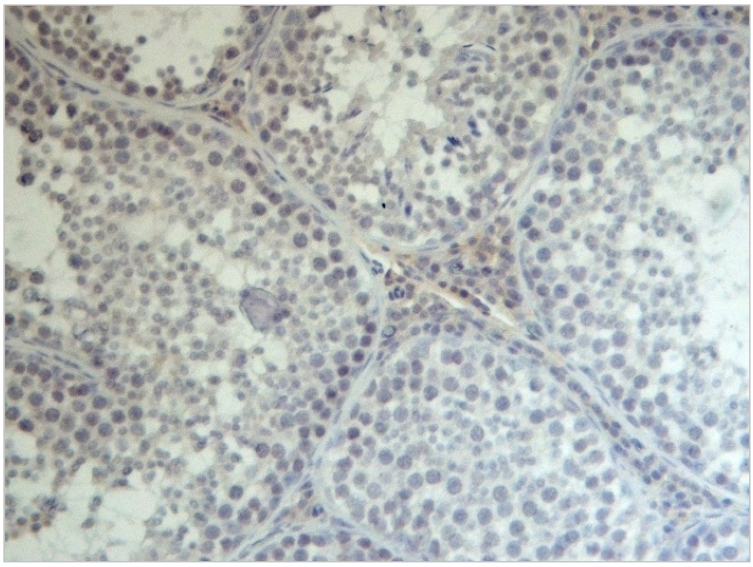

Fig. 11c: Negative control slide in the mature boar testis using non-specific mouse IgG isotypes. Magnification X200

4.1.1.5 ERα in 50 day old boars (immature)

For ERα immunoreactivity was observed in 90.6 ± 1.2% of the prespermatogonia (arrow 1) and 71.2 ± 2.6% of the Leydig cells (arrow 2) (Fig. 12 and Table 11a, b). Sertoli cells (arrow 3) were negative

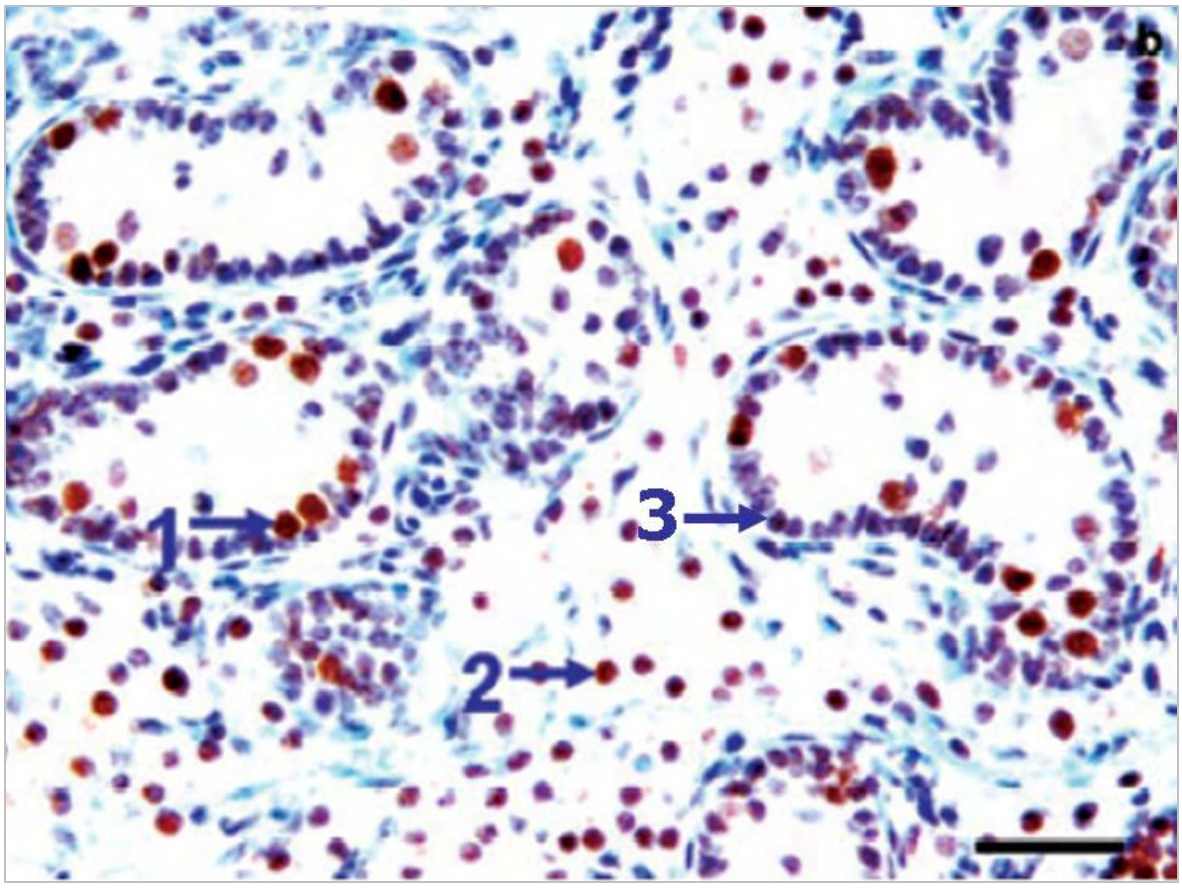

Fig. 12: Immunolocalisation of ERα in 50-day old boar testis. A strong immunoreaction was observed only in prespermatogonia (arrow 1) and in the Leydig cell (arrow 2) while the Sertoli cell (arrow 3) showed no reactivity. Mg=x400, bar = 50µm

Table 11a: Prespermatogonia staining positive for the ERα in 50 day old boars (E1-E5)

Prespermatogonia counted					% positive	
Boar	*slide*	*Positive*	*Negative*	*Total*	*slide*	*boar*
E1	A	29	5	34	85.29	
	B	16	1	17	94.12	
	C	33	2	35	94.29	91.23
E2	A	23	5	28	82.14	
	B	17	1	18	94.44	
	C	29	2	31	93.55	90.05
E3	A	23	4	27	85.19	
	B	23	3	26	88.46	
	C	21	1	22	95.45	89.70
E4	A	22	1	23	95.65	
	B	20	4	24	83.33	
	C	25	3	28	89.29	89.42
E5	A	31	2	33	93.94	
	B	27	4	31	87.10	
	C	24	1	25	96.00	92.35

Mean ($\bar{X}$) = 90.55, Standard deviation (SD) = 1.22

Table 11b: Leydig cells staining positive for the ERα in 50 day old boars (E1-E5)

Leydig cells counted				% positive		
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	44	13	57	77.19	
	B	34	17	51	66.67	
	C	35	19	54	64.81	69.56
E2	A	40	11	51	78.43	
	B	25	18	43	58.14	
	C	38	17	55	69.09	68.55
E3	A	29	12	41	70.73	
	B	23	11	34	67.65	
	C	31	12	43	72.09	70.16
E4	A	42	6	48	87.50	
	B	32	12	44	72.73	
	C	28	18	46	60.87	73.70
E5	A	24	14	38	63.16	
	B	36	9	45	80.00	
	C	31	8	39	79.49	74.22

Mean ($\bar{X}$) = 71.24, Standard deviation (SD) = 2.56

4.1.1.6 ERβ in 50 day old boars

In immature boars, 95.5 ± 3.5% of the prespermatogonia cells (arrow 1) (Table 12) showed a strong immunoreactivity for the ERβ (Fig 13). The Leydig- (arrow 2) and the Sertoli cells (arrow 3) were negative.

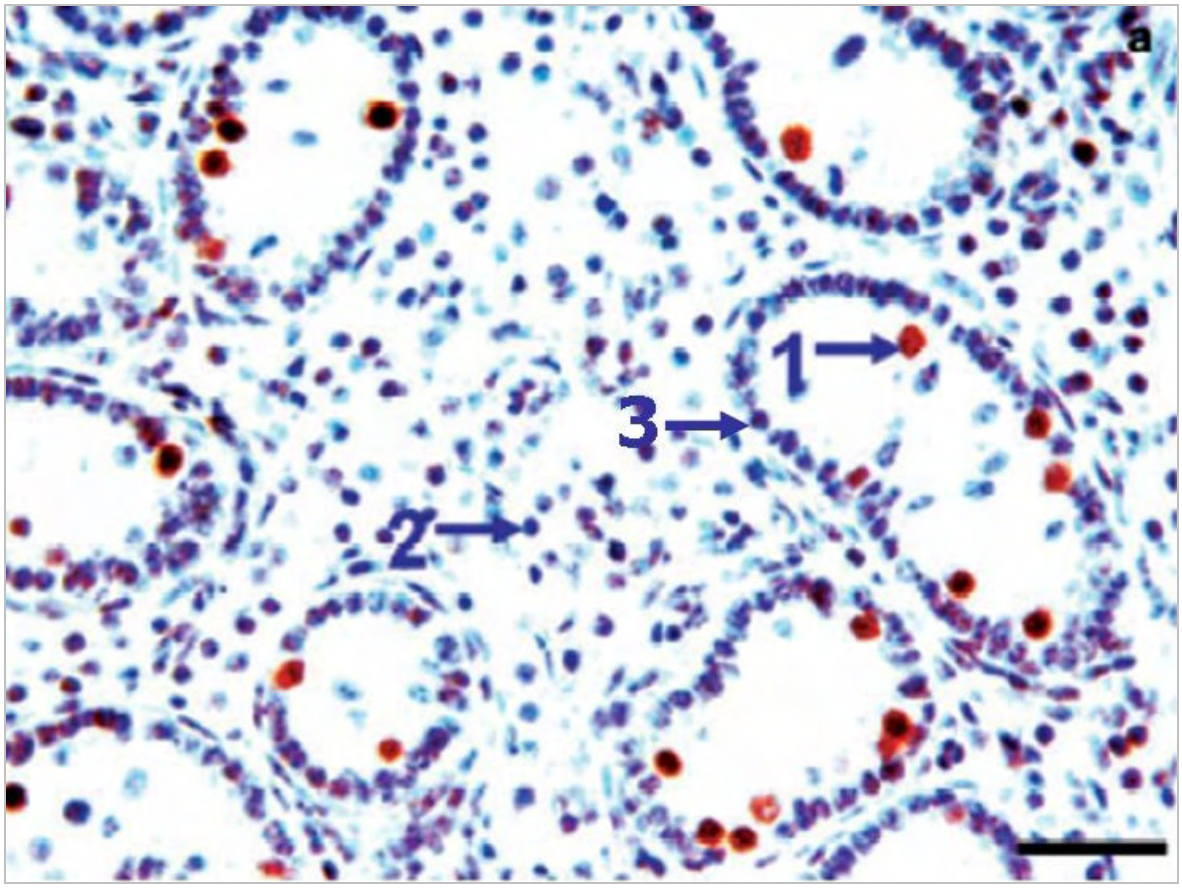

Fig. 13: Immunolocalisation of ERβ in 50-day old boar testis. A strong immunoreaction was observed only in prespermatogonia (arrow 1) while the Leydig cell (arrow 2) and the Sertoli cells (arrow 3) showed no reactivity (2). Mg=x400, bar = 50µm

Table 12: Prespermatogonia staining positive for the ERβ in 50 day old boars (E1-E5)

Prespermatogonia counted					% positive	
Boar identity	*Slide*	*positive*	*negative*	*total*	*Slide*	*boar*
E1	A	31	0	31	100,.0	
	B	17	1	18	94.44	
	C	37	0	37	100.00	98.15
E2	A	28	1	29	96.55	
	B	19	0	19	100,00	
	C	27	1	28	96.43	97.66
E3	A	20	4	24	83.33	
	B	24	2	26	92.31	
	C	25	1	26	96.15	90.60
E4	A	23	1	24	95.83	
	B	19	1	20	95.00	
	C	22	3	25	88.00	92.94
E5	A	30	0	30	100.00	
	B	19	1	20	95.00	
	C	28	0	28	100.00	98.33

Mean ($\bar{X}$) = 95.54, Standard deviation (SD) = 3.54

4.1.1.7 ERα in sexually mature boars (250 day old group)

As identified in fig. 14b, ERα showed a strong immunoreactivity in 93.6±2.7% (Table 13a, b) of the spermatogonia (arrow 1) and primary spermatocytes (arrow 2). With the exception of a few cells, a distinctly less intensive staining was observed in 51.4±0.27% (Table 13c) of the other germ cells (arrow 3) except elongating/ed spermatids (arrow 6), which stained clearly negative. Most of the Sertoli cells (arrow 4) stained weakly positive and 61.1±3.4% (Table 13d) of the Leydig cells (arrow 5) exhibited a weak

to distinct staining. The immunoreactivity observed showed no differences in respect to the stage of spermatogenesis (Fig.14a-c) i.e., no qualitative and/or quantitative changes with respect to seminiferous tubules were observed immediately after spermiation (phase I), during spermatid elongation (phase II) and prior to spermiation (phase III).

(a) Phase I of spermatogenesis (immediately after spermiation, no elongated spermatids)

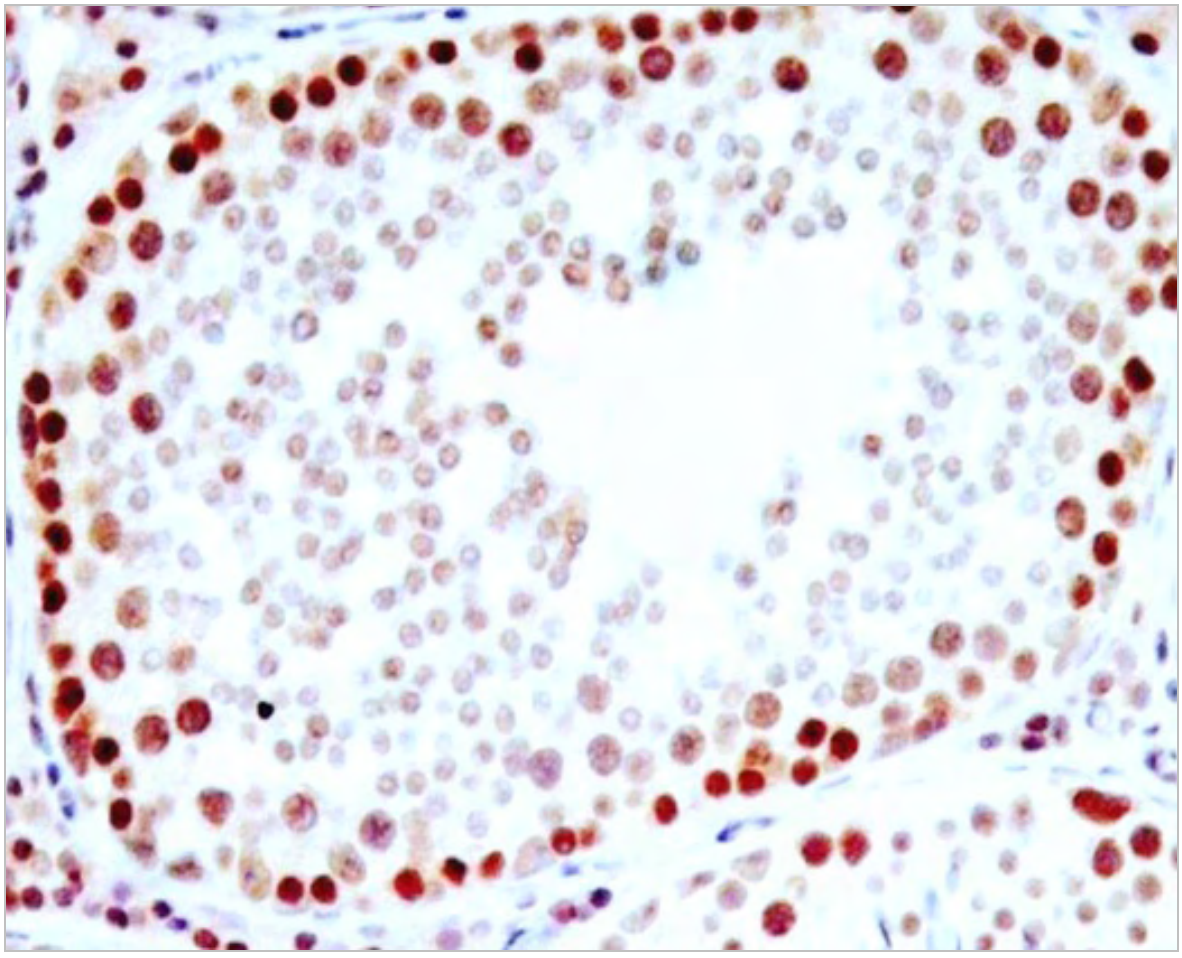

Fig. 14a: ERα in 250-day boar testis during phase I of spermatogenesis showing strong signals in spermatogonia and primary spermatocytes. Round spermatids, Sertoli cells and Leydig cells are weakly positive. Elongated spermatids are absent in this phase. Mg=400

(b) Phase II of spermatogenesis (elongating spermatids)

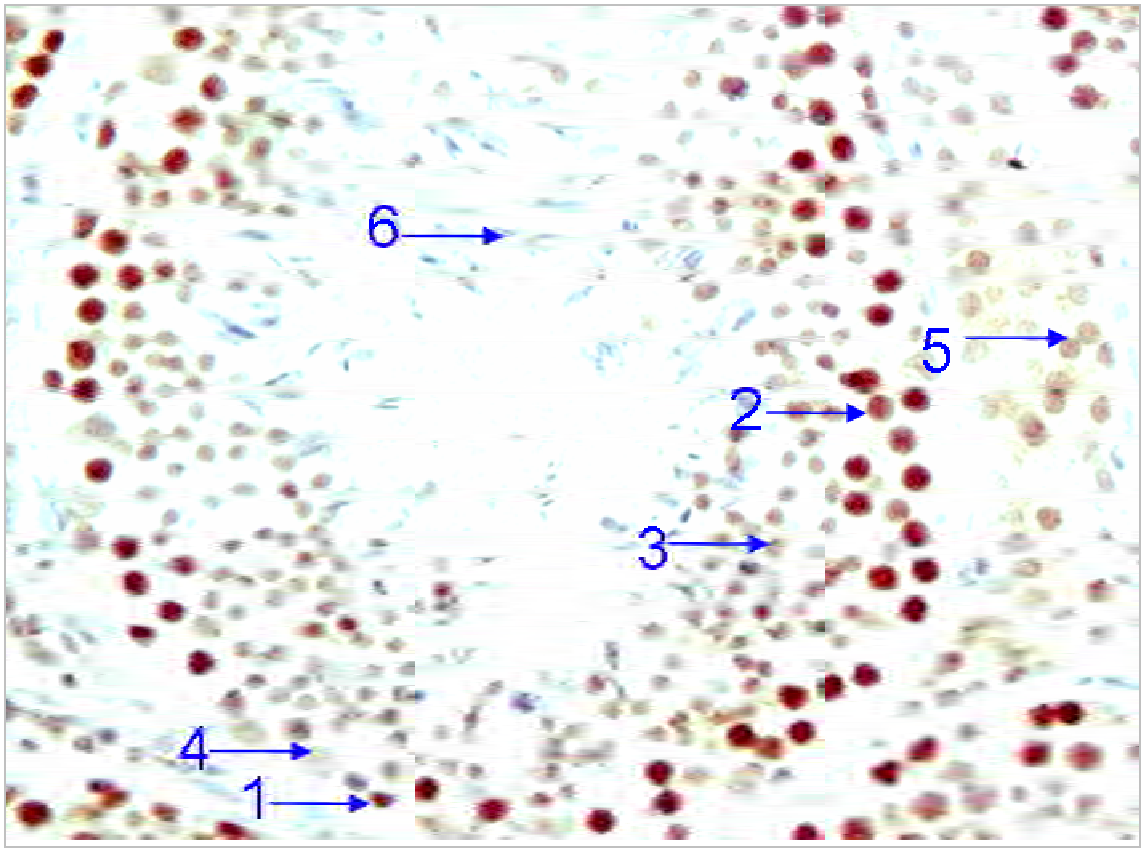

Fig. 14b: ERα in 250-day boar testis during phase II of spermatogenesis showing strong signals in the spermatogonia (arrow 1) and primary spermatocytes (arrow 2). Round spermatid (arrow 3), Sertoli- (arrow 4) and Leydig cells (arrow 5) were weakly positive while elongated spermatids (arrow 6) were distinctly negative. Mg=400

(c) Phase III of spermatogenesis (Fully elongated spermatids ready for release)

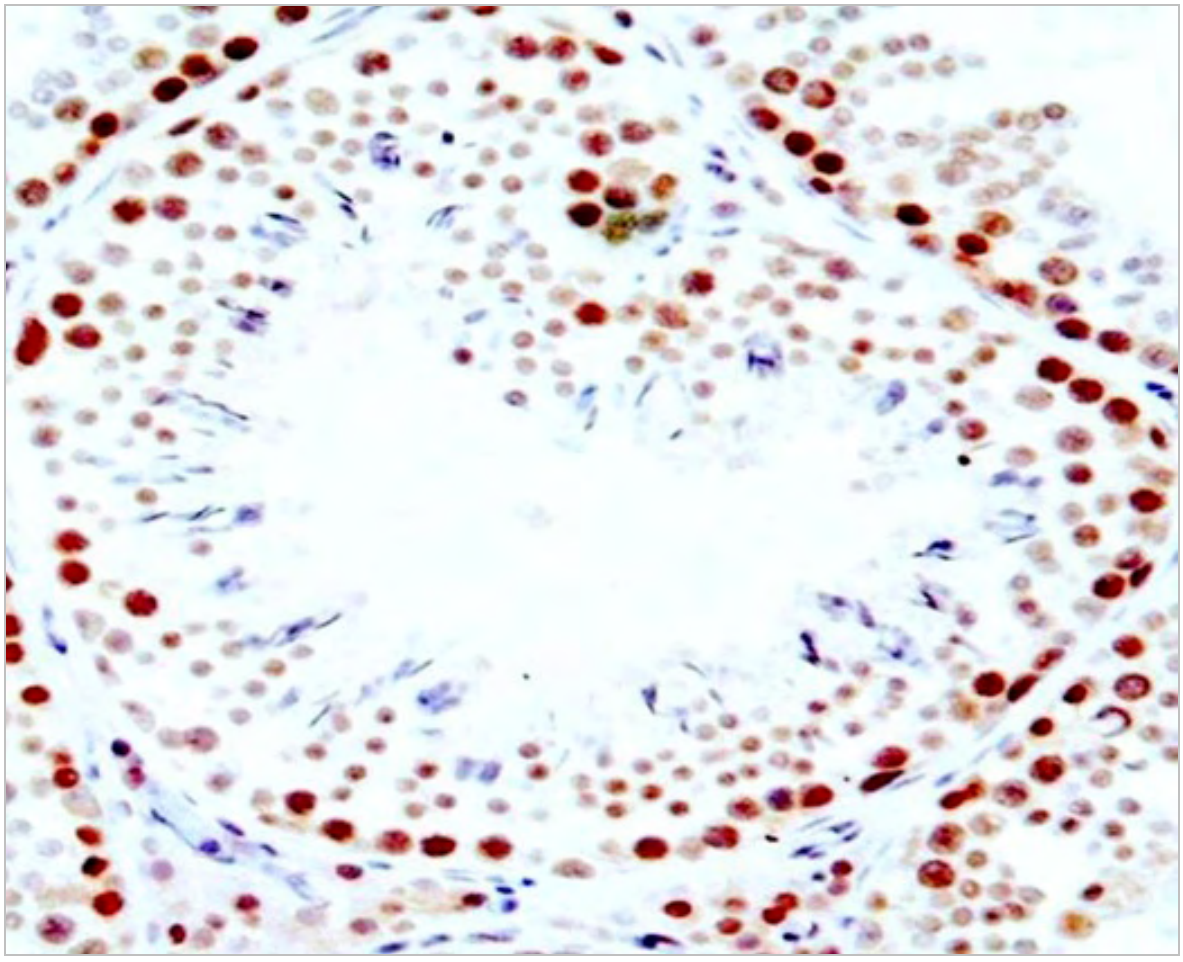

Fig. 14c: ERα in 250-day boar testis during phase III of spermatogenesis showing strong signals in spermatogonia and primary spermatocytes. Round spermatids, Sertoli cells and Leydig cells are weakly positive while elongated spermatid are negative. Mg=400

Table 13a: Spermatogonia staining positive for ERα in the mature boars

Spermatogonia counted						
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	46	4	50	92.00	
	B	45	5	50	90.00	
	C	47	5	52	90.38	90.79
E2	A	54	1	55	98.18	
	B	38	1	39	97.44	
	C	44	1	45	97.78	97.80
E3	A	42	1	43	97.67	
	B	47	5	52	90.38	
	C	43	3	46	93.48	93.85
E4	A	39	7	46	84.78	
	B	50	1	51	98.04	
	C	46	4	50	92.00	91.61
E5	A	49	1	50	98.00	
	B	47	5	52	90.38	
	C	48	3	51	94.12	94.17

Mean ($\bar{X}$) = 93.64, Standard deviation (SD) = 2.73

Table 13b: Primary spermatocytes staining positive for ERα in mature boars

Primary spermatocytes counted						
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	47	2	49	95.92	
	B	57	1	58	98.28	
	C	49	5	54	90.74	94.98
E2	A	56	5	61	91.80	
	B	58	6	64	90.63	
	C	54	1	55	98.18	93.54
E3	A	56	2	58	96.55	
	B	57	0	57	100.00	
	C	53	3	56	94.64	97.06
E4	A	52	6	58	89.66	
	B	53	3	56	94.64	
	C	48	3	51	94.12	92.81
E5	A	54	1	55	98.18	
	B	57	9	66	86.36	
	C	45	8	53	84.91	89.82

Mean ($\bar{X}$) = 93.64, Standard deviation (SD) = 2.69

Table 13c: Round spermatids and/or secondary spermatids) within the seminiferous tubules staining positive for ERα in mature boars

	Cells counted					
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	89	86	175	50.86	
	B	91	85	176	51.70	
	C	90	87	177	50.85	
						51.14
E2	A	85	80	165	51.52	
	B	74	72	146	50.68	
	C	77	71	148	52.03	
						51.41
E3	A	73	70	143	51.05	
	B	71	66	137	51.82	
	C	66	63	129	51.16	
						51.35
E4	A	76	71	147	51.70	
	B	94	90	184	51.09	
	C	90	87	177	50.85	
						51.21
E5	A	63	60	123	51.22	
	B	97	86	183	53.01	
	C	103	98	201	51.24	51.82

Mean ($\bar{X}$) = 51.37, Standard deviation (SD) = 0.27

Table 13d: Leydig cells staining positive for ERα in mature boars

Leydig cells counted						
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	78	52	130	60.00	
	B	72	48	120	60.00	
	C	66	62	128	51.56	
						57.19
E2	A	81	40	121	66.94	
	B	60	44	104	57.69	
	C	78	65	143	54.55	
						59.73
E3	A	83	57	140	59.29	
	B	81	26	107	75.70	
	C	68	54	122	55.74	
						63.57
E4	A	66	41	107	61.68	
	B	84	49	133	63.16	
	C	63	25	88	71.59	
						65.48
E5	A	79	51	130	60.77	
	B	51	29	80	63.75	
	C	67	58	125	53.60	59.37

Mean ($\bar{X}$) = 61.07, Standard deviation (SD) =3.37

4.1.1.8 ERα in the other groups (100-, 150- and 200-day old boars)

4.1.1.9 ERα in 100-day old boars

Day-100 old boars represented the sexually maturing group for which the tubules were predominantly filled with spermatogonia and primary spermatocytes with occasionally few visible elongated spermatids. In this group, apart from the myoid cells (arrow 1) and the elongating spermatids (arrow 2), virtually all the other cells within the seminiferous tubules were positive with the spermatogonia (arrow 3) and the primary spermatocytes (arrow 4) being intensively positive (Fig. 15a). Like in day-250 group the Leydig (arrow 5) and Sertoli cells (arrow 6) were also positive.

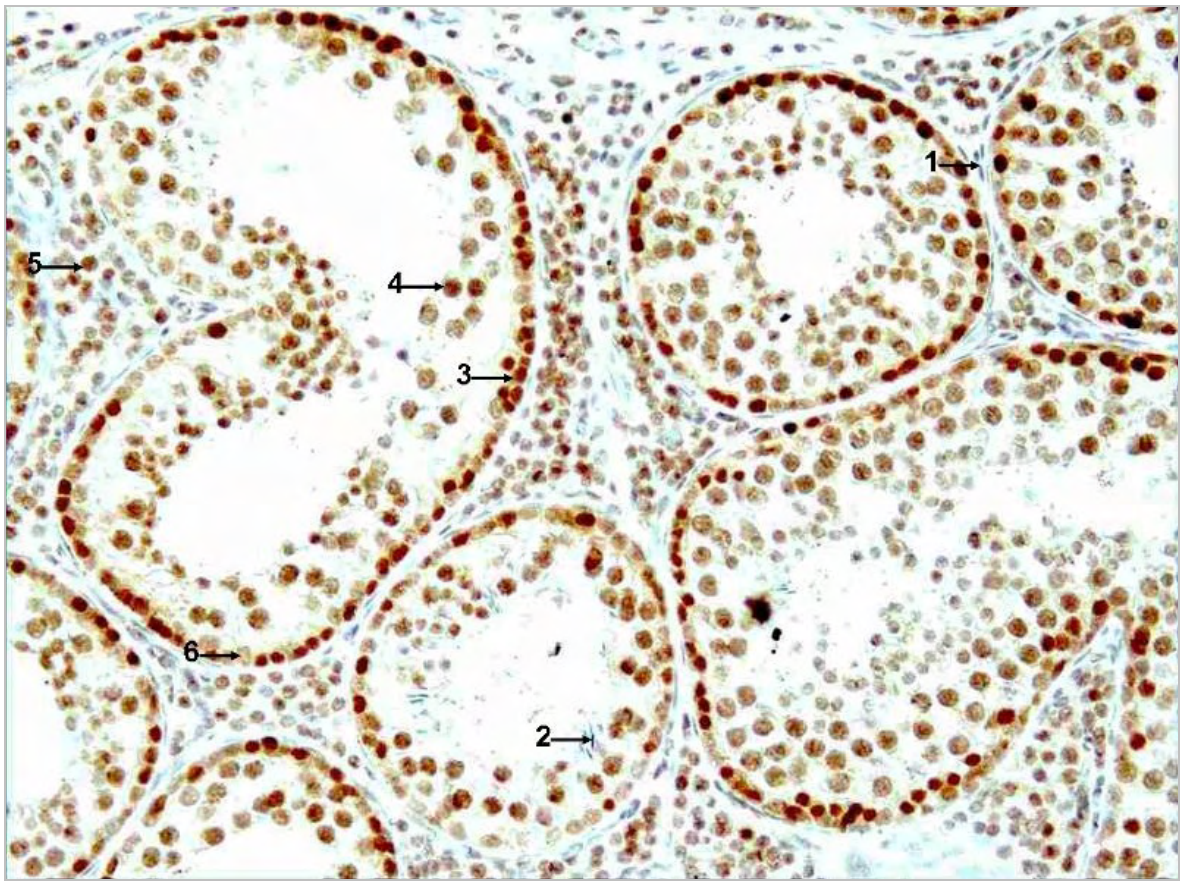

Fig. 15a: ERα in 100-day boar testis showing strong signals in spermatogonia (arrow 3) and primary spermatocytes (arrow 4) while weaker reactions were observed in round spermatids (arrow 4), Leydig- (arrow 5) and Sertoli cells (arrow 5). The myoid cells (arrow 1) and elongating spermatids (arrow 2) were negative. Mg=200

4.1.1.10 ERα in 150- and 200-day old boars

The pattern of ERα expression in day-150 group (Fig 15b) and day-200 group (Fig. 15c) was the same as in day-250 group. As shown for the day-150 group (Fig 15b), apart from the myoid cells (arrow 1) and elongated spermatids (arrow 2), all the other cells are positive. The spermatogonia (arrow 3) and primary spermatocytes (arrow 4) showed a strong immunoreaction. The round spermatids (arrow 5), Leydig- (arrow 7) and Sertoli cells (arrow 6) were weakly positive.

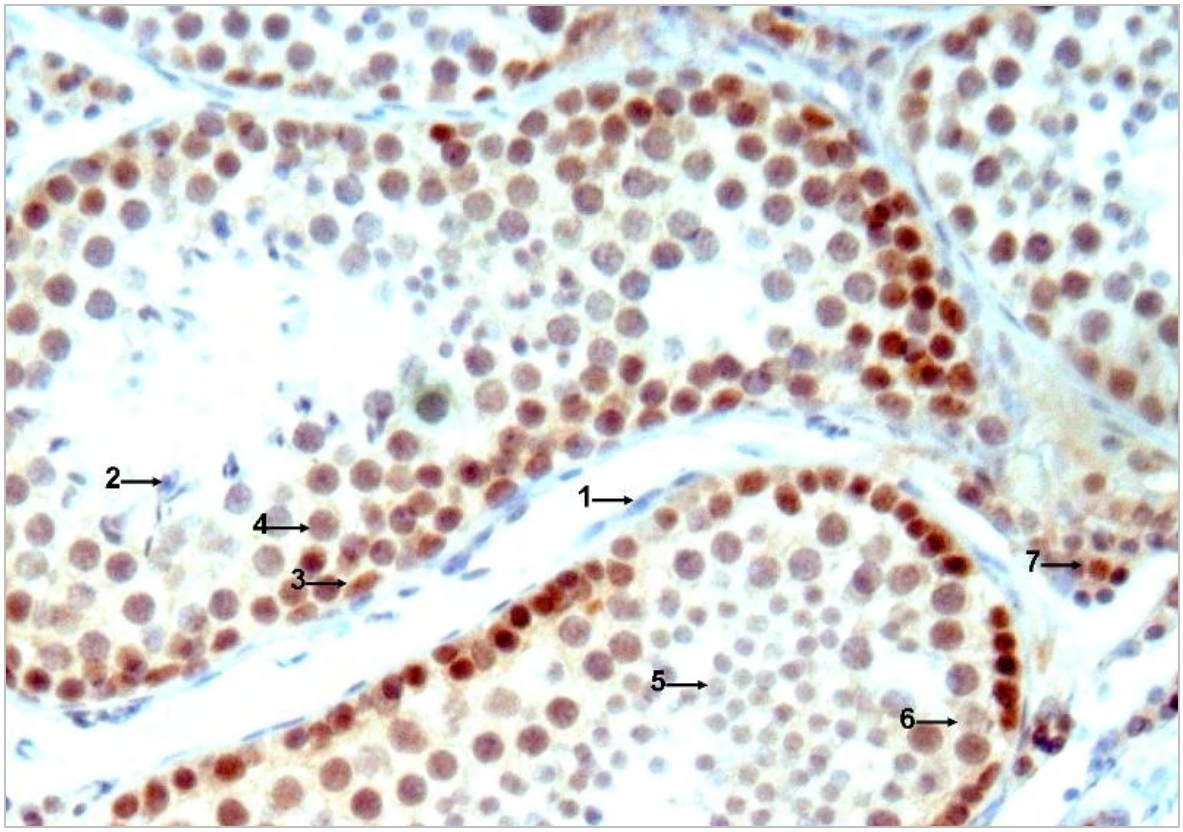

Fig. 15b: ERα in 150-day boar testis showing strong signals in spermatogonia (arrow 3) and primary spermatocytes (arrow 4) while weaker immunoreactions were observed in Leydig- (arrow 7) and Sertoli cells (arrow 6). Mg=400

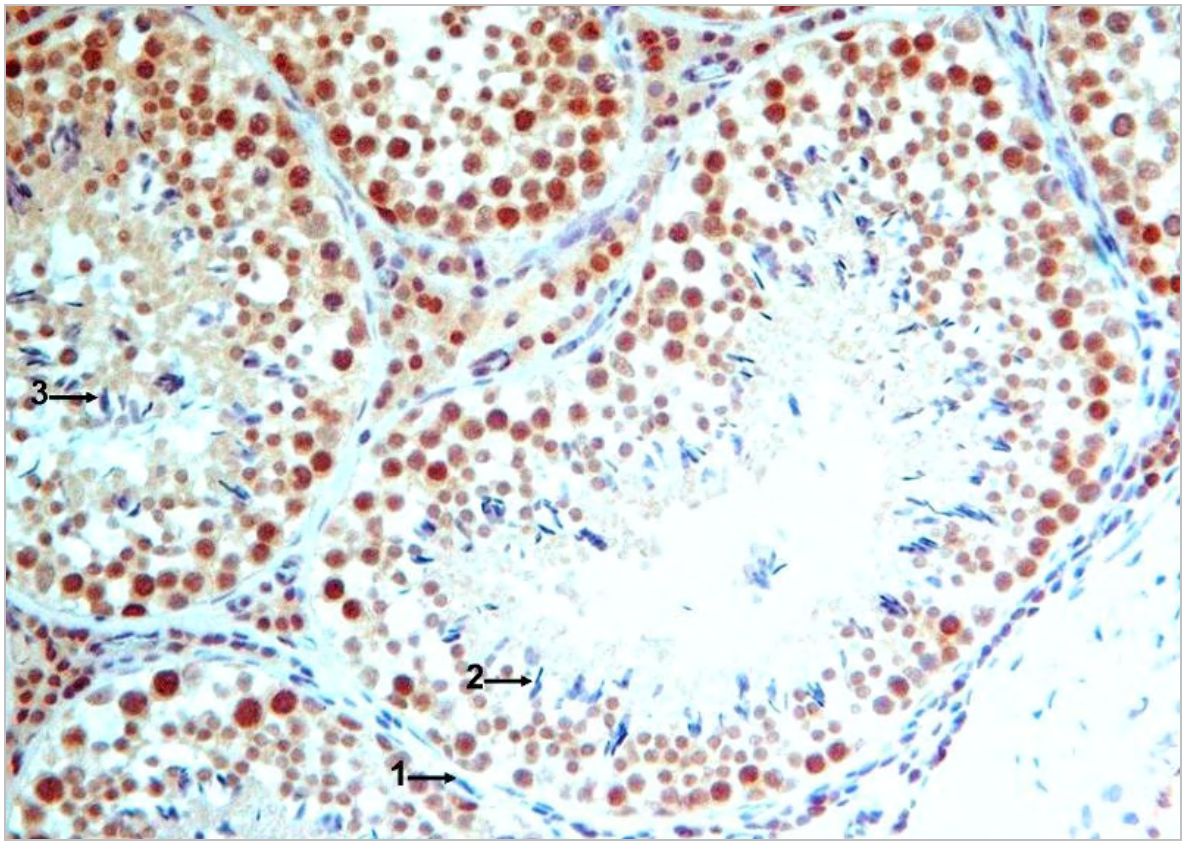

Fig. 15c: ERα in 200-day boar testis. Apart from the negative myoid cells (arrow 1), elongated- (arrow 2) and elongating spermatids (arrow 3), strong signals were observed in spermatogonia and primary spermatocytes, and weaker reactions in Leydig and Sertoli cells. Mg=200

4.1.1.11 ERβ in sexually mature boars (250 day old group)

With exception of elongating/ed spermatids (arrow 6) in the sexually mature group of boars virtually all (94.85% ± 1.6, see Table 14), the Sertoli (arrow 4), Leydig- (arrow 5) and germ cells (arrows 1-3) (see in Fig. 16b) – stained positive for the ERβ. Like it was the case for ERα, the pattern of ERβ expression was the same during the three phases of spermatogenesis (Fig. 16a–c).

(a) Phase I of spermatogenesis (immediately after spermiation, no elongated spermatids)

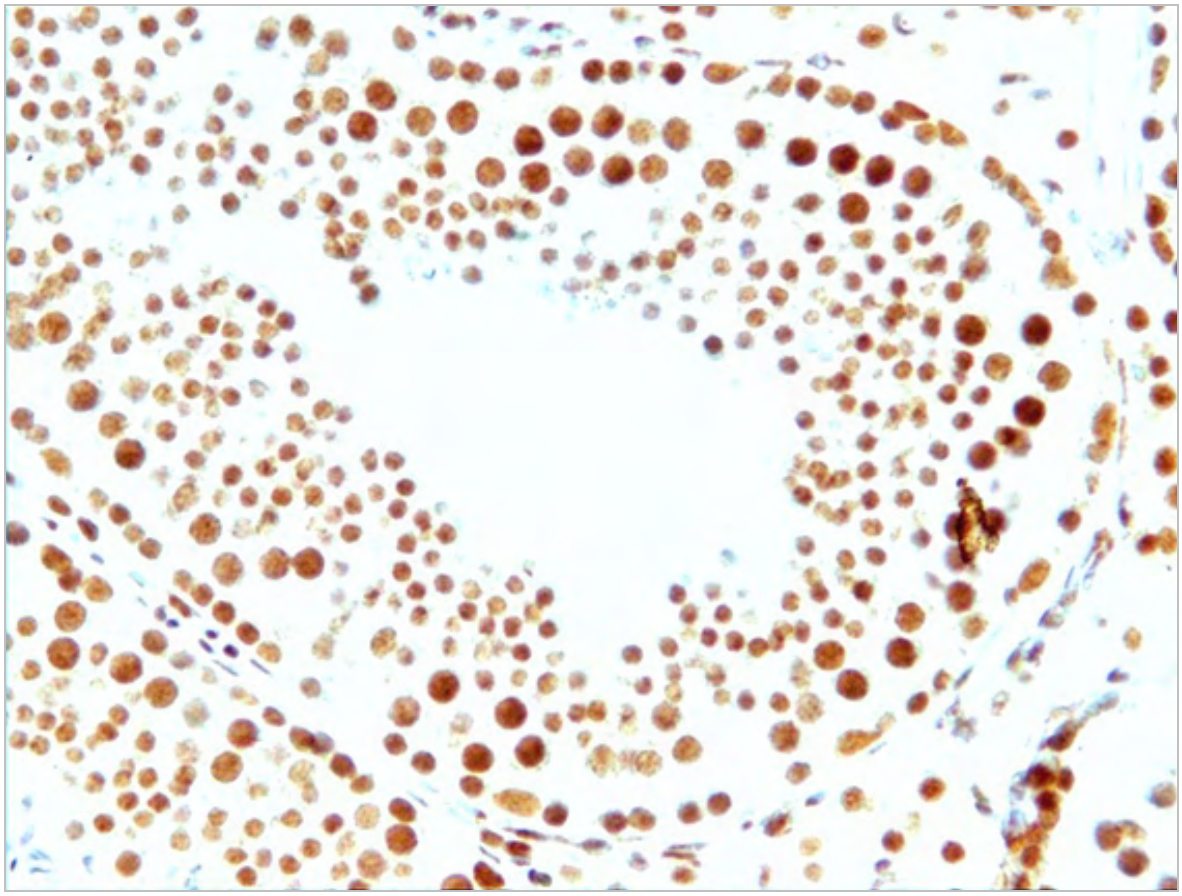

Fig. 16a: ERβ in 250-day boar testis during phase I of spermatogenesis showing signals in spermatogonia, primary spermatocytes, round spermatids, Sertoli cells and Leydig cells. Elongated spermatids are absent in this phase. Mg=400

(b) Phase II of spermatogenesis (elongating spermatids)

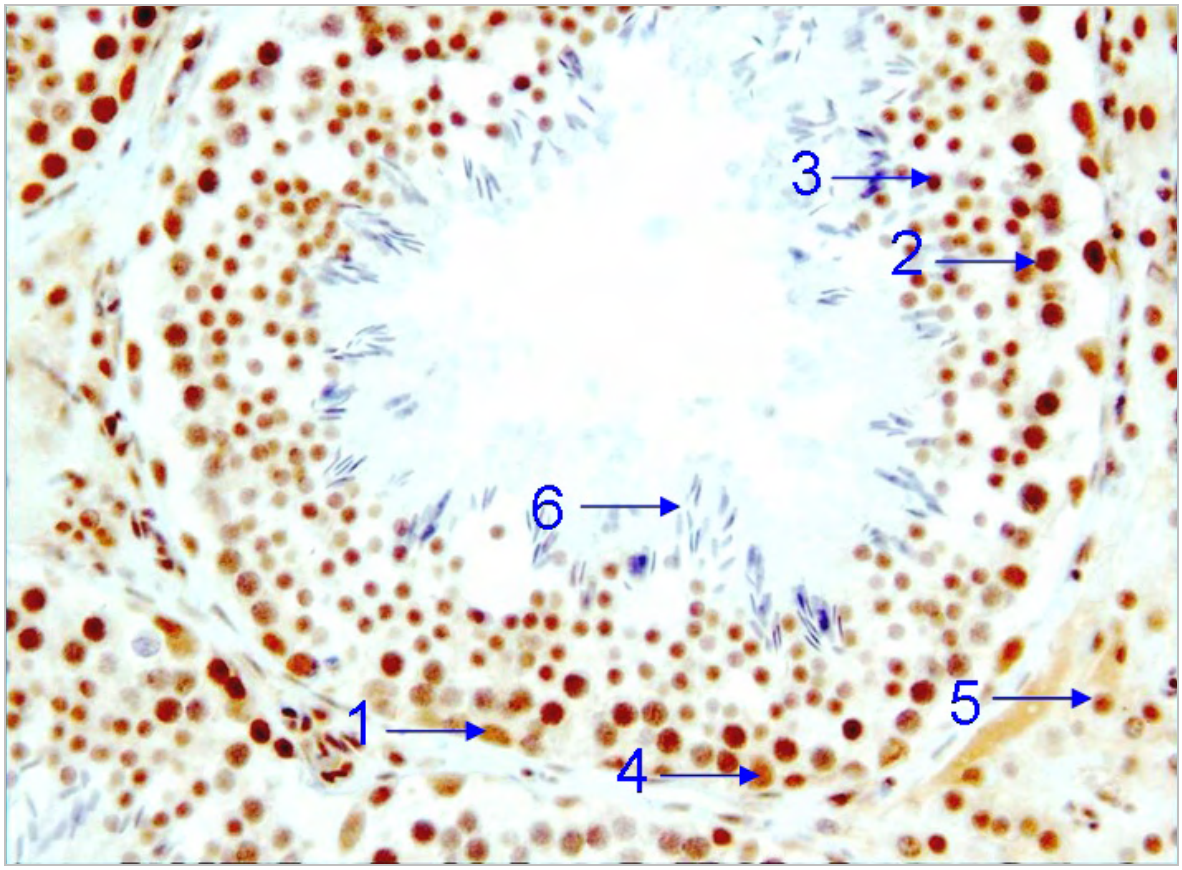

Fig. 16b: ERβ in 250-day boar testis during phase II of spermatogenesis showing signals in spermatogonia (arrow 1), primary spermatocytes (arrow 2), round spermatids (arrow 3), Sertoli- (arrow 4) and Leydig cells (arrow 5). Elongated spermatids (arrow 6) were distinctly negative (6). Mg=X400

(c) Phase III of spermatogenesis (Fully elongated spermatids ready for release)

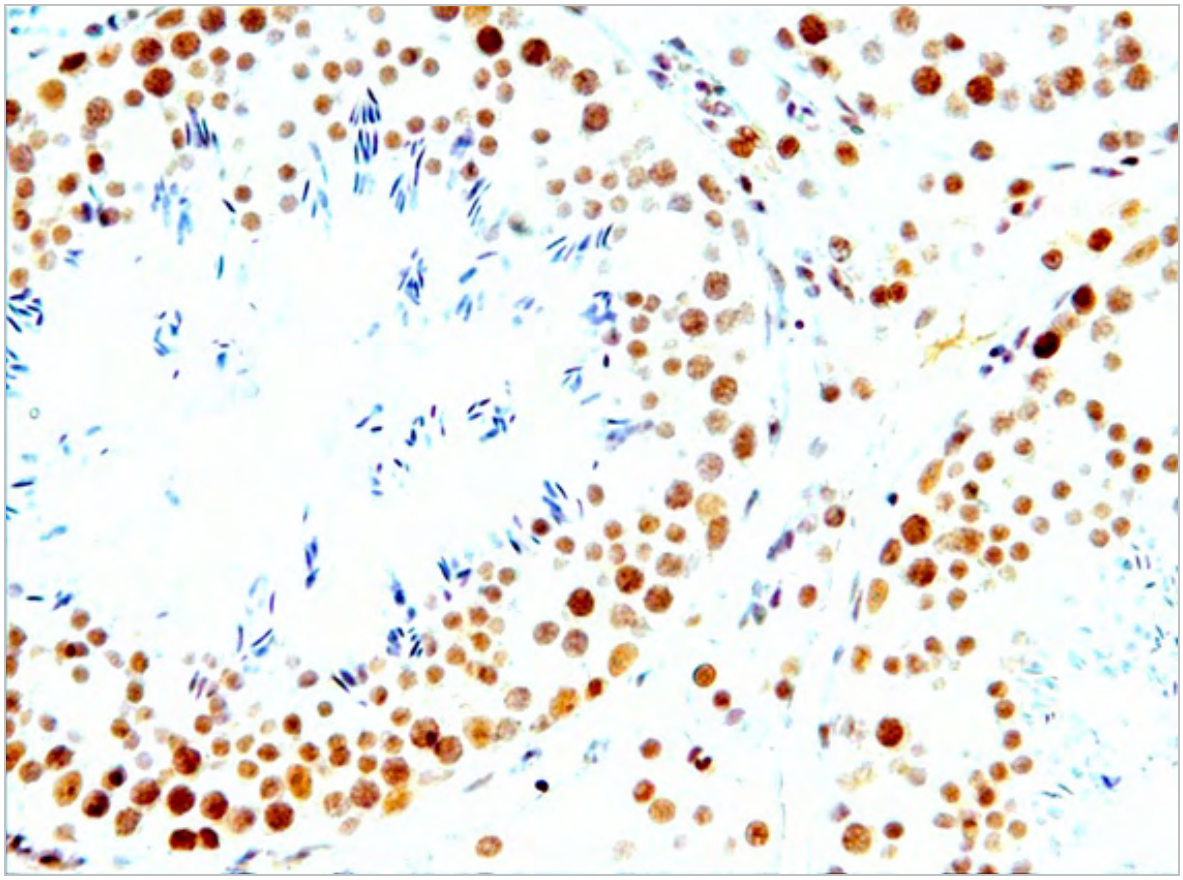

Fig. 16c: ERβ in 250-day boar testis during phase III of spermatogenesis showing signals in spermatogonia, primary spermatocytes, round spermatids, Sertoli cells and Leydig cells. Elongated spermatids are negative. Mg=400

Table 14: Germ cells (except elongating/ed spermatids), Sertoli- and Leydig cells staining positive for the ERβ in the seminiferous tubules of 250-day old boars (E1-E5)

Cells counted in mature seminiferous tubular cells							
Boar	*Slide*	*ERβ positive*	*ERβ negative*	*Total cells*	*%positive*	*Boar*	
E1	A	195	7	202	96.53	*Mean*	*SD*
	B	193	11	204	94.61		
	C	189	2	191	98.95		
						96.70	3.45
E2	A	180	12	192	93.75		
	B	164	2	166	98.80		
	C	149	17	166	89.76		
						94.10	6.43
E3	A	173	10	183	94.54		
	B	172	7	179	96.09		
	C	177	3	180	98.33		
						96.32	3.85
E4	A	175	7	182	96.15		
	B	174	22	196	88.78		
	C	169	5	174	97.13		
						94.02	6.53
E5	A	166	7	173	95.95		
	B	150	16	166	90.36		
	C	174	13	187	93.05	93.12	7.45

Mean ($\bar{X}$) = 94.85, Standard deviation (SD) = 1.57

4.1.1.12 ERβ in the other groups (100- 150- and 200- day old boars)

4.1.1.13 ERβ in 100- day old boars

As for ERβ in day-250 old boars, apart from the myoid cells (arrow 1) and the elongating spermatids (arrow 2), virtually all the other cells within the seminiferous tubules were equivalently positive (Fig. 17a), which includes the Sertoli cells (arrow 3) the spermatogonia (arrow 4), the primary spermatocytes (arrow 5) and round spermatid (arrow 6).

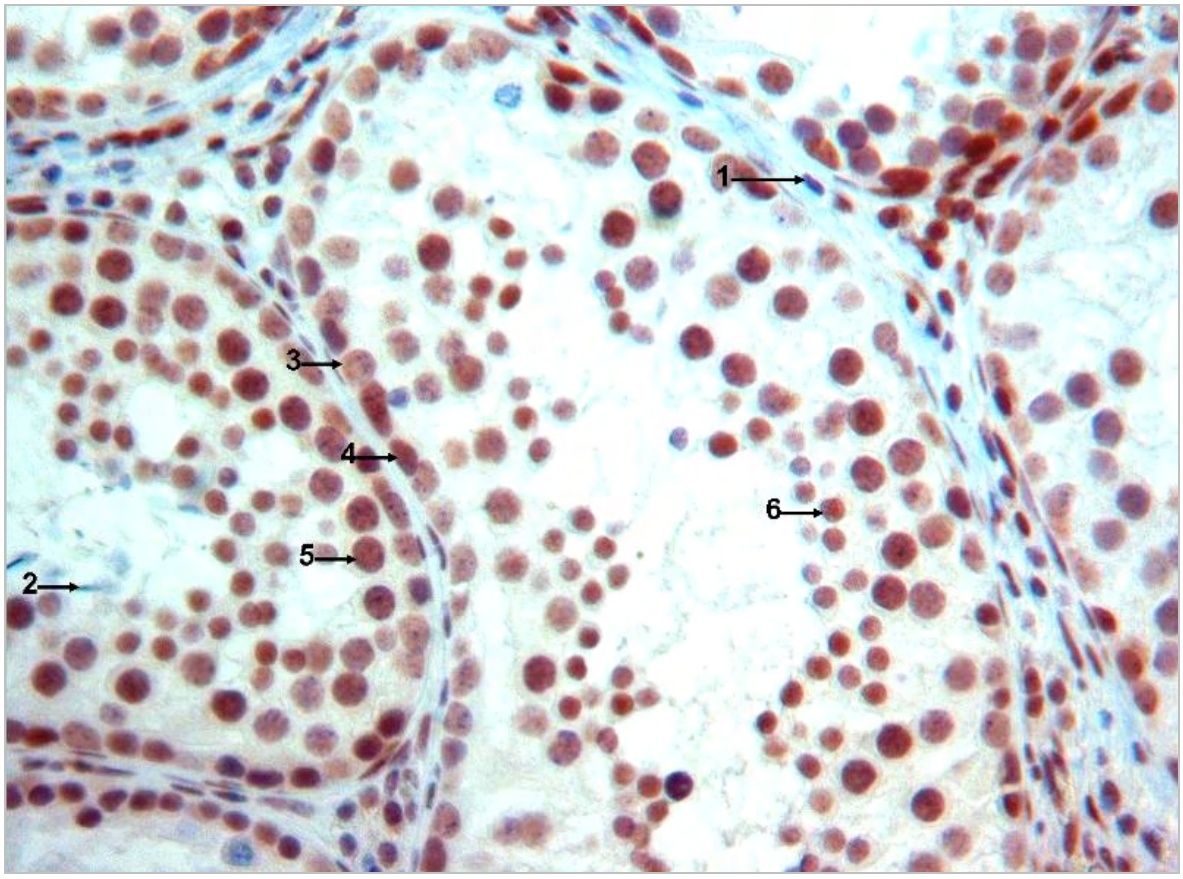

Fig. 17a: ERβ in 100-day boar testis showing signals in Sertoli cells (arrow 3), spermatogonia (arrow 4), primary spermatocytes (arrow 5) and round spermatids (arrow 6). Myoid cells (arrow 1) and elongating spermatids (arrow 2) are negative. Mg=400

4.1.1.14 ERβ in 150- and 200-day old boars

Like it was the case for day-250 group, in day-150 (Fig 17b) and day-200 groups (Fig. 17c), ERβ was expressed at similar intensity in all cell types except in elongating and elongated spermatids.

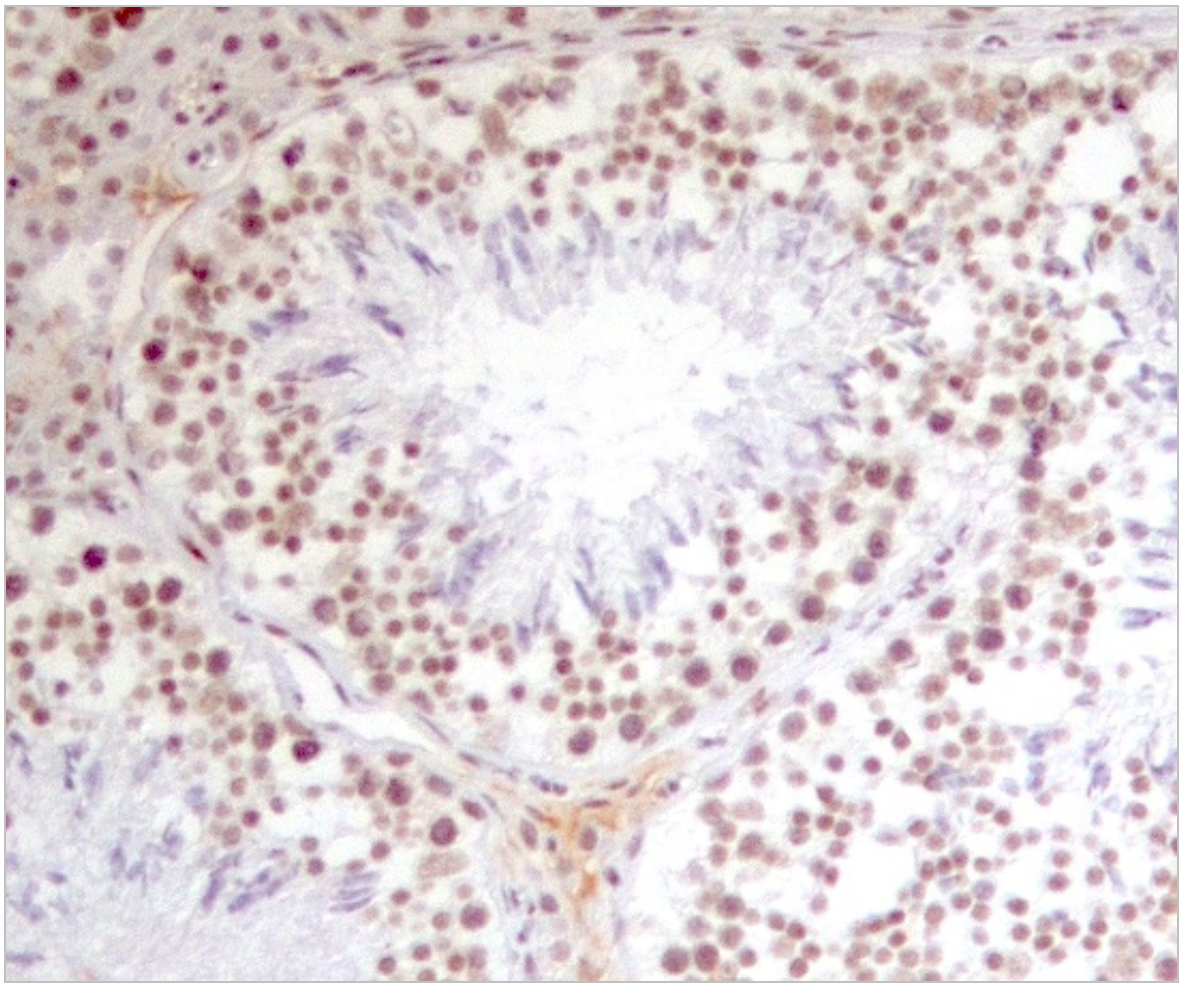

Fig. 17b: ERβ in 150-day boar testis showing signals in all cell types except in elongating/ed spermatids. Mg=X200

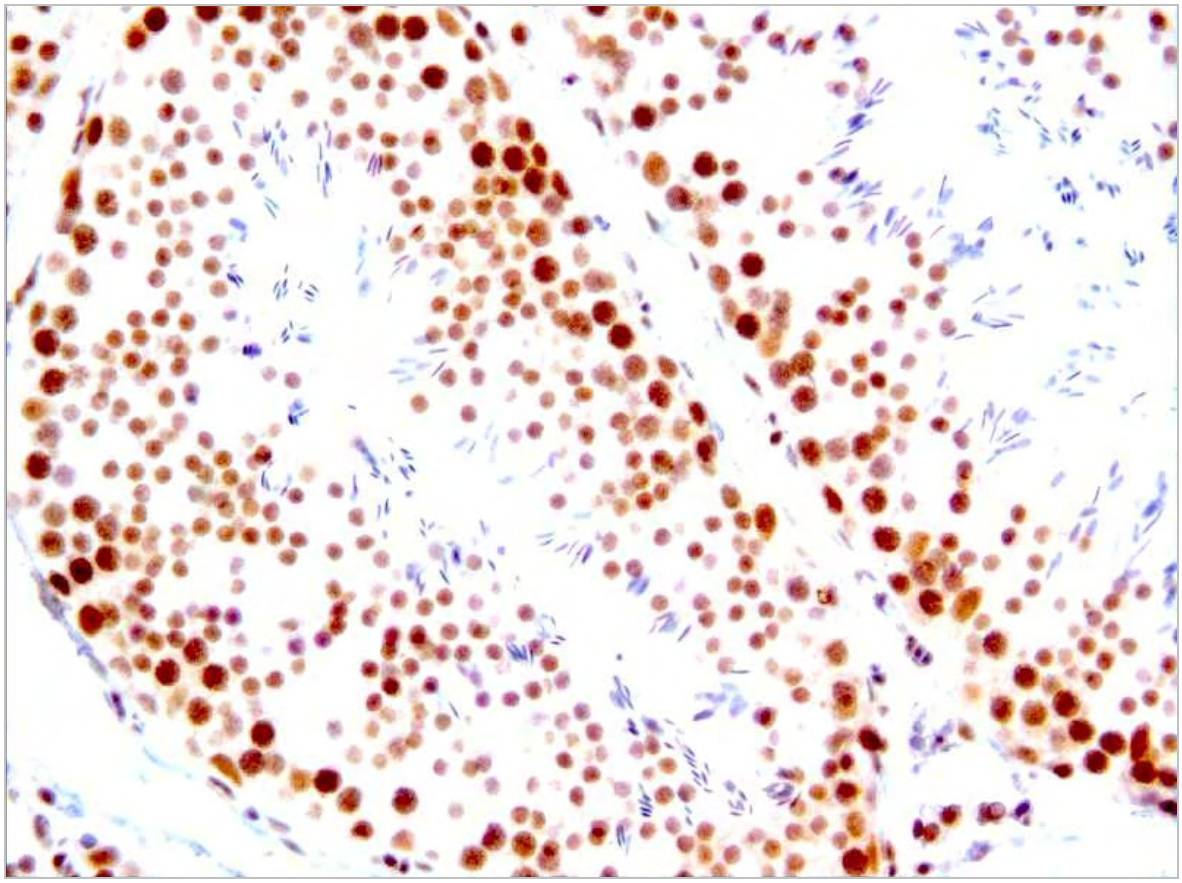

Fig. 17c: ERβ in 200-day boar testis showing signals in all cell types except in elongated/ing spermatids. Mg=200

4.1.2 Detection of ER-Specific mRNA

4.1.2.1 In vitro RT-PCR

One specific band of the expected base pairs corresponding to the respective ER isotype mRNA (Table 6) was detected during *in vitro* RT-PCR in all testicular samples from all age groups and porcine uterus (Fig.18). Sequencing of PCR products (Qiagen) yielded 95 and 92% similarity with the respective porcine mRNA sequences for ERα and ERβ, respectively.

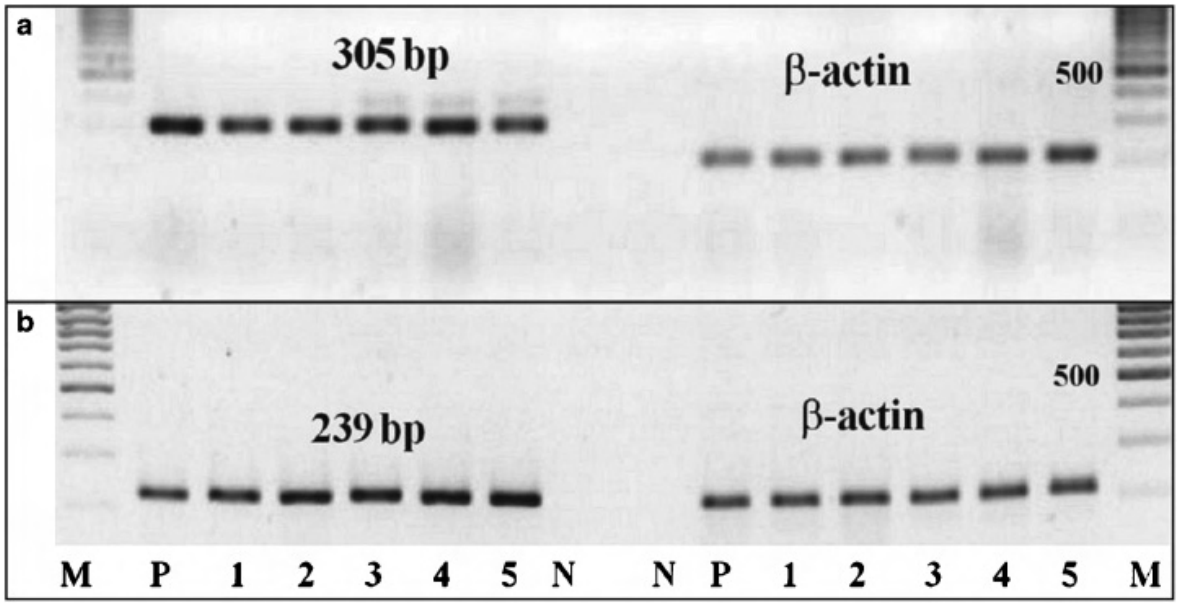

Fig. 18: Ethidium bromide-stained agarose gel of in vitro RT-PCR analysis for the presence of mRNA specific to (a) ERα and (b) ERβ. 1 = 50 days, 2 = 100 days, 3 = 150 days, 4 = 200 days and 5 = 250 days testicular samples; M, DNA ladder; P, porcine uterus (positive control tissue); N, autoclaved deionised distilled water (negative control sample)

4.1.2.2 ER localisation using in situ RT-PCR

When applying *in situ* RT-PCR, strong positive signals for the ERβ mRNA (Fig. 19a) were found in Sertoli and Leydig cells and germ cells except elongating/ed spermatids. In case of the ERα (Fig. 19b) the staining pattern observed corresponded to the results obtained by IHC; distinct cytoplasmic signals were restricted to the spermatogonia and primary spermatocytes, only weak and fewer signals showed up in round spermatids and Leydig cells, elongated spermatids were negative. Negative controls were devoid of non-specific staining (Fig. 19c) while very strong nuclear signals from the genomic DNA were observed in the positive controls (Fig. 19d).

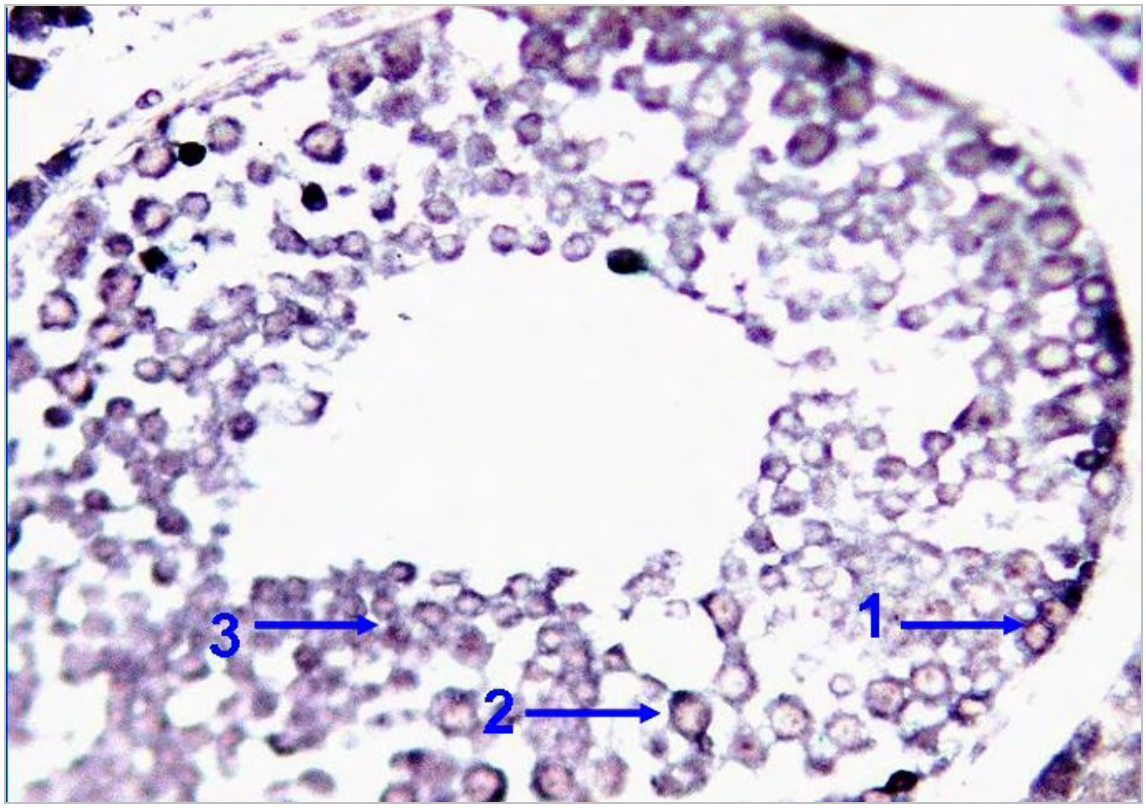

Fig. 19a: In situ RT-PCR for ERβ mRNA in 250-day old in mature boar testis showing distinct signals for ERβ-mRNA in spermatogonia (1), primary spermatocytes (2) and round spermatids (3). Mg=X400

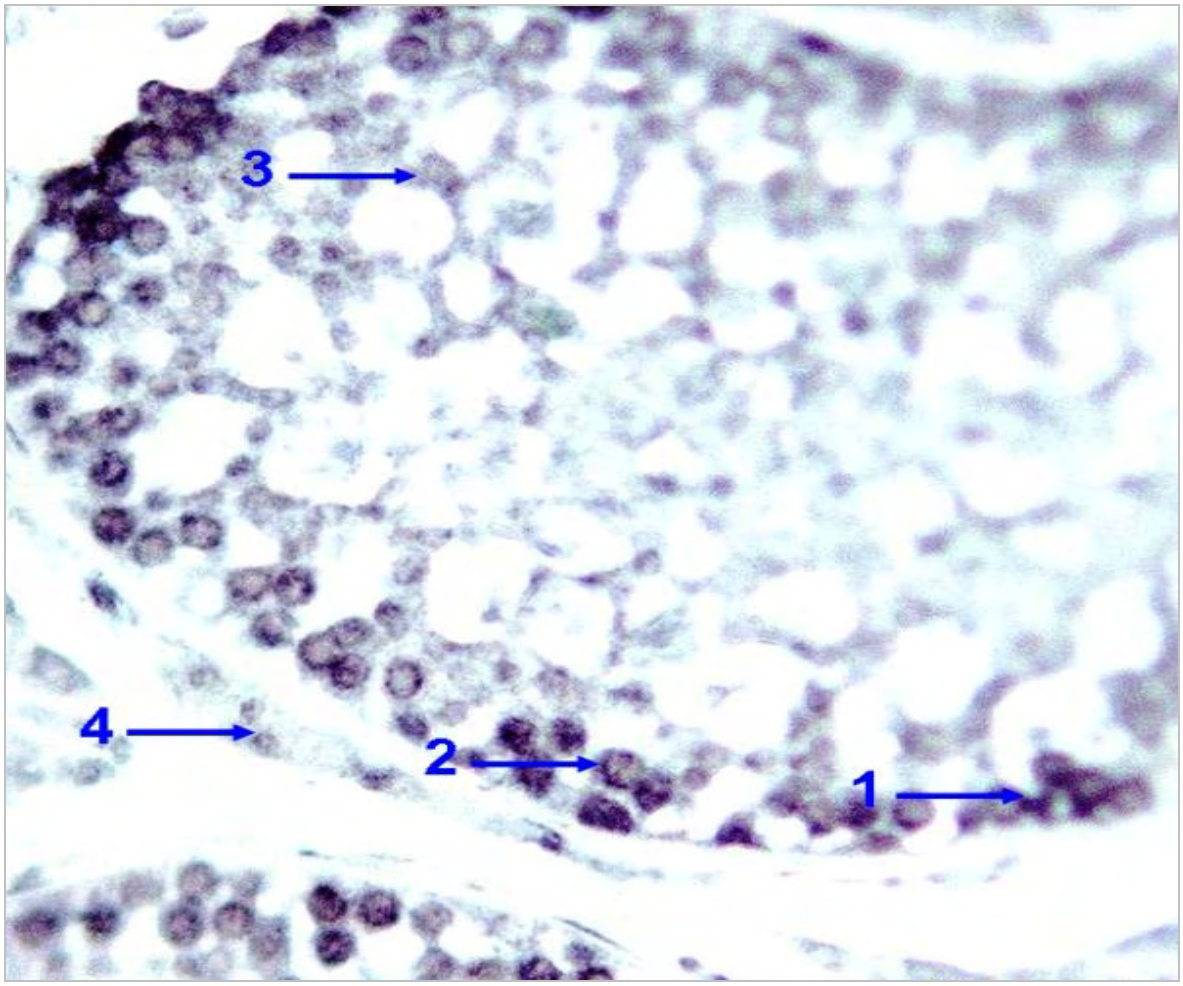

Fig. 19b: In situ RT-PCR for ERα mRNA in 250-day old boar testis. Strong cytoplasmic signals for ERα-mRNA in spermatogonia (1) and primary spermatocytes (2) while round spermatids (3) Leydig cells showed no and/ or very weak signals. Mg=X400

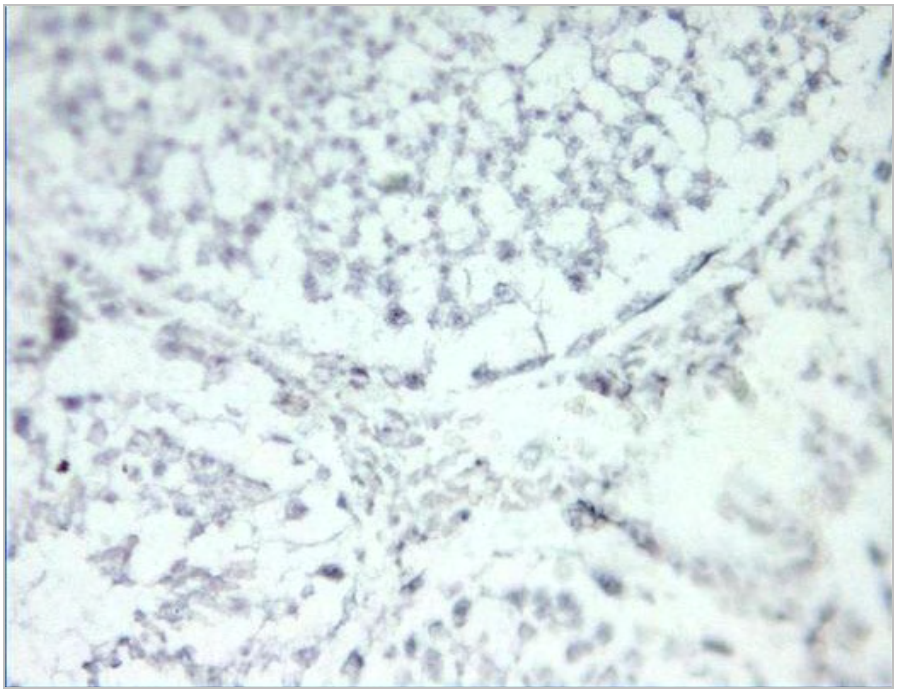

Fig. 19c: Negative control for in situ RT-PCR for estrogen receptor mRNA in 250-day old boar testis. No staining after **in situ**-RT-PCR (primers omitted). Mg=x200

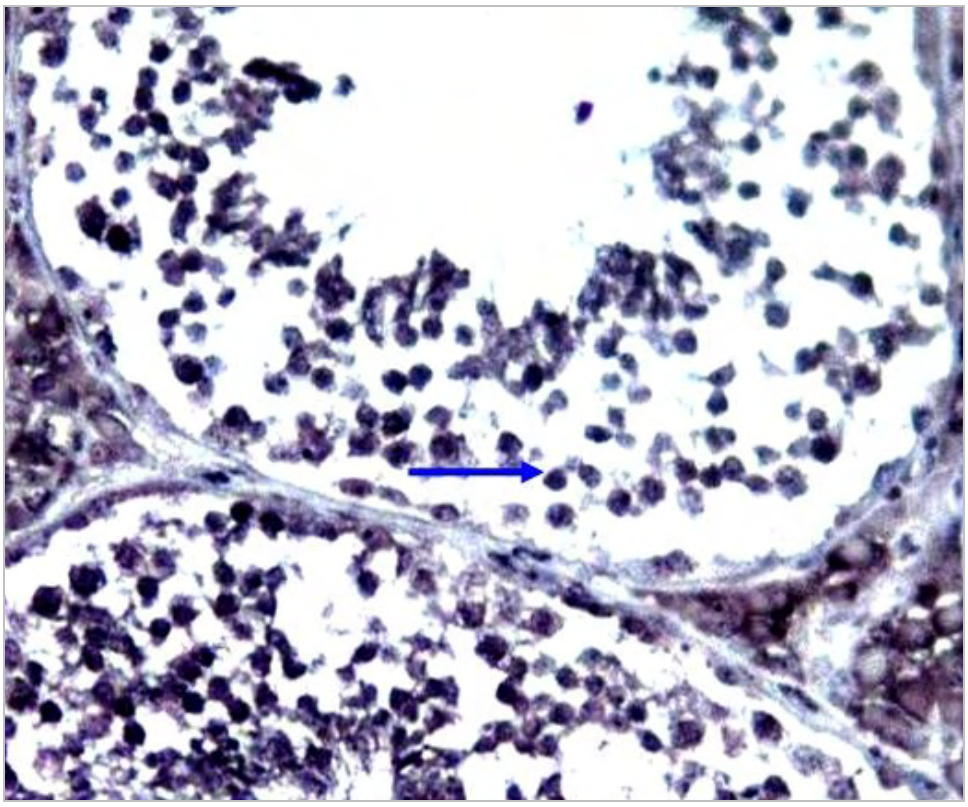

Fig. 19d: Positive control for in situ RT-PCR for estrogen receptor mRNA in 250-day old boar testis; positive control: omission of DNAse treatment showing nuclear signals due to amplification of genomic DNA sequences (blue arrow). Mg=X400

4.2 Expression of P450 aromatase by IHC

In all age groups the P450 aromatase immunoreactivity was found exclusively in the cytoplasm of Leydig cells. However an increase in both the number of positive cells and the intensity of the staining was observed with increasing age of the boars (Fig. 20a-e, Table 15a-c).

4.2.1 P450 aromatase in 50-day old boars

In this group of boars intense P450 aromatase immunoreactivity was found in 67.81%±2.29 of Leydig cells (arrow 1) while in the rest a weaker or no reaction (arrow2) was observed (Fig. 20a).

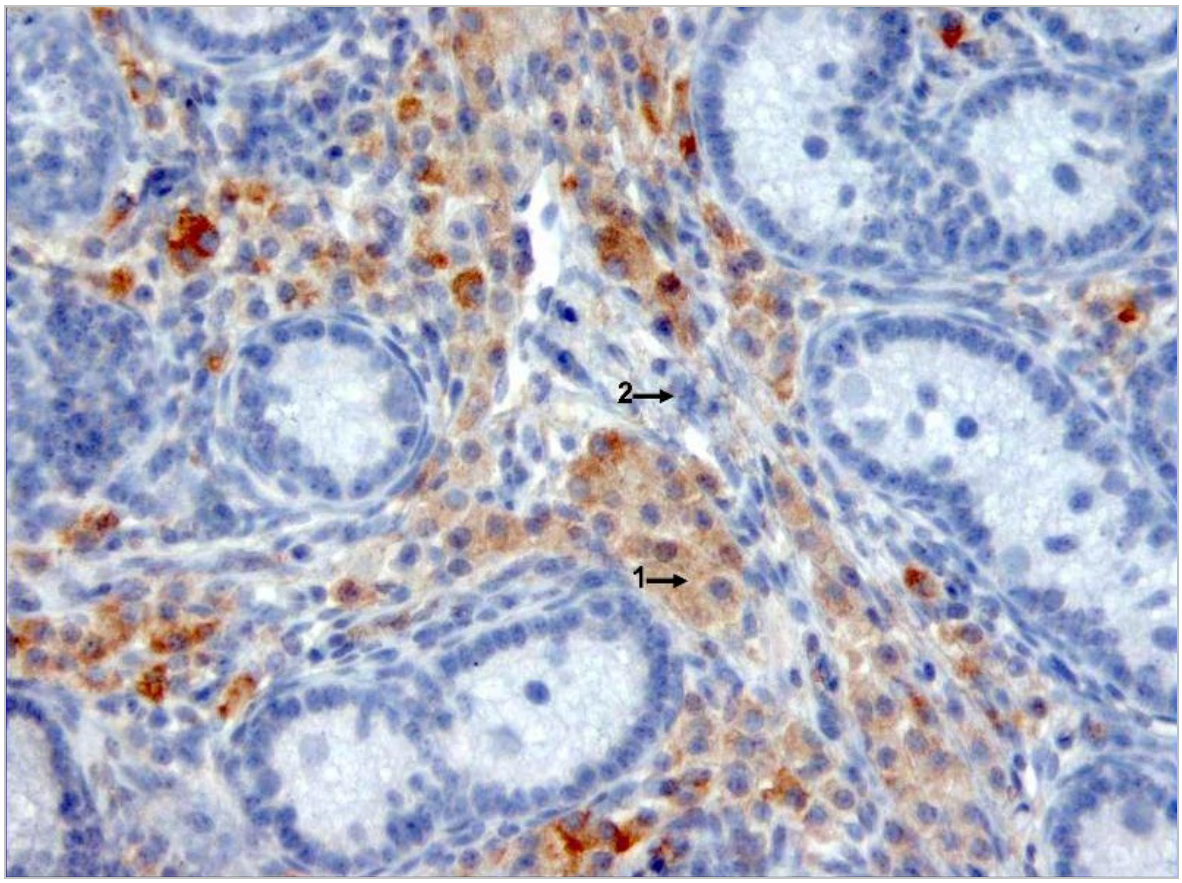

Fig. 20a: Immunolocalisation of P450 aromatase in the 50-day old boar testis showing strong positive signals in the cytoplasm of some of the Leydig cells (arrow 1) and weaker and/ or no signal in others (arrow 2). Mg=X200

Table 15a: Leydig cells staining positive for P450 aromatase in 50-day old boars

Leydig cells counted						
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	68	52	120	56.7	
	B	81	24	105	77.1	
	C	83	51	134	61.9	65.25
E2	A	81	38	119	68.1	
	B	65	33	98	66.3	
	C	82	45	127	64.6	66.32
E3	A	73	37	110	66.4	
	B	84	26	110	76.4	
	C	78	44	122	63.9	68.89
E4	A	86	40	126	68.3	
	B	73	32	105	69.5	
	C	65	21	86	75.6	71.12
E5	A	76	41	117	65.0	
	B	48	21	69	69.6	
	C	89	42	131	67.9	67.49

Mean ($\bar{X}$) = 67.81, Standard deviation (SD) = 2.29

4.2.2 P450 aromatase in 100-day old boars

In this group of boars intense P450 aromatase immunoreactivity was found in 75.84%±1.65 of Leydig cells (arrow 1) while in the rest a weaker or no reaction (arrow 2) was observed (Fig. 20b).

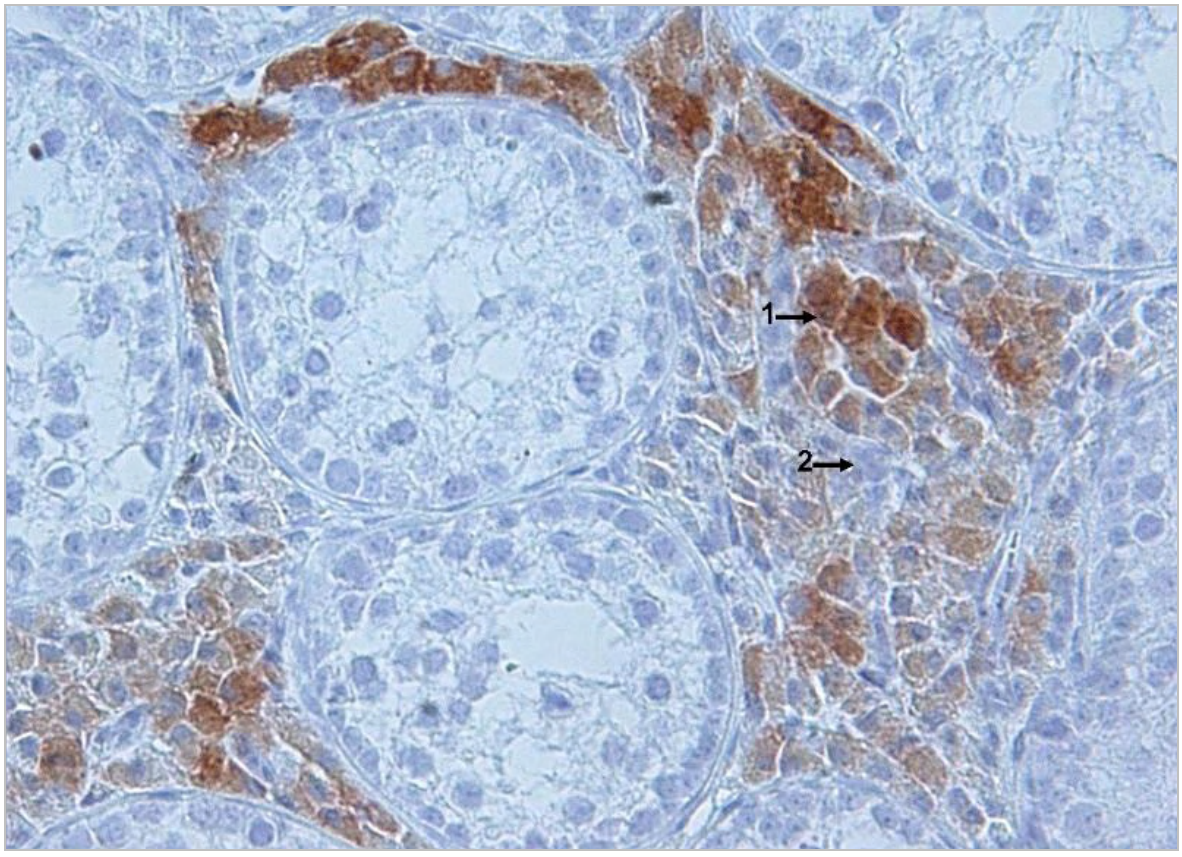

Fig. 20b: Immunolocalisation of P450 aromatase in the 100-day old boar testis showing strong positive signals in the cytoplasm of some of the Leydig cells (arrow 1) and weaker and/ or no signal in others (arrow 2). Mg=X200

Table 15b: Leydig cells staining positive for P450 aromatase in 100-day old boars

Leydig cells counted						
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	76	35	111	68.5	
	B	80	15	95	84.2	
	C	78	30	108	72.2	74.97
E2	A	84	29	113	74.3	
	B	79	33	112	70.5	
	C	77	25	102	75.5	73.45
E3	A	76	26	102	74.5	
	B	71	20	91	78.0	
	C	75	22	97	77.3	76.62
E4	A	72	20	92	78.3	
	B	69	21	90	76.7	
	C	64	18	82	78.0	77.66
E5	A	73	21	94	77.7	
	B	70	25	95	73.7	
	C	79	22	101	78.2	76.52

Mean ($\bar{X}$) = 75.84, Standard deviation (SD) = 1.65

4.2.3 P450 aromatase in 150-day old boars

In the 150-day old group of boars, the intensity of P450 aromatase immunoreactivity was stronger than for the day-50 and -100 groups and again the number of negative Leydig cells decreased (arrow 2), with the positive cells increasing to 87.52%±1.08 (arrow 1) (Fig. 20c).

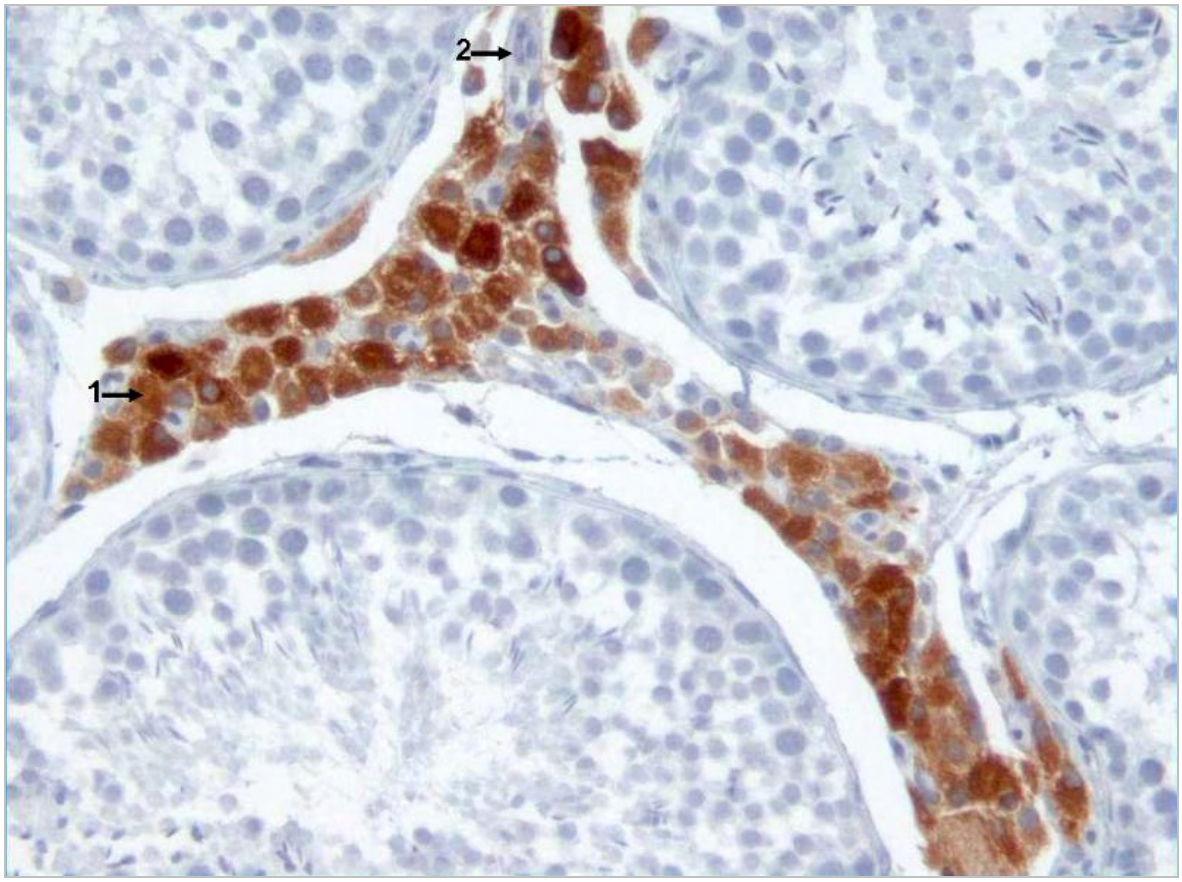

Fig. 20c: Immunolocalisation of P450 aromatase in the 150-day old boar testis showing positive signals in the cytoplasm of Leydig cells (arrow 1) and some cells with and/ or without signals (arrow 2). Mg=X200

Table 15c: Leydig cells positive for P450 aromatase in 150-day old boars

Leydig cells counted						
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	89	16	105	84.8	
	B	86	13	99	86.9	
	C	90	11	101	89.1	86.91
E2	A	92	12	104	88.5	
	B	87	15	102	85.3	
	C	93	8	101	92.1	88.61
E3	A	91	16	107	85.0	
	B	90	9	99	90.9	
	C	88	13	101	87.1	87.69
E4	A	94	10	104	90.4	
	B	89	14	103	86,4	
	C	91	12	103	88.3	88.38
E5	A	86	11	97	88.7	
	B	84	14	98	85.7	
	C	92	18	110	83.6	86.00

Mean ($\bar{X}$) = 87.52, Standard deviation (SD) = 1.08

4.2.4 P450 aromatase in 200- and 250-day old boars

In these boars the intensity of P450 aromatase immunoreactivity was stronger than for groups of younger animals and literally all Leydig cells were positive (arrow 1) (Fig. 20d).

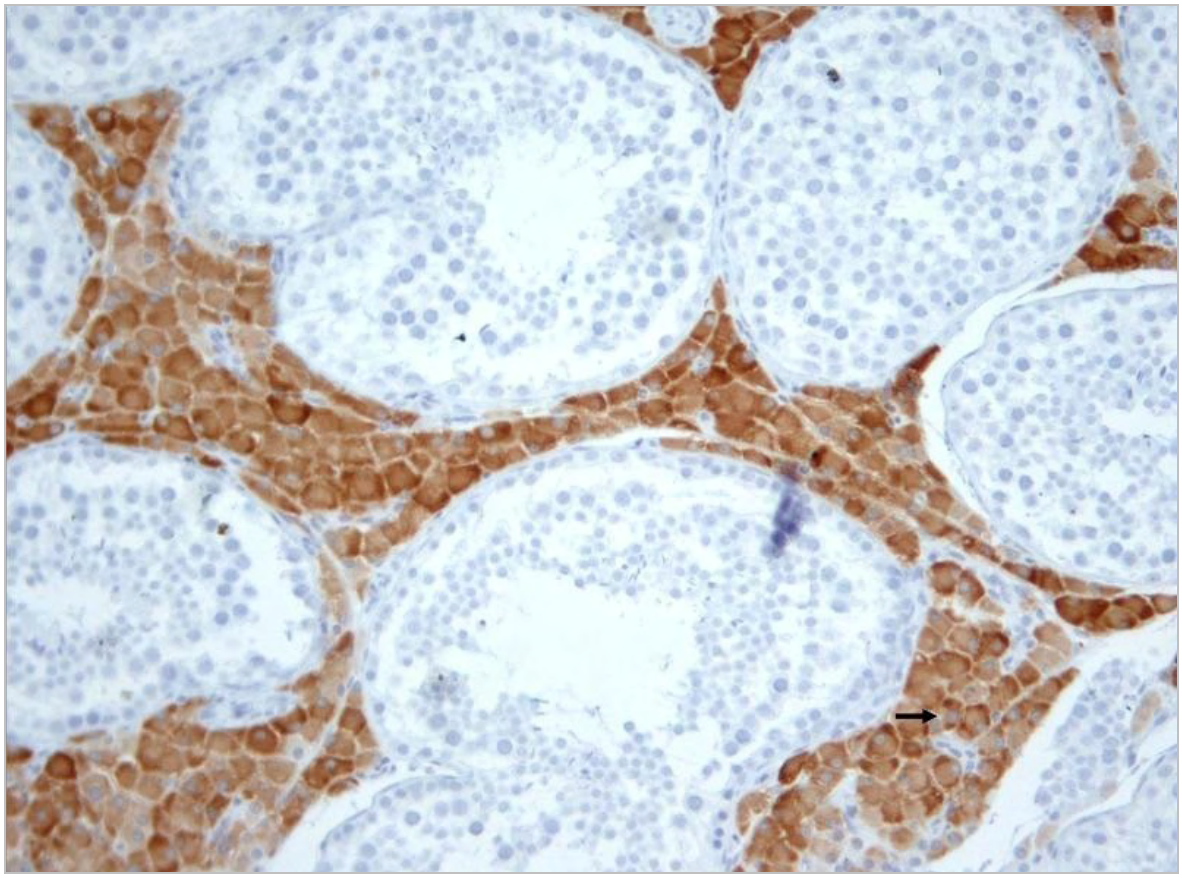

Fig. 20d: Immunolocalisation of P450 aromatase in the 200-day old boar testis showing positive signals in the cytoplasm of all Leydig cells (black arrow). Mg=X100

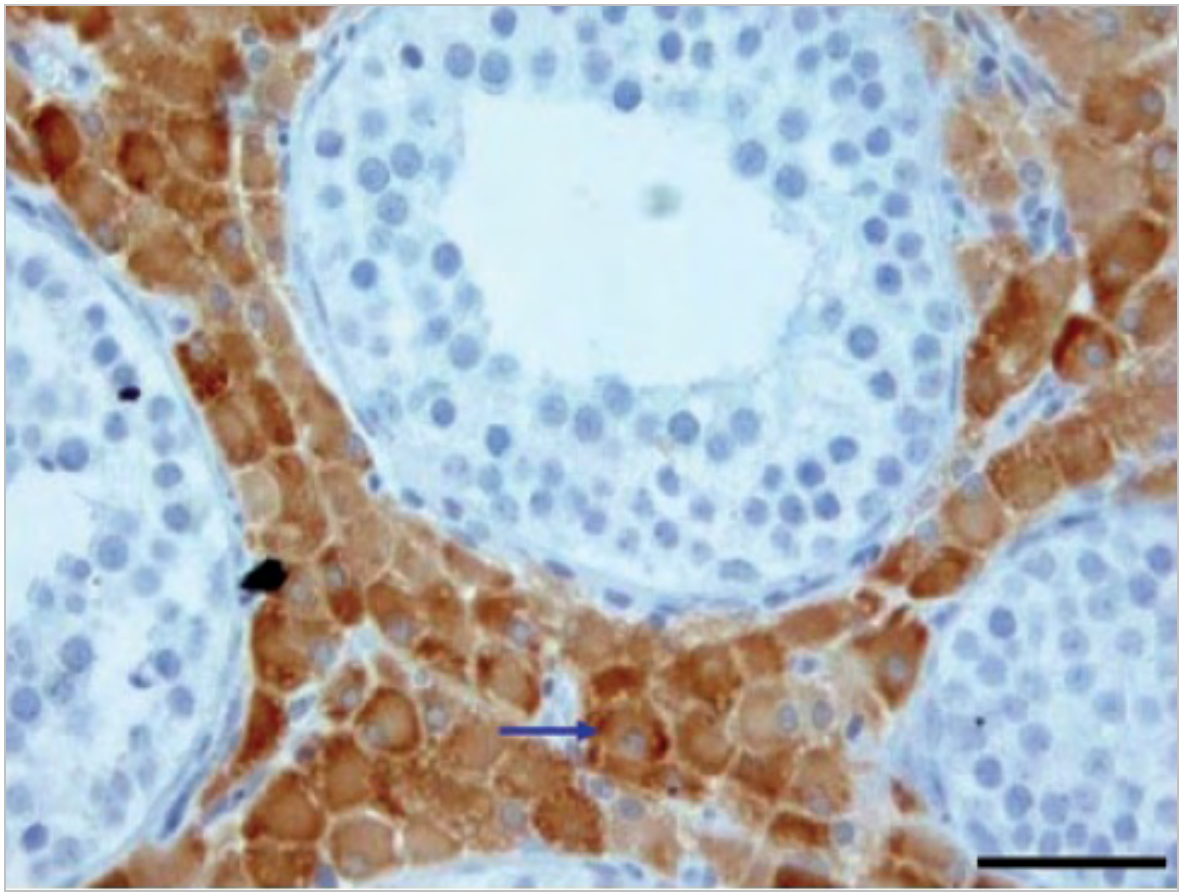

Fig. 20e: Immunolocalisation of P450 aromatase in the 250-day boar testis showing at higher magnification positive signals in the cytoplasm of all Leydig cells (blue arrow). Mg=X400

4.2.5 Detection of aromatase-Specific mRNA

In vitro RT-PCR detected one specific band of aromatase mRNA with the expected number of base pairs (Table 6) in samples from all age groups (Fig. 21).

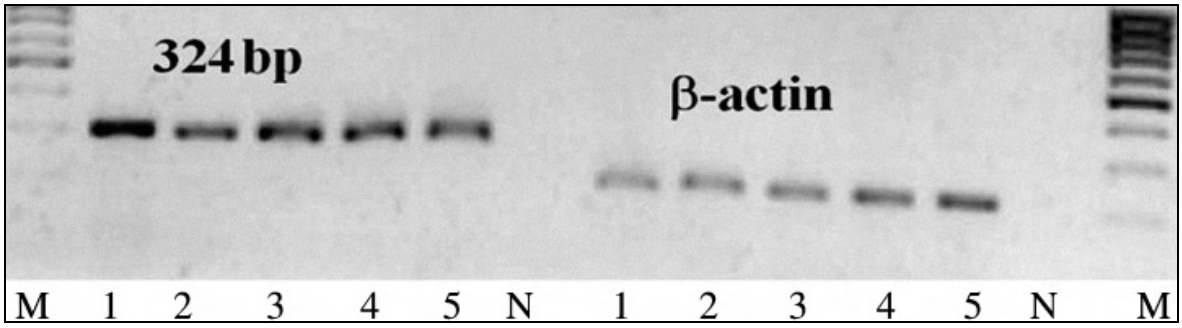

Fig. 21: Ethidium bromide-stained agarose gel of in vitro RT-PCR analysis for the presence of mRNA specific to P450 aromatase. 1 = 50 days, 2 = 100 days, 3 = 150 days, 4 = 200 days and 5 = 250 days testicular samples; M, DNA ladder; N, autoclaved double deionised water (negative control sample)

4.3 Expression of Steroid sulfatase and estrogen sulfotransferase

4.4.1 Immunolocalisation of Steroid Sulfatase (StS) by IHC

In all age groups (50-250 days) (Fig. 22a-e), StS was detected only in the cytoplasm of the Leydig cells. Apart from in day-50 group, in which 65.17±4.9% Leydig cells were positive (Table 16), StS expression in the other age groups remained relatively constant through out the age groups and literally all the Leydig cells were stained.

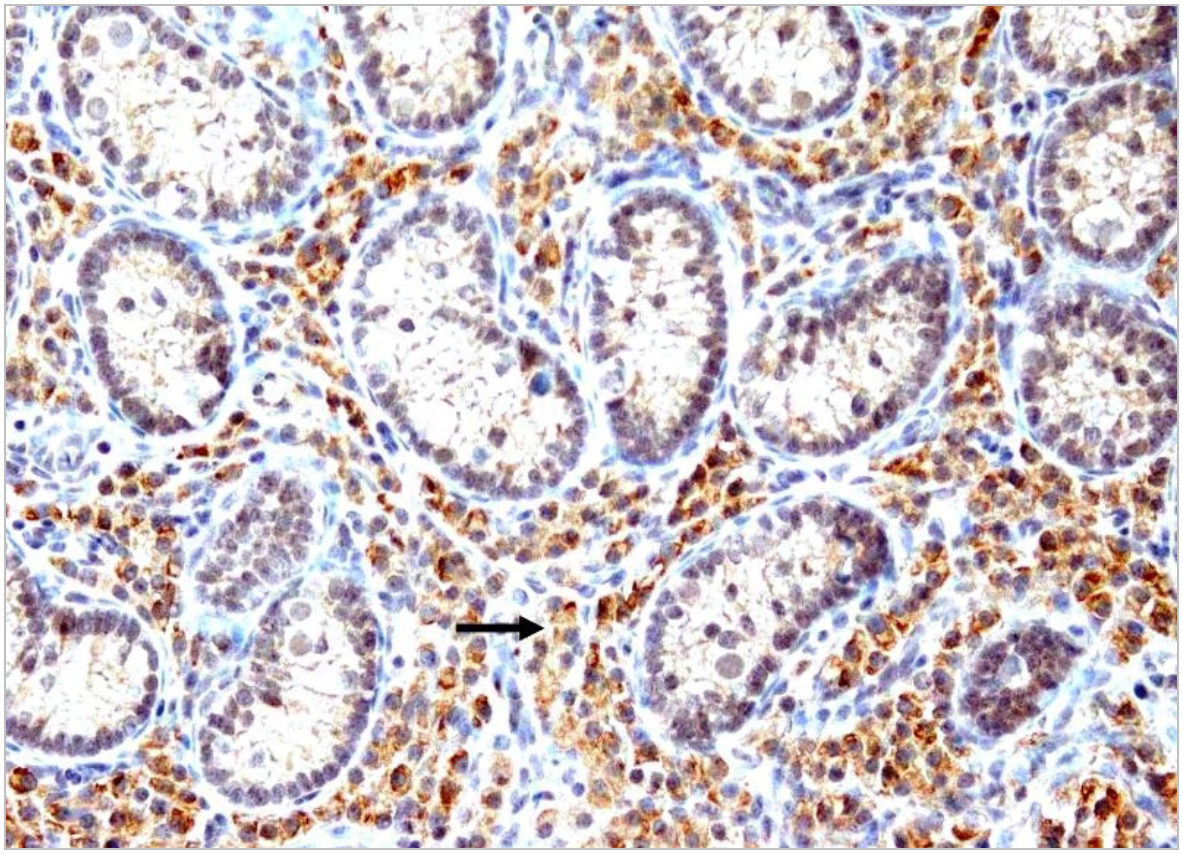

Fig. 22a: StS expression in 50-day old boar testis showing signals in the cytoplasm of the Leydig cells (black arrow). Mg=X100

Table 16: Leydig cells staining positive for StS in 50-day old boars

Leydig cells counted						
Boar	*Slide*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*
E1	A	71	37	108	65.7	
	B	78	40	118	66.1	
	C	69	47	116	59.5	63.78
E2	A	64	39	103	62.1	
	B	66	43	109	60.6	
	C	60	37	97	61.9	61.51
E3	A	68	48	116	58.6	
	B	79	47	126	62.7	
	C	75	52	127	59.1	60.12
E4	A	67	37	104	64.4	
	B	68	30	98	69.4	
	C	74	28	102	72.5	68.79
E5	A	71	30	101	70.3	
	B	74	28	102	72.5	
	C	75	29	104	72.1	71.65

Mean ($\bar{X}$) = 65.17, Standard deviation (SD) = 4.90

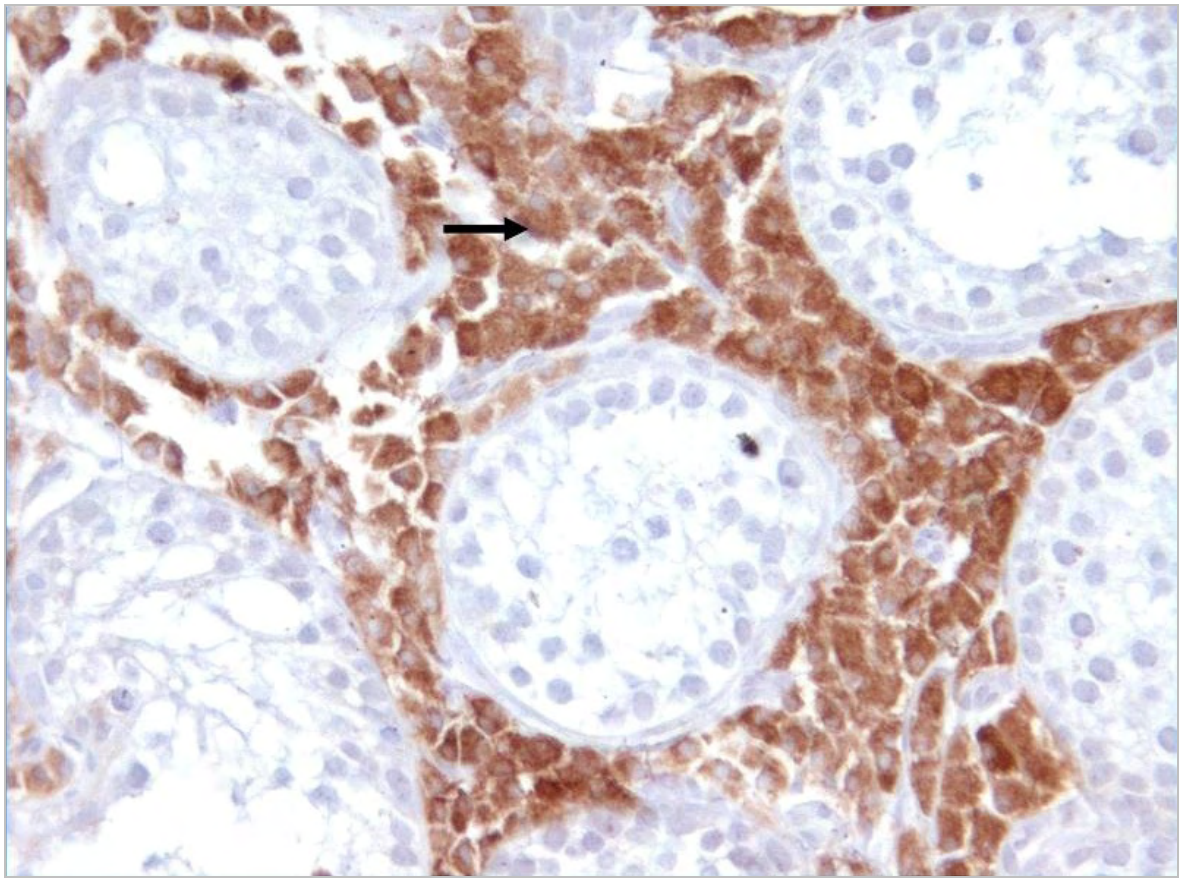

Fig. 22b: StS expression in 100-day old boar testis showing signals in the cytoplasm of the Leydig cells (black arrow). Mg=X200

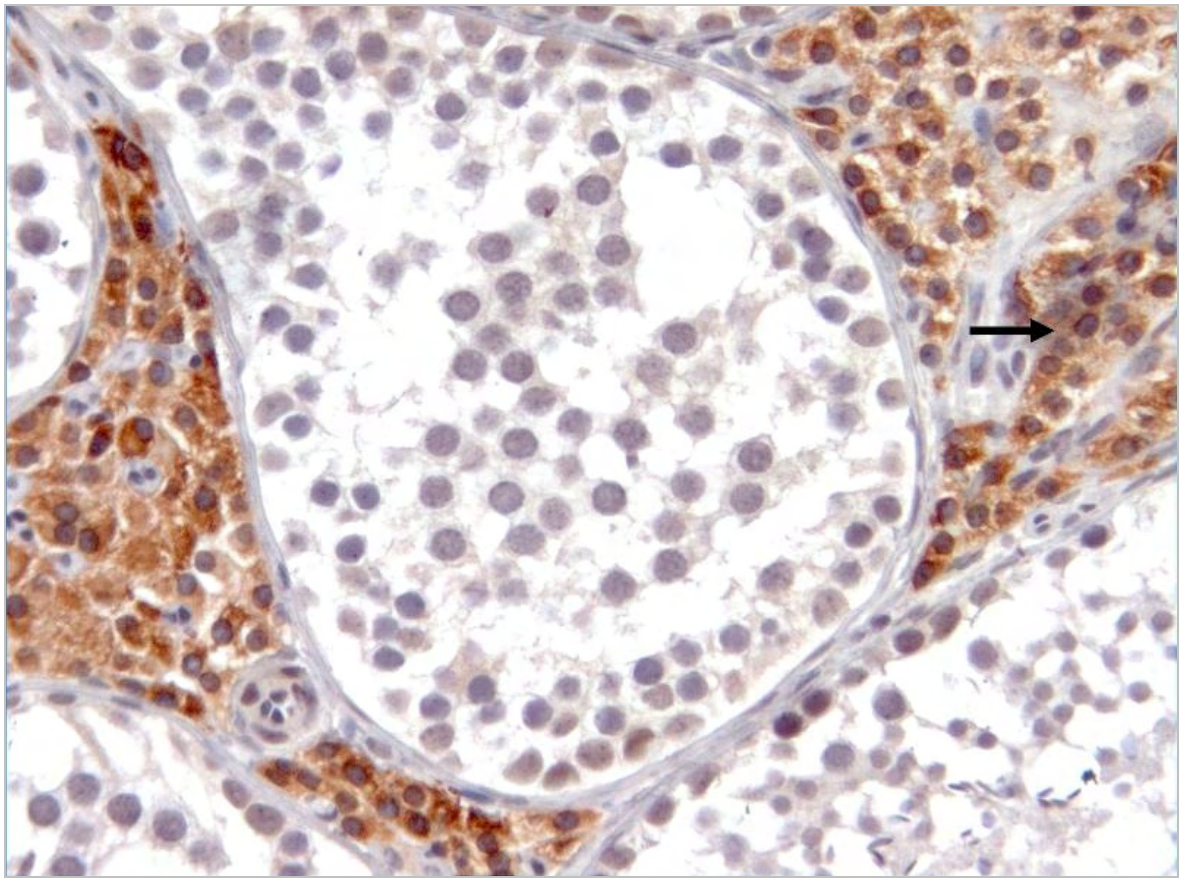

Fig. 22c: StS expression in 150-day old boar testis showing at a higher magnification signals in the cytoplasm of the Leydig cells (black arrow). Mg=X400

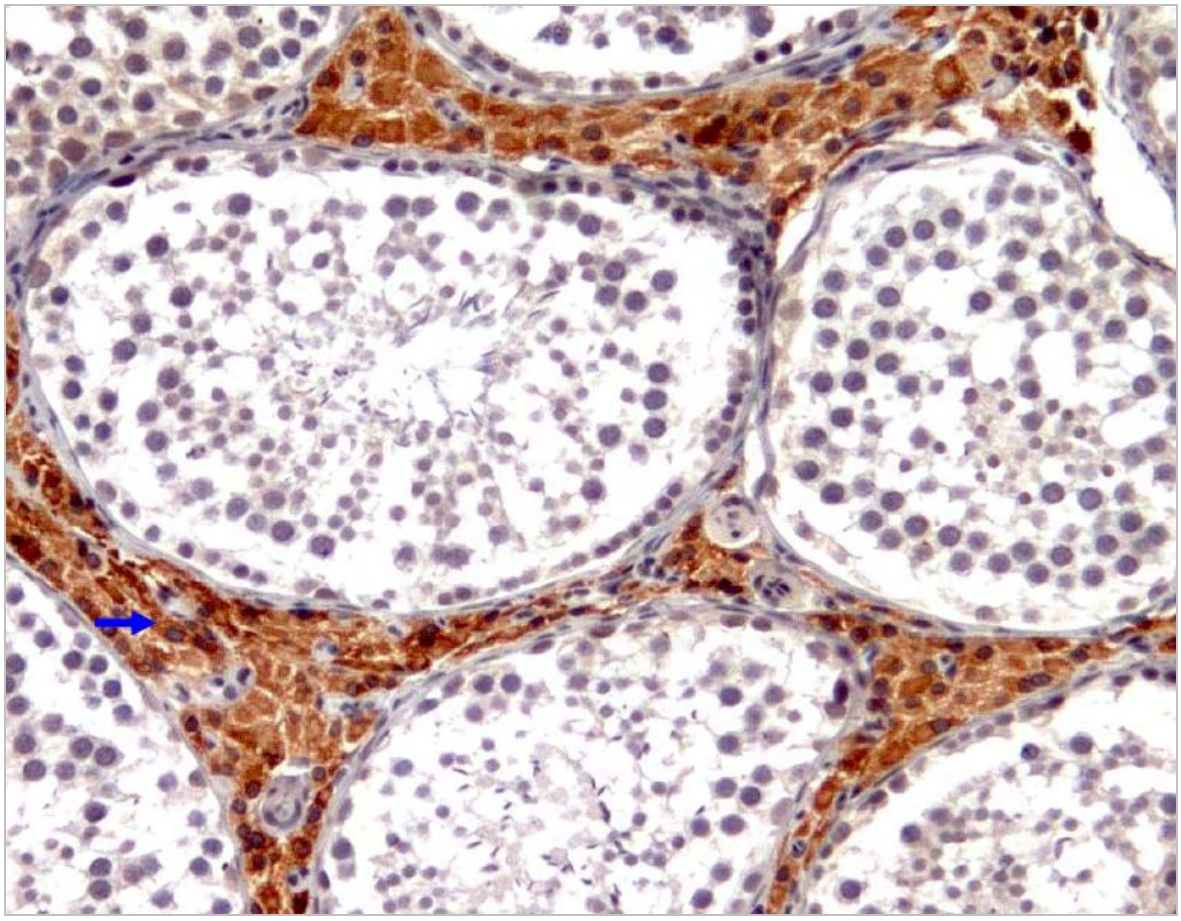

Fig. 22d: StS expression in 200-day boar testis showing signals in cytoplasm of the Leydig cells (blue arrow).Mg=X200

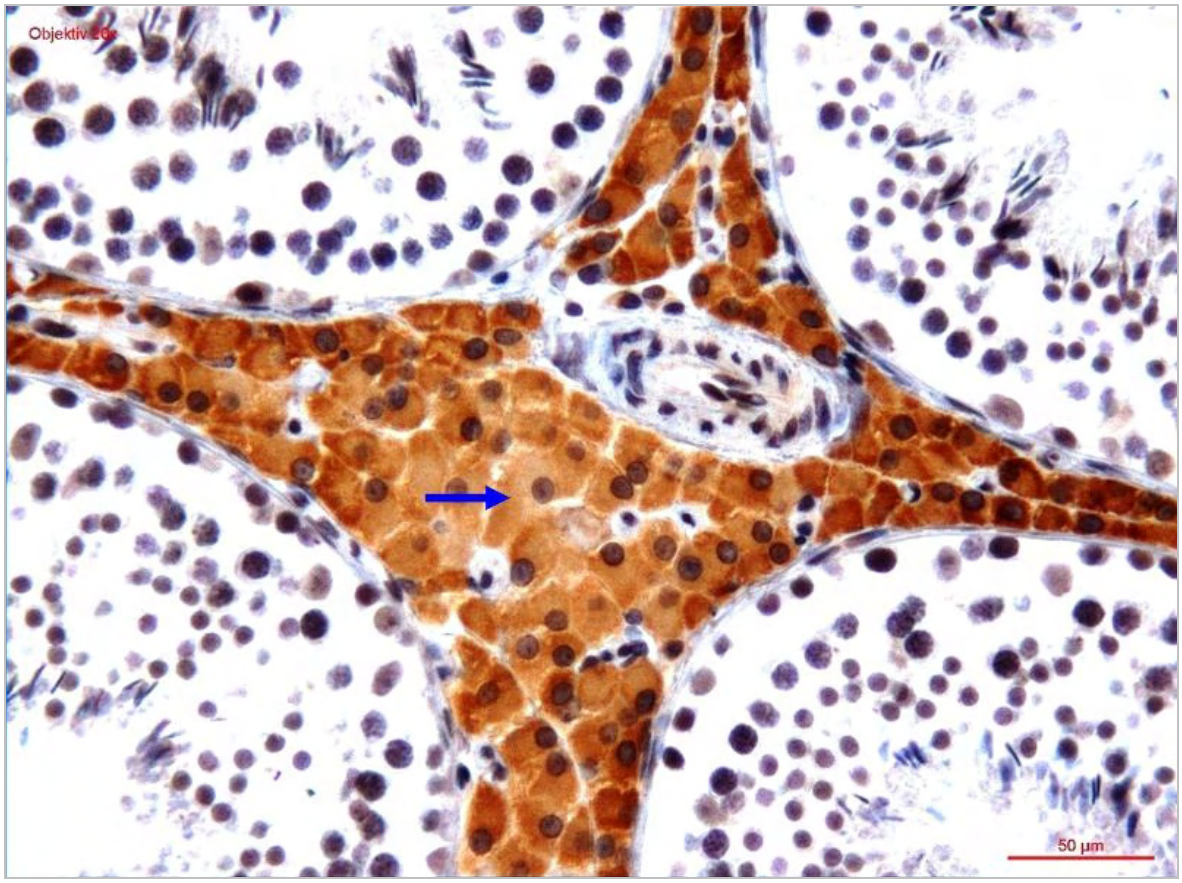

Fig. 22e: StS expression in 250-day old boar testis showing at a higher magnification signals in the cytoplasm of the Leydig cells (blue arrow). Mg=X200

4.4.2 Detection of Steroid Sulfatase-Specific mRNA

4.4.2.1 In vitro RT-PCR

Detection of a single StS-specific band by RT-PCR (Fig. 23) confirmed the presence of StS on the mRNA level.

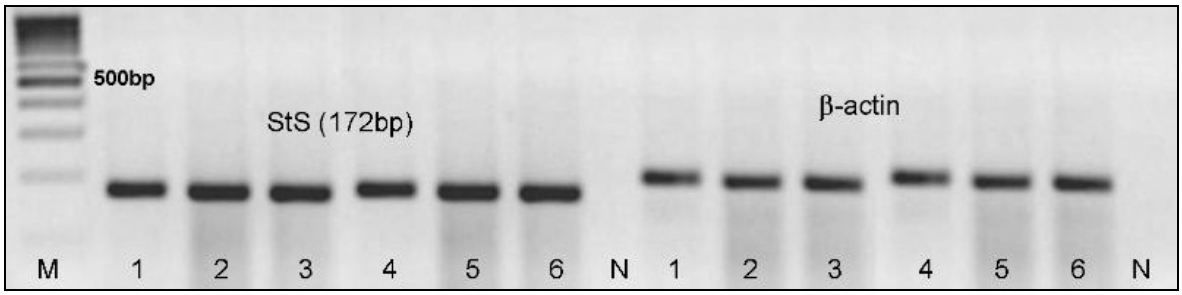

Fig. 23: Ethidium bromide-stained agarose gel of in vitro RT-PCR analysis for the presence of mRNA specific to StS. 1 = 50 days, 2 = 100 days, 3 = 150 days, 4 = 200 days and 5 = 250 days testicular samples; M, DNA ladder; N, autoclaved double deionised water (negative control sample)

4.4.2.2 ***Porcine StS gene sequence and in situ hybridisation***

As indicated in materials and methods (section 3.5.2), probes for *in situ* hybridisation were generated by using an appropppriate vector-system, inserted into competent E.coli. As the sequence of porcine StS was not known and in order to obtain specific primers for RT-PCR, an alignment of the human StS Open Reading Frame sequence (GenBank AccNo: M16505) against porcine genomic sequence available online was performed. The sequence resulting from this alignment was then used to design primers for PCR and to generate probes for in situ hybridisation. The Partial sequence of the Open Reading Frame for the Pig StS gene sequence (see below) was submitted to the gene bank (GenBank AccNo: DQ139314) and is available online (Mutembei et al., 2005a).

Partial sequence of the Open Reading Frame for the Pig StS

```
ctgggaagga ggcatccgag tcccaggcat cctgaggtgg ccaggagtga
tccaggcagg gctggagctt gatgcaccta caagcaacat ggacctgttt
cctacggtgg ccaacctcgc cggggcccct ctgccggaag acaggatcat
tgacggacgt gacctgatgc ccctgcttct agggcaaagc cagcattctg
atcatgagtt tctcttccac tactgcaact tctatctgaa cgccgtacgc
tggcacccac ggaacagcac gtccatctgg aaagccttct tcttcacacc
caagttctcc cccgagggcg ccaatggatg ctttgccaca cacgtgtgct
tctgtcacgg gcattccatc acccaccacg accctccttt gctgtttgac
atctcccaag acccgcgaga gacagacccg ttaactccga catccgagcc
ccggttctgg gagatcctgg ag
```

StS mRNA was detected only in the cytoplasm of the Leydig cells (Fig. 24 a) and there was no non-specific staining (Fig. 24b) in the negative control (antisense probe).

(a) The positive probe

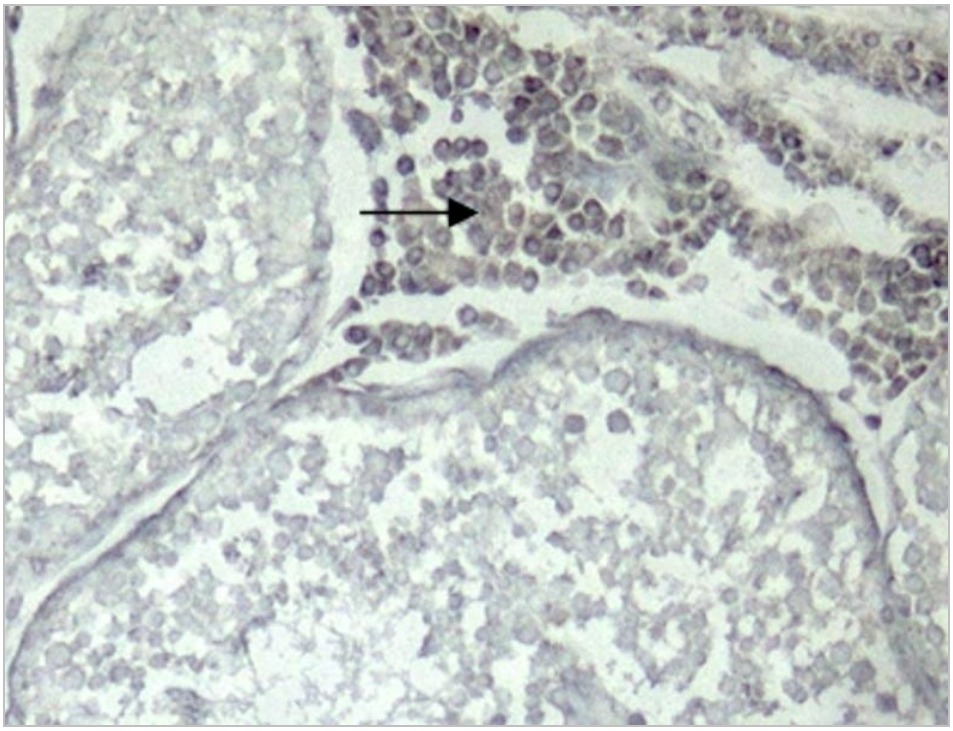

Fig. 24a: Expression of StS-specific mRNA in 250-day old boar testis showing positive signals in cytoplasm of the Leydig cells (black arrow).Mg=X200

(b) The negative probe

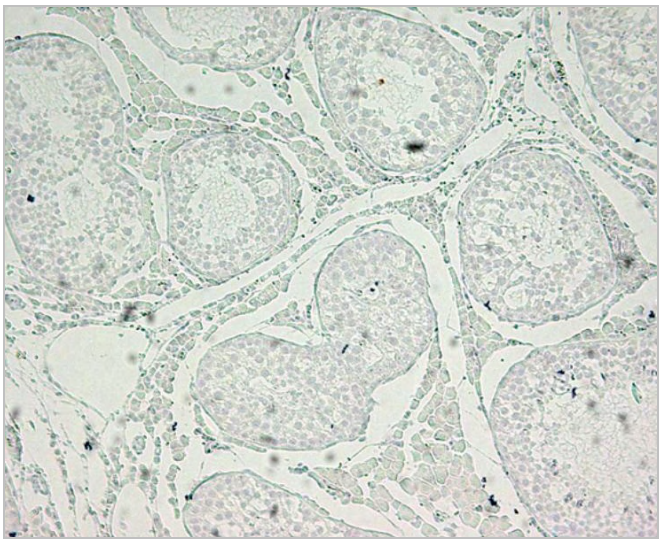

Fig. 24b: Negative control (antisense) for expression of StS-specific mRNA in 250-day old boar testis with no traces of non-specific signals. Mg=x100

4.4.3 Immunolocalisation of Estrogen sulfotransferase (EST) by IHC

In all age groups EST expression was restricted to the cytoplasm of the Leydig cells (Fig 25a-e). The intensity of expression and the number of positive cells remained relatively constant up to an age of 150 days with 71.87±1.66% of the Leydig cells being positive (Table 17a). Thereafter the number of positive cells decreased significantly (P≤0.05) to 57.21±0.56% in the day-200- and 250 age groups.

4.4.3.1 EST in 50-day old boars

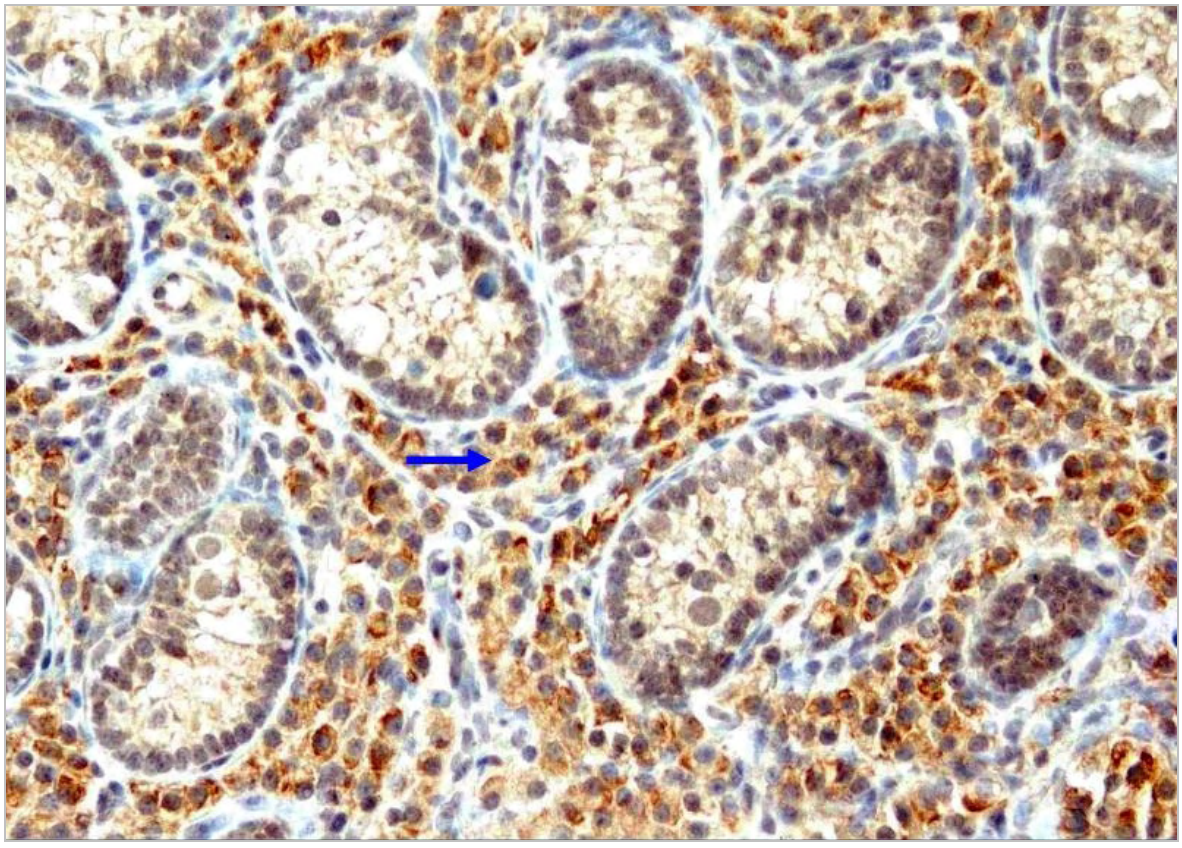

Fig. 25a: EST expression in 50-day old testis showing strong signals in cytoplasm of the Leydig cells (blue arrow). Mg=X200

Table 17a: Leydig cells staining positive for EST in 50-150 day old boars

Leydig cells counted							
Group	*Boar*	*Positive*	*Negative*	*Total*	*% Positive*	*Group mean*	*Group std*
Day 50	E1	83	31	114	72.8		
	E2	80	29	109	73.4		
	E3	81	30	111	73.0	73.06	0.31
Day 100	E1	80	35	115	69.6		
	E2	79	40	119	66,4		
	E3	88	31	119	73.9	69.97	3.76
Day 150	E1	79	32	111	71.2		
	E2	78	28	106	73.6		
	E3	81	30	111	73.0	72.58	1.25

Overall mean ($\bar{X}$) = 71.87, Standard deviation (SD) = 1.66

Table 17b: Leydig cells staining positive for EST in 200-250 day old boars

Leydig cells counted							
Group	*Boar*	*Positive*	*Negative*	*Total*	*%Positive*	*Average*	*SD*
Day 200	E1	63	45	108	58.3		
	E2	60	42	102	58.8		
	E3	59	47	106	55.7	57.61	1.66
Day 250	E1	65	46	111	58.6		
	E2	60	48	108	55.6		
	E3	58	45	103	56.3	56.81	1.57

Overall mean ($\bar{X}$) = 57.21, Standard deviation (SD) = 0.56

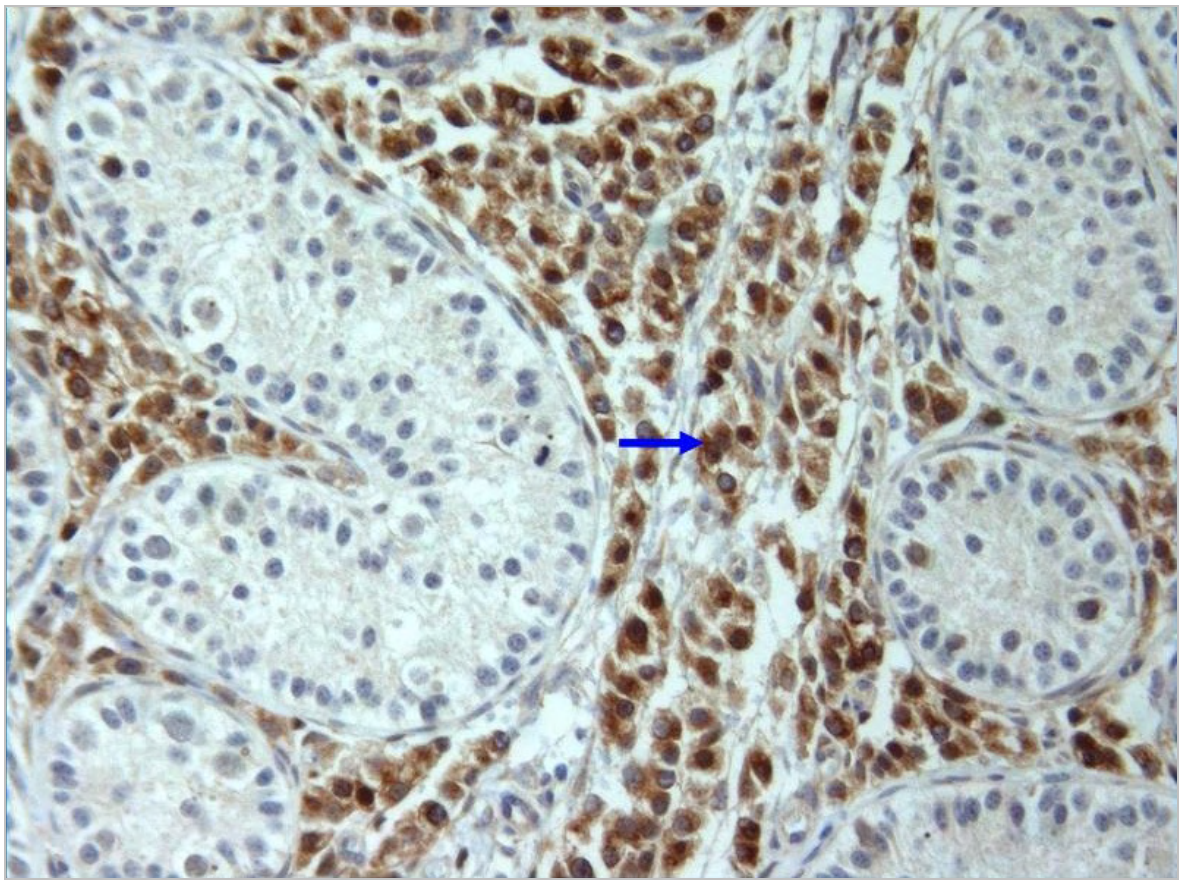

Fig. 25b: EST expression in 100-day old testis showing signals in cytoplasm of the Leydig cells (blue arrow). Mg=X200

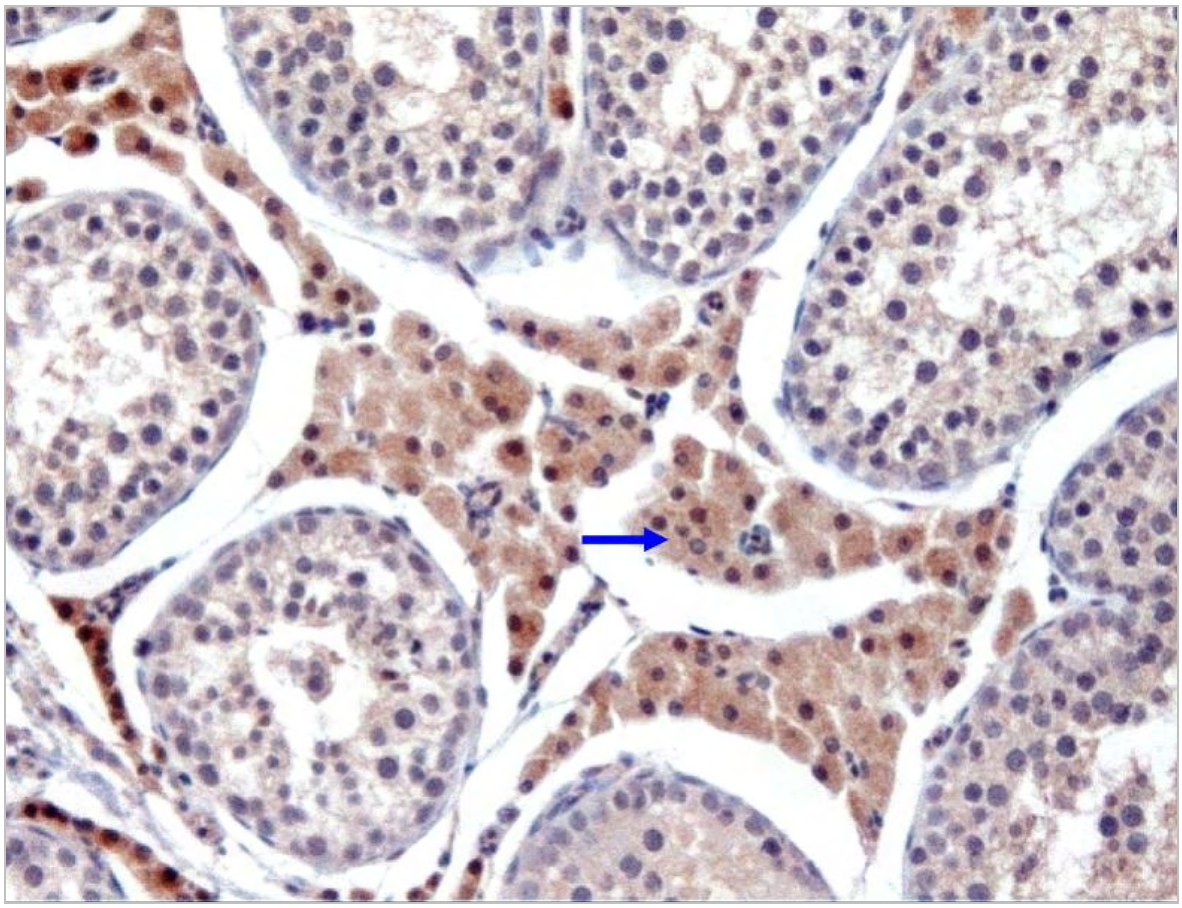

Fig. 25c: EST expression in 150-day old testis showing signals in cytoplasm of the Leydig cells (blue arrow). Mg=X200

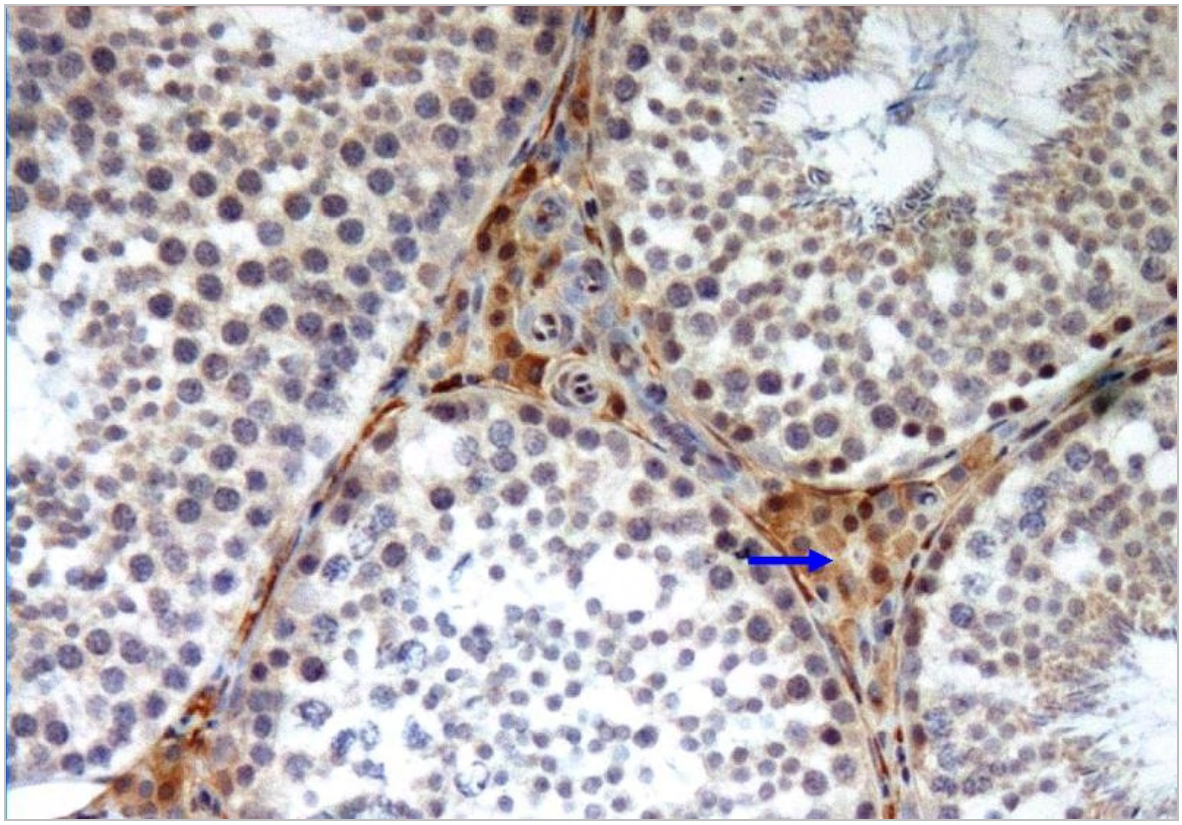

Fig. 25d: EST expression in 200-day old testis showing signals in cytoplasm of the Leydig cells (blue arrow). Mg=X200

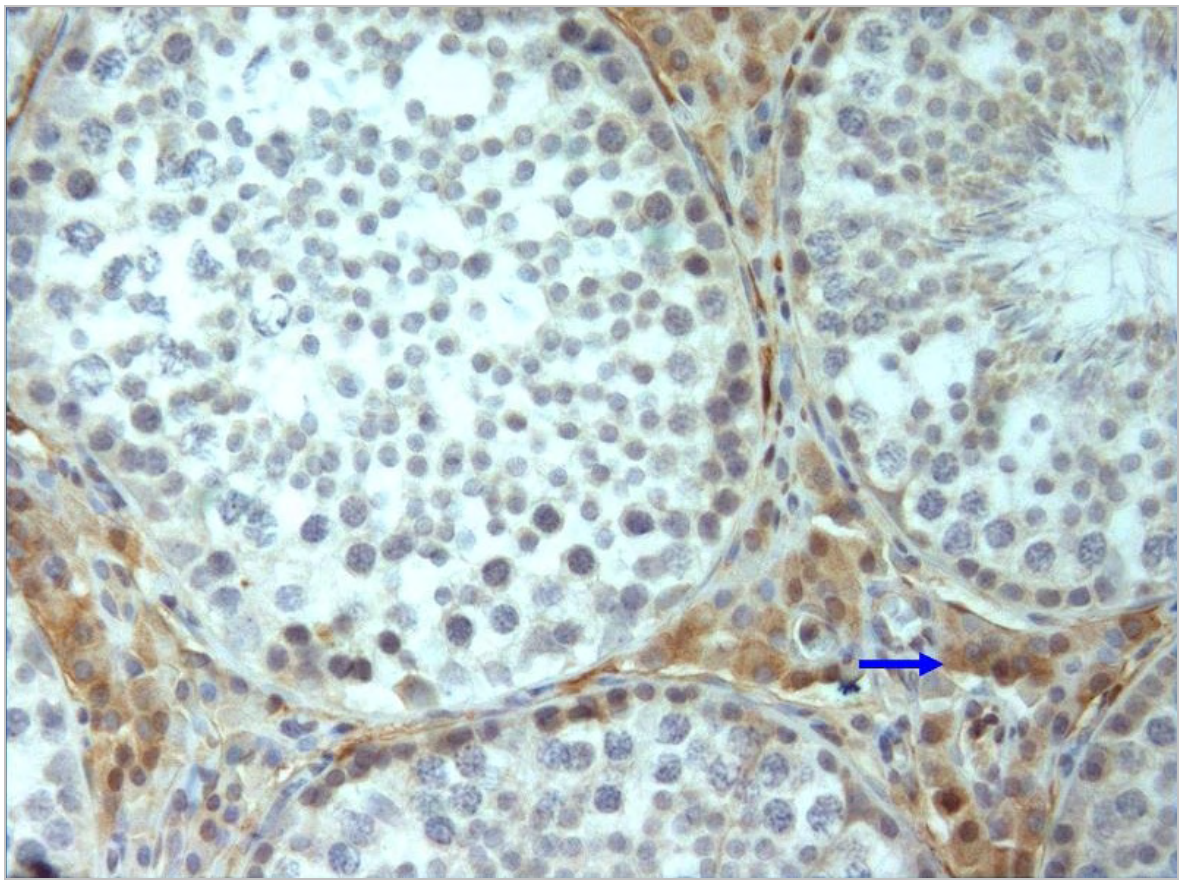

Fig. 25e: EST expression in 250-day old testis showing signals in cytoplasm of the Leydig cells (blue arrow). Mg=X200

4.4.4 Detection of Estrogen sulfotransferase-specific mRNA (RT-PCR)

4.4.4.1 In vitro RT-PCR

Detection of a single EST-specific band by RT-PCR (Fig. 26) confirmed the presence of EST on the mRNA level.

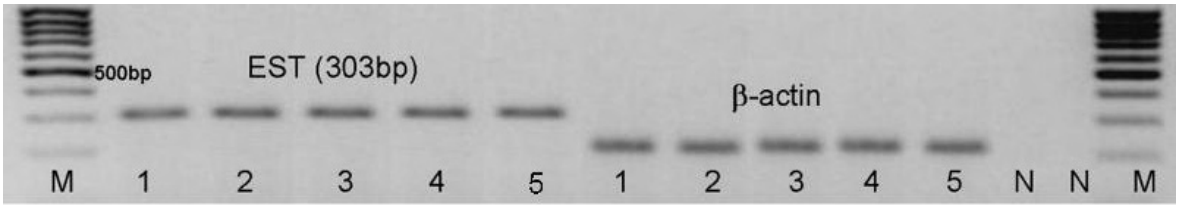

Fig. 26: Ethidium bromide-stained agarose gel of in vitro RT-PCR analysis for the presence of mRNA specific to EST. 1 = 50 days, 2 = 100 days, 3 = 150 days, 4 = 200 days and 5 = 250 days testicular samples; M, DNA ladder; N, autoclaved double deionised water (negative control sample)

5. DISCUSSION

5.1 Experimental design

Although the boar testis secretes high amounts of estrogens, up to date only little information has been available on their possible biological importance (Claus and Hoffmann 1980, Claus et al., 1985, Raeside et al., 1999, Rostalski et al., 2000). In particular no information was available on a testicular significance in this species before this study. Estrogens act via their receptors (ERs) and hence in order to assess their biological activities in the boar testis, the present study aimed at investigating for the ERα and ERβ as well as confirming expression of P450 aromatase (P450 arom), a key enzyme along the route of estrogen biosynthesis. In addition, expression patterns for two enzymes likely to alter bioavailability of testicular estrogens [steroid sulfatase (StS) and estrogen sulfotransferase(EST)] were studied. Immature and mature boars were tested to track the presence of these proteins and/or their mRNA with age of development and spermatogenesis.

5.2 Methods

5.2.1 Immunohistochemistry (IHC)

The present study utilized IHC to detect the cellular location of ERs, P450 arom, StS and EST proteins using appropriate antibodies (Table 4). This method is routinely applied to make use of its advantage of requiring very little probe material (Thorpe 1987, Pertschuk et al., 1990). However, unlike in vitro enzyme assay systems e.g. receptor-ligand binding tests that are usually quantitative, the IHC method is qualitative and also suffers a set back of the likelihood to detect non-specific signals from cross-reacting proteins. A further drawback is the fact that antigens may be masked due to tissue conservation, requiring the somewhat obscure procedures of antigen retrieval (see in page 60). In spite of these problems, IHC at present is the only applicable method for localisation of tissue proteins and can not be substituted by *in situ* hybridisation studies, which measure mRNA levels but do not determine whether the mRNA is translated to the protein. In the present study use of available antibodies directed against ERα and ERβ were expected to allow for discrimination between the ER subtypes. In addition to the declared specificity of a given antibody, IHC also requires further specifications, like the setting of negative control

sections using irrelevant isotype-specific antiserum from the same species in which the primary antibody was raised; absence of positive signals in the negative controls is an indicator of specificity. In addition, the observation that a reference tissue known to express the protein (positive control) would show the expected patterns of expression when applied, greatly support specificity of the binding of the antibody and argues for the presence of protein to be assessed in the probe tissue (Thorpe 1987).

In the present study porcine uterus was used as the reference tissue (Sierralta and Thole 1996) for ERs and the respective appropriate irrelevant isotype-specific isotypes (Table 4) were utilized to set up the negative controls. Mouse monoclonal antibody (clone HT277) directed against amino acids 532-554 of the pig ERα, domain E (Sierralta and Thole 1996), which is not shared by ERβ was used for ERα. For ERβ, a mouse monoclonal antibody against the highly conserved carboxy terminus of the human ERβ1 (clone PPG5/10) was used. It is worthy noting here that monoclonal types of antibodies are more specific than the polyclonal types and more so, both of the antibodies used for the present study have been previously utilized to detect the ER subtypes in other species using both western blot and IHC methods; the ERα antibody had been previously used to detect ERα in bovine uterus and placenta (Sierralta and Thole 1996, Schuler et al., 2002) and the ERβ antibody to detect ERβ in the pig epididymis (Carpino et al., 2004). No reference tissues were used for P450 arom studies because the interest was only to confirm the localisation of P450 aromatase (Conley et al., 1996). For StS and EST the respective enzyme activities have been demonstrated in boar testicular tissue in earlier studies (Rostalski et al., 2000).

5.2.2 In situ RT-PCR and in situ hybridisation

In situ RT-PCR detects incorporated DIG-labelled uridine triphosphate (11-dUTP) in the DNA amplified *in situ.* It is a relatively new molecular biological technique showing a high sensitivity (Sander et al., 1996, Schiller et al., 1998). It shares the advantage of *in situ* hybridization that enables precise localization and identification of individual cells that contain specific nucleic acid sequences of interest, however, it has the advantage of being faster, simpler and having reduced costs of running (Komminoth et al., 1992). In this technique, specific complementary primers are used to amplify the target gene-DNA in tissue sections into high molecular concatemers, which then prevents diffusion of the PCR

product out of the cell during thermocycling and enables detection of gene-specific DNA signals that are complementary to the targeted gene-mRNA (Nuovo 1996). In our study we used porcine ER-specific primers from the published pig mRNA sequences (Table 3).

5.2.3 In vitro RT-PCR and sequencing

In vitro RT-PCR is a molecular technique used routinely to qualitatively detect gene expression (mRNA) within tissues or organs. In many cases, the amount of product detected in the gel is not related to the amount of input DNA (mRNA in the tissue) making it just an indicator tool for detecting the presence or absence of a particular mRNA. Except for StS where a partial sequence of the Open Reading Frame had to be cloned (see 4.4.2.2), the present study utilized primers designed from published porcine-specific mRNA sequences (Table 3) and the PCR products were subsequently sequenced to confirm homology with those of the genes published in the gene bank.

5.3 Results

5.3.1 Estrogen Receptors

In the present study expression of the ERα and ERβ in the porcine testis (see a summary below in Table 18) was demonstrated at both the protein and mRNA level. Conventional RT-PCR detected mRNA for both receptor isotypes in all age groups and the type and distribution pattern of ER mRNA and ER protein expressing cells was identical when applying *in situ* RT-PCR and IHC. This indicates that both transcription and translation occur in the same cells; it also points to the specificity of the IHC method applied. Specificity of IHC was further confirmed by lack of non-specific signals in the negative controls and by the results observed in the positive control (porcine uterus), which were consistent with the findings described previously for this tissue (Sierralta and Thole 1996, Qualmann et al., 2000).

Table 18: Summarized Expression of ERα and ERβ in the boar testis.

Immature testis cell type	ERα	ERβ	Mature testis cell type	ERα	ERβ
Prespermatogonia	+++	+++	Spermatogonia	+++	+++
–			Primary spermatocytes	+++	+++
–			Round spermatids	+	+++
–			Elongated spermatids	–	–
Sertoli cell	–	–	Sertoli cell	+	+++
Leydig cell	++	–	Leydig cell	+	+++

+++/++/+/– indicate strong/moderate/very weak/no expression.

The observed expression of the ERβ (Table 18) in the nucleus of prespermatogonia in the testis of immature boars (50 days of age) is consistent with similar observation in neonatal rodents and prepubertal humans (Enmark et al., 1997, Saunders et al., 1998, Pelletier and El-Alfy 2000, Katzenellenbogen and Katzenellenbogen 2000, Takeyama et al., 2001). However, other than in the neonatal rodent, with ERβ expressing Sertoli and Leydig cells (Saunders et al., 1998, Katzenellenbogen and Katzenellenbogen 2000), or in the prepubertal human, with ERβ expressing Sertoli cells (Enmark et al., 1997, Pelletier and El-Alfy 2000, Takeyama et al., 2001), in the immature boar Leydig and Sertoli cells were negative.

Different from the ERβ (Table 18), the ERα was detected not only in the prespermatogonia but also in Leydig cells of the immature boar. This conforms to observation in the juvenile rat (O'Donnell et al., 2001) and prepubertal human (Enmark et al., 1997, Pelletier and El-Alfy 2000, Takeyama et al., 2001) where expression of ERα in prespermatogonia had been reported. However, unlike in the boar, the Leydig cells were negative in these two species (Enmark et al., 1997, Pelletier and El-Alfy 2000, O'Donnell et al., 2001, Takeyama et al., 2001). Another specific observation relates to the fact that ERβ seems to be the main ER isoform in the testis of immature rats and humans (Enmark et al. 1997, Pelletier and El-Alfy 2000, O'Donnell et al., 2001, Takeyama et al., 2001), while in the immature boar no differences were observed in the expression of the ERα and ERβ in prespermatogonia. These observations point towards differences between species. Thus in the immature boar both ER isotypes may be important factors during the postnatal testicular development and onset of spermatogenesis, but only the ERα influences Leydig cell functions.

With exceptions of elongating/ed spermatids, in sexually mature boars (100-250 days of age), the ERβ was clearly expressed in virtually all germ cells undergoing the development from spermatogonia to round spermatids. Also the majority of Sertoli and Leydig cells were ERβ-positive. This observation is in agreement with results reported for adult rodents, primates, goats, dogs, cats and human (Goyal et al., 1997, Pentikainen et al., 2000, Takeyama et al., 2001, Nie et al., 2002, Hess and Carnes 2004). There were only little variations in staining intensity and expression of the ERβ seemed rather homogeneous within and between cell types and there were no apparent relations to the stage of spermatogenesis. These data obtained on the protein level were confirmed on the mRNA level indicating, that transcription and translation of ERβ occurs in the positive staining cells. Similar and confirmatory evidences on ERβ data have been obtained by Rago et al. (2004).

With respect to the ERα, spermatogonia and primary spermatocytes showed a strong immunostaining with 93.6 ± 2.7% of these cells being positive while the secondary spermatocytes and round spermatids exhibited weak staining with 51.4 ± 0.27% of the cells being positive; again elongating/ed spermatids were negative. *In situ* RT-PCR confirmed the expression pattern for both the ERβ and ERα. These observations indicate that transcription and translation of the ERα in secondary spermatocytes and round spermatids may be down-regulated and/or that a part of the weakly immunostaining positive cells when applying IHC might be a result of a carry over of the receptor protein from the preceding developmental stages. Again, no relationship was observed between the IHC-staining pattern for the ERα and the stage of spermatogenesis. These data have been reported (Mutembei et al., 2005b) and to our knowledge, these are the second data reporting expression of ERα in germ cells of mammals, which agrees with a previous one on humans (Pentikainen et al., 2000). Similar and confirmatory evidences on these data have been obtained by Rago et al. (2004) and by Lekhkota et al. (2006). The predominant expression of ERα in the spermatogonia and primary spermatocytes of the boar so far seems to be unique for this species. However, in accordance with observations in other species (Hess and Carnes 2004), also in the boar Leydig and Sertoli cells express the ERα.

The results of the present study revealed a unique distribution pattern of ER in somatic and germ cells of young and adult boar testis, confirming the species-specific gonadal expression of estrogen receptors. However, as in other investigated species, the cellular ER distribution in boar testis appears to be developmentally regulated. This agrees

to the data demonstrating that puberty can also influence the ERβ expression in epithelial cells of boar epididymis (Carpino et al., 2004).

The involvement of estrogen receptors in development and functional activity of boar testes is unknown. Recently, it has been reported that there is a functional linkage between ERβ and embryonic growth of pigs (Kowalski et al., 2002). Therefore, our study represents a basic foundation to investigate the role of ERs in porcine male gonads. In fact, the variable ERα/ERβ immunostaining pattern in testicular somatic and germ cells as well as in immature and mature gonads suggests that estrogens could modulate spermatogenesis and testis development via a differential expression of the two estrogen receptor subtypes. Further studies investigating the expression patterns of the various ERβ isoforms in testicular cells could provide greater understanding of the gonadal maturation mechanism in pig.

The exact mechanism through which estrogen receptors regulate testicular function is unknown. However there is a general agreement that ERs function as dimers, and co-expression of ERα and ERβ in the same cell causes the formation homodimers (ERα/ERα and ERβ/ERβ) or heterodimers (ERα/ERβ), which affect ligand-specificity. For example, some agonists bind both ER subtypes with the same affinities, while others bind preferentially to ERα or ERβ (Dechering et al., 2000, Gronemeyer et al., 2004). The interactions between ERs, their ligands and estrogen responsive elements (EREs) are complicated by other factors, including the ability of ERβ to modulate ERα transcriptional activity and the recruitment of several protein co-activators and repressors by both ER sub types. Therefore, the relative amounts of ERα and ERβ in a given tissue (testis included) are key determinants of cellular responses to estrogen and other ER agonist and antagonist (Gustafsson 2003, Gronemeyer et al., 2004), particularly also in respect to the activity of environmental endocrine disruptors with estrogenic activity (Toppari et al., 1996, Cooper and Kavlock, 1997, Toppari et al., 1996).

In female organs and the prostate, it is now well established that ERs achieve genomic responses by regulating expression of target genes with either classical estrogen responsive elements (ERE) or with alternative promoter elements such as CRE and AP1 that bind heterologous transcription factors (Kushner et al., 2003). Mammalian ER target genes e.g. prolactin, cathepsin D and lactoferrin contain classical estrogen responsive elements (ERE) within the promoter regions that allow ERs to bind to DNA and regulate transcription. On the other hand, the cyclin D1 gene is a very important ER-induced gene

that is regulated at alternative promoter elements CRE and AP1. The cyclin D1 gene has been implicated as the principal mode for estrogen-mediated growth, both of breast cells in vitro and of mammalian epithelial cells in vivo (Peach et al., 1997). Moreover, the ER has the ability to mediate biological effects through non-transcriptional mechanisms mediated by protein-protein interactions between them and growth factors (Kuschner et al., 2003). Membrane-bound ERs signalling via protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) pathways have been shown to induce epithelial cell proliferations (Collin and Webb 1999). Thus, although the physiological significance of specific pathways in the testis remains to be elucidated, estrogen action in any particular tissue and organ is the result of activities mediated by an integration of several genomic and non-genomic pathways (Akingbemi 2005, Bjornstrom and Sjoberg 2005).

In some species e.g. rat, mice, monkey, man and horse (Shughrue et al., 1998), testicular estrogens have been shown to act at the hypothalamus to affect GnRH pulses, and at pituitary level to regulate gonadotropin (FSH and LH) secretion. Estrogen regulation of LH in these species occurs directly via the EREs contained on the promoter region of β-subunit of LH gene (Shupnik et al., 1988). On the other hand, in some species like it is the case for the boar, estrogens do not appear to play a regulatory role on gonadotropin secretion (Shupnik et al., 1988, At-Taras et al., 2006a). Recently it emerged clearly for the first time that not only that testis and sperm production are influenced by endogenous estrogen secretion in the boar, but that inhbiting estrogen synthesis can increase these parameters by over 20% (At-Taras et al., 2006b). Thus, in the boar, it is now evident that a direct rather than an indirect action of estrogens in the testis seems to occur. Testes of aromatase-inhibited boars initially exhibited delayed lumen formation, lower testicular weight, fewer detergent-resistant spermatids, and fewer Sertoli cells, but by 7 to 8 months, the boars had recovered and had larger testes (At-Taras et al., 2006b). Thus, reducing endogenous estrogen production in the boar causes a delay in testicular maturation/puberty that allows for a longer window for proliferation of Sertoli cells and maturation of Leydig cells, resulting in larger testes and higher spermatid production.

In summary, the biological significance of estrogen receptors expressed in the male genital tract and the testis still needs to be clarified and thus requires a careful interpretation. For example, although levels of ERβ are greater than those of ERα in the male gonad, mice lacking the ERβ are fertile and have normal genital tissues (Krege et al., 1998; Dupont et al., 2000). Otherwise mice lacking ERα are infertile and exhibit

remarkable morphological abnormalities of the reproductive system (Eddy et al., 1996, Hess et al., 2000). It has been proposed that ERβ could act as a negative regulatory partner for ERα (Weihua et al., 2000) and a "Ying-Yang" balance between ERα and ERβ suggested for normal functioning of the testis (Lindberg et al., 2003). In the human, in some individuals a lack of ERα expression has been reported in both immature and adult testes (Makinen et al., 2001, Gaskell et al., 2003) and normal testes and normal sperm density have been observed in an ERα-deficient man (Smith et al., 1994). Therefore ERβ could be the receptor mediating the effect of estrogens in human testes. The recent discovery of different human ERβ isoforms with distinct cell specific expression patterns (Gaskell et al., 2003, Saunders et al., 2002, Scobie et al., 2002) makes the interpretation of estrogen receptor expression in male gonads even more difficult. Furthermore, the obvious differences in the phenotypes of ArKO, ERαKO, ERβKO and ERαβKO mice, as were determined in early studies, implies that none of the two ER-subunits is the sole mediator of estrogen action. For example, ERα inactivation had no effect on number of spermatogonia whereas ERβ inactivation increased the number of spermatogonia by more than 50% in neonatal mice (Delbes et al., 2004). In this case, ERβ seems to be a modulator of ERα function, a function that is generally implicated for ERβ in the testis. However, in general, both the ERα and the ERβ seem to have varying functions that also could be species dependent.

5.3.2 Aromatase

In agreement with previous observations (Conley et al., 1996; Carreau et al., 1999; Fraczek et al., 2000), our data confirm that the expression of aromatase is restricted to the Leydig cells, which also express the ERα and ERβ. Thus, in the boar testicular estrogens seem to be autocrine and/or intracrine factors controlling Leydig cell function while Sertoli cell function and spermatogenesis might be affected by paracrine mechanisms. Although in some species, like rodents and equines, where aromatase is also expressed in other cell lines like germ cells, in the boar aromatase is solely expressed by the Leydig cells.

5.3.3 StS and EST

As has been shown for the mouse, rat and man (Song et al., 1995, 1997, Munroe and Chang 1997), the present study revealed that also in the boar, data from EST is expressed in Leydig cells. Leydig cells also expressed StS and like it was the case for the

rat and the mouse, this enzyme is also coexpressed with the aromatase system in the boar (Conley et al. 1996; Carreau et al. 1999, Fraczek et al. 2000, Mutembei et al., 2005b). Thus, in the boar, the Leydig cells houses EST, StS and aromatase. For a functional interpretation of these observations further studies are necessary. However, colocalisation of StS and aromatase in the Leydig cell seems to suggest that StS enzyme might be important in the hydrolysis of precursor steroids during testicular steroidogenesis. However, a direct role in the hydrolysis of E1S to E1, as it was demonstrated in human breast cancer cells (Chandra 2003) cannot be ruled out.

Based on the facts that cultured Leydig cells of the boar (Reaside 1983) have been shown to conjugate estrone and EST activity has been demonstrated in the boar testicular homogenates (Rostalski 2005), it was not surprising that Leydig cells expressed EST. However, the biological significance of this expression needs careful interpretation. In the boar conjugation of estrogen occurs not only within the Leydig cells (Reaside 1983) but also in the periphery (Booth 1984) and in the epididymis, vas deferens, seminal vesicle and the prostate (Reaside et al., 1999). Therefore, in accordance to Claus et al. (1985), who found higher concentrations of E1 than of EIS in testicular tubular fluid, and in respect to the lower activity of EST determined in the testis compared to the epididymis (Rostalski et al., 2000) and the local circulatory system (Rerkamnuaychoke et al., 1991, Pinart et al., 2001a), rather the epididymis than the testis seems to be the origin of the high E1S-concentrations determined in the testicular vein plasma (Rostalski 2005, Claus et al., 1987). Probably conjugations within the Leydig cells might be only for homeostasis purposes. In any case, if active conjugation of estrogens occurs within the Leydig cells, the conjugated estrogens would be either locked in there or require active transporters for their exocytosis.

6. THESIS ABSTRACT

Estrogen action is mediated via estrogen receptor proteins (ERs). ERs occur in two forms, the classical ERα subtype and the novel ERβ discovered in 1996. These proteins are also widely expressed in the male reproductive tract. Recent data from transgenic mice deficient in estrogen receptors or aromatase, points to an essential physiological role of estrogens for male fertility, which may, however, also be severely hampered by the administration of estrogens and xenoestrogens during fetal and neonatal development. These observations raise the question on the mechanisms of action of estrogens in the male.

Together with the stallion the boar secretes remarkably high amounts of testicular estrogens. The present study was based on the hypothesis that these estrogens are likely to have local activity and hence designed to provide basic information on sources and targets of estrogens in the boar testis to set a foundation for further studies on their biological significance. In order to achieve this information, expression and localisation of the ERα and the ERβ, cytochrome P450 aromatase, steroid sulfatase (StS) and estrogen sulfotransferase (EST) in the testes of immature and mature boars was assessed.

Immunohistochemistry (IHC) was applied to show cellular expression of proteins and either one-step *in situ* RT-PCR or *in situ* hybridisation was applied to localize cytological mRNA. Gene expressions were evaluated using qualitative RT-PCR.

Testicular samples were collected from 25 healthy Large White boars. The animals were randomly obtained from 5 litters, allotted to five age groups (5 animals per group) and castrated at ages of 50, 100, 150, 200 and 250 days. Samples for IHC were preserved in phosphate buffered 4% formalin while those for RT-PCR were snap frozen in liquid nitrogen and stored at -80^{o}C. Porcine uterine samples serving as positive controls were collected from the local slaughter house.

The ER expression patterns showed qualitative and quantitative differences between and within immature (50 days old) and sexually mature (100–250 days old) animals. The ER immunoreaction signals were observed only in nuclei. In 50-day old boars, 90.6 ± 1.2% of prespermatogonia and 71.0 ± 2.6% of the Leydig cells showed a strong staining for ERα; 95.5 ± 3.5% of the prespermatogonia but none of the Leydig and Sertoli cells were ERβ-positive. In 100-250-day old boars a strong staining for the ERβ was observed in virtually all Sertoli, Leydig and germ cells, except for the elongating/ed spermatids, which were clearly negative; for the ERα, strong immunoreaction signals were

restricted to spermatogonia and primary spermatocytes with 93.6 ± 2.7% of these cells being positive; distinctly less intensive signals were observed in 51.4 ± 0.27% of the secondary spermatocytes and/ or round spermatids, Sertoli- and Leydig cells. *In vitro* RT-PCR was positive for both receptors and results of *in situ* RT-PCR matched those obtained by IHC. P450 aromatase, steroid sulfatase and estrogen sulfotransferase expressions were restricted to the cytoplasm of Leydig cells, both at protein and mRNA localisation.

The present study confirmed that Leydig cells of the boar are the only source of estrogens which could likely have local autocrine and/or paracrine activities via the available ER receptors (ERα and ERβ). Apparently, availability of biologically active free estrogens seems to be modulated by the enzymes steroid sulfatase and estrogen sulfotransferase, both of which are expressed in the Leydig cells. The variable ERα/ERβ immunostaining pattern in testicular somatic and germ cells of the gonads of immature and mature boars suggests that estrogens could modulate spermatogenesis and testis development via a differential expression of the two estrogen receptor subtypes. To validate these hypotheses further functional studies and studies dealing with subcellular localisation of the regulatory factors determined are necessary.

7. ZUSAMMENFASSUNG

Die genomische Wirkung der Östrogene wird über nukleare Östrogen-Rezeptoren (ERs) vermittelt, deren Expression auch im männlichen Reproduktionstrakt nachgewiesen wurde. Die ERs kommen in zwei Formen vor, dem klassischen ER-α und dem erstmals 1996 nachgewiesenen ER-β. Untersuchungen an transgenen Mäusen (ER Knockout, Aromatase Knockout) haben die essenzielle Bedeutung der Östrogene für die männliche Fortpflanzung dargelegt, wobei ebenfalls bekannt ist, dass die Applikation östrogen-wirksamer Verbindungen, z.B. von Xenoöstrogenen, während der Embryonal- oder Fetalperiode zu schwerwiegenden Störungen der männlichen Reproduktion führen kann. Diese Beobachtungen werfen die Frage nach den dabei zugrunde liegenden Wirkmechanismen auf.

Neben dem Hengst zeichnet sich der Eber durch eine bemerkenswert hohe Produktion testikulärer Östrogene aus, für die auch eine direkte Wirkung im Hoden unterstellt werden kann. Ziel der vorliegenden Studie war es daher, Herkunft und Zielzellen von Östrogenen im Eberhoden zu definieren, um Anhaltspunkte für eine mögliche biologische Wirkung testikulärer Östrogene zu erhalten und damit eine Grundlage für weiterführende Untersuchungen zu schaffen. Dazu sollte die Expression und Lokalisation der Östrogenrezeptoren ER-α und ER-β, der Aromatase (Cyp P450 arom), und der Enzyme Steroidsulfotransferase (Inaktivierung) und Steroidsulfatase (Aktivierung) dargestellt werden.

Zur Darstellung der Expression auf der Proteinebene diente die Immunhistochemie (IHC). Qualitative RT-PCR, One - Step *in situ* RT-PCR und *in situ* hybridization (ISH) dienten der Feststellung der Expression und Lokalization auf mRNA Ebene. Zur Verfügung standen 25 Eber aus 5 Würfen, von denen nach randomisierter Zuteilung zur Gewinnung der Hoden jeweils 5 Tiere im Alter von 50, 100, 150, 200 und 250 Tagen kastriert worden waren. Gewebeproben wurden aus dem Hodenparenchym entnommen und für die IHC, One – Step *in situ* RT-PCR und ISH in 4%-igem Formol nach Lillie für 20-24 Stunden fixiert und in Paraffin eingebettet; für die RT-PCR wurden die Proben schockgefroren (-196 ^{o}C) und danach bei -85 ^{o}C aufbewahrt. Als Kontrollgewebe dienten am lokalen Schlachthof gewonnene Schweineuteri.

Die Expression der Östrogenrezeptoren zeigte qualitative und quantitative Unterschiede sowohl zwischen den noch nicht geschlechtsreifen (50 Tage) und den geschlechtsreifen Tieren (100-250 Tage) als auch innerhalb der Altersgruppen.

IHC-positive Signale waren auf die Kerne beschränkt, im Falle des ERα waren bei den 50 Tage alten Tiere 90,6 ± 1,2 % der Präspermatogonien und 71,0 ± 2,6 % der Leydigzellen positiv, im Falle des ERβ 95,5 ± 3,5 % der Präspermatogonien, die Leydigzellen und Sertolizellen zeigten keine positiven Signale. Bei den 100-250 Tage alten Tieren ergab die IHC positive Signale für den ERβ in praktisch allen Sertoli-, Leydig- und Keimzellen, ausgenommen der elongierten/elongierenden Spermatiden, die eindeutig negativ waren. Im Falle des ER-α waren deutliche IHC-positive Signale auf die Spermatogonien und primären Spermatozyten beschränkt, die zu 93,6 ± 2,7% positiv waren. Positive Signale von allerdings wesentlich geringer Intensität ergaben sich für die sekundären Spermatozyten, runden Spermatiden, Sertoli- und Leydigzellen, die zu 51,4 ± 0,27% positiv waren. Die *in-vitro* RT-PCR war positiv für beide Rezeptoren, die in situ one – step RT-PCR bestätigte die Ergebnisse der IHC. Die Expression der P450-Aromatase, der Steroidsulfatase und der Steroidsulfotransferase war auf das Zytoplasma der Leydig-Zellen beschränkt, sowohl auf der Protein-, als auch auf der mRNA- Ebene.

Die auf die Leydigzellen beschränkte Expression der Aromatase bestätigt, dass allein diese Zellen im Hoden vom Eber zur Synthese von Östrogen befähigt sind, denen im Hinblick auf die dort ebenfalls festgestellte Expression des ERα und ERβ mit hoher Wahrscheinlichkeit eine autokrine Wirkung zukommt. Dabei scheint die Verfügbarkeit von freiem, biologisch aktiven Estron durch die Expression der Steroidsulfatase und der Östrogensulfotransferase moduliert zu werden. Dies gilt sowohl für eine mögliche autokrine Wirkung, bezogen auf die Funktion der Leydigzellen, als auch für eine zu unterstellende parakrine Wirkung, bezogen auf die ERα- und ERβ-positiven Zellen im restlichen Hodengewebe. Die unterschiendliche Expression des ERα und ERβ in den somatischen Zellen und Keimzellen des Hodens bei immaturen und maturen Ebern legt nahe, dass den Östrogenen eine die Spermatogenese modulierende und die Entwicklung der Hoden betreffende Wirkung zukommt. Zur Untermauerung dieser Thesen sind weiterführende funktionelle Studien sowie Studien zur subzellulären Verteilung der erfassten Regelfaktoren notwendig.

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9. ANNEXES

Annex 1: Summary of immunohistochemistry protocol

Day 1
Alcohol (2x Xylol 5min, then 2min: 2x99%, 2x95%, 2x70%, 2x30%)
5 min under running tap water; rinsing
1x5 min 10mM (7.0 pH) Citrate buffer (pre-incubation at Room temperature)
3x5 min Citrate buffer (heating at 560 Watt Microwave, covered in pre-heated buffer)
1x20 min in Citrate buffer cooling
5 min under running tap water; rinsing
30 min 0.3% H_2O_2 in Methanol (2,5ml H_2O_2 30%+250ml Methanol)
5 min with IHC buffer; rinsing
Section drain and dry and wells made around sections using PAP-Pen
20 min in a moist chamber incubation with blocking Serum; 70µl/section
Drain and dry PAP-Pen
Over night (20h) incubation with primary antibody at 4°C; 90µl/section, in a moist chamber
Day 2
Drain antibody, then 2x10 min with ICC buffer; rinsing
Drain and dry PAP-Pen, then apply secondary boitynylated antibody for 30 min, 90µl/section
Drain Ab
10 min in ICC buffer rinse
Drain and dry PAP-Pen, then 30 min ABC-System (Vector Laboratories, Burlingame, CA94010, USA), 70µl/section (5ml ICC buffer +2 drops of sol.A +2 drops of sol. B, note: must be set30 min before
Drain and dry PAP-Pen
10 min in ICC buffer rinse
Drain and dry PAP-Pen
Nova RED for 15 min, 90µl/section (5ml distilled water+3 drops of sol.1+ 2drops of sol 2+ 2 drops of sol.3+ 2 drops of H_2O_2 ; mixed well after every sol. added. (Vector Laboratories, Burlingame, CA94010, USA)
Drain and pap pen
10 min under running water rinsing
Counter stain with Hematoxylin, 30 sec.;
5 min under running water rinsing
Alcohol series (2X2min 80%, 2X2min 96%, 2X2min 99%,2X10min Xylene)
Mounting in Eu-Kit (Assistant, D-35520 Osterode)

Annex 2: Summary of RNeasy Mini Kit RNA extraction procedure

1) Prepare 3ml RTL buffer + 30µl β-mercaptoethanol in a sterile glass tube
2) Tissue preparation (as described above)
3) Add 200 mg of the tissue in the glass tube with RTL+mercaptoethanol buffer (1 above)
4) 3X30 sec ultraturrax homogenise
5) Centrifugation; 10 min at RT at a speed of 4000-5000 g.
6) 2 ml supernatant transferred to another glass tube + 2 ml %70 Ethanol (Vortex)
7) Transferred the whole liquid into pink tubes, (specific for PCR- tissue preparation) and centrifugation; 10 min at RT at a speed of 4000-5000 g.
8) Liquid that flows through the filter bottom is poured away, then 4ml RW1 is added and centrifugation; 10 min at RT at a speed of 4000-5000 g.
9) Liquid that flows through the filter bottom is again poured away, then 2.5ml RPE Buffer is added and centrifugation; 2 min at RT at a speed of 4000-5000 g.
10) Liquid that flows through the filter bottom is again poured away, then 2.5ml RPE Buffer is added and centrifugation; 2 min at RT at a speed of 4000-5000 g.
11) Then the mRNA filter (pink filter) is transferred into another tube and 150µl RNAse free water is added and given an allowance of 1-10 min (wait for 1-10 min), then centrifugation; 3 min at RT at a speed of 4000-5000 g.
12) The liquid that flows through is not poured away this time round! (This is your RNA)
13) One can choose to repeat step 11; that is, add a further 150µl RNAse free water into the filter (wait for 1-10 min), then centrifugation; 3 min at RT at a speed of 4000-5000 g.
14) Transfer the probe to the eppendorf tubes.

Annex 3: Protocol for ligation using pGEM-T-Vector

1. Briefly centrifuge the pGEM®-T Vector and Control Insert DNA tubes to collect contents at the bottom of the tubes		
2. Set up ligation reactions as described below. **Note:** Use 0.5ml tubes known to have low DNA-binding capacity and vortex the 2X Rapid Ligation Buffer vigorously before each use.		
	Standard Reaction	Control Reaction
T4 DNA Ligase 10X Buffer	1µl	1µl
pGEM®-T Vector (50ng)	1µl	1µl
PCR product (or pGEM®-T Vector Control)	Xµl	2µl
T4 DNA Ligase (1 Weiss unit/µl)	1µl	1µl
dH2O to a final volume of	10µl	10µl
3. Mix the reactions by pipetting and incubate the reactions 1 hour at room temperature.		

Annex 4: Bacteria transformation protocol

1. Prepare 2 LB/ampicillin/IPTG/X-Gal plates for each ligation reaction. Equilibrate the plates to room temperature prior to plating
2. Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 2µl of each ligation reaction to a sterile 1.5m- microcentrifuge tube on ice.
3. Remove tube(s) of frozen Competent *E. coli* cells from–70°C storage and place in an ice bath until just thawed (about 5 minutes) Mix the cells by **gently** flicking the tube.
4. **Carefully** transfer 50µl of cells into each tube prepared in Step 2
5. **Gently** flick the tubes to mix and place them on ice for 20 minutes. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C (**Do Not Shake**) Immediately return the tubes to ice for 2 minutes
6. Add 950µl room temperature SOC medium to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid (LB broth may be substituted, but colony number may be lower).
7. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
8. Plate 100µl of each transformation culture onto duplicate LB/ampicillin/ IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 × g for 10 minutes, resuspended in 200µl of SOC medium, and 100µl plated on each of 2 plates.
9. Incubate the plates overnight (16–24 hours) at 37°C. In our experience, approximately 100 colonies per plate are routinely seen when using competent cells that are 1 × 108cfu/µg DNA, if 100µl is plated. Longer incubations or storage of plates at 4°C (after 37°C overnight incubation) may be used to facilitate blue colour development. White colonies generally contain inserts; however, inserts may also be present in blue colonies.

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