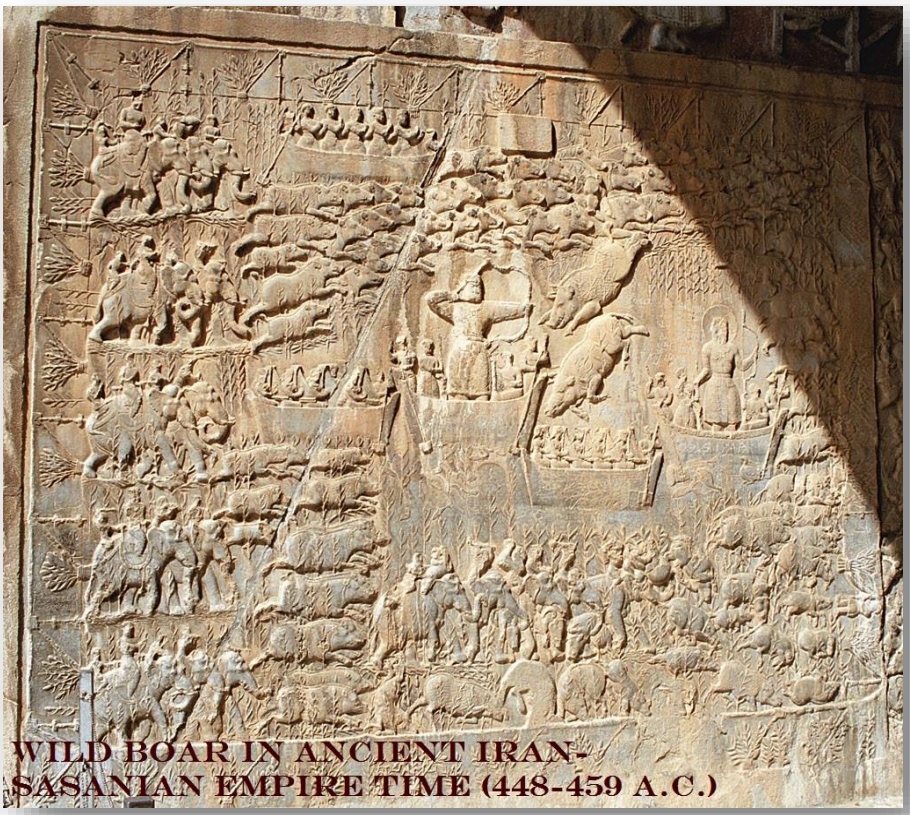
Investigations into Metabolism, Transport and Function of Sulfonated Steroids in the Porcine Testicular-Epididymal Compartment

Yaser Dezhkam



INAUGURAL DISSERTATION

Submitted to the Faculty of Veterinary Medicine

in Partial Fulfillment of the Requirements

for the PhD-Degree

of the Faculties of Veterinary Medicine and Medicine

of the Justus Liebig University Giessen

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by

Yaser Dezhkam

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From the Clinic for Obstetrics, Gynecology and Andrology for Large and Small Animals with Ambulatory Service

Faculty of Veterinary Medicine, Justus-Liebig-University Giessen

First Supervisor: Prof. Dr. Gerhard Schuler

Second Supervisor: Prof. Dr. Klaus Steger

Committee Members: Prof. Dr. Gerhard Schuler	**(Internal Thesis Examiner)**
Committee Member: Prof. Dr. Marion Piechotta	**(External Thesis Examiner)**
Other committee Members: Prof. Dr. Klaus-Dieter Schlüter	**(Chairman of Oral Panel)**
Committee Member: Prof. Dr. Klaus Steger	**(Chairman of Oral Panel)**

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DECLARATIONS

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

Yaser Dezhkam

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LIST OF ABBREVIATIONS

3ß-HSD	3β-Hydroxysteroid dehydrogenase-Δ5/4-isomerase (syn.: HSD3B1)
17ß-HSD	17ß-Hydroxysteroid dehydrogenase
ASBT	Apical sodium-dependent bile acid transporter
ANOVA	Analysis of variance
A4	Androstenedione
ABP	Androgen binding protein
ACTH	Adrenocorticotropic hormone
APS	Ammonium persulfate
AR	Androgen receptor
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate
CTP	Cholesterol transport protein
CYP17	17α-Hydroxylase-C17/20-lyase
ddH_2O	Double distilled water
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
E1	Estrone
E1S	Estrone-3-sulfate
E2	17ß–Estradiol
E2S	17ß-Estradiol-3-sulfate
EB	Epididymal body
EH	Epididymal head
EST	Estrogen sulfotransferase (this term is used when referring to estrogen sulfotransferase activity without exact identification of the underlying enzyme)
ESD	Ethane dimethan sulfamate
ER	Estrogen receptor
ET	Epididymal tail
GPX	Glutathione peroxidase
GXXGXXK	P-loop related motif
HEX	Hexosaminidase

HSD3B1	3β-Hydroxysteroid dehydrogenase-Δ5/4-isomerase (syn.: 3β-HSD)
IHC	Immunohistochemistry
I.U.	International unit
LC-MS-MS	Liquid chromatography tandem mass spectrometry
LH	Luteinizing hormone
MW	Molecular weight
OATPs	Sodium-independent organic anion transporting polypeptides
P450scc	Side-chain cleavage enzyme (CYP11A1)
P5	Pregnenolone
P5S	Pregnenolone-3-sulfate
PAPS	3'-Phosphoadenosine-5'-phosphosulfate (syn.: adenosine 3′-phosphate 5′-phosphosulfate)
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline triton
RABP	Retinoic acid binding protein
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulfate
SOAT	Sodium dependent organic anion transporter (syn.: solute carrier family 10 member 6; SLC10A6)
STS	Steroid sulfatase
StAR	Steroidogenic acute regulatory protein
SULT	Cytosolic sulfotransferase
SULT1E1	Estrogen sulfotransferase
SULT2B1	Pregnenolone/cholesterol specific sulfotransferase
SULT2A1	DHEA specific sulfotransferase
T	Testosterone
TE	Testis
TEMED	Tetramethylethylenediamine
tBME	Tert-butyl methyl ether
Tris	Tris (hydroxymethyl)-aminomethane
XLI	X-linked ichthyosis
WB	Western blot

1. Introduction

Sulfonation is the conjugation of a substrate with a sulfuryl group ($-SO_3^{1-}$), whereas sulfation refers to the transfer of an SO_4^{2-} group (Huxtable 1986). However, contrary to the official nomenclature compounds structured $RO-SO_3^{1-}$ are still commonly named as sulfates of RO-H. Thus, sulfonates of estrogens or neutral hydroxysteroids are frequently but inaccurately called steroid sulfates.

Sulfonation of steroids abolishes their capability to bind to and thus to activate classical nuclear steroid receptors (Hähnel et al. 1973, Kuiper et al. 1997) and it dramatically increases their polarity, thus reducing their distribution volume (Back et al. 1981, Strott 1996, Raftogianis et al. 2000). So far, sulfonation of steroids has been commonly considered as an important mechanism leading to their inactivation and excretion via bile or urine (Dawson et al. 2010).

On the other hand, due to an enhanced protein binding, the half-life of sulfated steroids in the systemic circulation is longer and they may circulate in substantially higher concentrations compared to their free counterparts. Thus, sulfonated steroids may form a pool of precursors, from which after hydrolysis catalyzed by the enzyme steroid sulfatase, active free steroids may be formed directly or after additional subsequent conversions. In comparison to the aromatization of androgens, this *sulfatase pathway* has been identified as the major route of the local production of bioactive estrogens in human hormone-dependent breast cancer tissue (Santner et al. 1984, Reed et al. 2004).

In contrast to free steroids commonly considered to freely distribute in tissues by passive diffusion (Mendel 1989, Willnow & Nykjaer 2010), the transmembrane transport of the substantially more polar sulfated steroids obviously depends on more or less specific transport mechanisms. Thus, the cellular import or export of sulfated steroids may provide mechanisms for their directed transport within and between tissues. Several steroid sulfate transporter candidates have been suggested (Geyer et al. 2004, Bortfeld et al. 2006, Ugele et al. 2008), however, their actual role in the cellular uptake or export of sulfated steroids under in vivo conditions is still widely unclear. Howsoever, in combination steroid sulfatase and steroid sulfate transporters may be important factors in the local control of steroid hormone effects in specific target cells, which may uptake sulfonated steroids and utilize them for the production of bioactive steroids to stimulate their specific intranuclear receptors (intracrine way of steroid effects). A considerable number of studies is now available on the intratumoral production of estrogens in human breast cancer tissue from sulfonated precursors. However, to date only very little information is available on sulfatase pathways in a physiological setting. A particular

situation exists in the human placenta, which is virtually devoid of 17α-Hydroxylase-C17/20-lyase (CYP17) expression and thus with relation to estrogen synthesis largely depends on the supply of C19 precursors provided in sulfonated form by the fetal and maternal adrenals. The role of placental steroid sulfatase in human placental steroid metabolism becomes obvious in cases of placental steroid sulfatase deficiency, which are characterized by very low levels of placental derived estrogens (Lykkesfeldt et al. 1984).

Among males of our domestic mammalian animals, the boar is very exceptional in that it exhibits extraordinarily high blood concentrations of numerous sulfated steroids including dehydroepiandrosterone sulfate and estrone sulfate, of which the synthetic pathways, transport and the biological roles are still unclear. Comparative measurements in the testicular artery and vein clearly showed that the steroid sulfates addressed so far do not primarily result from peripheral inactivation of free steroids but are mainly produced in the testicular-epididymal compartment (Hoffmann et al. 2010, Setchell et al. 1983). Measurement of their concentrations in testicular tissue or cultured Leydig cells provided evidence that they are original products of the testis, probably of the Leydig cells. However, when measuring estrogen sulfotransferase activity in tissue homogenates, no activity was detectable in the testis, whereas significant activity was found in the epididymis (Hoffmann et al. 2010, Zdunczyk et al. 2012). These observations suggest that free estrogens produced in the testis may be sulfonated to a substantial extent in the epididymis. However, to date there is no experimental confirmation of this concept and also the mechanisms accomplishing this hypothetical transfer of free estrogens from the testis to the epididymis while preventing their significant leakage into the systemic circulation are fully unclear.

Interestingly, in the porcine testicular-epididymal compartment not only the production of high amounts of sulfonated steroids was observed but also a considerable expression of steroid sulfatase (Mutembei et al. 2009, Hoffmann et al. 2010, Zdunczyk et al. 2012). In the porcine testis, the expression of steroid sulfatase is restricted to the Leydig cells, where the role of this enzyme is fully unclear (Mutembei et al. 2009). Anyhow, the co-localization of steroid sulfatase expression and the production of high amounts of sulfonated steroids in the porcine testicular-epididymal compartment suggest that in this organ system the sulfonation of free steroids and the hydrolysis of sulfonated steroids may be important mechanisms in the local control of the availability of active free steroids and their effects. Thus, in this work, which is a sub-project of the DFG research group *Sulfated Steroids in Reproduction (FOR1369)*, the porcine testicular-epididymal compartment was used as a model to study the sulfatase pathway in a physiological setting addressing the sulfonation of free steroids and the transport and hydrolysis

of sulfonated steroids. Moreover, another aim of this study was to obtain new information on specific traits of reproductive endocrinology in boars.

This study addresses four major issues:

1) The secretion patterns of androstenedione (A4), testosterone (T), estradiol-17β (E2), estrone (E1), estradiol-17β-3-sulfate (E2S), estrone sulfate (E1S), pregnenolone sulfate (P5S) and dehydroepiandrosterone sulfate (DHEAS), which are considered to be key steroids with respect to the hypothesized sulfatase pathway. It was further hypothesized that qualitative differences in the secretion pattern like phase shiftings or different degrees of decoupling might occur when synthesis of free steroids and their sulfonation are differently regulated in different organs (testis vs. epididymis) or in the case of differential transport (blood, lymph, rete testis fluid). As these effects might become particularly obvious in the case of significant fluctuations of production rates, thus, in addition to long-term measurements in unstimulated animals, hCG application was used to induce a more dynamic situation. Although a significant amount of data on steroid levels is available in boars (for review see Raeside et al. 2006) including profiles after hCG stimulation, a holistic view from long-term measurements of free and sulfonated steroids with high frequent sampling including a significant number of animals is still missing. Moreover, in order to characterize more precisely the immediate testicular output of free and sulfonated steroids, comparative measurements in arterial and venous blood vessels penetrating the testicular capsule were performed. For the first time, newly developed LC-MS-MS methods were used for the measurement of P5S, DHEAS, E1S, E2S, T and A4 in blood plasma, which allows the analysis of free and intact sulfonated steroids with currently the highest specificity and good sensitivity and without the need for chemical modification of the analyte (Galuska et al. 2013).

2) Expression patterns of SULT1E1, SULT2A1, SULT2B1 and steroid sulfatase in the testis and different segments of the epididymis. In humans, among the many members of the superfamily of cytosolic sulfotransferases (SULTs), SULT1E1 has been identified as estrogen specific. SULT2A1 and SULT2B1 are considered as the relevant enzymes for the sulfonation of 3β-hydroxysteroids.

3) Sulfonation of E1, dehydroepiandrosterone (DHEA) and pregnenolone (P5) in cytosolic preparations from the testis and different segments of the epididymis.

4) Hydrolysis of E1S, DHEAS and P5S in cytosolic preparations from the testis and different segments of the epididymis.

2. Literature review

2.1 Anatomy, histology and functions of the testis

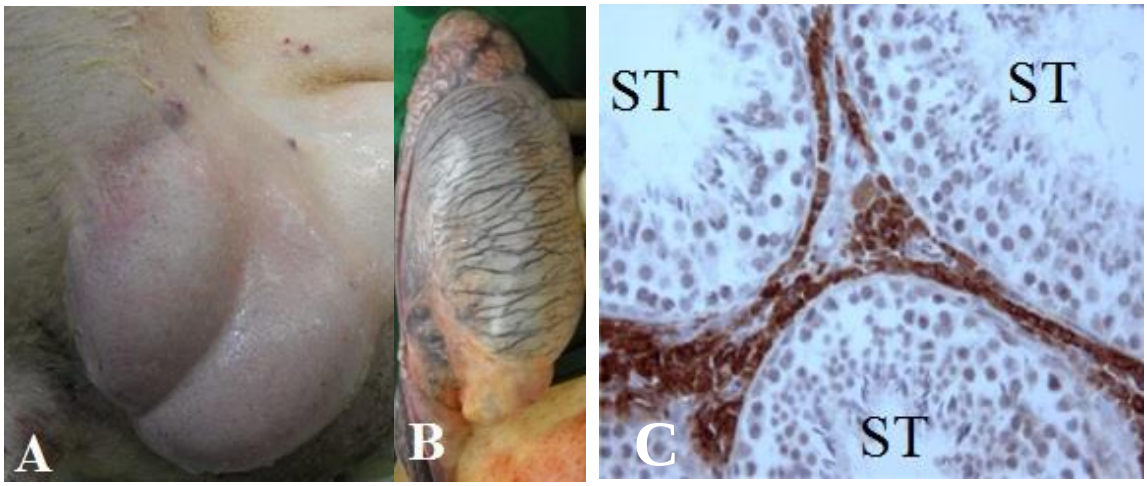


Figure 1. Porcine testis. **A,** Porcine scrotum with testes. **B,** Porcine testis exposed after incision of the scrotum. **C,** Histomorphology of the porcine testis. The Leydig cells situated in the interstitium between the cross-sectioned seminiferous (ST) tubules are identifiable by their distinct immunostaining for steroid sulfatase.

2.1.1 General information and gross morphology of the testis

The testes are the male gonads, which in adult boars are approximately 10 cm in length, 5 to 6.5 cm in width and have a weight of 400 g each (Amann 1970, Dym & Cavicchia 1978) (Fig. 1A & B). They have basically two functions: the production of sperm cells and of hormones (Bonet et al. 2013). Thus, the testis is divided into two compartments. The first compartment consists of vascularized interstitial tissue formed mainly by Leydig cells, macrophages, lymphoactive vessels and connective tissue while the second compartment consists of the seminiferous tubules that contains Sertoli and germ cells (Fig. 1C) (Lee et al. 2009).

The testes have a strong capsule named “tunica albuginea”. The testicular parenchyma is composed of numerous lobules mainly consisting of contorted seminiferous tubules, which drain through the tubuli seminiferi recti, the rete testis and the efferent ductules into the epididymis (Russell et al. 1990). The lobules are separated by connective tissue septa (septula testis) originating from the mediastinum testis. A lobule of the testis consists of one or several seminiferous tubules, which end and start at the rete testis (Schatten & Constantinescu 2007) with exception of mouse seminiferous tubules, which terminate in blind ends (Huber & Curtis 1913). In most mammals seminiferous tubules are flexuous with a diameter between 150 to 300

µm and are lined with the stratified germinal epithelium (Russell et al. 1990). For information on the vascular and lymph systems of the testis see section 2.4.1.

The testes are situated in the scrotum (Fig. 1A), which keeps the temperature of the testis about 2-8^{o}C below the normal body temperature (Stone 1981). Other important factors controlling testicular temperature are the cremaster muscle and the vascular architecture of the spermatic cord. The cremaster muscle is found in the spermatic cord and contracts or relaxes in order to pull the testis closer to the body in cold weather or let them hang further away in hot weather. The specific arrangement and structures of the testicular artery and vein form a counter-current heat exchanger to pre-cool the arterial blood entering the testis (see chapter 2.4.1).

2.1.2 Seminiferous tubules, spermatogenic epithelium and spermatogenesis

2.1.2.1 Seminiferous tubules

The convoluted seminiferous tubules are also called tubuli seminiferi convoluti or contorti. They are entangled running seminiferous tubules, which are mainly located in the periphery of the testis. There they form the main component of the testicular parenchyma (Noguchi et al. 2013). In rats in the unfolded state each tubule would have a length of about 50-60 cm (Wing & Christensen 1982).

The convoluted seminiferous tubules are lined by the germinal epithelium, which basically consists of two cell types: 1) the somatic supporting Sertoli cells and 2) the germ cells, which are in various stages of spermatogenesis.

The epithelium of the seminiferous tubules is externally surrounded by a basement membrane, beyond which myoepithelial cells are found whose contraction causes the sperm transport via the tubuli seminiferi contorti and recti in the direction of the rete testis.

2.1.2.2 Sertoli cells

Sertoli cells are the only somatic cell type physiologically occurring in the spermatogenic epithelium. Before the animal reaches the reproductive age, they are the predominant cell type of the seminiferous tubule. During the reproductive age, Sertoli cells make up about 10% of the population in the epithelium. The timing of Sertoli cell mitosis and proliferation varies between species based on age at puberty and postnatal development (Gondos & Berndston 1993). In primates, Sertoli cells undergo two phases of replication due to the extended timing of puberty while rodents undergo a single phase of Sertoli cell proliferation (Sharpe et al. 2003).

Sertoli cells extend from the base to the apex of the seminiferous epithelium, and are in direct physical contact with all the types of germ cells. Along the lateral surfaces of the Sertoli cells are elaborate intercellular junctions. A specific feature of the Sertoli cell under light-microscopic preparations is the pale-staining nucleus, which is not readily recognizable, because it is situated among the more numerous and prominent types of germ cell nuclei (Lunstra et al. 2003). In mammals, the size of the mature Sertoli cell ranges from 2000 to 7000 μm^3, and the size of the Sertoli cell nucleus is approximately 600 μm^3, with a range from 250 to 850 μm^3 (Lunstra et al. 2003). Sertoli cell nuclei in mammals are usually located close to the periphery of the seminiferous tubule, near the basal lamina of the tubule. The elongated or ovoid nucleus contains a predominantly euchromatic chromatin pattern.

Boars contain two populations of Sertoli cells (Chevalier 1978). Type A Sertoli cells have fine structural characteristics similar to Sertoli cells observed in other mammals including typical junctions with adjacent Sertoli cells and columnar morphology. Type B Sertoli cells are less numerous and are found only in close apposition to the basement membrane of the seminiferous epithelium. They are small cells with dark nuclei and limited cytoplasm that look similar to spermatogonia using light microscopy (McCoard et al. 2001).

As with structure, also the functions of Sertoli cells are widely consistent between mammalian species.

a) They play an essential role in ‘’fine tuning’’ of spermatogenesis. Expansion of the spermatogonia population is limited by Sertoli cells. Each Sertoli cell supports a defined number of germ cells, thus the number of Sertoli cells determines testicular size. Sertoli cells are controlling the many morphological and functional changes that germ cell undergo during spermatogenesis (Griswold 1995). The paracrine interactions between the Sertoli cell and the germ cells, at each maturation stage, are including the maintenance of mitochondrial morphology in spermatocytes (de Kretser et al. 1998).

b) They create a highly specialized environment in which spermatogenesis proceeds by the secretion of essential products (de Kretser et al. 1998) including binding and transport proteins (Sylvester et al. 1993), extracellular matrix and functional proteins (Russel & Peterson 1985), proteases and protease inhibitors (Fritz et al. 1993), growth factors (Benahmed 1996), and energy substrates such as lactate (Grootegoed & Den Boer 1987). Lactate production in Sertoli cells predominantly is under the control of the endocrine system including FSH, insulin (Espositoet al. 1991), and IGF-I (Borland et al. 1984). In turn, the capacity of the germ cells to modulate Sertoli cell activity is well established (de Kretser et al. 1998).

c) Blood-testis (spermatogenesis)–barrier: specialized junctions between pairs of Sertoli cells are the crucial part of the blood-testis-barrier. This divides the germ epithelium into a basal and an adluminal compartment. The basal compartment contains spermatogonia and in the adluminal compartment are the spermatocytes, spermatids and spermatozoa. The tight-junctions between Sertoli cells separate the more differentiated stages of germ cells from getting into contact with the blood circulation or the lymphatic system. This is important because the immune system would produce antibodies against the antigens expressed on the membrane of the spermatozoa or their haploid precursors, leading to an autoimmune orchitis and thus to sterility (Dym & Fawcett 1970, Fawcett et al. 1970, Setchell & Waites 1975). Moreover, the network formed by Sertoli cells controls the transfer of substances into the adluminal compartment (Tarulli et al. 2012).

d) Mediation of androgen effects on the seminiferous epithelium: generally in mammals androgens are essential for the initiation and maintenance of spermatogenesis (Verhoeven et al. 2010). The effect of androgens on the spermatogenesis is mediated by the androgen receptor (AR), which belongs to the superfamily of nuclear receptors. Its genetic and pharmacological disruption is manifested in spermatocytes as meiotic arrest (Holdcraft & Braun 2004). However, investigations on AR expression in the seminiferous epithelium in a broad spectrum of mammalian species yielded negative or highly questionable results, whereas they are readily detectable in Sertoli cells (Hazra et al. 2013). Thus, the effects of androgens on the seminiferous epithelium are obviously mediated by AR expressed in Sertoli cells. Convincing evidence for this concept comes from studies in Sertoli cell specific AR knockout mice (Kerkhofs et al. 2009, Zhou et al. 2011, Chojnacka et al. 2012).

2.1.2.3 Germ cells and spermatogenesis

Spermatogenesis is the process by which spermatozoa are produced from male primordial germ cells through mitosis and meiosis. It comprises four principles: a) stem cell renewal from the mitosis process, b) stem cell reinforcement by dissociation and mitosis, c) chromosomal numbers depletion by meiosis, d) spermiogenesis (Knobil & Neill 2006). The term spermatocytogenesis describes the part of spermatogenesis involved with the mitotic and meiotic divisions of germ cells, while spermiogenesis refers to the maturation of spermatozoa from spermatids.

2.1.2.3.1 Spermatogonia

Spermatogonia proliferation is the initial step for spermatogenesis (Russell et al. 1990). Diploid spermatogonia are the first cells along the route of spermatogenesis and situated in contact with basal membrane of the seminiferous tubule (Drumond et al. 2011).

In boars just prior to puberty, undifferentiated germ cells called gonocytes differentiate to form type AO spermatogonia. These are the precursor sperm cells from which all other cells of the spermatogenic process originate. There is some evidence that the number of AO spermatogonia is directly related to the sperm production capacity of adult males, as Sertoli cells. In adult boars, AO spermatogonia differentiate into A1 spermatogonia which divide progressively to form various types of immature sperm cells (Hafez et al. 2004).

2.1.2.3.2 Primary spermatocytes

They lie in the cell layer luminal to the spermatogonia and in size are larger than spermatogonia. The final mitotic division during spermatocytogenesis provides the primary spermatocytes. The average number of mitotic cell divisions occurring between the A1 spermatogonia and the primary spermatocytes is a subject of some controversy (Hafez et al. 2004). The number of primary spermatocytes originating from a single division of AO spermatogonia may differ between individual mammalian species.

2.1.2.3.3 Secondary spermatocytes

These cells arise from the first meiotic division and rapidly enter the second meiotic division. The secondary spermatocytes have a haploid number of duplicated chromosomes (2N): (Montgomery 1912). They are spherical and smaller than primary spermatocytes. They are located between primary spermatocytes and spermatids, close to the lumen of seminiferous tubules (Nicander 1967, Dym & Fawcett 1971, Holstein & Roosen-Runge 1981).

2.1.2.3.4 Spermatids, spermiogenesis and sperm release

The haploid round spermatids emanating from the secondary spermatocytes after the second meiotic division are transformed into spermatozoa by a series of morphological changes referred to as spermiogenesis.

Important maturational changes that spermatozoa undergo during spermiogenesis include condensation of nuclear material, formation of the sperm tail and development of the acrosomal

cap, which covers the anterior part of the nucleus and contains important enzymes for fertilization (Hafez et al. 2004). During most of spermiogenesis the sperm cells appear to have their heads imbedded in Sertoli cells. In reality, the membrane of the Sertoli cell actually is wrapped around the sperm head. Communication and exchange of materials between the Sertoli and developing sperm cells occur via intercellular bridges. The actual release of spermatozoa into the lumen of the seminiferous tubule is called spermiation. The elongated spermatids are gradually extruded or pushed out of the Sertoli cell into the lumen of the seminiferous tubule until only a small cytoplasmic stalk connects the head of the sperm to the residual body in the Sertoli cell. Breakage of the stalk results in the formation of a cytoplasmic droplet in the neck region of the sperm. These commonly are referred to as proximal cytoplasmic droplets (Williams 2009).

The initial forms of spermatids are small with very light staining in nucleus (Dadoune 1994), while the development of spermatids into spermatozoa results in a smaller and darker nucleus. The changes in spermatids will continue from a simple rounded cells to the differentiated spermatozoon based on complex morphological, physiological and biochemical changes (Gilbert 2000). Spermatozoa are morphologically divided into three main parts of head, neck and tail. In porcine spermatozoa, which are 50 µm in total length, the head, neck and tail are 5, 10 and 35 µm, respectively.

2.1.2.4 Peritubular cells

The spermatogenic epithelium is situated on layers of stromal peritubular cells (fibroblasts and myoid cells) (Tung & Fritz 1986). The organization of the peritubular cell layers differs among species (Christl 1990). In larger animals, such as the rabbit (Leeson & Forman 1981), ram (Bustos-Obergon & Courot 1974), bull (Wrobel et al. 1979) and in humans (Ross & Long 1966), peritubular cells are arranged in five to seven layers. Testosterone is an important factor in the regulation of myoid cell function and differentiation (Bressler & Ross 1972). Peritubular cells are known to act as a partial permeability barrier helping to create the blood-testis barrier. Myoid cells play a role in contractions of the seminiferous tubule to aid the transport of spermatozoa in the tubular lumen (Yazama et al. 1997).

Peritubular cells contact with the basal surface of the Sertoli cells are known to cooperate in the production and formation of a complex extracellular matrix (Raychoudhury et al. 1992).

2.1.3 Tubuli seminiferi recti, rete testis and ductuli efferentes

The rete testis in the boar like some other species (bull, goat, and dog) is formed as a central zone within the testis (Hees et al.1987), while in man, rat, mouse, hamster, and bird the rete testis is found at the testis margin and usually forms an extratesticular portion that connects to the ductuli efferentes (Reid & Cleland 1957, Cooper & Jackson 1972, Amann et al. 1977).

The delineation between rete testis and the efferent duct is abrupt, evidenced by the epithelium changing from low cuboidal to columnar in a sharp transition and a marked increase in peritubular capillaries (Amann et al. 1977, Robaire & Hermo 1988). Nevertheless, in some species such as the boar, there is no clear demarcation of the beginning. Wystub et al. (1989) described a flowing transition at different levels, such that both kinds of epithelia are recognized in the same area of the proximal efferent ductules.

The ductuli efferentes tubules conduct the sperm cells from the rete testis to the epididymal head. They have been called variously as ductuli efferentes, ductuli efferentes testis, ductuli efferentia, vasa efferentes or tubuli efferentes.

In general these ductules arise separately from the rete testis near the tunica albuginea. They are originally formed from the mesonephric (Wolffian) duct (Setchell 1978). In the boar, the approximately 15–20 efferent ductules originate from the rete testis near its upper pole. The ductuli efferentes are divided into a testicular and an epididymal segment. The wall of the ductules is formed by a single layer of columnar epithelium supported by a thin layer of smooth muscle and connective tissue. The ductular epithelium is composed of the nonciliated principal cells and a population of ciliated cells. Detailed descriptions of the histology of efferent ductules for different species are available in the literature (e.g. see the reviews by Hamilton 1975, Robaire & Hermo 1988). Disturbance of the contact formation between the rete testis and mesonephric tubules during ontogenesis may result in the formation of blind ending diverticula named ductuli aberrantes (synonym: aberrant ducts, ductus aberrantes, vasa aberrantes; Blom & Christensen 1960). Two kinds of aberrant ductuli were distinguished by Hemeida et al. (1978): 1) blind-ending tubules that originate from the rete testis and the testicular segment of the efferent ductules and 2) blind aberrant ductules which arise from the epididymal segment. Both kinds of abnormalities have been reported in the boar, goat, ram, bull, and stallion (Blom & Christensen 1960, Hemeida et al. 1978).

Morphological and biochemical studies suggest that the efferent ductules not only enable the transport of the sperm cells from the testis into the epididymis but also fulfill other

physiologically important functions such as the absorption of fluid and other substances (Ilio & Hess 1994).

2.1.4. Leydig cells

Leydig cells are situated in the interstitium surrounding the seminiferous tubules. They are polygonal or ovoid with a relatively big nucleus (Penchev 2011). In contrast to most mammalian species, which exhibit a biphasic pattern of Leydig cell development (Ge et al. 1996), pigs exhibit three phases of Leydig cell development. The first occurs immediately after the gonadal differentiation and is characterized by a large number of mature cells, providing increased concentrations of testicular testosterone. The second phase spans the period between 3 weeks before until 3 weeks after birth. During this phase, the interstitium consists mainly of hyperplastic mesenchymal cells. Between 3 and 13 weeks of age, regression of the secondary Leydig cells is observed until the onset of puberty, when the third phase of activity commences, resulting in the adult population of Leydig cells (Van Straaten & Wensing 1978, Ford et al. 1980, Allrich et al. 1983, Lunstra et al. 1986, Penchev 2011). One third of the total adult boar testicular volume consists of Leydig cells (Wagner & Claus 2004). Generally the secretion of testosterone needed for the maintenance of spermatogenesis and other reproductive functions is considered as the primary function of Leydig cells (Herrera et al. 1983). Indeed, in adult boars they produce considerable amounts of numerous other steroids including estrogens and steroidal pheromones (Raeside et al. 2006, Davidoff et al. 2009; see sections 2.9).

2.2 Anatomy, histology and function of the epididymis

2.2.1 Anatomy and histomorphology

The epididymis is a comma shaped, elongated structure firmly attached to the testis via the mesepididymis. It is formed by a single, tightly-coiled tube connecting the efferent ductules originating from the testis to the deferent duct. The length of the unraveled epididymis varies between species and can be as long as 40 m in bulls or 80 m in stallions. Its main functions are transport, maturation and storage of sperm cells (Cosentino 1986, Stoffel et al. 1990). Traditionally the epididymis is divided in three parts: head (caput), body (corpus) and tail (cauda). Every segment has different physiological functions (Cosentino 1986, Dacheux et al. 2005, Turner 2008, Joseph et al. 2011). In all mammalian species examined to date each region of the epididymis is further organized into lobules separated by connective tissue septa. These septa have been proposed to provide a functional separation between lobules that allows

selective expression of genes and proteins within individual lobules (Turner et al. 2003), resulting in a highly specific regionalization of the epithelium and the luminal protein secretion within the three main epididymal segments (França et al. 2005, Henderson et al. 2006, Turner 2008). The intraluminal compartment is delimitated by a secretory pseudo-stratified epithelium. Histological characteristics allows for the easy identification of the anterior and posterior extremities of the mammalian epididymis. The thickness of the epididymal epithelium varies with the thickest portion in the proximal caput and the thinnest in the caudal region. Conversely, the luminal diameter and the thickness of the peritubular smooth muscle increases from the proximal to the distal regions (Lasserre et al. 2001, Toshimori 2003).

2.2.1.1 Epithelial cell types of the epididymis and epididymosomes

2.2.1.1.1 Principal cells

Principal cells appear in the epithelium along the entire epididymal duct but show structural differences between different regions (Hamilton 1975, Robaire & Hermo 1988). They are the most frequent epithelial cells which cover 65%-80% of the total epithelial cell population. They are predominantly responsible for the secretion of proteins into the epididymal lumen (Hamilton 1975, Sun & Flickinger 1979). These columnar cells exhibit prominent stereocilia at their apical side which extend into the ductal lumen. Ultrastructurally, the supranuclear region of this cell type contains large stacks of Golgi saccules, mitochondria, multivesicular bodies and apically dilated membranous elements, while the infranuclear region is densely packed with rough endoplasmic reticulum (Robaire et al. 2000, Dacheux et al. 2005). These cells also form tight junctions with one another at the lateral side, forming as such the blood-epididymis barrier (Cornwall 2009) which allows an intraluminal milieu with a specific composition of electrolytes and macromolecules.

2.2.1.1.2 Epididymosomes

After their release from the spermatogenic epithelium of the testis, spermatozoa must undergo significant maturation processes in the epididymis to obtain their full functional capacities, e.g. their ability to bind to the zona pellucida. However, due to the high compaction of the DNA in the head of sperm cells after the replacement of histones by protamines, transcription and translation virtually come to rest. Thus, concerning their post-testicular maturation the sperm cells practically fully depend on the epididymal environment, which provides a complex spectrum of compounds as a function of particular epididymal segments. These compounds

may be attached to or integrated into specific domains of the cellular membrane or may even cross the cell membrane to exert their function at specific intracellular structures of the sperm cell. However, as they are derived from extracellular sources, the mechanisms usually directing cellular products after intracellular synthesis to their specific cellular compartments or targets may be unsuitable during epididymal maturation of spermatozoa. Thus, obviously alternative pathways of secretion by epithelial cells of the epididymis and specific targeting of the secreted products on or into sperm cells may exist, and it has been suggested that apocrine secretion, which is observed to extensively occur from the surface of the epididymal epithelium and provides small membrane vesicles named epididymosomes, may be involved in these processes (Sullivan & Saez 2013). Epididymosomes arise from blebs in the apical part of the principal cells which undergo fragmentation into 20-100 nm diameter vesicles upon detachment (Belleannée et al. 2013). They exhibit a complex composition of proteins and other substances such as various lipids. However, the mechanisms underlying the target-oriented transfer of macromolecules from the epididymosomes to the maturing sperm cells are widely unknown and still under research. The presence of epididymosomes has been described in various species including hamsters (Legare et al. 1999), rats (Fornes et al. 1995, Grimalt et al. 2000), mice (Rejraji et al. 2006, Griffiths et al. 2008), sheep (Ecroyd et al. 2004, 2005), cattle (Frenette & Sullivan 2001, Frenette et al. 2002), and humans (Thimon et al. 2008). An extensive use of the apocrine secretion pathway has also been described in other reproductive organs such as the prostate, seminal vesicles (Aumüller et al. 1997), coagulating gland (Groos et al. 1999), vas deferens (Manin et al. 1995, Hermo & Jacks 2002, Rejraji et al. 2006) and in the uterus (Griffiths et al. 2008).

2.2.1.1.3 Basal cells

Basal cells form about 15-20% of the total epithelial cell population of the epididymis (Robaire et al. 1988, Marengo & Amann 1990, Adamali & Hermo 1996). They are triangular or flat cells which are located in the base of the epididymal epithelium. Their nuclei are elongated or round shaped, and they are in close association with the overlying principal cells or other basal cells through the presence of cytoplasmic extensions (Robaire et al. 2000, Cornwall 2009). Some findings have suggested that the basal cells may have a role within the defense mechanisms against pathogenic agents and in the regulation of electrolytes by the epididymal epithelium. However, the exact functions of these cells are not yet definitely clear (Robaire et al. 2006).

2.2.1.1.4 Other cell types of the epididymal epithelium

Other cell types, such as apical, narrow, clear and halo cells, have also been described in the epithelium of the epididymal duct in rabbit (Orgebin-Crist 1967), boar (Holtz & Smidt 1976), ram (Fournier-Delpech 1997), mouse (Pavlok 1974, Hoppe 1975), rat (Dyson 1973), and hamster (Horan & Bedford 1972). Narrow and apical cells are shaped oval to spherical. They are responsible for H^+ secretion and bicarbonate resorption (Cooper & Hamilton 1977, Hermo et al. 1992, Robaire et al. 2006). Clear cells are present in all three main regions of epididymis and form only 5% of the total epithelial cell population (Sun & Flickinger 1979, Hermo et al. 1988, Flickinger et al. 1988, Vierula et al. 1995). They are responsible for the reabsorption of proteins excreted by the epididymal epithelium. Halo cells are small cells with a narrow rim of clear cytoplasm present as intraepithelial cells throughout the epididymal epithelium. They are believed to play a role in the immunological barrier of the male reproductive duct and have been postulated to be lymphocytes or monocytes (Dym & Romrell 1975, Flickinger et al. 1997, Dacheux et al. 2005).

2.2.2 Functions of the epididymis

The epididymal duct is a channel for transport, concentration and storage of the spermatozoa. Moreover, under the control of androgens the secretions of the epididymal epithelium provide an environment within the intraluminal compartment enabling important maturational processes of spermatozoa that leave the testis in a structurally and functionally immature state. Motility and various other essential sperm cell functions such as the capability for capacitation are only acquired during passage of the epididymis (Toshimori 2003, Sullivan 2004, Ecroyd et al. 2005, Dacheux et al. 2005).

2.2.2.1 The blood-epididymis barrier

The spermatozoa are immunogenic as they express proteins on their surfaces that would be recognized as nonself if they would get into contact with the immune system (Robaire & Hermo 1988, Dacheux et al. 2005). Tight junctions between the adjacent epididymal epithelial cells form the blood-epididymal barrier and restrict the passage of a number of ions, solutes, and macromolecules through the epididymal epithelium. This barrier serves as an extension of the blood-testis barrier which keeps blood and ductular fluid in separate compartments (Hinton et al. 1995) and maintains a specific luminal microenvironment for the maturing spermatozoa

(Hinton & Hernandez 1985), thus protecting spermatozoa from toxic substances and immunoglobulins (Qiu et al. 1992).

2.2.2.2 Reabsorption of tubular fluid

The volume percentage of sperm cells in the epididymal ductal fluid increases significantly from 2% in the efferent ductules to about 50% in the epididymal tail, indicating a substantial reabsorption of fluid in the epididymis. Observations in male estrogen receptor α (ERα) knockout mice showed that fluid reabsorption mainly occurs in the efferent ductules, where ERα is highly expressed (Hess et al. 2000, Hess 2000, 2003). Male ERα knockout mice exhibited markedly dilated efferent ductules with a flattened, degenerated epithelium obviously resulting from a significant congestion of ductular fluid. Further evidence for a role of estrogens in the control of epididymal fluid reabsorption comes from studies applying a potent estrogen receptor antagonist (ICI 182780) (Hess 2000). Moreover in mice it has been shown that estrogens participate in the regulation of the expression of key molecules involved in the epididymal transport of ions and water (Joseph et al. 2010). Different from the knockout of ERα, of which the expression is especially high in the efferent ductules and the epididymal head, no alterations of testicular-epididymal morphology or functions were found after the knockout of ERβ, which is more uniformly expressed along the epididymis. Although the epididymal expression patterns of ERα and ERβ were found to be conserved in several species investigated so far including the pig, currently it is unclear to which extent the well-established role of estrogens in the control of epididymal fluid reabsorption in mice may be extrapolated to other mammalian species (Hess et al. 2000, Hess 2000, 2003).

2.2.2.3 Microenvironment for maturation

Mammalian spermatozoa originate from extensive cellular differentiations transforming round spermatids into highly polarized and motile cells. Most of these complex biochemical and physiological processes and morphological changes take place in the seminiferous epithelium of the testis in close contact and interactions with Sertoli cells during the spermiogenesis process. However, after spermiation testicular spermatozoa are still immotile and incapable of fertilization. Thus, they need considerable postgonadal differentiation to be able to fertilize. Spermatogenesis in the testis is generally under the genomic regulation of the gamete. However, concomitant with DNA condensation in the elongated spermatids the transcription process in the germinal DNA decreases and finally virtually ceases. Thus, during the subsequent passage

of the epididymis the presence of a specific environment obviously plays an essential role in inducing or controlling the final sperm differentiation. Different maturational processes may occur in specific segments of the epididymal duct, which is mirrored by progressive changes in the composition of the tubular fluid during its passage. It is shown that repeated ejaculations, which remove spermatozoa from distal epididymis, do not affect the rate of sperm transport along the rest of the epididymis (Kirton et al. 1967). The caudal portion of epididymis is the main sperm cell reservoir enabling a maximal survival time of spermatozoa. This microenvironment contributes ions, organic solutes, and proteins to a luminal fluid milieu specialized for sperm storage (Rodriguez-Jimenezet al. 2003).

In boar, the proximal section of epididymis is characterized by the synthesis and secretion of glutathione peroxidase (GPX), hexosaminidase (HEX) and an RNase-train A (train A), an RNase A-like protein without RNase activity.

Secretion of clusterin and the presence of cathepsin L, lactoferrin and NCP2/CTP/HE1 proteins have been described in middle caput. Secretion of α-mannosidase, retinoic acid binding protein (E-RABP) and an unidentified train E are characteristic for the distal caput and proximal corpus. Train H was the only protein found to be specific for the cauda epididymis (Dacheux et al. 2005).

Moreover, addition or loss of membrane components may be associated with the remodeling of the sperm membrane during epididymal maturation (Dacheux et al. 2005). Important sperm cell functions such as acrosome reaction, capacitation, ovum binding and penetration are mediated by sperm membrane (Breitbart et al. 2005). In response to the epididymal secretions (enzymes, proteins and glycoproteins), sperm maturation involves morphological and biochemical changes in the sperm surface (Robaire et al. 2000, Ecroyd et al. 2005, Dacheux et al. 2005, Sullivan et al. 2005). Within this processes some proteins bind to the sperm (Von Horsten et al. 2007), and some of them will later bind to the zona pellucida (Ellerman et al. 1998) or the plasma membrane of the oocyte (Flesch & Gadella 2000). Another process occurring with spermatozoa during the passage of the through the epididymis is the loss of cytoplasmic droplets. During normal spermatogenesis most of round spermatid's cytoplasm is phagocytized by Sertoli cells. However, commonly a small cytoplasmic residue remains as a small droplet attached to the neck of immature spermatozoa after their release from the germinal epithelium. During the epididymal transit the cytoplasmic droplet migrates away from the neck to the annulus which marks the end of the midpiece, where it commonly detaches from the sperm cell (Cooper 2011). There are different reports among species for the epididymal location where this process takes place (Cooper et al. 2003). In boars only very few ejaculated spermatozoa

still possess cytoplasmic droplets (Kato et al. 1996). The retention of cytoplasmic droplets in a significant proportion of ejaculated spermatozoa has been shown to be associated with infertility in bulls (Amann et al. 2000, Thundathilet al. 2001) and boars (Kuster et al. 2004).

2.2.2.4 Storage of sperm cells in the epididymis

After important post-testicular maturational processes during passage of the epididymal head and body, the sperm cells are stored in the epididymal tail until their consumption during ejaculation or the elimination of overaged spermatozoa in the absence of sexual activity. Although depending on the species a minor proportion of fertile sperm cells may also reside in the deferent duct and excretory ducts of accessory sex glands, the epididymal tail is by far the most important storage organ for mature sperm cells (Jones 1999).

A low epithelium and a wide lumen containing masses of mature spermatozoa are the morphological equivalents for the sperm storage function of the distal epididymal tail. After ejaculation spermatozoa advanced in the vas deferens are returned to the distal epididymis, which additionally shows the importance of the cauda epididymis as sperm storage site (Jones 1999).

The spermatozoa stored in cauda epididymis are basically capable of motility but are maintained in a state of quiescence in most animals (Bedford 1975). On ejaculation the sperm are activated to full motility by the secretions of the accessory sex glands. Immediately after ejaculation the spermatozoa are incapable of fertilization until incubated for several hours in the female tract; this transition is referred to as capacitation. During capacitation, motility pattern changes to a less progressive “hyperactivated” behavior characterized by frequent changes of the direction. After capacitation sperm can undergo the acrosome reaction needed for fertilization (Martin-Hidalgoet al. 2013).

2.2.2.5 Control of epididymal differentiation and functions by androgens and estrogens

During embryogenesis the epididymis develops from the Wolffian duct (mesonephric duct), a paired organ connecting the primitive kidney, the mesonephros, to the cloaca. In males further organs originating from Wolffian structures are the vas deferens and the seminal vesicles, whereas the prostate forms from the urogenital sinus. For the development of male urogenital structures from the Wolffian duct its exposition to androgens during fetal development is essential (Imperato-McGinley & Zhu 2002). In females, in the absence of testosterone, the

Wolffian duct widely regresses with the exception of a few remnants which may persist (e.g. Gartner´s duct).

Testosterone and 5α-dihydrotestosterone (DHT) are the two most important naturally occurring androgens. Testosterone, the primary androgen synthesized and secreted by the testes, can be converted to DHT in target organs by two 5α-reductase enzymes (type 1 and 2, SRD5A1, SRD5A2; see also chapter 2.5.6). Traditionally, DHT has been regarded as the "stronger" androgen in comparison to testosterone due to its significantly higher binding affinity to the AR. However, despite the existence of only one AR receptor type, testosterone and DHT may have qualitatively different effects which results from a differential conformational change of the AR after binding of each of the two ligands, leading to the recruitment of different sets of AR cofactors (Imperato-McGinley & Zhu 2002, Askew et al. 2007). Northern blot analysis along the human epididymis have shown the SRD5A2 transcript in the midcaput, distal caput, corpus, and proximal cauda of the epididymis while it has not been identified in the testis, proximal caput, and distal cauda regions (Mahony et al. 1998). The same authors have shown that both types of 5α-reductase are present in human epididymis with evidence of higher mRNA level of type SRD5A2 compared to type SRD5A1. Similar results have been obtained in rat epididymis, where SRD5A2-mRNA is also expressed at higher levels; it is predominantly expressed in the caput segment of the epididymis (Normington & Russell 1992, Viger & Robaire 1996).

During the fetal phase, development and differentiation of Wolffian duct derived structures such as the epididymis, seminal vesicles, and vas deferens is primarily considered as testosterone-dependent (Imperato-McGinley & Zhu 2002). Puberty is important to maintain morphology and secretory function in epididymis (Pujol et al. 1979, Bedford 1994, Pearl et al. 2007, Lydka et al 2011). In the postpubertal epididymis the high expression of ARs points to an important role of androgens for differentiation and functions in this organ also during adulthood (Belgorosky et al. 2008). In mature rats DHT has been identified as the androgen present in the nuclei of epididymal cells (Tindall et al. 1972). Treatment of rats with dual 5α-reductase inhibitors suppressing the activity of both SDR5A1 and -2 resulted in pronounced effects on epididymal gene expression and functions such as altered expression of genes essential to the formation of the optimal luminal microenvironment that is required for proper sperm maturation and a significant decrease in the percentage of progressively motile and morphologically normal spermatozoa in the cauda epididymis. Moreover, mating females to treated males resulted in fewer successful pregnancies and a higher rate of pre-implantation loss (Robaire & Henderson 2006).

In the male reproductive tract estrogens may be produced in the testis, spermatozoa and epididymis (Carreau et al. 1999, Shayu & Rao 2006, Mutembei et al. 2009). High expression of ERα has been detected in the epithelium of the efferent ductules and of the caput epididymis, and their role in luminal pH maintenance, fluid absorption and smooth muscle contractility has been suggested (Hess et al. 2000, Zhou et al. 2002, Joseph et al. 2010). In mice, after knockout of ERβ no effects on function and morphology of the epididymis could be demonstrated (Rosenfeld et al. 1998). As impressively evident from the observations in αERKO mice, estrogens may be important regulators of epididymal function. However, the exposition of the epididymis to estrogens may vary significantly between species depending on their source (Leydig cells, Sertoli cells, germ cells) and the spectrum of estrogens produced (Hess et al. 2001). Based on the detection of aromatase expression there is now evidence from several species that estrogens may be produced locally in the epididymis (Hess et al. 2001).

2.3 Vas deferens

The vas deferens (syn.: ductus deferens) is a muscular tube connecting the distal end of the epididymis, from which it is in direct continuity, with the pelvic part of the urethra. From its origin at the epididymal tail it passes upward along the posterio-medial border of the testis to become part of the spermatic cord. After passing through the inguinal canal, it enters the abdominal cavity, courses over the pelvic brim into the pelvic cavity, where it ends behind the urinary bladder. Near its termination, in some mammalian species it becomes dilated into a portion named the ampulla. The course of the final part of the vas deferens exhibits species specific peculiarities. In some species (man, horse, domestic ruminants) it unites with the excretory duct of the ipsilateral seminal vesicle to form a short ejaculatory duct, which passes through the prostate gland and empties into the dorsal part of the urethra. In the boar, in most cases vas deferens and the excretory duct have separate endings. Also species without seminal vesicles such as the dog and cat have independent endings of the vas deferens into the urethra (Knobil & Neill 2006).

The wall of the vas deferens is composed of a mucosa, a massive smooth muscle coat (the muscularis), and an adventitia. Similar to that of the epididymal tail, the pseudostratified columnar epithelium of the vas deferens is composed of tall columnar epithelial cells possessing long microvilli (stereocilia) that extend into the lumen, and a discontinuous layer of round or pyramidal basal cells situated on the basal lamina. Lymphocytes frequently occur between the epithelial cells. However, in comparison to the epididymis, the lumen of the vas deferens is

clearly more irregular in outline due to the formation of deep longitudinal mucosal folds that are particularly prominent towards the urethral end. The muscularis is formed of an intermediary circular layer, enclosed by inner and outer longitudinal layers. During ejaculation, coordinated rhythmic contractions and relaxation of these muscular layers propel the spermatozoa along the vas deferens due to adrenergic mechanisms. However, numerous substances may modulate the release of noradrenaline from sympathetic innervations (Schatten 2007).

In comparison to other male reproductive organs, for the vas deferens relatively little information is available concerning its functions. However, structural features consistent with a high synthetic and excretory activity and the absorption of material from the lumen suggest that the vas deferens is not a mere conduit for sperm transport, but is an important organ contributing actively to the maintenance of sperm structure, maturation, survival and viability (Knobil & Neill 2006).

2.4 The vascular and lymph system of the testis and epididymis

2.4.1 The vascular and lymph system of the testis

Arterial blood supply of the testis occurs predominantly via the testicular artery, which arises directly from the abdominal aorta and descends through the inguinal canal reflecting testicular descend, whereas the scrotum and the rest of the external genitalia are supplied by the internal pudendal artery, a branch of the internal iliac artery. In addition to the major blood supply by the testicular artery, the testis may have collateral blood supply as the distal part of the testicular artery often anastomoses with the cremasteric artery (a branch of the inferior epigastric artery) and the deferential artery (a branch of the inferior vesical artery, which is a branch of the internal iliac artery). After reaching the testis near its upper pole, the testicular artery runs down the epididymal margin of the organ just under the capsule in a reasonably straight course to the distal part of the testis. Soon after rounding the distal pole it forms several branches which aspire to free margin of the testis. The terminal branches of the testicular artery eventually enter a vascular network situated in the deeper layer of the tunica albuginea formed by winding arteries, with the exception of the posterior part of the testis on both sides of the testicular artery, which are not covered with these vessels (Polguj et al. 2011). The arterial supply of the testicular parenchyma follows the lobular structure in that centripetal arteries arising from the superficial vascular network enter the testicular septa and run in a relatively straight course to near the mediastinum, where they form coils and turn back as centrifugal (recurrent) arteries. On their

way towards the testicular surface within the lobules, the centrifugal arteries give rise to segmental arteries, which originate at a regular distance from each other, branch within the interstitial tissue and finally give rise to a capillary network and the venous system (Setchell & Main 1975, Myrén & Einer-Jensen 1992).

The venules draining the interstitial tissue empty into intralobular veins, which empty into veins in the septa (Setchell & Main 1975, Myrén & Einer-Jensen 1992). The venous blood flow in the septa exhibits considerable differences between mammalian species. In the boar, most of the veins run centrifugally towards the surface to empty into a subalbugineal plexus, which finally join an intra albugineal plexus and superficial veins running to the proximal pole of the testis, which final drain into branches of the pampiniform plexus (Setchell & Main 1975, Myrén & Einer-Jensen 1992).

Despite a significant number of studies on the fine structure of testicular lymphatic spaces and the walls of lymphatic vessels, information on the gross architecture of the lymphatic system in the testis is sparse and in part conflicting. Moreover, information so far available indicates significant species specific and individual differences. In general, lymphatic vessels may reach the testicular surface via the testicular septa, which empty into a superficial plexus, or via the rete testis. Superficial collecting lymph vessels originate from the proximal extremity and the epididymal border of the testis. These lymph vessels may ascend along the testicular artery thereby reducing their number (Itoh et al. 1998, Heinzelbecker et al. 2014). However, as shown in the rabbit, testicular efferent lymphatic vessels may not only course along the testicular artery, but to a minor extent also along the ductus deferens and/or cremasteric artery (Heinzelbecker et al. 2014).

2.4.2 The vascular and lymph system of the epididymis

The angio-architecture of the epididymis is in part species specific. However, basically epididymis blood supply is derived from two sources: the testicular artery and the deferential artery. In the boar two branches (rami epididymales) origin from the testicular artery immediately proximally to or after the beginning of its highly convoluted part in the vascular cone. Soon after their origin from the testicular artery, the epididymal branches form a widely ramified network, which supplies predominantly the epididymal head and the proximal body. However, some arteries originating from this network run to the distal body and the proximal tail to supply also these parts of the epididymis. From the deferential artery epididymal branches originate already proximally of the vascular cone and run to the hind part of the epididymis

together with branches of the rami epididymales. Only the hind part of the epididymal tail is exclusively dependent on supply from the deferential artery. Anastomoses between branches from the testicular artery and the deferential artery may already occur within the region of the vascular cone. The venous blood vessels follow the arteries (Stoffel et al. 1990).

No specific information was found concerning the architecture of the lymphatic system in the porcine epididymis. In rats it has been described that the epididymis is drained by lymphatics emerging separately from different regions of the head, body and the tail and which converge to form a single vessel draining into the main testicular trunk. The pattern of lymphatic drainage in general is very similar to that of the arterial blood supply. Anastomoses occur at various levels between lymphatics draining the testis and the epididymis (Pérez-Clavier et al. 1982).

2.4.3 Architecture of the vascular cone of the spermatic cord

The testicular vascular cone is a specialized anatomical structure located in the distal part of the spermatic cord which is formed mainly by a highly sinuous and coiled section of the testicular artery juxtaposed between fine networks of testicular veins that form the pampiniform plexus. Its main physiological role is the heat transfer between the warm arterial blood and the cool venous blood flowing in opposite directions within adjacent vessels occurring through a counter-current mechanism, as maintenance of testicular temperature some centigrades below body temperature is essential for normal spermatogenesis in most mammalian species. Detailed information on the vascular architecture in the boar spermatic cord is available from a study by using corrosion casts. In sexually mature boars the spermatic cord is about 17-20 cm in length. In its upper fifth part, the testicular artery is relatively straight, whereas in the remaining distal part it is extensively convoluted. During the upper straight part of the testicular artery and the first 5-8 loops numerous thin branches arise forming a network for the arterial supply of the pampiniform plexus and the epididymis (Böttcher & Lange 1987, Rerkamnuaychoke et al. 1990, Stoffel et al. 1990; see also section “vascularization of the epididymis” (2.4.2))

The convoluted part consists of retrograde and spiraled loops of which the diameter steadily decreases towards the upper pole of the testis. Thus, the convoluted part has the form of a cone with the tip directed towards the testis. Despite the high degree of convolution and retrograde looping, a bifurcation was not found. The convoluted part is covered by venous plexus. The layers of veins covering the artery vary in number according to the distance of the artery from the surface of the vascular cone. Parts of the artery running close to the surface of the cord are covered by only one layer of veins. In the deeper part of the cord, there are at least three venous

layers between its loops (Rerkamnuaychoke et al. 1990). After passage of the pampiniform plexus, the testicular artery penetrates the tunica albuginea near the proximal (cranial) testicular pole and runs under the epididymis and the tunica albuginea towards the caudal testicular pole (Böttcher & Lange 1987; see also section "vascularization of the epididymis" (2.4.2)).

The convoluted part of the testicular artery is embedded in the pampiniform plexus. This complex venous network emerges mainly from the back of the testis with additional tributaries from the epididymis (Heinze & Ptak 1976). It is composed of different types of veins characterized by different luminal diameter and structure of the vessel wall. The large type I veins form a network on the most outer surface of the spermatic cord. The medium-sized type II veins run as a single layer of anastomosing veins along the testicular artery. The type III veins, also classified as medium-sized, form networks of several layers between type II venous networks; anastomosing type II and III veins form the great part of the pampiniform plexus. The small type IV veins arise from type II and III veins and form networks between the larger veins or between larger veins and the testicular artery. As they exhibit a thin fenestrated epithelium and may deeply penetrate into the media of the testicular artery, arterio-venous exchange may be efficient despite the thick arterial media.

In addition to its function as a heat exchanger, the testicular vascular cone has also been suggested to act as a countercurrent mechanism of hormone transfer, especially for maintaining a high concentration of testosterone in the testis. In addition to passive diffusion, a transfer between the testicular artery and the pampiniform plexus has been suggested to occur via arterio-venous anastomoses (Einer-Jensen & Hunter 2005). It has been described in different species including bovine (Amann & Ganjam 1976), human (Bayard et al. 1975), rhesus monkey (Dierschke et al. 1975), rams (Noordhuizen-Stassen et al. 1985) that the concentration of testosterone in the testicular artery by side of pampiniform plexus is higher than that in the systemic circulation. However, as the difference was generally low, the physiological relevance of these observations is unclear.

2.5 Steroid hormones and steroidogenesis

Steroids are characterized by their sterane (cyclopentanoperhydrophenanthrene) backbone, which is a hypothetical structure consisting of four condensed ring molecules (A-D). Natural steroids are produced from cholesterol, from which basically five major classes of steroids can be synthetized 1) glucocorticoids (21 carbon atoms, C21), 2) mineralocorticoids (C21), 3) progestogens (C21), 4) androgens (C19) and 5) estrogens (C18), which are further characterized

by an aromatic A ring. Progestogens, androgens and estrogens are also named sex steroids. However, biological activity does not always correlate with this nomenclature. For example, 19-nortestosterone, a C18 steroid, is a potent androgen (Attardiet al. 2010) Moreover, androst-5-ene-3β, 17β-diol and 5α-androstane-3β, 17β-diol, both are C19 steroids with a saturated A-ring, possess considerable estrogenic activity (Chen et al. 2013).

Steroids are a very important class of hormones involved in the regulation of growth, differentiation, metabolism and many other crucial bodily functions. Glucocorticoids primarily affect metabolism of proteins, carbohydrates and lipids by increasing concentrations of circulating glucose, fatty acids and amino acids. Their actions are generally metabolically antagonistic to insulin, i.e., glucose uptake, glycolysis and protein synthesis are depressed and protein degradation is increased (Gower 1979). Depending on the species, cortisol or corticosterone is the most important endogenous glucocorticoid. Mineralocorticoids primarily affect transport of electrolytes and distribution of water in tissues (Gower 1979). The most potent mineralocorticoid is aldosterone. Among numerous functions, the sex hormones affect secondary sex characteristics, control the reproductive cycle in females and cause growth and development of the accessory reproductive organs in males (Mankidy et al. 2014). Basically, two pathways are available for the synthesis of androgens and estrogens, the Δ4-pathway and the Δ5-pathway (Fig. 2). The Δ4-pathway is the predominant route of steroidogenesis in rodents (Slaunwhite & Samuels 1956, Bell et al. 1968), whereas in pigs (Vihko 1974, Booth 1975), dogs (Eik-Nes 1970), rabbits (Hall et al. 1964), human and higher primates (Preslock 1980, Rey et al. 1995) the Δ5-pathway are found more predominant. As a specific feature of the porcine species, in the boar testis high amounts of steroidal pheromones (Δ16-steroids) are produced (Vihko 1974).

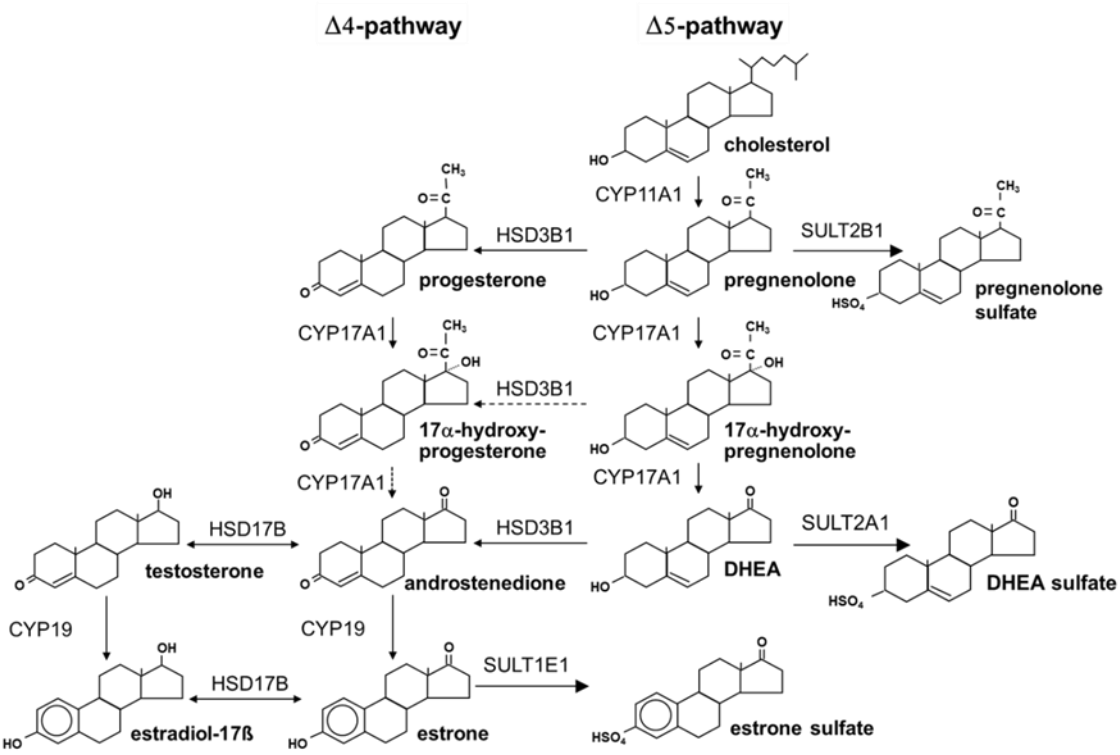


Figure 2. Steroidogenic pathways (Δ4- and Δ5-pathway).

2.5.1 Initiation of steroidogenesis and steroid acute regulatory protein (StAR)

The original substrate for steroidogenesis is cholesterol (Fig. 3). The predominant source of cholesterol for steroid hormone synthesis may vary depending on the species and the steroidogenic tissues (Christenson & Devoto 2003). Steroidogenic cells have access to cholesterol mainly from one of the following three sources: a) from de novo cholesterol synthesis from acetate, b) from the serum in the form of circulating lipoproteins that are then processed to yield either esterified cholesterol for storage in lipid droplets or free cholesterol for immediate steroid biosynthesis or, c) from the hydrolysis of cholesterol ester stored within the lipid droplets of steroidogenic cells (Christenson & Devoto 2003). Cholesterol is an important component of biological membranes. The cholesterol-binding capacity of a membrane, as well as the ability of cholesterol to enter and exit the membrane, depends on the chemical nature of the membrane phospholipids (Miller & Auchus 2011). For more detailed information on cholesterol sources, receptors and the mechanisms involved in the uptake and utilization of cholesterol comprehensive reviews are available (Azhar et al. 2002, 2003, Rigotti et al. 2003, Christenson & Devoto 2003).

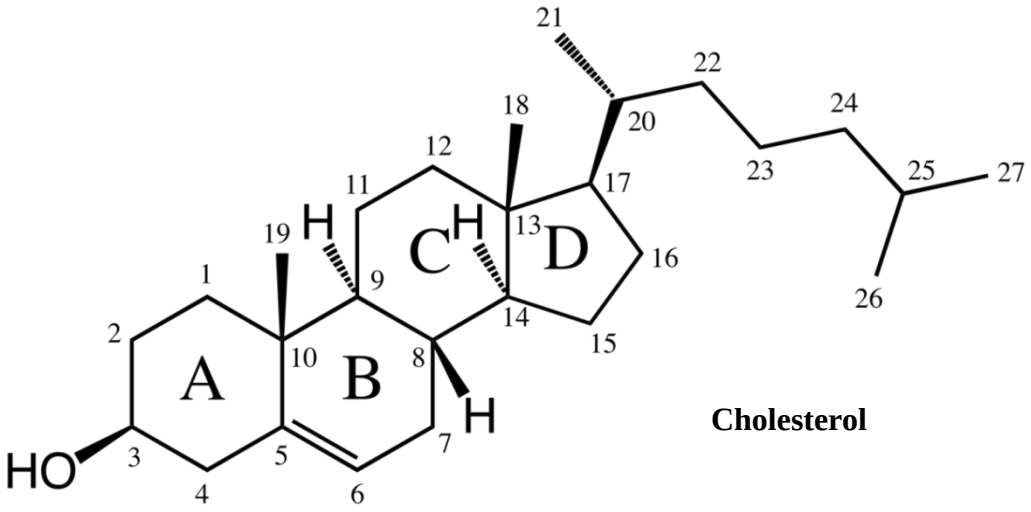


Figure 3. The structure of cholesterol with numbering of the C-atoms. Letters label the cyclohexane rings A, B and C, and the cyclopentane ring D.

A first crucial step in steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane, where the first and rate-limiting enzyme of the steroidogenic cascade, the cholesterol side-chain cleavage enzyme (P450scc, CYP11A1) is localized. The StAR protein, a mitochondrial phosphoprotein, is an essential component in the regulation of adrenal and gonadal steroid biosynthesis as it is a crucial component of the machinery mediating this transport. It was initially described as a 30 and 37 kDa phosphoprotein in tropic hormone treated mouse Leydig cells (Stocco et al. 2005). In gonadal and adrenal steroidogenesis the StAR dependent transport of cholesterol to the inner mitochondrial membrane is rapidly stimulated by gonadotrophins (gonads) or adrenocorticotropic hormone (ACTH) (adrenal) via their membrane-bound G-protein coupled receptors activating cAMP dependent signal cascades. The exact mechanism of StAR is still not fully understood (activation by phosphorylation vs. rapid genomic mechanism). In addition to the activation of adenylate cyclase it has also been shown that the protein kinase C (PKC) signaling, as has the mitogen-activated protein kinase (MAPK) family of Ser-Thr kinases, plays an important role in regulating StAR expression and steroidogenesis (Gyles et al. 2001, Seger et al. 2001, Manna et al. 2002, Martinelle et al. 2004, Jo et al. 2005, Martinat et al. 2005). StAR protein is generally expressed in steroidogenic cells with the exception of the human trophoblast, where a different protein similar to StAR obviously has a StAR-like function (Yivgi-Ohana et al. 2009). Moreover, StAR expression has

also been detected in cells without a quantitatively significant steroid hormone production (Sugawara et al. 2006).

2.5.2 Cytochrome P450 side chain cleavage (CYP11A1)

Conversion of cholesterol to pregnenolone is the initial enzymatic reaction in the biosynthesis of all steroids (Jefcoate 1992). This conversion is catalyzed by the cytochrome P450 side chain cleavage enzyme (P450scc, CYP11A1). This enzyme is located on the inner mitochondrial membrane (Farkash et al. 1986, Geuze et al. 1987). There is a high expression of this enzyme in Leydig cells (O'Shaughnessy & Murphy 1991), whereas, in Sertoli cells its expression is only low (Ford et al. 1999). Conversion of cholesterol to pregnenolone includes three separate monooxygenation reactions: hydroxylation of C22 followed by the hydroxylation of C20 to produce 20, 22 R-hydroxycholesterol (Boyd & Simpson 1968, Burstein & Gut 1976); the reaction is finalized by the cleavage of this hydroxylated intermediate to yield pregnenolone and isocaproic aldehyde. In rabbit (Yang et al. 1993) or mouse (Hu et al. 2002) inactivation of the CYP11A1 eliminates all steroidogenesis giving evidence that P450scc is the only enzyme capable of converting cholesterol to pregnenolone.

2.5.3 3β-Hydroxysteroid dehydrogenase-Δ5/4-isomerase (3β-HSD, HSD3B)

The key enzyme during the synthesis of all active steroid hormones is 3β–HSD. This membrane-bound enzyme belongs to the family of oxidoreductases, which oxidize hydroxyl groups with NAD+ or NADP+ as an electron acceptor. It converts 3ß-hydroxy-5-ene into 3-keto-4-ene steroids (Lorence et al. 1990). 3β-HSD is expressed in a high number of tissue types in the body: adrenal, ovary, testis, placenta, liver, breast, prostate, skin, brain, kidney, cardiovascular system, blood cells, and adipose tissue. Depending on the steroidogenic organ this enzyme is localized in the endoplasmic reticulum or in the mitochondria (Cherradi et al. 1993, Sauer et al. 1994). Depending on the species, several isoenzymes may occur. The numbers of 3β–HSD isoenzymes identified are two in humans and rats (HSD3B1, HSD3B2) and six in mice (HSD3B1-6) (Abbaszade et al. 1997, Simard et al. 1993), whereas in pigs so far only one isoform was described (Von Teichman et al. 2001). Different from the subcellular localization mentioned above, Nakajin et al. (1994) have found 3β–HSD expression for the first time in cytosolic fractions from the pig testis (Nakajin et al. 1994). Conley et al. (1994) have investigated the expression of 3β-HSD in several porcine fetal tissues. In fetal testis, expression of 3β-HSD was only low in comparison to the fetal adrenal gland at all stages of development.

In the boar testis, in addition to its role in the synthesis of testosterone and estrogens, 3β-HSD is also a key enzyme in the synthesis of the boar taint compound Δ16-androstenone (Rasmussen et al. 2013).

2.5.4 17α-Hydroxylase-C17/20-lyase (CYP17, P450c17)

This enzyme is responsible for two catalytic reactions in both the Δ4- and Δ5-pathway (Fig. 2): 1) 17α-hydroxylation at C17; 2) cleavage of the remaining side chain between C17 and C20 (lyase activity). These two reactions are catalyzed by a single enzyme as was shown by cloning of CYP17 into CYP17 negative cells (Miller 2007). This enzyme belongs to the large family of cytochrome P450 situated in the endoplasmic reticulum (microsomal fraction). Furthermore, it is an important key enzyme of steroidogenesis controlling the flow of substrates through the cascade of steroidogenic enzymes: a) no CYP17 expression: only synthesis of C21 steroids (progesterone, aldosterone and corticosterone) b) 17α-hydroxylase activity only: synthesis of cortisol c) 17α-hydroxylase plus lyase activity: synthesis of androgens and estrogens (Conley & Bird 1997). Efficiencies of the two activities of CYP17 are dependent on the transfer of electrons provided by NADPH to the enzyme via its redox partner, cytochrome P450 reductase (CPR). The lyase activity is significantly stimulated by the presence of an allosteric modulator, cytochrome b5. The preference of the Δ4- and Δ5-pathway depends on the species (Stanczyk 2009). In general, irrespective of the species, CYP17 efficiently converts progesterone and pregnenolone to their 17α-hydroxylated metabolites. However, in some species such as primates and ruminants the lyase activity on the Δ4-pathway is only minimal (conversion of 17α-hydroxyprogesterone into androstenedione). In rat CYP17 can convert both 17α-hydroxypregnenolone and 17α-hydroxyprogesterone to C19 steroids, whereas in guinea pig CYP17 has a much higher affinity for 17α-hydroxyprogesterone compared to 17α-hydroxypregnenolone (Tremblay et al. 1994). In addition to its role in the synthesis of androgens and estrogens, in the boar testis, CYP17 has an additional important function which is the conversion of pregnenolone into androstadienol thereby yielding the precursor for the synthesis of steroidal pheromones (Δ16-steroids) via its andien-ß-synthase activity. This additional catalytic function of CYP17 depends on the presence of high levels of cytochrome b5 (Davis & Squires 1999). Two isoforms of cytochrome b5 have been previously identified in pig. It has been shown that CYB5B has the potential to stimulate the production of 17α-hydroxypregnenolone and DHEA by porcine CYP17A1 at a level similar to CYB5A, while significantly reducing the production of 16-androstene steroids. Thus, increasing the expression

of CYB5B and decreasing the expression of CYB5A would maintain the production of androgens, while decreasing the production of 16-androstene steroids (Billen & Squires 2008). Consequently it has been suggested that in porcine Leydig cells CYP17/b5 may act as a key enzyme system that regulates the production of androgens and estrogens versus 16-androstene steroids (Billen & Squires 2008).

2.5.5 17β-Hydroxysteroid dehydrogenases (17β-HSD, HSD17B)

These enzymes catalyze the interconversions of 17-keto- into 17ß-hydoxy-steroids with the direction of this reversible reaction depending on the concentration of the substrate (Oshima & Ochiai 1973, Hall 1988). Four 17β-HSD isoenzymes (types I-IV) have been described. The type I 17β-HSD converts estrone to 17β-estradiol using NADPH as cofactor and is also known as estrogenic 17β-HSD. It is a soluble form and was initially purified by Jarabak et al (1962) from human placenta. Immunochemical analyses have confirmed the presence of the type I enzyme in the syncytiotrophoblast of human placenta, the granulosa cells of human ovary, the epithelial cells of human breast, and the endometrium (Poutanen et al. 1993). Type II 17β-HSD catalyzes the oxidation of testosterone and 17β-estradiol to form androstenedione and estrone, respectively. This type is a microsomal form and uses NAD^+ as a cofactor; its principal function is to inactivate circulating androgens and estrogens. The expression pattern of the type II 17β-HSD mRNA in human tissues supports its role as a steroid hormone inactivator (Andersson et al. 1995). Type III 17β-HSD was also found in microsomal fractions where it reduces androstenedione to testosterone; it uses NADPH as a cofactor. The cDNA encodes a protein of 310 amino acids. Pairwise comparisons between the 17β-HSD isoenzymes types I-III show that their amino acid identities are approximately 23% (Wu et al. 1993), indicating that these enzymes are members of the same family. The 1.3-kb mRNA encoding type III 17β-HSD has been detected only in the testis, which is consistent with its role in the formation of testicular androgens. Type IV 17β-HSD has been found in human, mouse and pig. It uses NAD^+ as a cofactor and is principally involved in the oxidation and therefore inactivation of estrogens and androgens (Carstensen et al. 1996). In pigs, immunofluorescence detected the type IV 17β-HSD in granulosa cells as well as in Leydig cells and Sertoli cells. The cDNA encodes an 80-kDa protein featuring domains not present in other 17β-HSDs (Markus et al. 1995).

2.5.6 5α-Reductase (steroid-5-alpha-reductase, SRD5A)

Testosterone can be converted to the more potent androgen 5α-dihydrotestosterone (DHT) in specific androgen target tissues, through the function of the enzyme 5α-reductase (Russell & Wilson 1994). In mammals, two isoenzymes, type 1 and type 2, have been identified encoded by two different genes, SRD5A1 and SRD5A2 (Andersson & Russell 1990, Russell & Wilson 1994, Zhu et al. 1998). Both isoenzymes are microsomal NADPH-dependent enzymes. In human, SRD5A2 is mapped to chromosome region 2p23, and SRD5A1 to chromosome region 5p15 (Jenkins et al. 1991). The homology in amino acid composition between two human isoenzymes is approximately 50%. In comparison to SRD5A1, SRD5A2 has a higher affinity for testosterone, while SRD5A1 has lower affinity but a high capacity for this steroid. Moreover SRD5A1 is less sensitive to the competitive 5α-reductase inhibitor finasteride (Russell & Wilson 1994). In adults, the expression of SRD5A2 is relatively high in epididymis, prostate, seminal vesicle and genital skin, while SRD5A1 is highly expressed in nongenital skin, liver and certain brain regions (Russell & Wilson 1994, Zhu et al. 1998). Both human isoenzyme genes have 5 exons and encode a highly hydrophobic 254 and 259 amino acid protein, respectively (Kim et al. 2006, Bentz et al. 2007).

2.5.7 Cytochrome P450 aromatase (CYP19)

The aromatase enzyme is also a member of the large cytochrome P450 family possessing both heme and substrate binding regions. This enzyme is nearly 58 kDa in size (Hinshelwood et al. 1993, Corbin 1999, Conley 2001). Aromatase uses the androgenic substrates androstenedione, testosterone and 16-hydroxytestosterone with high specificity, and converts them to the respective estrogens, estrone, estradiol-17ß and estriol. As among the substrates and products of this enzyme are powerful hormones, alterations in its activity have profound effects on estrogen and androgen physiology. The products of aromatase, estrogens, have been traditionally regarded primarily as female sex hormones. However, there is now evidence in several species that estrogens are also essential regulators of male reproductive functions. In mammals there is only one aromatase gene with the exception of the suiformes (Corbin et al. 1999, 2004, 2007). In domestic pigs, three aromatase isoforms have been identified (Conley et al. 1997, 2009). In the testis, depending on the species aromatase may be expressed in Leydig cells, Sertoli cells and/or germ cells, indicating considerable differences in the role of testicular estrogens between species. Testicular aromatase expression pattern may change between the neonatal age and adulthood. For example, in rodents, aromatase activity is high in Sertoli cells

before sexual maturity, but becomes more prominent in Leydig cells in adult animals (Carreau et al. 2007). In humans, both Leydig and Sertoli cells produce estrogens in vitro (Carreau et al. 2008). Aromatase activity has also been demonstrated in spermatogenic cells of several species, including humans (Conley 2001, 2009, Carreau & Hess 2010). Moreover, in human and rat, spermatozoa express aromatase and actively synthesize estrogens within the lumen of the epididymis (Lambard et al. 2003). Therefore, sperm may serve as the source of estrogens that target estrogen receptors present in efferent ductules and epididymal epithelia. In aromatase-null males, testicular morphology appears to be normal in young animals, but age-dependent disruption of spermatogenesis, spermatid degeneration, and reduction in testis weight, and hypertrophy of Leydig cells have been described, indicating a crucial role of aromatase in normal spermatogenesis (Carreau & Hess 2010). Several studies have clearly established an association between aromatase and sperm count and motility. Lower sperm concentration and motility have been associated with aromatase polymorphisms causing low enzyme activity in normozoospermic men (Lazaros et al. 2011). Decreased sperm motility is common in aromatase-deficient men and in aromatase knockout mice (Carreau et al. 2003). In aromatase-deficient adult men, spermatogenesis varies from normal to highly reduced (hypospermatogenesis) (Maffei et al. 2007). In fact, it has been proposed that the quantification of aromatase in ejaculated sperm could be used as an indicative parameter of sperm function and spermiogenesis to evaluate male infertility (Carreau et al. 2001, Carreau et al. 2008). More strikingly, in ejaculated spermatozoa, aromatase localizes to the midpiece, tail, and the annular region located at the limit between the acrosomal membrane and the nucleus (Galeraud-Denis et al. 2009, Carreau et al. 2010). In human spermatozoa aromatase is still active after ejaculation (Aquila et al. 2002). The dual localization of aromatase at the acrosomal membrane and the midpiece, along with the presence of estrogen receptors in sperm, provides evidence for a potential role of estradiol in energy production, motility, capacitation and acrosomal reaction (Lazaros et al. 2011). In boar, testicular expression pattern of aromatase has been reported in several studies. Consistently, by means of immunohistochemistry in neonatal and adult pigs it was exclusively detected in Leydig cell (Conley et al. 1996, Carreau et al. 1999, Fraczek et al. 2001, Mutembei et al. 2005).

In addition to classical steroidogenic organs, in both sexes, aromatase is found in a number of extra-gonadal sites, including bone, breast, adipose tissue and brain. This tissue-specific expression of aromatase maintains tight local control over the synthesis and action of estrogens. In this context, aromatase converts circulating ‘pro-hormones’, i.e. androgens, into estrogens,

which then act locally to regulate tissue function (Bulun et al. 2005, 2009, Chen et al. 2009, Santen et al. 2009).

2.6 Sulfonated steroids

2.6.1 Enzymatic reaction of sulfonation

Sulfonation is the conjugation of a substrate with a sulfuryl group ($-SO_3^{1-}$), whereas sulfation refers to the transfer of an SO_4^{2-} group (Wang & James 2006). However, contrary to the official nomenclature compounds structured $RO-SO_3^{1-}$ have been commonly named as sulfates of ROH. Thus, sulfonates of estrogens or neutral hydroxysteroids are frequently but inaccurately called steroid sulfates.

During sulfonation, the sulfonate acceptor (R-OH) and the cosubstrate 3'-phosphoadenosine-5'-phosphosulfate (PAPS) bind to a sulfotransferase with subsequent release of the sulfonated product and desulfonated PAPS, i.e. 3'-phosphoadenosine-5'-phosphate (PAP). PAPS thus serves as the universal sulfonate donor molecule required for all sulfonation reactions (Venkatachalam 2003). In mammals, all tissues are able to carry out the synthesis of PAPS (Bannister et al. 2011). PAPS synthesis requires a ready supply of sulfate, which is available from the diet as well as catabolism of proteins and sugar sulfates (Wang & James 2006). PAPS is formed in the cytosol where the sulfation of most xenobiotics occurs. Brzeznicka et al. (1987) measured concentrations of PAPS in various organs of the mouse, hamster, rabbit and dog. The results indicate that irrespective of the species the highest levels occur in the liver and range from approximately 15 nmol/g tissue for dogs to approximately 80 nmol/g tissue for rats, the concentration of PAPS in various other tissues varies between 6 and 20 nmol/g tissue, with no significant species or sex differences. The level of PAPS in several human tissues, including liver, lung, kidney, ileum, and colon, ranged from approximately 4 nmol/g tissue in the lung to approximately 23 nmol/g tissue in the liver (Cappiello et al. 1989).

Sulfonation of biomolecules is catalyzed by sulfotransferases, which are characteristically high-affinity and low-capacity enzymes. As a consequence, the activity of sulfotransferases is 2–3 orders of magnitude slower than that of phosphotransferases (Wang & James 2006). According to their substrate specificity, two major classes of sulfotransferases have been identified: the cytosolic sulfotransferases (SULTs), which sulfonate small endogenous and exogenous compounds, such as hormones, bioamines, drugs and various xenobiotic agents, and the membrane associated sulfotransferases, sulfonating larger biomolecules, such as carbohydrates

and proteins (Sakakibara 1998, Chapman et al. 2004, Blanchard et al. 2004). As only SULTs are relevant for steroids, the membrane bound sulfotransferases will not be addressed further.

2.6.2. Effects of steroid sulfonation and possible roles of sulfonated steroids

Steroid sulfonation plays an important role in the inactivation and excretion of steroids. It abolishes their binding to classical nuclear receptors, and considerably increases their polarity. Sulfonated steroids are thus hardly able to penetrate biological membranes by passive diffusion. As a result their distribution volume in the body is substantially reduced (Strott 1996, 2002). Sulfonation of steroids increases their binding to plasma proteins, predominantly to albumin. Nevertheless, sulfonated steroids are efficiently eliminated in the kidney, thus they are found at high concentrations in the urine (Dawson 2012).

Sulfonation abolishes the biological activity of steroids by preventing their binding to classical nuclear steroid receptors (Strott 1996). However, there is now clear evidence that sulfonated steroids may be more than just inactivated metabolites destined for excretion. They have longer half-time in plasma in comparison to their free counterparts and they may circulate in significantly higher concentrations. During the past three decades, especially from studies on steroid metabolism in human breast cancer tissue increasing evidence came up that free active steroids may be produced locally from sulfonated precursors in cells expressing the enzyme steroid sulfatase ("sulfatase pathway"; see chapter 2.8.3).

Whereas there is now a considerable number of studies on the role of the sulfatase pathway in the progression of estrogen-dependent breast cancer tissue including clinical trials using a sulfatase inhibitor (Nakata et al. 2003, Pasqualini 2004, Secky et al. 2013), virtually no information is available on the occurrence and role of sulfatase pathways in physiological settings with the exception of the situation in feto-placental unit in human pregnancy, where the production of estrogens in the syncytiotrophoblast largely depends on the provision of sulfonated C19-precursors by the fetal and maternal adrenals (Fritz & Speroff 2010).

The role of sulfonated precursors for human placental estrogen productions becomes obvious in cases of an inherited steroid sulfatase deficiency, which are characterized by very low levels of pregnancy-associated estrogens (Lykkesfeldt et al. 1984). Under physiological conditions the role of a sulfatase pathway may consist in the limitation of steroid effects to a subset of potentially responsive cells, which in addition to the specific receptor are characterized by the expression of steroid sulfatase and an uptake transporter, providing additional levels for local regulatory mechanisms. Moreover sulfonation of steroids enables the transport of high amounts

of steroidal substrates from a producing tissue into a target cell without interfering with the function of other steroidogenic tissues. Different transporter proteins have been shown or suggested to mediate the transport of sulfonated steroids through the plasma membrane of cells, including the sodium-dependent organic anion transporter (SOAT, SLC10A6, Geyer et al. 2006), the sodium-independent organic anion transporting polypeptides (OATPs) and the ASBT (apical sodium-dependent bile acid transporter (Moitra et al. 2011).

As mentioned above, sulfonation of steroids abolishes their capacity of interacting with classical nuclear steroid receptors. However, they may have direct effects at other receptor types.

Sulfonation in the brain modulates the nongenomic actions of neurosteroids on GABA A, N-methyl-D-aspartate, glutaminergic and σ-opioid receptors, usually in opposing ways (Kríz et al. 2008). For example, pregnenolone sulfate is a picrotoxin-like antagonist, whereas unconjugated pregnenolone is a barbiturate-like agonist. In addition, DHEA sulfate stimulates acetylcholine release from the hippocampus but unconjugated DHEA does not. These findings may be relevant to the association of pregnenolone sulfate and DHEA sulfate with enhanced cognitive function in animals (Kríz et al. 2008). These findings, together with the detection of SULT1A1, SULT1E1, SULT2A1, SULT2B1 and steroid sulfatase in the fetal and adult brain, suggests that sulfonation and deconjugation of neurosteroids contributes to neurodevelopment and maintenance of brain function. Another cytosolic sulfotransferase, SULT4A1, is most highly expressed in selective regions of the brain. However, its substrate and physiological role is yet unknown (Liyou et al. 2003, Minchin et al. 2008).

Sulfonated steroids are possibly moved through the plasma membrane of cells by several different transporter proteins. The ATP binding cassette (ABC) proteins transporters are ubiquitously expressed and are mostly considered responsible for the efflux of steroid substrates, whereas SOAT and OATPs mediate tissue-specific bi-directional transport of steroid sulfates across the plasma membrane of cells (Geyer et al. 2006, Roth et al. 2012, Moitra et al. 2011). SOAT transports steroid sulfates, including estrone-3-sulfate, pregnenolone sulfate and DHEA sulfate. Four families of OATP (OATP1, OATP2, OATP3 and OATP4) have been shown to transport DHEA sulfate and estrone-3-sulfate. The OATP1 genes are expressed throughout the body, with highest expression levels for sub-family member OATP1A2 in the brain, liver, lung, kidney and testis. OATP1B1 and OATP1B3 are specifically expressed in the liver and OATP1C1 in the brain and testis. The OATP2 sub-family member OATP2B1 is expressed in numerous tissues, including liver, syncytiotrophoblasts of the placenta, mammary gland, heart, skeletal muscle and endothelial cells of the blood-brain barrier. The OATP3A1_v1

transporter is expressed in the germ cells of the testis, as well as in the choroid plexus and frontal cortex. Two OATP4 sub-family members have been identified in the following tissues: OATP4A1 in heart, lung, liver, skeletal muscle, kidney, pancreas and syncytiotrophoblasts in the placenta; whereas OATP4C1 is localized to the basolateral membranes of renal proximal tubules. Whilst certain sulfonated steroids (i.e. DHEA sulfate and estrone-3-sulfate) have been used to test the substrate specificity of the above ABC, SOAT and OATPs, further studies are required to investigate all known naturally occurring (as well as synthetic) steroid sulfate substrates (Dawson 2012).

2.7 Cytosolic sulfotransferases (SULTs)

SULTs sulfonate small endogenous and exogenous compounds, such as hormones, bioamines, drugs, and various xenobiotic agents. To date, 13 human cytosolic SULTs have been identified (Allali-Hassani et al. 2007). The significant substrate overlap between SULTs caused a lot of confusion in early naming schemes, and the nomenclature of these enzymes has a long and confusing history. Accordingly, in many older studies based on the measurement of enzyme activities only, it does not come clear which of the SULTs definitely contributed to the substrate conversions measured. Similarly, the significance of studies based on immunological methods (immunohistochemistry, Western blot) may have been impaired by the potential reactivity of the antibodies used with more than one of the structurally closely SULTs. Only after the availability of more precise biochemical, biophysical, and genetic methods this issue has been resolved (Chapman et al. 2004). However, many different names for the same enzyme may still be encountered in the literature.

The SULTs characterized so far have been recognized as structurally closely related members of a gene family. Their current nomenclature is based on 45% of the amino acids that members of this family have in common in their structure. Isoforms within a subfamily are labeled using Arabic numbers following the subfamily designation. In human, three SULT families have been characterized: (1) the SULT1 family (“phenol family”) including eight subfamilies: A1, A2, A3, A4, B1, C2, C4, and E1; (2) the SULT2 family (“hydroxysteroid family”) consisting of the SULTs 2A1 and 2B1; and (3) the “brain-specific” SULT4 family (Pasqualini 2009). An overview on human SULT superfamilies and some structural features and substrate preferences of individual SULTs are presented in Table 1.

Table 1: The human cytosolic SULT superfamily (according to Pasqualini 2009).

SULT	common name	chromo-some	number of amino acids	substrate preference (endogenous)	sequence identities with SULT1A1
SULT1 (phenol) family					
SULT1A1	P-PST/-1 TS-PST H-PST HAST1/2	16q11.2-12.1	295	phenols estrogens	
SULT1A2	ST1A2 HAST4 TS-PST2	16q11.2-12.1	295	phenols	95.6%
SULT1A3	M-PST TL-PST HAST3 hEST-1	16q11.2	295	phenols catecholamines	92.9%
SULT1A4		16q12.1		not known	99% homology with SULT1A3
SULT1B1	ST1B2	4q11.13	296	thyroid hormones	53.4
SULT1C2	HAST5 SULT1C1	2q11.2	296	phenols	52.2
SULT1C4	hSULT1C	2q11.2	296	phenols	52.2
SULT1E1	hEST/-1	4q13.2	294	estrogens (high affinity)	50.1

Table 1 (continued): The human cytosolic SULT superfamily (according to Pasqualini 2009).

SULT	common name	chromosome	number of amino acids	substrate preference (endogenous)	sequence identities with SULT1A1
SULT2 (hydroxysteroid) family					
SULT2A1	DHEA-ST HST	19q13.3	285	3β-hydroxysteroid DHEA	34.6
SULT2B1-v1	hSULT2B1a	19q13.3	350	DHEA, pregnenolone	36.3
SULT2B1-v2	hSULT2B1b		365	DHEA, cholesterol	36.9
SULT4 (brain specific) family					
SULT4A1-v1	hBR-STL	22q13.1-13.2	284	not known	34.2
SULT4A1-v2					

2.7.1 SULTs relevant to the sulfonation of steroids

Among the many SULTs identified, only SULT1E1 (estrogens) and the SULTs 2A1 and 2B1 (neutral hydroxysteroids) are now considered as relevant concerning the sulfonation of steroids under in vivo conditions. More detailed information on these SULTs is presented in the following sections. In addition to SULT1E1, other members of the SULT1 family such as SULTS 1A1 and 1A3 may have some activity for estrogens but only at clearly supraphysiological concentrations (Ebmeier & Anderson 2004), thus these SULTs will not be addressed further.

2.7.1.1 SULT1E1

Among several enzymes catalyzing the sulfonation of phenols or steroids, only SULT1E1 has been shown to be highly specific for estrogens (Chapman et al. 2004). SULT1E1 is a small soluble enzyme found in cytoplasm that catalyzes the sulfonation of various estrogens to the corresponding estrogen sulfates (Fig. 4) (Chapman et al. 2004, Alnouti & Klaassen 2006). The

approximate molecular weight of a SULT1E1 molecule is 34 kDa (Strauss & Barbieri 2014); a homodimer of two SULT1E1 molecules is considered as the enzymatically active form (Strott 2002, Cook et al. 2009). SULT1E1 consists of 295 amino acids (Pasqualini 2009). The amino acid sequence of SULT1E1 is highly conserved between mammalian species as shown in Table 2.

Table 2: Amino acid identities between SULT1E1 (composed of 295 amino acids) of various mammalian species (according to Strott 1996).

Species	Amino acid identities	%
Rat/mouse	260	88
Human/guinea pig	241	81
Human/mouse	225	76
Guinea pig/mouse	220	74
Human/bovine	215	73
Human/rat	212	72
Guinea pig/bovine	208	70
Guinea pig/rat	203	69
Mouse/bovine	200	68
Rat/bovine	194	66

A theoretical study on the catalytic mechanism of SULTE1 stated that the reaction proceeds in several steps: at first the charge on PAPS is neutralized, then the O-S bond is lengthened, and finally the nucleophile is activated by deprotonation (Strott 1996). The sequence motif GxxGxxK is present in all cytosolic SULTs and has shown to be important for PAPS binding (Gamage et al. 2006, Driscoll et al. 1995). The phosphosulfate binding loop of SULTs provides the major binding site for the 5'-phosphate group of PAP (Kakuta et al. 1997). In mouse, the 3'-phosphate group of PAP interacts with two conserved regions of sequence including residues 257–259 located at the beginning of the P-loop related motif (GxxGxxK) region (Yoshinari et al. 2001).

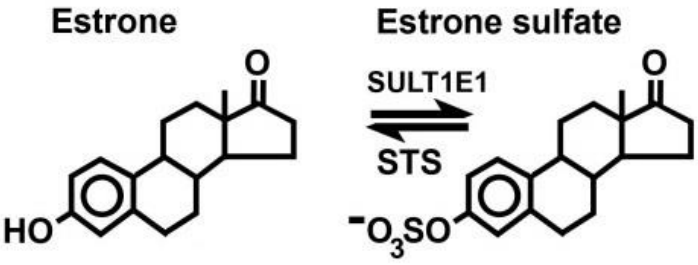


Figure 4. Sulfonation of estrone to estrone-3-sulfate by SULT1E1 enzyme.

As mentioned above, older studies are mainly based on determination of enzyme activities and antibodies elicited against enzyme preparations purified from various tissues. Estrogen sulfotransferase (EST) activity has been detected in male and female reproductive tissues, liver, kidney, brain, and the adrenal cortex (Hobkirk 1985). Molecular mass of EST has been determined in different species including mouse (30 kDa; Hobkirk et al. 1983), pig (31 kDa; Brooks et al. 1987), guinea pig (34 kDa; Hobkirk & Glasier 1992), cattle (36 kDa; Moore 1988) and human (36 kDa; Hondoh et al. 1993). In the mouse, EST is highly expressed in the testis but not the adrenal, whereas in the guinea pig the opposite is the case (Hobkirk & Glasier 1992). After molecular characterization of SULT1E1, definite information on its expression pattern became available in various species (Blanchard et al. 2004). SULT1E1 expression has been found in human fetal liver, lung and kidney (Coughtrie 2002). It is also present on immunoblots applying cytosols of human jejunum, secretory endometrium but not of proliferative endometrium (Forbes-Bamforth & Coughtrie 1994, Falany et al. 1995).

To date, no information is available on the expression of SULT1E1 in the porcine testicular-epididymal compartment. When testing porcine testicular and epididymal tissue homogenates for their capacity to convert E1 into E1S significant activity was found in the epididymis (Hoffmann et al. 2010, Zduńczyk et al. 2012), whereas no considerable conversion was detectable in the testis. These observations suggest that rather the epididymis than the testis may be the primary source of the high amounts of sulfonated estrogens produced in the porcine testicular-epididymal compartment. In contrast, by means of immunohistochemistry using a polyclonal antiserum against "murine testis-specific estrogen sulfotransferase" (Song et al. 1995), considerable positive staining was found in a proportion of Leydig cells, with a higher proportion and staining intensity in premature compared to adult boars (Hoffmann et al. 2010). However, as this antiserum was elicited against an enzyme preparation in a different species, the specificity of this antiserum for porcine SULT1E1 remained unclear.

2.7.1.2 SULT2A1

The number of SULT2A genes may vary across species with only one SULT2A gene found in humans, whereas at least three SULT2A genes exist in the rat (Blanchard et al. 2004) and two in the mouse and also in the guinea pig. SULT2A1 is a major SULT isoform involved in the sulfonation of neutral steroids, which is capable of sulfonating both 3α- and 3β-hydroxysteroids (Gamage et al. 2006). As DHEA is considered as predominant steroidal substrate of SULT2A1, it was originally named DHEA sulfotransferase. However, it has a broad substrate reactivity

and can also sulfonate alcoholic hydroxyl groups at other positions of steroids, such as at C17 of testosterone. At high substrate concentrations, even 17β-estradiol may be sulfonated at its phenolic 3-hydroxyl as well as at its alcoholic 17-hydroxyl group. In addition to steroids, SULT2A1 is involved in the sulfonation of many other molecules such as bile acids and many dietary xenobiotics and drugs (Sinclair et al. 2006).

Steroid sulfation in humans and higher primates is unique from that in many other mammalian species due to the synthesis and secretion of large amounts of adrenal androgens, with DHEAS being by far the most abundant adrenal androgen. High levels of DHEAS are secreted by the fetal adrenal as well as by the adult adrenal after adrenarche. DHEAS is present at micromolar quantities in the plasma of young adults and decreases slowly thereafter with age (Dharia & Parker 2004). Thus, in humans, who synthesize large amounts of DHEAS during fetal development, SULT2A1 shows the highest expression level in fetal adrenal gland. In adult humans, the protein level follows the order liver > adrenal > duodenum. In adult human liver, the enzyme has been suggested to play an essential role in the protection from toxic endogenous and exogenous compounds, as in addition to O-sulfation it obviously also mediates the N-sulfation of amine containing drugs and environmental chemicals. Although SULT2A1-mRNA has been detected in many tissues, no or only extremely low enzyme levels have been detected in other human tissues (Glatt & Meinl 2004, Senggunprai et al. 2009). Much less information is available on the expression pattern of SULT2A1 in other species.

Sulfonation is a phase II conjugation reaction responsible for the biotransformation of many compounds facilitating their excretion. However, certain compounds such as benzylic alcohols derived from polycyclic aromatic hydrocarbons as well as the drugs or drug metabolites may be activated to mutagens by SULT2A1 (Glatt 2000).

In the boar SULT2A1 has recently found considerable interest as potential candidate gene for the development of genetic markers for selecting animals with low boar taint, as SULT2A1 has been identified to be a key enzyme involved in the sulfonation of 16-androstene steroids. In the boar, 16-androstene steroids have pheromonal properties and are the most abundant steroids produced by the testes. In intact postpubertal males high levels of 16-androstene steroids may accumulate in adipose tissue and produce an inacceptable unpleasant odor when their meat is heated. As free 16-androstene steroids are the forms that accumulate in adipose tissue, sulfonation by SULT2A1 may play an important role in the prevention of boar taint. Consistently, Sinclair et al. (2006) could show that animals with high concentrations of 5α-androstenone (5α-androst-16-en-3-one) in fat had significantly lower SULT2A1 activity in both

testis and liver. However, in a later study, Grindflek et al. (2010) obtained contradictory results with an up-regulation of SULT2A1 gene expression level in high androstenone animals.

2.7.1.3 SULT2B1

The human SULT2B gene family consists of a single gene capable of generating two functional transcripts. The two different 5´-terminal sequences for these isoforms are encoded by different 5´-exons as a result of a combination of the initiation of transcription at alternative locations plus alternative splicing. This results in the translation of two separate proteins, SULT2B1a (350 amino acids) and SULT2B1b (365 amino acids), with the last 342 amino acids being identical. The two isoforms differ at the amino termini with a unique amino acid stretch of 23-residues in SULT2B1b vs. a unique 8-residue sequence in SULT2B1a. Another distinguishing feature of the SULT2B isoforms in comparison to other SULTs is a 53-amino acid proline-serine rich C-terminal extension that possesses 17 proline and 10 serine residues (Her et al. 1998, Falany & Rohn-Glowacki 2013). Thus, the SULT2B1 molecule is larger than other SULTs with an apparent SDS-PAGE molecular mass of approximately 48 kDa compared with a 34 kDa mass for SULT2A1 or SULT1E1 (Meloche & Falany 2001). In humans the SULT2B1 gene is located at the same chromosomal loci as the SULT2A1 gene suggesting that they may have evolved from gene duplication even though with 48% the homology (amino acids) between SULTs 2A1 and 2B1 is relatively low (Otterness et al. 1995, Falany et al. 2006).

Human SULT2B1 shows a distinct stereo-specificity for 3β-hydroxysteroid conjugation (Meloche & Falany 2001). This is different from SULT2A1, which also sulfates 3α-hydroxysteroids, the 3-phenolic hydroxyl of estrogens and the 17-hydroxyl group of testosterone and estradiol-17ß (Falany et al. 1989). Important steroidal substrates for SULT2B1 are cholesterol, pregnenolone, DHEA, androstenediol and 5α-androstane-3β, 17β-diol. However, characterization of the human SULT2B1 isoforms has been limited by the inability to isolate reliably active SULT2B1b from tissues or cells. Due to its prominent expression in skin and prostate (see below), it has been suggested that important physiological roles of SULT2B1 are the synthesis of cholesterol sulfate in the epidermis and the local control of androgen activity in the prostate (Falany et al. 2006, Falany & Rohn-Glowacki 2013).

On the mRNA level expression of SULT2B1a and SULT2B1b was detected in various human tissues. However, the expression of SULT2B1b-mRNA is generally more abundant and more widespread than that of SULT2B1a-mRNA. Among many tissues tested, SULT2B1b-mRNA was found in liver, colon, small intestine, ovary, uterus and prostate. The expression of

SULT2B1a was more restricted and only detected in colon, ovary and fetal brain (Geese & Raftogianis 2001). However, on the protein level, by means of Western blot no SULT2B1a expression was found, whereas SULT2B1b could be detected in skin, prostate, placenta and lung (He et al. 2004). These observations suggest that SULT2B1a and SULT2B1b expression in human tissues is controlled at both the transcriptional and translational levels, and that SULT2B1a-mRNA may only be translated in specific tissues or at specific developmental times (Falany et al. 2006).

Different from other members of the SULT family, for which only a cytosolic localization has been reported, for SULT2B1 depending on the tissue also a nuclear localization has been described. In prostate and lung, SULT2B1b was only found in the cytosol of epithelial cells. In term placenta, SULT2B1b is localized predominantly in nuclei of syncytiotrophoblasts, whereas in normal breast and breast cancer tissues, the enzyme is present in both cytosol and nuclei (He et al. 2004, 2005). It has been suggested that serine phosphorylation of the proline-serine-rich carboxy-terminal extension plays a role in altering protein-protein interactions that may be involved in cellular translocation (He & Falany 2006, Falany et al. 2006). Moreover, results from site-directed mutagenesis to phospho-mimetic Ser348Asp indicates, that serine phosphorylation in addition to nuclear localization increases catalytic activity (Salman et al. 2011).

In comparison to humans, only little information is available on SULT2B1 in other species. However, information obtained so far in mice (Shimizu et al. 2003) and rats (Falany et al. 2006) indicate some species specific differences in structure, tissue distribution pattern and substrate preference.

As SULT2A1 (see chapter 2.7.1.2), in the boar also SULT2B1 has recently found considerable interest as potential candidate gene for the development of genetic markers for selecting animals with low boar taint (Moe et al. 2007, Duijvestein et al. 2010). Mo et al. (2007) investigated SULT2B1 expression in the testis and liver of Duroc and Landrace pigs in relation to androstenone levels in subcutaneous adipose tissue. In liver and testis the presence of an approximately 45 kDa SULT2B1 immunoreactive band was detected. Expression of the hepatic SULT2B1 protein was negatively related to the androstenone concentration in Duroc, but not in Landrace pigs, whereas expression of the testicular SULT2B1 was negatively related to the androstenone concentration in both breeds investigated. From their results the authors concluded that the mechanisms regulating androstenone concentrations in pig adipose tissue are breed specific.

2.8 Steroid sulfatase and sulfatase pathway

2.8.1 Steroid sulfatases

Sulfatases represent a large protein family that is involved in heterogeneous processes, ranging from the degradation of macromolecules to hormone biosynthesis and the modulation of developmental cell signaling (Hanson et al. 2004). They hydrolyze sulfate esters from different sulfated substrates such as steroids, carbohydrates, proteoglycans and glycolipids (Sardiello et al. 2005). Thirteen human sulfatase genes have been reported to date (Hopwood & Ballabio 2001). As a member of this family, steroid sulfatase (STS) plays a pivotal role in the formation of biologically active steroids from inactive steroid sulfates (Reed et al. 2005).

2.8.2 Physico-chemical and structural properties

As other sulfatases investigated so far at its catalytic site STS contains a Ca-formylglycine (FGly) residue that is essential for enzyme activity (Schmidt et al. 1995). FGly formation occurs post-translationally by the oxidation of a cysteine residue that is conserved in all eukaryotic sulfatases as well as in most prokaryotic sulfatases (Dierks et al. 1997). Thus, impairment of this post-translational modification affects the enzyme activity of all sulfatases.

STS is an insoluble membrane bound hydrolytic enzyme with a molecular weight of 62 kDa, with an unknown number of isoforms. After targeting to the secretory pathway and extensive glycosylation, sulfatases are transported to their final subcellular compartment lysosome, Golgi complex or endoplasmic reticulum (ER) - or are extruded to the extracellular matrix (Hopwood & Ballabio 2001, Hanson et al. 2004). Accordingly, STS is commonly considered as a microsomal enzyme primarily localized in the ER. However, other subcellular localizations have also been described. STS was found to a large extent in the rough endoplasmic reticulum. Besides, in cultured human fibroblasts, immunohistochemistry proved that the enzyme exists in Golgi cisternae and in the trans-Golgi. It was also found at the plasma membrane (Nardi et al. 2009, Fietz et al. 2013), endosomes and multivesicular endosomes. These structures may be the sites where sulfated estrogen and/or androgen precursors are hydrolyzed (Steckelbroeck et al. 2004, Kriz et al. 2008).

STS cleaves the sulfate groups of the 3β-position of sterols and steroids. Its best known natural substrates are dehydroepiandrosterone sulfate (DHEAS) and cholesterol sulfate, although the enzyme is also known to hydrolyze other steroid sulfates, such as pregnenolone sulfate and androstenediol-3-sulphate. Moreover, it cleaves the sulfate group from the phenolic OH group of estrogen sulfates (Hernandez-Martin 1999, Wang et al. 2014, Wlcek et al. 2014).

In humans STS is widely distributed throughout fetal and adult tissues or cells. Among other sites, STS activity has been detected in brain, liver, adrenal cortex (Honour et al. 1985), placenta (France et al. 1969), skin (Koppe et al. 1978), testicles (Payne et al. 1972), ovary (Haninget al. 1992), leucocytes (Meyer et al. 1982, Okano et al. 1988), prostate and kidney (Reed et al. 2005). Moreover, STS activity has been detected in most tissues of the female reproductive tract. It is present in ovarian tissues from pre- and postmenopausal women, suggesting that in the ovary sulfated precursors, such as DHEAS, could be used as precursors for the formation of androgens and estrogens. Support for this concept was obtained from the finding that relatively high STS activity was detected in ovarian follicles, stroma, and corpus luteum, which were capable of utilizing DHEAS as a substrate for the production of DHEA, androstenedione, and testosterone (Hanning et al. 1990). DHEAS is present in high concentrations in follicular fluid in close proximity to the ovarian cells involved in steroidogenesis. Using human granulosa cells obtained from women undergoing treatment for in vitro fertility, significant conversion of DHEAS to DHEA was detected, confirming the presence of STS activity in these cells (Bonseret al. 2000). By means of immunohistochemistry in the boar testis STS is exclusively detectable in Leydig cells (Mutembei et al. 2009). High STS and EST activities in the epididymis suggest that the local availability of biologically active estrogens in the epididymis may result also from the interplay between estrogen supply and their metabolism (Zdunczyk et al. 2012).

Information on the physiological role of STS in humans became predominantly available from cases of inherited STS deficiency. As the STS gene is localized on the X-chromosome, generally males manifest the disease, while heterozygous female carriers do not present it. However, a case in a homozygous female has also been described (Thauvin-Robinet 2005). As the major clinical symptom of the X-linked recessive STS deficiency is a generalized scaling of the skin, with large, polygonal, dark brown scales, the condition is named X-linked ichthyosis (XLI). STS is needed to hydrolyze cholesterol sulfate, which acts as glue between epidermal cells, thus STS deficiency prevents the normal exfoliation of superficial keratinized cells. Routine histopathology in XLI typically shows moderate hyperkeratosis with mild acanthosis and partial accentuation of the granular cell layer (Kubilus et al. 1979). Extracutaneous manifestations are also frequent, especially corneal opacities and cryptorchidism (Fernandes et al. 2010). Due to the crucial role of STS in human placental estrogen synthesis, pregnancies with offspring affected by XLI are characterized by low levels of pregnancy-associated estrogens. Due to a lack of CYP17 in the human placenta, placental estrogen production largely depends on exogenous C19 precursors, which are provided in sulfonated form by the fetal and

maternal adrenals (feto-placental unit, Diczfalusy 1953). Since the initial discovery of a relationship between STS and X-linked ichthyosis by Koppe et al. (1978) and Shapiroet al. (1978) many studies have been performed to map the gene and to study the genetic abnormalities in affected patients (Hernandez-Martin 1999).

2.8.3 Sulfatase pathway

Steroid sulfates are inactive at classical nuclear steroid receptors. Thus in the past they have been primarily regarded as inactivated forms destined for excretion. However, steroid sulfates occurring at relatively high concentrations in the systemic circulation may constitute a potential reservoir of precursors for the local steroid hormone biosynthesis in specific target cells which in addition to specific receptors also express STS. The hydrolysis of the sulfate moiety by STS is the first step of this metabolic pathway (sulfatase pathway), which is now considered as an important route for the synthesis of bioactive steroids in addition to the de novo synthesis of free steroids. The sulfatase pathway has found increasing interest during the last years especially in relation with breast cancer in postmenopausal women, and concerning the role of STS in the progression of human hormone-dependent breast cancer now much information is available (Sasano et al. 2006, Tsunoda et al. 2006, Stanway et al. 2007). After the cease of cyclic ovarian function, in mammary neoplastic tissue estrogens may be produced from extraovarian precursors. A large contribution to intratumoral levels of active estrogens comes from the hydrolysis of sulfonated estrogens and from the aromatization of circulating androgens of adrenal origin (Reed et al. 1979). One importance of STS in intratumoral estrogen production lies in its crucial role in the conversion of E1S to E1 (Pasqualini 2004). The relatively weak estrogen E1 could be further metabolized to the biologically highly active E2 by the action of 17β-HSD type I (Poutanen et al. 1995). Additionally, in androgen metabolism, STS may hydrolyze DHEAS or androstenediol sulfate there by providing important precursors for the production of aromatizable C19-steroids (Nakata et al. 2003, Suzuki et al. 2003). The importance of STS in intratumoral estrogen production has prompted the development of new potent STS inhibitors (Kriz et al. 2008), which have recently entered clinical trials (Purohit & Foster 2012).

2.9 Testicular steroidogenesis in boars

As in other mammals, also in the boar the testis acts as an endocrine gland providing testosterone which in adult males is essential for the maintenance of numerous reproductive

functions. As a peculiarity of the porcine species, in addition to the production of androgens, the boar testis synthesizes high amounts of steroidal pheromones (see chapter 2.9.1) and a broad spectrum of sulfonated steroids (see chapter 2.9.2), which also may circulate in the peripheral circulation in intriguingly high concentrations.

2.9.1 Steroidal pheromones

The boar testes secrete large amounts of C-16 unsaturated androgens. These steroids act as pheromones when they are excreted (Billen et al. 2009). Attempts to identify chemical compounds responsible for boar taint in pork were initiated by Lerche (1936), who described the parotid gland as processing the bad odor (Lerche 1936). The androst-16-ene (16-androstene) steroids are the most abundant steroids produced by the pig testes, reaching total levels of approximately 0.6 mg/g of testicular tissue. The 16-androstenes are synthesized primarily in Leydig cells of boar testes along with other androgens and estrogens, with lesser contributions from the adrenals (Gower 1984). CYP17A1 catalyzes the key regulatory step in the formation of the 16-androstene steroids from pregnenolone by the andien-beta synthase reaction. It has been shown that the two forms of cytochrome b5 (CYB5A and CYB5B) exert different effects on the three activities of porcine CYP17A1 and that CYB5B does not stimulate the andien-beta synthase activity of CYP17A1 (Billen et al. 2009). The 16-androstenes produced in the testes, 5α-androstenone, 3α-androstenol and 3β-androstenol, are released into the systemic circulation via the spermatic vein (Gower et al. 1970, Saat et al. 1972). Due to their hydrophobic property, circulating 16-androstenes are then transported to fat tissue where they are stored (Bonneau 1982, Brooks & Pearson 1986). Androstenone storage in fat is reversible. Castrating mature boars results in a progressive decline in serum and loin fat concentrations of androstenone (Claus 1976, Cliplef et al. 1985, Grinwich et al. 1988). The apparent half-life of fat androstenone ranges from 4–14 days in boars of 100 kg (220 lb) of live weight (Claus 1976, Bonneau et al. 1982). Androstenone and other 16-androstene steroids are probably catabolized in the liver (Claus 1979, Fish et al. 1980). In young boars (100 kg, 220 lb), androstenone is eliminated mainly through the urine, in the form of 5β-androstenol, and in trace amounts through feces (Bonneau & Terqui 1983). In adult boars, 5β-androstenol and, to a lesser extent, 5α-androstenol are the only 16-androstenes that are eliminated in urine (Saat et al. 1972, Gower et al. 1970, Gower et al. 1972).

The 16-androstenes in the circulatory system are also transported to the salivary glands, where they are excreted during the mating process (Gower et al. 1972, Claus 1979). Accordingly, boar

salivary glands contain high concentrations of 5α-androstenone, 3α- and 3β-androstenol (Patterson 1968). When a mature boar is aroused by the presence of an estrous female or an unfamiliar boar, he champs copious amounts of frothy saliva. This excessive salivation provides a medium for the release of large amounts of 16-androstenes into the environment. These odorous steroids, in particular, 5α-androstenol and 5α-androstenone, act as signaling pheromones and trigger the mating stance in the estrous female, or indicate to the other boar that his status is being challenged (Melrose et al. 1971, Perry et al. 1980). Exposing prepubertal gilts to these pheromones also advances the onset of puberty (Brooks & Cole 1970). These compounds also contribute to dominance hierarchies in the agonistic behaviors of pigs. Aggressive boars have a higher concentration of salivary androstenone than control boars (Booth 1980). Spraying androstenone around newly regrouped growing pigs reduced fighting (McGlone et al. 1986).

Steroidal pheromones and skatol are considered as the most important components of boar taint, but other substances have also been suggested to play a role in the overall perception of boar taint such as indole (Annor-Frempong et al. 1998), 4-phenyl-3-buten-2-one and short fatty acids (Sole & García-Regueiero 2001, Rius et al. 2005).

2.9.2 Sulfonated steroids in boars

Among the males of mammalian domestic animal species the boar is rather exceptional in that it exhibits intriguingly high levels of numerous sulfonated steroids in the systemic circulation which may reach concentrations up to several hundreds of nmol/l (Baulieu et al. 1967, Claus and Hoffmann 1980, Schwarzenberger et al. 1993, Zamaratskaia et al. 2004, Raeside et al. 2006). Data from measurements in testicular vein (Baulieu et al. 1967, Gower et al. 1970, Raeside and Howells 1971, Setchell et al. 1983, Raeside et al. 2006, Hoffmann et al. 2010) or in lymph fluid of the spermatic cord (Setchell et al. 1983) clearly identified the testicular-epididymal compartment as the predominant source of sulfonated steroids in boars. High concentrations measured in testicular tissue point to the testis as their primary site of production (Ruokonen and Vihko 1974), and results from in vitro experiments using purified Leydig cells indicate that they are the main producers of sulfonated steroids in boars (Raeside & Renaud 1983, Raeside et al. 1992, Sinclair et al. 2005). Sulfonated steroids in boars can be mainly related to intermediates of "core steroidogenesis" such as P5S, DHEAS or androstenediol sulfate (Baulieu et al. 1967, Raeside & Howells 1971, Tan & Raeside 1980, Schwarzenberger et al. 1993, Zamaratskaia et al. 2004), estrogens (Claus and Hoffmann 1980, Schwarzenberger

et al. 1993, Zamaratskaia et al. 2004) and to steroids arising from the synthesis of steroidal pheromones (3-enol sulfate of 5α-androst-16-en-3-one, 5α-androst-16-en-3α-yl sulfate, 5α-androst-16-en-3ß-yl sulfate; Gower et al. 1970, Sinclair et al. 2005, Raeside et al. 2006). A list of testicular steroid sulfates identified in boars is included in Table 3. As far as available (total conjugated estrogens: Claus & Hoffmann 1980; DHEAS: Tan & Raeside 1980), data from long-term measurements in adult boars point to diurnal profiles similar to that of testosterone characterized by more or less regular pulses of low frequency (approximately 5 pulses per day). Age related profiles of sulfonated steroids (E1S, E2S, DHEAS) showed a pattern similar to free estrogens, testosterone and other free C19-steroids with a pronounced peak around an age of four weeks, a decline to basal levels at an age of 8 weeks followed by another increase associated with the onset of puberty (Schwarzenberger et al. 1993, Zamaratskaia et al. 2004).

So far, no definite information is available on the synthetic pathways of sulfonated steroids produced in the porcine testicular-epididymal compartment. Commonly, sulfonated steroids are considered to arise from the activity of specific SULTs (see chapter 2.7.1). For SULTs 2A1 and 2B1, the expression of protein and mRNA has been reported in the porcine testis (Sinclair et al. 2006, Moe et al. 2007). However, their cellular distribution and their contribution to the synthesis of individual steroid sulfates in the porcine testis are still unclear. Cooke et al. (1983) characterized the sulfonation of DHEA and P5 by cytosolic fractions from boar testis, but no information on the identity of the underlying enzymes is available from this study. Hobkirk et al. (1989) described the partial characterization of sulfotransferase activity for DHEA, androstenediol, P5 and E1 in cytosol prepared from porcine Leydig cells. By means of chromatofocusing estrogen sulfotransferase was clearly separated from the neutral steroid sulfotransferase, but again no information on the identity of the underlying enzymes is available. Moreover, the Michaelis-Menten constant (K_m) of estrogen sulfotransferase using E1 as a substrate was reported as 4 µm, which is significantly higher than the values around 5-10 nmol measured for the substrates E1 or E2 when the catalytic activity of the estrogen specific SULT1E1 was characterized after cloning of the enzyme (Falany et al. 1995, Zhang et al. 1998). In contrast to Hobkirk et al. (1989), in a study by Hoffmann et al. (2010) using testicular homogenates, estrogen sulfotransferase activities were not significantly different from background level. However, considerable estrogen sulfotransferase activities were detected in the porcine epididymis, which was later confirmed by Zdunczyk et al. (2012). These observations challenge the concept that in the porcine testicular-epididymal compartment sulfonated estrogens mainly originate from the sulfonation of free estrogens by testicular SULT1E1 and bring up the question whether the epididymis may significantly contribute to the

production of sulfonated steroids in boars. In addition to the sulfonation of free steroids, steroid sulfates may also be products of a sulfate pathway, which means from conversions of sulfonated precursor steroids potentially starting from cholesterol sulfate. In studies using samples from various steroidogenic tissues including boar testis, conversions of Δ5-3β-yl steroid sulfates (cholesterol sulfate, P5S, 17α0H-P5S) into products which still contain the sulfate group have been described. One study was found reporting the direct conversion of DHEAS into E1S, which was observed in human placental tissue (Oertel et al. 1967). However, these studies do not provide definite information on the efficiency of the sulfate pathway in comparison to the corresponding conversions of free counterparts and on its physiological relevance or specific role.

To date, no specific information is available on the physiological role of the sulfonated steroids produced in the porcine testicular-epididymal compartment. In addition to a role as waste products arising from the sulfonation of active free steroids and their precursors, they could be substrates for sulfatase pathways in the testicular epididymal compartment or elsewhere in the organism (Mutembei et al. 2009, Hoffmann et al. 2010, Zdunczyk et al. 2012). Finally, for sulfonated steroids a role as regulators of steroidogenesis (Neunzig & Bernhardt 2014) and direct effects independent from classical nuclear steroid receptors (Shihan et al. 2013) have been suggested.

Table 3: Steroids conjugated as monosulfates in boar testis tissue (adopted from Raeside et al. 2006).

C21	Pregnenolone 3α-hydroxy-5α-pregnan-20 one 3β-hydroxy-5α-pregnan-20 one 3β,17α-dihydroxy-5β-pregnan-20 one
C19	5α-androst-16-en-3α-ol 5α-androst-16-en-3β-ol 5,16-androstadien-3β-ol androsterone epiandrosterone dehydroepiandrosterone (DHEA) 5α-androstane-3α,17α-diol 5α-androstane-3α,17β-diol 5α-androstane-3β,17α-diol 5α-androstene-3β,17β-diol 5-androstane-3α,17β-diol 5-androstane-3β,17α-diol 5-androstane-3β,17β-diol
C18	estradiol-17β estrone

2.10 Hypotheses and aims of this study

Inactivation of steroid hormones by sulfonation usually occurs in target cells (Meyers et al. 1983) or in the liver (Duanmu et al. 2006). In boars, however, preceding studies clearly showed that the intriguingly high levels of sulfated steroids are not primarily resulting from peripheral metabolization but are unequivocally produced in the testicular-epididymal compartment (Baulieu et al. 1967, Setchell et al. 1983, Hoffmann et al. 2010). However, up to date their biological roles and the pathways underlying their synthesis are still fully unclear. Concerning the sulfonation of estrogens, no activity of estrogen sulfotransferase was detectable in testicular tissue in a previous study, whereas significant activity was found in the epididymis (Hoffmann et al. 2010, Zdunczyk et al. 2012). This observation suggests that free estrogens produced in the testis may be sulfonated to a substantial extent in the epididymis. However, to date there is no experimental evidence for this concept and the also mechanisms accomplishing this hypothetical transfer of free estrogens from the testis to the epididymis while preventing their significant leakage into the systemic circulation are fully unclear.

Thus, aim of the first part of this study was to get further “in vivo” insights into formation, transport and function of sulfated steroids within the testicular-epididymal compartment in boars by assessing simultaneously the secretion patterns of androstenedione, testosterone, estrone, estradiol, estrone sulfate, estradiol-17ß-sulfate, pregnenolone sulfate and dehydroepiandrosterone sulfate. It was hypothesized that qualitative differences in the secretion pattern like phase shifting or different degrees of decoupling might occur when synthesis of free steroids and their sulfonation are differently regulated in different cell types or organs (testis vs. epididymis) or in the case of differential transport (blood, lymph, rete testis fluid). As these effects might become particularly obvious in the case of significant fluctuations of production rates, thus, in addition to long-term measurements in unstimulated animals, hCG application was used to induce a more dynamic situation. Although a significant amount of data on steroid levels is available in boars (for review see Raeside et al. 2006) including profiles after hCG stimulation, a holistic view from long-term simultaneous measurements of free and sulfated steroids with high frequent sampling including a significant number of animals is still missing. Moreover, in order to characterize more precisely the immediate testicular output of free and sulfated steroids, comparative measurements in arterial and venous blood vessels penetrating the testicular capsule were performed.

In the second part of this work, the expression of enzymes involved in the synthesis and hydrolysis of sulfonated steroids and their activities were addressed to obtain further information on the formation and metabolism of sulfonated steroids in the porcine testicular-epididymal compartment. In addition to steroid sulfatase and the estrogen specific sulfotransferase SULT1E1, the expression of SULT2B1, considered as an important sulfotransferase for hydroxysteroids based on observations in other species, was characterized on the protein level in the testis and various segments of the epididymis using Western blot and immunohistochemistry. Moreover, the hydrolysis of E1S, DHEAS and P5S as well as the sulfonation of estrone, DHEA and P5 were assessed in microsomal and cytosolic fractions prepared from testis and epididymal tissue samples.

The long-term objective of the underlying project is

- to extend our knowledge of the specific reproductive endocrinology in boars and
- to obtain basic information with respect to the suitability of the boar as a model for the study of the sulfatase pathway.

3. Material and Methods

3.1 Animal experiments and sample collection

All animal experiments were in accordance with the relevant regulations and were approved by the competent authority (Regierungspräsidium Giessen, permit No. V54-19c-20-15(I) Gi 18/14-No. 32/2010).

3.1.1 Collection of blood samples

3.1.1.1 Long-term profiles in unstimulated boars

Blood samples from six unstimulated boars (UB1-6, German Landrace x Pietrain) aged 8-11 (mean: 9.1±1.2 months) were collected from the jugular vein which was cannulated via the ear vein following the technique described by Niiyama et al. (1985) for sequential blood sampling from unrestrained pigs. However, for the application of the catheter the animals were deeply sedated using an intramuscular application of 2 mg/kg BW azaperone (Stresnil – Janssen Animal Health, Neuss) and 20 mg/kg BW ketamine (Ursotamin 100 mg/kg – Serumwerk Bernburg AG, Bernburg, Germany). Insertion of the catheter was made from the middle or lateral auricular vein by using a trocar-cannula type needle (CavafixCerto, 45 cm/16 and CertoSplittocan 5 cm/14G – B. Braun Melsungen AG, Melsungen, Germany). The free end of the catheter was connected to a feeding tube (Rüsch No. 5, WdT e.G., Garbsen, Germany) used as an extension which was stitched down at several sites of the ear, neck and back (Fig. 5). Immediately after application of the catheter the collection of blood samples was started, which was between 9:00-9:45 a.m. A total number of 20 samples were taken in 20 min intervals. Blood was withdrawn from the Luer cone of the extension into heparinized 9 ml collection syringes (Monovette – Sarstedt, Nümbrecht, Germany), which were centrifuged immediately after filling and the resulting plasma was stored on ice until freezing at -20°C. After collection of the blood sample, the catheter and its extension were filled with 5 ml heparinized saline (20 I.U./ml), which was thoroughly removed immediately prior to the collection of the next sample. The animals were somnolent at the beginning of the sampling procedure, started to wake up gradually after about one hour and were fully conscious after about another hour.

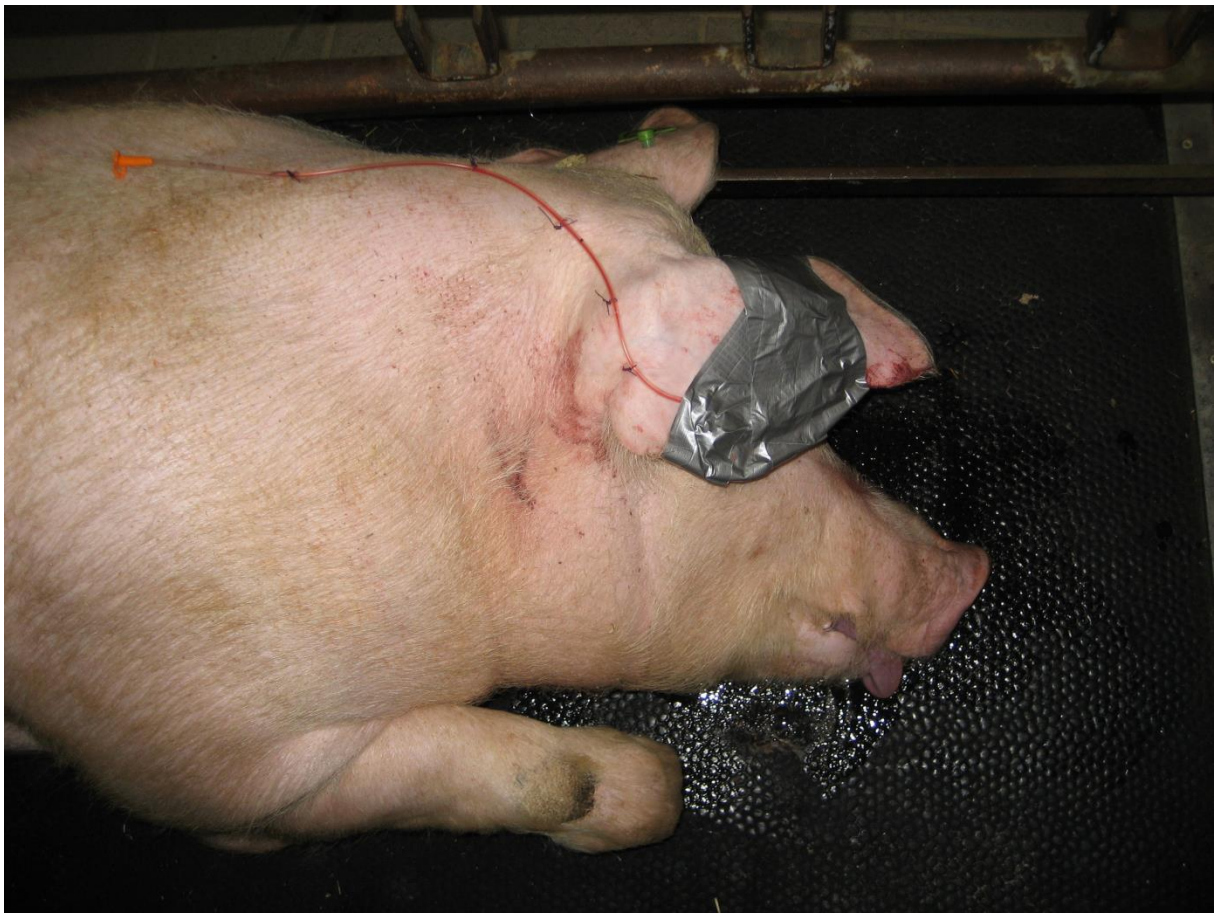


Figure 5. Placement of catheter and extension for frequent blood sampling in boars.

3.1.1.2 Long-term profiles in hCG-stimulated boars

Sample collection was performed as described above for unstimulated boars. In a first trial, 1500 I.U. hCG (Ovogest – Intervet, Unterschleissheim, Germany) were applied intravenously to three nine month old boars (SB1-3, German Landrace x Pietrain) after two prestimulatory samples taken 20 minutes apart. After hCG application, blood sample collection was continued in 20 min. intervals for another 6 hours (protocol 1). In a second trial, in order to better define the prestimulatory steroid levels and to closer assess the hormonal changes immediately after hCG-stimulation, from four German Landrace x Pietrain crossbred boars (SB4-7) aged between 11-15 months (mean: 12.2±1.4 months) four prestimulatory samples were taken in 20 min. intervals, and the sampling interval was reduced to 10 min during the first hour after the application of 1500 I.U. hCG; thereafter, another 16 samples were collected in 20 min. intervals (protocol 2). Finally, in a pilot experiment using an 11.5 month old Large White boar (SB8), the hCG dosage was increased to 10000 I.U. to exert maximum stimulation and the poststimulatory sampling intervals were further reduced to increase the resolution of the

hormonal profiles (protocol 3). Sampling was in 5 min. intervals from 15 min. before to 15 min. after hCG stimulation, followed by sampling in 3 min. during the following hour. A last sample was taken 90 min. after hCG stimulation.

3.1.1.3 Sample collection from local blood vessels of the testicular-epididymal compartment

Blood samples were collected from local blood vessels of the testis and the spermatic cord from four 11-19 (mean: 14.4±2.9) months old boars (two German Landrace x Pietrain crossbreds, two Large Whites). The animals were anaesthetized as described above with increased dosages of ketamine in case of insufficient analgesia. The testis and spermatic cord were exposed through a median prescrotal incision. Then blood samples were collected from one to three small veins running in the testicular capsule near the proximal pole of the organ (capsular vein), from the terminal part of the testicular artery running on the testicular surface near the distal pole (capsular artery), and from a vein of the proximal part of the pampiniform plexus, herein referred to as testicular vein (Fig. 6). Moreover, a blood sample was obtained from testicular artery proximal to the pampiniform plexus either by puncture or by collection from strong pulsatile arterial bleeding after transection of the spermatic cord. After completion of blood sampling and subsequent tissue sampling (section 3.1.2), the animals were euthanized following standard veterinary procedures.

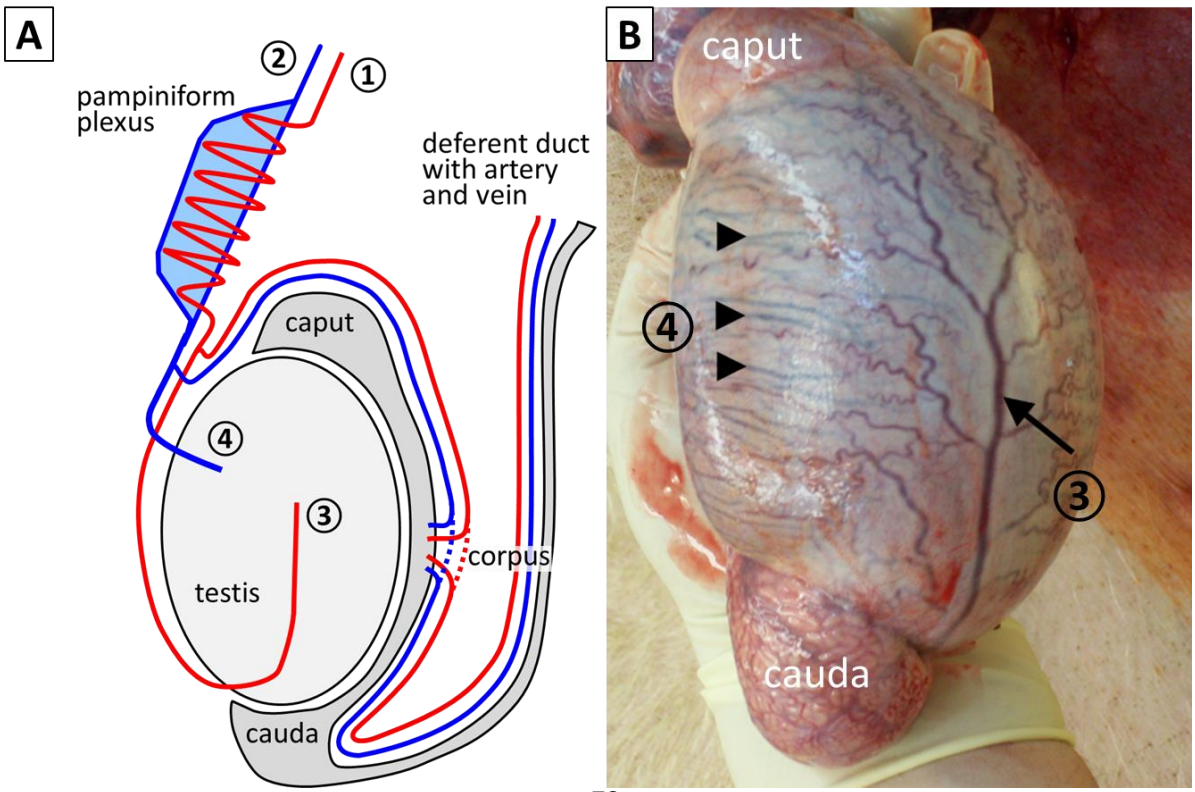


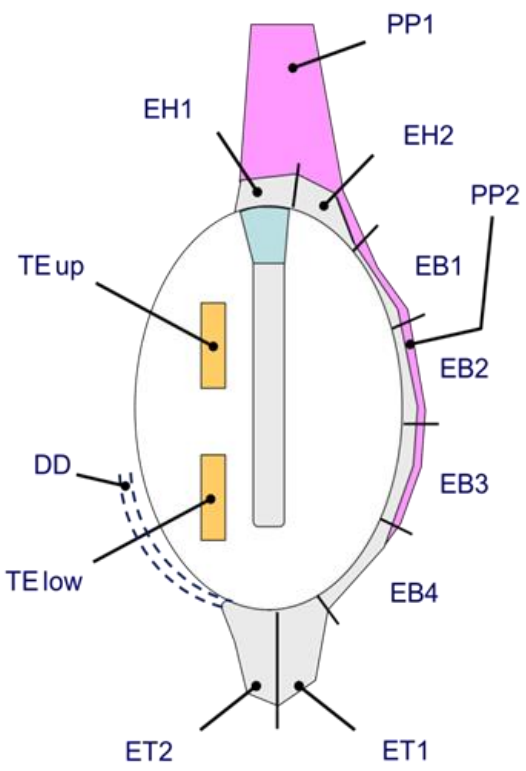
Figure 6. Localizations of sampling from local blood vessels. A) Schematic presentation of major blood vessels of the testicular-epididymal compartment (adopted and modified from Einer-Jensen & Hunter (2005)) and localizations of blood sampling. B) Photo of a porcine testis showing the distal part of the testicular artery ("capsular artery", arrow) with its terminal branches and small veins emanating from the testicular tissue ("capsular veins", arrowheads). Up to three venous samples were collected from the individual animals.

1 -testicular artery prior to the passage of the pampiniform plexus; 2 -testicular vein; 3 -terminal part of the testicular artery; 4 -capsular veins.

3.1.2. Collection and processing of tissue samples

Tissue samples from defined localizations of the testicular-epididymal compartment (Fig. 7) were collected from five 11-19 (mean: 14.4±2.9) months old German Landrace x Pietrain crossbred boars with (see section 3.1.1.3) or without preceding collection of samples from local blood vessels. Anesthesia and surgery was performed as described above (section 3.1.1.1) and the spermatic cord was cut close to the inguinal ring after compression with a big arterial clamp. After the stop of blood reflux from the spermatic cord, the testis with adjoining tissues was placed into a beaker with chilled phosphate buffered saline (PBS) placed in a styrofoam box filled with crunched ice and transported within five minutes to the laboratory, where the preparation of tissue samples was started immediately. For immunohistochemistry, tissue samples were taken from defined localizations specified in Fig. 7 and were conserved overnight in 10% neutral phosphate buffered formalin. For measurement of enzyme activities, small tissue pieces (about 1 cm x 1 cm x 0.5 cm) from the same localizations were wrapped in aluminum foil and put on a block of dry ice in a styrofoam box. After freezing, the tissue samples were stored at -80°C until further analysis.

After removal of the testis with epididymis and the distal part of the spermatic cord, the animals were euthanized following standard veterinary procedures.



TE up, TE low: upper/lower half of testis

EH1, EH2: proximal/distal segment of epididymal head

EPB1-4: segments of epididymal body, from proximal to distal

ET1, ET2: proximal/distal segment of epididymal tail

DD: deferent duct

PAM1: pampiniform plexus of the spermatic cord

PAM2: pampiniform plexus, part attached to the testicular surface

Figure 7. Localizations of tissue sampling.

3.2 Steroid measurements

The primary aim of the steroid measurements performed in this project was to characterize the secretion patterns of sulfonated steroids by the porcine testicular-epididymal compartment and their interrelationships to the production of free steroids. At the beginning of this work, only radioimmunological methods (RIAs) established in the endocrine laboratory of the Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Justus-Liebig University, Giessen were available, including only one method for the measurement of a sulfonated steroid, which was estrone sulfate (E1S). With the start of this project, methods based on liquid chromatography-tandem mass spectrometry (LC-MS-MS) for the measurement of various free and sulfonated steroids in blood plasma were established and validated in the collaborating Steroid Research & Mass Spectrometry Unit, Division of Pediatric Endocrinology & Diabetology, Center of Child and Adolescent Medicine, Justus-Liebig-University, Giessen (head: Prof. Dr. S. Wudy), and were applied to the blood samples collected from boars. Thus, eventually data were available for estrone (E1), estradiol-17ß (E2) and cortisol (F) from RIA only, for testosterone (T) and E1S from both RIA and LC-MS-MS, and for androstenedione

(A4), pregnenolone sulfate (P5S), dehydroepiandrosterone sulfate (DHEAS) and estradiol-17ß-3-sulfate (E2S) from LC-MS-MS only.

Data from parallel measurements of T and E1S using RIA and LC-MS-MS were evaluated with respect to the consistency between the two methods (see section 4.1). For presentation and evaluation of secretory profiles (see sections 4.2 and 4.3), E1S and T data obtained from LC-MS-MS measurements were used.

3.2.1 Radioimmunological measurements of steroid concentrations

Measurements of T, E1, E1S, E2 and F were performed in the laboratory of the Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Justus-Liebig University, Giessen applying validated inhouse RIAs, which were set up as competitive assays as described by Hoffmann et al. (1973). As primary antibodies, inhouse produced polyclonal rabbit antisera were applied (for detailed information see Table 4). As tracers ^{3}H-labelled ligands were used. All measurements were performed in duplicates as equilibrium assays (T, E1S, F) or as a sequential assay (E1, E2). Prior to radioimmunological determination, free steroids were extracted from plasma samples using organic solvents. The separation between free and antibody bound ligands was achieved by charcoal adsorption of free steroids followed by centrifugation. For the calculation of hormone concentrations, which was based on standard curves integrated in each assay, the software of the liquid scintillation counter (LS 5000TD, Beckman, Munich) was used. In addition to the respective standard curve, in each assay reference samples (reagent blank, pooled plasma samples with low and high hormone concentrations) were included for quality control. The determination was repeated when - in terms of counts per minute (cpm) - a difference of more than 10% occurred between the duplicates. Moreover, in case of concentrations exceeding the highest standard curve point, the sample was re-tested using a smaller volume of plasma (T, E2) or an adequate dilution of the reconstituted sample after extraction (E1S). Specific information on the individual assay procedures is provided in the following sections.

Table 4: Information on the antisera used in the radioimmunological determinations of steroids.

	antiserum for the detection of			
	estrone	testosterone	estradiol-17ß	cortisol
Name	E1-6-CMO-BSA-GI II	GI-testosterone I	E2ß-rabbit pool I	GI-COL-IV
antigen used for immunization	estrone-6-carboxy-methyl-oxim-BSA	4-androstene-17ß-ol-3-one-11-hemisuccinate-BSA	1,3,5 (10) estratrien-3,17ß-diol-6-O-carboxy-methyloxim- BSA	4-pregnene-11ß,17α, 21-triol-3,20-dione-3-O-carboxy-methyloxin-BSA
working dilution	1:115 000	1:100 000	1:17 000	1:12 000
cross reactivity (%) with:				
androstenedione	<0.01	0.84	<0.01	<0.01
cortisol	<0.01	<0.01	<0.01	100.00
dehydroepiandrosterone	0.23	<0.01	<0.01	<0.01
5α-dihydrotestosterone	<0.01	47.00	<0.01	<0.01
estradiol-17ß	2.27	0.04	100.00	<0.01
estradiol-17α	0.65			
equilenin	12.40			
equilin	3.92			
estriol	0.03	0.01	0.68	
estrone	100.00	<0.01	1.30	
pregnenolone	<0.01	<0.01	<0.01	<0.01
progesterone	<0.01	0.02	<0.01	<0.01
testosterone	<0.01	100.00	<0.01	<0.01
17αOH-pregnenolone				3.80
17αOH-progesterone	<0.01			<0.01

3.2.1.1 Radioimmunological measurement of testosterone

Measurement of testosterone was performed as described in detail by Landeck (1997) and Röcken et al. (1995). 0.1 ml plasma was extracted twice with 2 ml toluene. Separation of the organic from the aqueous phase was achieved by freezing in a dry ice/ethanol bath (approximately -50°C). The pooled extracts were taken to dryness in a vortex evaporator (Micro Dancer - Zinsser Analytic GmbH, Frankfurt, Germany); the dried residues were re-dissolved in 100 µl of BSA buffer and introduced into the radioimmunological determination. The standard curve consisted of 8 points covering a range between 10-2560 fmol/tube. The interassay and intraassay coefficients of variation were 8.2% and 9.0%, respectively. The minimum detectable concentration was at 0.1 ng/ml.

3.2.1.2 Radioimmunological measurement of estradiol-17ß

Plasma concentrations of free estradiol-17ß were measured with a sensitive inhouse RIA set up as a sequential assay (Strecker et al. 1979) as previously described (Hoffmann et al. 1992, Klein et al. 2003). Before RIA 0.25 ml plasma was extracted twice with 2.5 ml toluene. The pooled extracts were tried in a vortex evaporator (Micro Dancer - Zinsser Analytic GmbH, Frankfurt, Germany) and re-dissolved in 0.1 ml assay buffer. After the addition of 0.4 ml antiserum dilution to the samples and standards (0.5 - 32 pg per tube), a first 'overnight' incubation (minimum 12 h) at 4°C was performed. The tracer (^{3}H-estradiol) was then added and the tubes were again incubated for 45 min at 4°C. After separation of free and bound steroid by addition of 0.2 ml of ice-cold charcoal and centrifugation for 15 min at 2400 g and 4°C, 0.6 ml of the supernatant was taken to measure the radioactivity of the antibody-bound fraction. The minimum detectable concentration was at 2 pg/ml. Intra- and inter-assay coefficients of variation were 7.1 and 17.6%, respectively.

3.2.1.3 Radioimmunological measurement of estrone

E1 was measured using a sequential assay (Strecker et al. 1979) basically following the method described above for the measurement of estradiol-17ß. 0.25 ml plasma was extracted twice with 3 ml toluene, and the pooled extracts were taken to dryness in a vortex evaporator (Micro Dancer - Zinsser Analytic GmbH, Frankfurt, Germany). To ensure re-dissolution of the extracted analyte in an aqueous medium after evaporation of the organic solvent, 0.08 ml 0.1 M NaOH solution were pipetted into the glass tubes containing the dried residues to convert E1 into its more polar phenolate sodium salt. After incubation for 10 min at 37°C and intense

vortexing, neutralization and adjustment to assay conditions were achieved by the addition of 10 µl 0.8 N HCl and of 10 µl concentrated phosphate buffer. The standard curve consisted of 7 points covering a range between 1-64 pg/tube The mean interassay coefficient of variation was 12.4% at a relative binding (B/B_0) of 50%. The lower limit of sensitivity was at 8 pg/ml.

3.2.1.4 Radioimmunological measurement of estrone sulfate

Measurement E1S was performed following a previously published method (Gentz 1994, Hoffmann et al. 1996). With this method, E1S was measured as free E1 after extraction of free E1 present in the samples, followed by enzymatic conversion of E1S into E1. Firstly, 0.4 ml plasma was extracted twice with 3 ml toluene to remove free E1. Then 50 µl 3ß-glucuronidase arylsulfatase (from Helix pomatia, Boehringer Mannheim, 1:25 diluted in 0.9% NaCl solution) were added to the remaining aqueous phases after adjustment of the pH to 4.8 with 150 µl acetate buffer. After overnight incubation in a shaking water bath, the samples were again extracted twice with 3 ml toluene and the pooled extracts were taken to dryness in a vortex evaporator (Micro Dancer - Zinsser Analytic GmbH, Frankfurt, Germany). As described above for the measurement of free E1, to ensure efficient re-dissolving of the analyte, 0.16 ml 0.1 N NaOH solution were pipetted into the glass tubes containing the dried residues to convert E1 into its more polar phenolate sodium salt, and neutralization and adjustment to assay conditions were achieved by the addition of 20 µl 0.8 N HCl, and 20 µl concentrated phosphate buffer. The standard curve consisted of 9 points covering a range between 20-3200 fmol/tube. The interassay and intraassay coefficients of variation were 12.5% and 9.4%, respectively. The minimum detectable concentration was at 0.1 ng/ml.

3.2.1.5 Radioimmunological measurement of cortisol

Radioimmunological determinations of cortisol levels were performed as previously described (Richert-Hanauer et al. 1988). 0.1 ml plasma was extracted twice with 2 ml ethyl acetate, and the pooled extracts were taken to dryness in a vortex evaporator. The dried extracts were re-dissolved in 1 ml assay buffer, and 0.1 ml of the resulting solution was used in the radioimmunoassay. The standard curve covered the range 20-2560 fmol/tube. Interassay and intraassay coefficients of variation were 11.6% and 7.9%, respectively. The minimum detectable concentration was at 0.7 ng/ml.

3.2.2 Measurement of free and sulfonated steroids by liquid chromatography-tandem mass spectrometry

The measurements of A4, T, P5S, DHEAS, E1S and E2S using isotope dilution liquid chromatography tandem mass spectrometry (LC-MS-MS) were performed as previously published (Galuska et al. 2013) by the Steroid Research and Mass Spectrometry Unit, Center of Child and Adolescent Medicine, Faculty of Medicine, Justus-Liebig-University, Giessen (head: Prof. Dr. Stefan Wudy). Briefly, the samples were mixed and equilibrated with deuterated internal standards (d4E2S, d4P5S, d3T, d7A4 and d6DHEAS). All the internal standards except d6DHEAS were purchased from C/D/N Isotopes Inc. (Quebec, Canada). Unlabeled standards and d6DHEAS were obtained from Sigma-Aldrich (Taufkirchen, Germany).

After protein precipitation and removal, the samples were evaporated, re-dissolved in water and extracted using SepPak C18 cartridges (Waters Corporation, Milford, MA, USA). Methanolic eluates were split into two identical volumes, evaporated and reconstituted in different solutions for free steroids or sulfated steroids. Steroids were chromatographically separated with a C18 reverse-phase column (Thermo Fisher Scientific, Dreieich, Germany) adapted to a HPLC system (1200 SL systems, Agilent, Waldbronn, Germany).

Sulfated steroids (E1S, E2S, DHEAS, P5S) were ionized in negative mode using electrospray ionization (ESI) whereas free steroids (T, A4) were ionized in positive mode by atmospheric pressure chemical ionization (APCI). Detection and quantification were performed with a triple quadrupole mass spectrometer (TSQ Quantum Ultra, Thermo, Dreieich, Germany) in tandem mass configuration.

As the method for the measurement of A4 was developed after the start of hormone measurements, it was not included in profiles from one unstimulated and one stimulated boar, respectively. During the final stage of this study, an LC-MS-MS based method for the measurement of 17ß-estradiol-3-sulfate (E2S) became available and was applied to the samples from two hCG-stimulated boars and to samples collected from peripheral blood and local blood vessels of four boars during tissue sampling.

3.3 Immunohistochemical detection of steroid sulfatase, SULT1E1 and SULT2B1

3.3.1 Preparation of formalin-fixed, paraffin embedded tissue

After 20-24 hours of fixation in 10% phosphate–buffered formalin, the tissue samples were transferred in 0.1 M phosphate buffer for removal of the fixative and stored in this solution for 1-2 weeks at 4°C. During this time, the buffer was changed every other day. After that, the tissue samples were dehydrated by incubation in an ascending ethanol series (30%, 50%, and 70%), with each step taking 24 h. Finally, the samples were embedded in paraffin.

3.3.2 Immunostaining procedure

For immunostaining of tissue samples collected from defined localizations of the testicular-epididymal compartment (see Fig. 7) indirect immunoperoxidase staining methods were employed using the streptavidin–biotin technique for signal enhancement following standard procedures as described below. Specific information depending on the individual target molecule (STS, SULT1E1, SULT2B1) is presented in Table 5.

Slices of 5 µm were prepared from formalin-fixed paraffin embedded tissues with a microtome (Reichardt Jung AG, Heidelberg) and placed onto super frost®plus glass slides (MenzelGläser, J1800 AMNZ, Thermo Scientific). The sections were dried overnight at room temperature before staining or storage.

At the beginning of the staining procedure the slides were deparaffinized by incubation for 2×4 min in xylene, rehydrated in graded ethanol (99%, 96% and 70%; 2 x 2 min for each step) and then washed for 5 min in running tap water (5 min.). Antigen retrieval was performed using steaming as follows: the slides were placed in jars filled with 10 mM citrate buffer pH 6.0, which were heated in the vapor phase over boiling water in a pot equipped with a lid made of glass. The temperature in the jars was controlled by observation of a thermometer placed in a reference jar filled with buffer. Heating was stopped after exposition of the slides to temperatures between 98-100°C for 20 min, and the lid of the pot was opened. The slides were cooled for ca. 20 min at room temperature and washed for 5 min under running tap water. Then they were incubated for 30 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Thereafter, sections were washed for 5 min in ICC buffer (phosphate buffered saline pH 7.4 with 0.3% triton X) and covered for 20 min with diluted normal goat serum (from goat anti-rabbit IgG VECTASTAIN ABC Kit, LINARIS BiologischeProdukte GmbH, Dossenheim, Germany, 3 drops of serum in 10 ml ICC buffer) to block non-specific

binding sites. Following drainage of blocking serum, the respective primary antibody – appropriately diluted in ICC buffer - was applied and sections were incubated overnight (20-24 h) in a moist chamber at 4°C. On the following day, the primary antibody was removed and the slides were washed with ICC buffer for 2×10 min. After that the diluted biotinylated secondary antibody (from goat anti-rabbit IgG VECTASTAIN ABC Kit, LINARIS BiologischeProdukte GmbH, Dossenheim, Germany; 25 µl diluted in 5 ml ICC buffer) was applied for 30 min at room temperature. Following draining of the secondary antibody, the sections were washed twice for 5 min with ICC buffer, then covered with streptavidin-peroxidase complex (from goat anti-rabbit IgG VECTASTAIN ABC Kit, LINARIS BiologischeProdukte GmbH, Dossenheim, Germany) and incubated for 30 min. After washing with ICC buffer (2 changes 5 min each), the sections were incubated with substrate solution (NovaRed substrate kit, Vector Laboratories, Burlingame, USA). Finally, they were washed under running tap water for 5 min, counterstained with diluted hematoxylin solution (Thermo Fisher, Osterode, Germany, dilution 1:4 in aqua bidest.), dehydrated in graded ethanol (70%, 96% and 100%; 2 minutes each) and xylene (2x3 minutes) and were mounted in Histokit (Assistent, Osterode, Germany). For each animal, all slides were stained during a single run to avoid effects of inter-assay variability. Negative controls were set up with purified IgG from a nonimmunized rabbit (Invitrogen, Darmstadt) instead of the specific primary antiserum at an equal protein concentration.

Table 5: Information on the antisera used for the immunohistochemical detection of SULTs and steroid sulfatase.

target molecule	source/reference	immunogen	working dilution	host	type of antibody
SULT1E1	MediMabs	recombinant bovine SULT1E1	1:2000	rabbit	polyclonal
SULT2B1	GeneTex	fragment corresponding to amino acids 30-287 of human SULT2B1	1:2000	rabbit	polyclonal
steroid sulfatase	Dr. B. Ugele; (Dibbelt & Kuss 1986; Dibbelt et al. 1989)	human placental steroid sulfatase	1:2000	rabbit	polyclonal
negative control	Invitrogen	irrelevant	1:2000	rabbit	purified IgG

3.3.3 Semiquantitative evaluation of immunostaining

Semi-quantification of immunostaining for STS, SULT1E1 and SULT2B1 was performed in the tissue samples collected at defined localizations (Fig. 7) from three 11-19 (mean: 14.4±2.9) months old boars with the examiner blinded for the animal. In the evaluations two aspects of immunoreactivity were evaluated: the cell types/structures affected and mean staining intensity, which was classified as +++/++/+/- for intense/moderate/weak/no immunostaining. For all evaluations a microscope Leitz DMRB (Leica, Wetzlar, Germany) was used.

3.4 Preparation of subcellular fractions by differential centrifugation

For the measurement of enzyme activity, cytosolic (measurement of SULT activities) and microsomal fractions (measurement of STS activity) were prepared from frozen (-80°C) tissue samples basically following the method by Moran et al. (2002). From the frozen tissue samples, small pieces with an approximate total weight of 0.5 g, were removed using a scalpel blade and thawed in Eppendorf cups placed on ice. After thawing, they were transferred into a Potter glass

hand homogenizer and homogenized in 5 ml in microsomal buffer (pH 7.4) with the lower part of the homogenizer placed in ice. The resulting suspension was centrifuged for 10 min at 15000 g in a precooled centrifuge (4°C) to remove remaining particles of connective tissue, cellular debris and nuclei. The supernatants from this centrifugation step were centrifuged in a precooled Beckman L-70 ultracentrifuge (Beckman Coulter GmbH, Col 95L40) at 100000 g for 1 h at 4°C. The supernatant (cytosolic fraction) was decanted and stored at -80°C until further analysis. The resulting pellet (microsomes) was re-suspended in 500 µl of solubilization buffer (pH 7.4) and also stored at -80°C.

3.5 Measurement of protein concentrations in subcellular fractions

Measurement of protein concentrations in subcellular fractions were performed using the Pierce BCA protein assay kit (purchased from Thermo Fischer Scientific GmbH, Dreieich, Germany; Smith et al. 1985) using a modified version of the standard protocol provided by the supplier. Briefly, microsomal (30 µl) or cytosolic samples (20 µl) were applied into 12 x 75 mm glass tubes and diluted with 50 µl ddH_2O, respectively. Standards ranging between 0-100 µg/tube were prepared as described by the supplier in a volume of 50 µl ddH_2O. Standard solutions were then supplemented with 20 µl microsome buffer for the measurement in cytosols and with 30 µl solubilization buffer for the measurement in microsomal fractions. Thereafter, 2 ml of the dye solution prepared according to the instructions of the supplier were added to each standard and sample and the tubes were shortly vortexed. After incubation for 30 minutes at 37°C in a waterbath and cooling at room temperature for 5 min. the absorbance was measured in a photometer at a wavelength of 562 nm. Finally, the protein concentrations were calculated using the standard curve included in each experiment.

3.6. Detection of steroid sulfotransferase, SULT1E1 and SULT2B1 in subcellular fractions using Western blot

Western blot experiments were performed using samples from three 11-19 month old German Landrace x Pietrain cross-bred boars (mean: 14.4±2.9) in order to further characterize the expression of STS, SULT1E1 and SULT2B1 in the porcine testicular epididymal compartment (for sites of tissue sampling see Fig. 7) on the protein level and to provide evidence for the specificity of the primary antisera used in immunohistochemistry. As STS is commonly considered as a microsomal enzyme, microsomal fractions obtained from differential

centrifugation as described in section 3.4 were used for the detection of this enzyme. In the Western blots for the detection of SULT1E1 and SULT2B1, the corresponding cytosol fractions were applied as the sulfonation of small biomolecules such as steroids is well known to be catalyzed by cytosolic enzymes.

A 10% polyacrylamide separating gel was prepared by mixing 2.5 ml 30% acrylamide solution (Roth GmbH & Co., Karlsruhe, Germany), 2.8 ml separating gel buffer, 2.05 ml ddH_2O, 75 µl 10% SDS solution, 60 µl 10% ammonium persulfate (APS) (Roth GmbH & Co, Karlsruhe, Germany) and 6 µl TEMED (Roth GmbH & Co, Karlsruhe, Germany). The resulting solution was poured in a pre-assembled dual gel caster (Hoefer Inc. Holliston, USA) and then 1 ml isopropanol was added on top to obtain an even surface. The solution was allowed to solidify for 45 min, then the isopropanol layer on the surface was discarded and the gel surface was washed with ddH_2O. The remaining water was removed by help of filter paper. Polyacrylamide collecting gel was prepared by mixing 417 µl 30% acrylamide solution (Roth GmbH & Co, Karlsruhe, Germany), 313 µl collecting gel buffer, 1.73 ml ddH_2O, 25 µl 10% SDS solution, 13 µl 10% APS (Roth GmbH & Co, Karlsruhe, Germany) and 3 µl TEMED (Roth GmbH & Co., Karlsruhe, Germany). This solution was poured onto the separating gel and for the formation of wells the plastic comb was inserted. After solidification (approx. 40 min), the plastic comb was pulled out and the wells were washed with ddH_2O. For electrophoresis, 20 µg of microsomal or cytosolic protein were mixed with 5 µl 3x loading dye, boiled for 3 min and then immediately placed on ice till loading on to gel. The processed samples were then loaded on the gel situated in a dual gel caster fitted in a buffer chamber (Hoefer, Inc. Holliston, USA) which was filled with cold (4°C) SDS electrode buffer. Initially electrophoresis was run at 300 V and 15 mA for about 15 min until the protein had migrated into the separating gel and was then continued at 300 V and 25 mA for about 1 hour. During electrophoresis, the buffer in the chamber was constantly kept cold by running cold water through the cooling jacket of the chamber. After electrophoresis, the gel and a polyvinylidene fluoride membrane (Millipore Corporation, USA) were fitted in a gel holder cassette which was then placed into a transblot tank (BioRad Laboratories, Segrate, Italy). The transblot tank was filled with chilled transfer buffer, and electroblotting was performed at 100 V and 300 mA for 1 hour. During blotting the tank was constantly kept cold by the accessory cooling unit. After the blotting procedure the polyvinylidene fluoride membrane was removed from the gel holder cassette and incubated in blocking buffer (5% skim milk in PBS) overnight at 4°C to block non-specific sites. On the next day, the membrane was briefly washed with PBST buffer (0.25% Tween-20 in PBS) and then incubated for 2 hours in the respective primary antibody solution. Primary antibodies used

for this experiment were same as used for immunohistochemistry, i.e. a polyclonal antisera produced in rabbits against human placental STS (Dibbelt and Kuss 1986, Dibbelt et al. 1989), bovine recombinant SULT1E1 (Frenette et al. 2009) and human SULT2B1 (He et al. 2004). Dilution of primary antisera in blocking buffer was 1:5000 for STS, 1:4000 for SULT1E1 and 1:2000 for SULT2B1. Thereafter, membranes were washed in PBST buffer (3 changes 7 min. each), and then incubated for 50 min with the secondary antibody (biotinylated anti-rabbit antibody; from Vectastain Elite ABC Kit, PK 6101, LINARIS Biologische Produkte GmbH, Dossenheim, Germany) at a dilution of 1:3000 in blocking buffer. Thereafter, the membranes were again washed in PBST buffer (3 changes 7 min. each) and then covered with streptavidin-peroxidase complex (Vectastain Elite ABC Kit, PK 6101, LINARIS Biologische Produkte GmbH, Dossenheim, Germany) for 30 minutes. After washing with PBST buffer (3 changes 7 min. each), the membranes were developed in substrate solution (NovaRed substrate kit, Vector Laboratories, Burlingame, USA) for 50 to 90 seconds. Finally, the membrane was washed extensively in ddH_2O and air dried before images were captured using a digital camera. Using the above-mentioned method for the development of the membranes, initially no specific band was detected for SULT2B1. Thus, in subsequent experiments, a more sensitive chemiluminescence-based method was applied (AceGlow Ultrasensitive Chemiluminescence Substrate, detection of immunoreaction and documentation of results with Fusion Solo VilberLourmat, both from PEQLAB Biotechnologie GmbH, Erlangen) following the protocol provided in the instruction manual of the substrate kit.

3.7 Measurements of the activities of steroid sulfatase and of sulfotransferases relevant to phenolic and neutral steroids in subcellular fractions.

Activities of STS and SULTs relevant for steroids were measured in processed tissue samples collected from defined localization of the testicular-epididymal compartment (Fig. 7) of 3-4 11-19 months old German Landrace x Pietrain crossbred boars. Hydrolysis of sulfonated steroids (STS activity) was measured using E1S, DHEAS and P5S as substrates. As STS is commonly considered as a microsomal enzyme, these measurements were performed in microsomal fractions which were obtained from differential centrifugation as described in section 3.4. Concomitantly, in the corresponding cytosolic fractions the sulfonation of E1, DHEA and P5 were assessed, as the sulfonation of small biomolecules such as steroids is well known to be catalyzed by cytosolic sulfotransferases (SULTSs). In humans SULT1E1, SULT2A1 and SULT2B1 have been identified as the most relevant SULTs for the sulfonation of estrogens,

DHEA and P5, respectively (see section 2.7, Table 1). However, as in the pig no definite information is available which of the many SULTs considerably contribute to the sulfonation of the individual steroids, with respect to enzyme activities the terms "estrogen sulfotransferase", "DHEA sulfotransferase" and "P5 sulfotransferase" will be used.

Basically, a similar type of assay was applied for the measurements of enzyme activities. After incubation of the processed tissue sample (microsomal or cytosolic fraction) and the substrate, the enzymatic reaction was stopped by heating and the organic solvent tert-butyl methyl ether (tBME) was added. The analysis was based on the differential distribution of free and sulfonated steroids in the aqueous phase versus the organic phase. Whereas the free steroids mainly enter the organic phase due to their lipophilic character, the highly polar sulfonated steroids accumulate in the aqueous phase. For analysis, in a first step free steroids were extracted from the samples using the above-mentioned organic solvent, yielding fraction 1. Then, the remaining aqueous phase was subject to enzymatic hydrolysis (Helix pomatia, Boehringer Mannheim), followed by a second extraction yielding fraction 2 (sulfonated steroids). In order to reduce the experimental efforts and to allow a high throughput of samples, a radioactive tracer (approx. 20000 cpm tritium-labeled steroid per tube) was added to the steroid used as substrate, and the conversion of the substrate was calculated from the decrease or increase of ^{3}H-activity observed over the time of incubation in one of the two phases, depending on the respective enzyme to be measured and the substrate used. In order to test for non-enzymatic conversion of the substrate, in each assay medium blanks and tissue blanks (heat inactivated cytosol or microsomal fraction) were included as negative controls. All measurements were done in duplicates. Specific information on the individual measurements is provided in the following sections.

3.7.1. Measurements of steroid sulfatase activity

3.7.1.1 Optimization of the steroid sulfatase assay

The measurement of STS in microsomal fractions was basically performed following a method established in a previous study (Mutembei et al. 2009). However, in order to optimize this method with respect to the aim of this study, which was the comparison of the STS activities in the testis and different segments of the epididymis, pilot experiments were performed to identify optimal experimental conditions concerning the amount of substrate, the amount of microsomal protein and the time of incubation. Moreover, for reason of convenience (considerably shorter time for evaporation), toluene was replaced by tBME as the organic solvent after confirmation

of adequate extraction rates for the relevant free steroids, E1, DHEA and P5. Parallel incubations in the presence or absence of a protease inhibitor cocktail (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) gave no evidence for an impairment of the results by proteolysis. Thus, the protease inhibitors were not included in the final protocol. Finally, efforts were made to reduce the high "conversion rates" frequently found in previous studies in tissue blanks, medium blanks and in samples in which the enzymatic reaction was immediately stopped after pipetting of the components (incubation time 0 min). Observations in preliminary experiments suggested that this problem originated from a carry-over of sulfonated steroids into fraction 1 during the first extraction. Heating of the samples after incubation followed by centrifugation was found to widely overcome this problem.

The results of the preliminary experiments led to the final protocol specified in chapter 3.7.1.2 (Table 6). Results of a preliminary experiment showing the time-dependent hydrolysis of E1S under the conditions of the final assay procedure over an incubation time of 2 hours is presented in Fig. 8.

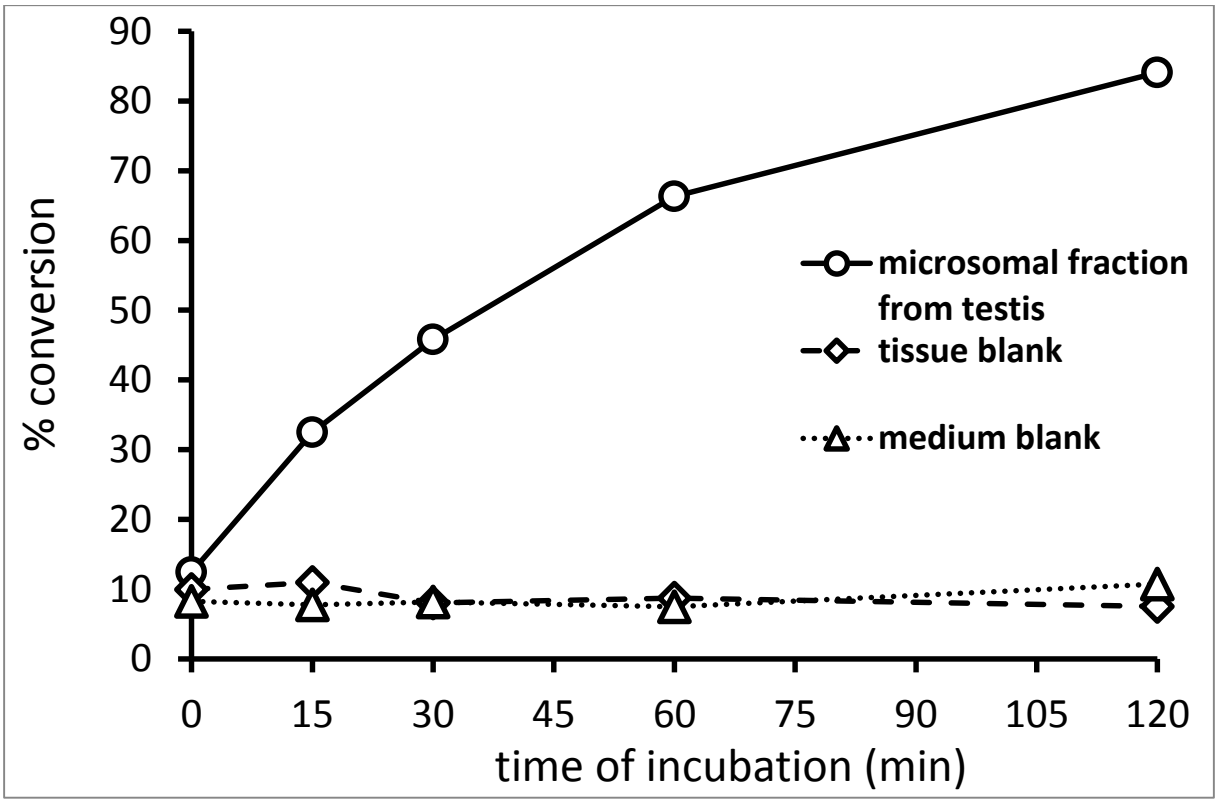


Figure 8. Steroid sulfatase enzyme activity assay in a testicular microsomal fraction – results from a preliminary experiment. The experiment was carried out following the final protocol specified in chapter 3.7.1.2 (Table 6). The figure shows the hydrolysis of estrone sulfate over time. Medium blank: microsomal protein was replaced by

Ringer/HEPES-buffer; tissue blank: incubation of heat-inactivated microsomal protein.

3.7.1.2 Final steroid sulfatase assay procedure

The experimental setup of the STS activity measurements is presented in Table 6. After pipetting of the components, Ringer/HEPES-buffer, labeled and non-labelled substrate and microsomal fraction into 15 ml Wheaton reaction tubes (purchased from MAGV Laborbedarf, Rabenau-Londorf, Germany), incubation was performed in a shaking water bath (TypeWB-24; Medax Nagel KG, Kiel, Germany) for 0, 30 and 60 min. After the respective incubation time, the samples were boiled for 15 min in a customary cooking pot, centrifuged for 10 minutes at 3000 g and an aliquot of 800 µl was transferred into new 15 ml Wheaton reaction tube. The samples were overlaid with 3 ml of tBME, vortexed for 30s and shortly centrifuged (3000 g). After freezing of the aqueous phase in a dry ice/ethanol bath, the supernatants were decanted into small glass tubes (RIA glass tubes, VWR Germany). This extraction step was repeated, and the pooled extracts were evaporated in a vortex evaporator at 60^0C (Micro-Dancer, Hettich, CH-8806 Bach, Switzerland). Then 1 ml scintillation fluid (Aquasafe, Zinsser Analytic, Frankfurt) was added into the tubes, followed by vortexing, and their content was transferred into liquid scintillation (LSC) vials (Aquasafe, Zinsser Analytic, Frankfurt). This step was repeated again. Finally, the ^{3}H-activity present in the LSC-vials was measured in a ß-scintillation counter (Beckman GmbH, Krefeld).

For a total ^{3}H-balance, following first extraction 50 µl 3ß-glucuronidase arylsulfatase (Helix pomatia, Boehringer Mannheim, 1:25 diluted in 0.9% NaCl solution) was added to the remaining aqueous phases. After overnight incubation in the shaking water bath, the samples were again extracted twice with 3 ml of tBME and the pooled extracts were taken to dryness in a vortex evaporator. Finally, the dried extracts were re-dissolved in scintillation fluid as described above and ^{3}H-activity was measured.

The percentage of hydrolyzed E1S was calculated from the increase of ^{3}H-activity in the fraction of free steroids (F1) as

$$\frac{\text{Sample (cpm in F1)} - \text{MB (cpm in F1)}}{\text{TA (cpm in F1)} - \text{MB (cpm in F1)}} * 100$$

TA = total activity (cpm added to the reaction tubes); MB = medium blank

Table 6: experimental setup of steroid sulfatase activity measurements.

	^{3}H-E1S[1]	E1S	microsomal fraction	ringer-HEPES buffer	total volume
volume	125 µl	61 µl	125 µl		
sample	0.27 pmol	9.73 pmol	200 µg	689 µl	1 ml
tissue blank	0.27 pmol	9.73 pmol	200 µg[2]	689 µl	1 ml
medium blank	0.27 pmol	9.73 pmol	none	814 µl	1 ml

[1] equivalent to 16000 cpm per tube

[2] heat-inactivated

3.7.2 Measurements of estrogen sulfotransferase activity

3.7.2.1 Optimization of the estrogen sulfotransferase assay

As described in chapter 3.7.1.1 for the optimization of the STS assay, also for the estrogen sulfotransferase assay preliminary experiments were performed to identify suitable experimental conditions with respect to the amount of cytosolic protein, substrate and incubation time. Moreover, in order to obtain information on the amount of the co-substrate PAPS needed for the maximal stimulation of E1 sulfonation, cytosolic protein prepared from epididymal head was incubated in the presence of four different amounts of PAPS per tube, which were 50 nmol, 10 nmol, 2 nmol and 0.4 nmol. As no substantial difference was found between the four levels of PAPS supplementation concerning the sulfonation of E1, in the final protocol 0.4 nmol PAPS were included per tube. In the absence of PAPS supplementation, sulfonation of E1 was low and not different from the controls, medium blank and tissue blank.

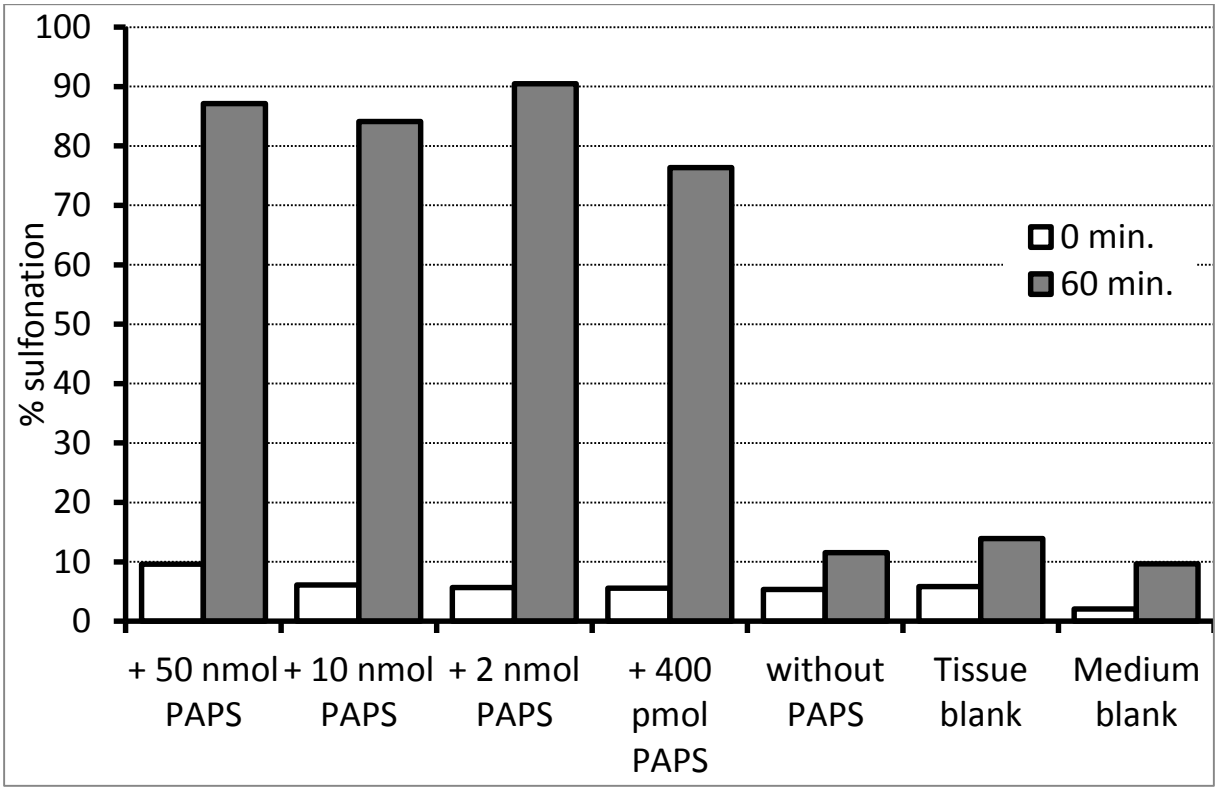


Figure 9. Sulfonation of 10 pmol estrone by 200 µg cytosolic protein prepared from epididymal head – results from a preliminary experiment in which the effect of different amounts of the co-substrate PAPS was tested. The experiment was otherwise carried out following the final protocol specified in chapter 3.7.2.2 (Table 7). Medium blank: microsomal protein was replaced by Ringer/HEPES-buffer; tissue blank: incubation of heat-inactivated cytosolic protein.

3.7.2.2 Final estrogen sulfotransferase assay procedure

The experimental setup of the estrogen sulfotransferase measurements is presented in Table 7. After pipetting of the components, Ringer/HEPES-buffer, labeled and non-labeled substrate, PAPS and cytosolic fraction into 15 ml glass vials (Wheaton reaction tubes, purchased from MAGV Laborbedarf, Rabenau-Londorf, Germany), incubation was performed in a shaking water bath for 0, 10 and 20 minutes. After the respective incubation time, the samples were boiled for 15 minutes in a customary cooking pot, centrifuged for 10 minutes at 3000 g and aliquots of 800 µl were transferred into new 15 ml Wheaton reaction tubes. The further analytical procedure was performed as described in section 3.7.1.2 for STS assay using tBME as organic solvent.

The percentage of sulfonated E1S was calculated from the increase of ^{3}H-activity in the fraction of sulfonated steroids (F2) as

$$\frac{\text{sample (cpm in F2)} - \text{TB (cpm in F2)}}{\text{TB in F1 (cpm)} + \text{TB in F2 (cpm)}} * 100$$

TB = tissue blank (heat inactivated microsomal protein)

Table 7: Experimental setup of estrogen sulfotransferase activity measurements.

	^{3}H-E1[1]	E1	PAPS	cytosol	Ringer/ HEPES buffer	total volume
volume	125 µl	61 µl	100 µl	125 µl		
sample	0.27 pmol	9.73 pmol	0.4 nmol	200 µg	589 µl	1 ml
tissue blank	0.27 pmol	9.73 pmol	0.4 nmol	200 µg[2]	589 µl	1 ml
medium blank	0.27 pmol	9.73 pmol	0.4 nmol	none	714 µl	1 ml

[1] equivalent to 16000 cpm per tube

[2] heat inactivated

3.7.3 Measurement of DHEA sulfotransferase activity

The experimental setup of the DHEA sulfotransferase activity measurements was basically analogous to the estrogen sulfotransferase assay and is presented in Table 8.

After pipetting of the components, Ringer/HEPES-buffer, labeled and non-labeled substrate, PAPS and cytosolic fraction into 15 ml Wheaton reaction tubes (purchased from MAGV Laborbedarf, Rabenau-Londorf, Germany), incubation was performed in a shaking water bath for 0, 10 and 20 minutes. After the respective incubation time, the samples were boiled for 15 min. in a customary cooking pot, centrifuged for 10 min. at 3000 g and aliquots of 800 µl were transferred into new 15 ml Wheaton reaction tubes. The further analytical procedure was performed as described in section 3.7.1.2 for the steroid sulfatase assay using tBME as organic solvent.

The percentage of sulfonated DHEA was calculated from the decrease of ^{3}H-activity in the fraction of free steroids (F1) as

$$\frac{\text{TB (cpm in F1)} - \text{sample (cpm in F1)}}{\text{TB (cpm in F1)}} * 100$$

As a positive control, microsomal protein prepared from a human term placenta was included.

Table 8: experimental setup of the DHEA sulfotransferase activity measurements.

	^{3}H-DHEA[1]	DHEA	PAPS	cytosol	Ringer/ HEPES buffer	total volume
volume	125 µl	61 µl	100 µl	125 µl		
sample	0.27 pmol	9.73 pmol	0.4 nmol	200 µg	589 µl	1 ml
tissue blank	0.27 pmol	9.73 pmol	0.4 nmol	200 µg[2]	589 µl	1 ml
medium blank	0.27 pmol	9.73 pmol	0.4 nmol	none	714 µl	1 ml

[1] equivalent to 16000 cpm per tube

[2] heat inactivated

3.7.4 Measurement of pregnenolone sulfotransferase activity

The experimental setup of the P5 sulfotransferase activity measurements is basically analogous to the sulfotransferase assay for E1 and DHEA and is presented in Table 9. After pipetting of the components, Ringer/HEPES buffer, labeled and non-labeled substrate, PAPS and cytosolic fraction into 15 ml Wheaton reaction tubes (purchased from MAGV Laborbedarf, Rabenau-Londorf, Germany), incubation was performed in a shaking water bath for 0, 10 and 20 minutes. After the respective incubation time, the samples were boiled for 15 min. in a customary cooking pot, centrifuged for 10 min. at 3000 g and aliquots of 800 µl were transferred into 15 ml Wheaton reaction tubes. The further analytical procedure was performed as described in section 3.7.1.2 for STS.

The percentage of sulfonated P5 was calculated from the decrease of ^{3}H-activity in the fraction of free steroids (F1) as

$$\frac{\text{TB (cpm in F1)} - \text{sample (cpm in F1)}}{\text{TB (cpm in F1)}} * 100$$

Table 9: experimental setup of the P5 sulfotransferase activity measurements.

	^{3}H-P5[1]	P5	PAPS	Cytosol	Ringer/ HEPES buffer	total volume
volume	125 µl	61 µl	100 µl	125 µl		
sample	0.27 pmol	9.73 pmol	0.4 nmol	200 µg	589 µl	1 ml
tissue blank	0.27 pmol	9.73 pmol	0.4 nmol	200 µg[2]	589 µl	1 ml
medium blank	0.27 pmol	9.73 pmol	0.4 nmol	none	714 µl	1 ml

[1] equivalent to 16000 cpm per tube

[2] heat inactivated

3.8. Statistical evaluations

3.8.1 Evaluation of correlations between radioimmunological vs. LC-MS-MS measurements of testosterone and estrone sulfate.

For concentrations of T and E1S in unstimulated (boars UB1-6) and stimulated boars (SB1-7), data were available from measurements using RIA and LC-MS-MS. In order to evaluate the correlation between the two methods, a linear regression analysis was performed, including the calculation of the correlation coefficient r, of the tail probability P and of the regression line (Microsoft Excel).

3.8.2 Pairwise cross-correlation analyses between steroid profiles and calculation of biserial correlation functions.

This part of the statistical evaluations was performed by the Unit of Biomathematics and Data Processing, Faculty of Veterinary Medicine, Justus-Liebig-University under the supervision of Dr. Klaus Failing. To cope with the right-skewed distribution of hormone data, they were

transformed logarithmically to obtain an approximated normal distribution prior to statistical evaluation. In unstimulated boars (UB1-6) data over the complete sampling period were used. In stimulated boars (SB1-7), data from samples collected after hCG application were analyzed. For evaluation of the relationships between the secretory profiles of the free and sulfonated steroids assessed, pairwise cross correlation analyses were performed for the individual unstimulated boars (UB1-6) and the stimulated boars sampled according to protocol 1 (SB1-3) and protocol 2 (SB4-7) (calculation of the cross correlation coefficient r and the tail probability P, program BMDP6D, BMDP Statistical Software Inc., Cork, Ireland).

Additionally, in order to test for phase shiftings, the secretory profiles were pairwise shifted relative to one another in 20 min intervals in both directions (from -80 min to +80 min) within the cross correlation analysis (calculation of biserial correlation function, BMDP6D, BMDP Statistical Software Inc., Cork, Ireland). A maximum of r following shifting to either side would indicate that secretion of one of the steroids determined is ahead of the other one. This type of analysis was performed with pooled data from unstimulated (UB1-6; for result see section 4.2) and stimulated boars (SB1-7; for result see section 4.3), respectively. Results from the calculation of biserial functions in individual animals are presented in the appendix (chapter 9.1).

3.9 Solutions, reagents, material and equipment

3.9.1 Solutions and buffers

3.9.1.1 Solutions and buffers used in radioimmunoassay

Phosphate buffer (pH 7.2; 78.6 mM)

Na_2HPO_4	8.356 g
KH_2PO_4	2.686 g
NaN_3	0.325 g
ddH_2O	ad 1000 ml

BSA buffer (pH 7.2; 78.6 mM; 0.1 BSA)

Bovine serum albumin (BSA)	1.0 g
Phosphate buffer	ad 1000 ml

Hydrolysis buffer (0.625 M Na-acetate buffer pH 4.8)

1 N NaOH	3 volumes
1 N CH_3COOH	5 volumes

(1 N CH_3COOH: 60.05 g glacial acetic acid ad 1000 ml ddH_2O)

Charcoal solution (0.50% Norit A, 0.05% Dextran)

Norit A	0.50 g
Dextran	0.05 g
ddH_2O	ad 100 ml

^{3}H-Estrone tracer solution used in RIA (^{3}H-estrone in BSA buffer; approx. 10000 cpm/0.1 ml):

^{3}H-Estrone [2,4,6,7-3H(N)] stock solution:	57 µl
BSA buffer:	10 ml

^{3}H-Testosterone tracer solution used in RIA (^{3}H-testosterone in BSA buffer, approx. 10000 cpm/0.1 ml)

^{3}H-Testosterone [1,2,6,7-3H(N)] stock solution:	72 µl
BSA buffer:	20 ml

^{3}H-Cortisol tracer solution used in RIA (^{3}H-cortisol in BSA buffer, approx. 10000 cpm/0.1 ml)

^{3}H-Cortisol [1,2,6,7-3H(N)] stock solution:	90 µl
BSA buffer:	20 ml

3.9.1.2 ^{3}H-Steroid stock solutions used in RIA and enzyme assays

^{3}H-steroid	Labeling	specific activity	Supplier
DHEA	[1,2-3H(N)]	50 Ci/mmol (1.86 TBq/mmol)	American Radiolabeled Chemicals
DHEAS	[1,2,6,7-3H(N)]	70.5 Ci/mmol (2.62 TBq/mmol)	Perkin Elmer
Estrone	[2,4,6,7-3H(N)]	73.1 Ci/mmol (2.71 TBq/mmol)	Perkin Elmer
Estrone sulfate	[6,7-3H(N)]	57.3 Ci/mmol (2.10 TBq/mmol	Perkin Elmer
Pregnenolone	[7-3H(N)]	22.9 Ci/mmol (0.85 TBq/mmol)	Perkin Elmer
Pregnenolone sulfate	[7-3H(N)]	20.0 Ci/mmol (0.74 TBq/mmol)	Perkin Elmer
Progesterone	[1.2.6.7-3H(N)]	99.1 Ci/mmol (3.67 TBq/mmol)	Perkin Elmer
Testosterone	[1,2,6,7-3H(N)]	70.0 Ci/mmol (2.59 TBq/mmol)	Perkin Elmer
Estradiol-17β	[2,4,6,7-3H(N)]	89.2 Ci/mmol (3.30 TBq/mmol)	Perkin Elmer
Cortisol	[1,2,6,7-3H(N)]	70.0 Ci/mmol (2.59 TBq/mmol)	Perkin Elmer

The ^{3}H-steroids (9.25 MBq) were supplied in 250 µl ethanol. The ^{3}H-steroid stock solutions were prepared from the original preparations by adding methanol to a final volume of 10 ml. They were stored at -30°C.

3.9.1.3 Solutions and buffers used for the preparation of subcellular fractions

Preparation of buffer stock solutions:

1 M K_2HPO_4

K_2HPO_4	3.482 g
ddH_2O	ad 20 ml

1 M KH_2PO_4

KH_2PO_4	1.361 g

ddH_2O	ad 10 ml

CHAPS= 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

CHAPS	0.769 g
potassium phosphate/glycerol buffer (microsome buffer, s. below)	ad 5 ml

Microsome buffer pH 7.4	
1 M K_2HPO_4 (stock solution)	6.000 ml
1 M KH_2PO_4 (stock solution)	1.485 ml
glycerol	15 ml
ß-mercaptoethanol	26.25 µl
ddH_2O	ad 75 ml
check pH	

Solubilization buffer pH 7.4	
1 M K_2HPO_4 (stock solution)	0.802 ml
1 M KH_2PO_4 (stock solution)	0.198 ml
glycerol	2.000 ml
ß-mercaptoethanol	3.5 µl
250 mM CHAPS	40 µl
ddH_2O	ad 10 ml
check pH	

3.9.1.4 Steroid substrate solutions used for the measurements of enzyme activities

3.9.1.4.1 Unlabeled steroids

A) Estrone sulfate

Estrone sulfate potassium salt (MW 388.52): MAKOR Chemical (cat no: 2033, batch 30-125

B) Preparation of stock solution 1:

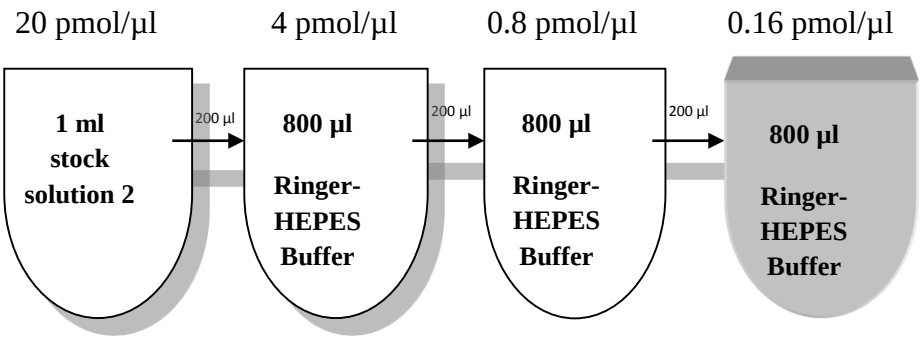
1 mg = 0.002574 mmol = 2.574 µmol

Dissolve 10 mg E1S in 10 ml methanol = 2.574 µmol/ml (nmol/µl)

Preparation of stock solution 2:

Add 77.7 µl stock solution 1 + 9922.3 µl Ringer/HEPES buffer (concentration: 200 nmol/10 ml = 20 pmol/µl)

Preparing of working dilution (9.73 pmol in 61 µl)



B) Dehydroepiandrosterone sulfate

5-Androsten-3β-ol-17-one sulfate sodium salt (MW 368.48): Paesel-Lorei GmbH (batch 1980)

Preparation of stock solution 1:

1 mg = 0.002713 mmol = 2.713 µmol

Dissolve 10 mg DHEAS in 10 ml methanol = 2.713 µmol/ml (nmol/µl)

Preparation of stock solution 2:

Add 73.7 µl stock solution 1 + 9926.2 µl Ringer/HEPES buffer

Concentration: 200 nmol/10 ml (20 nmol/ml = 20 pmol/µl)

C) Pregnenolone sulfate

5-Pregnen-3β-ol-20-one sulfate, sodium salt (MW 418.52): Paesel-Lorei GmbH (Cat.28-115-Q5545, L568)

Preparation of stock solution 1:

1 mg = 0.002389 mmol = 2.389 µmol

Dissolve 10 mg pregnenolone sulfate in 10 ml methanol = 2.389 µmol/ml (nmol/µl)

Preparation of stock solution 2:

Add 83.7 µl stock solution 1 + 9916.3 µl Ringer/HEPES buffer

Concentration: 200 nmol/10 ml (20 nmol/ml = 20 pmol/µl)

D) Estrone

1,3,5 (10)-Estratrien-3-ol-17-one (MW 270.37): Paesel-Lorei GmbH

Preparation of stock solution 1:

1 mg = 0.003698 mmol = 3.698 µmol

Stock solution 1 = 10 mg estrone in 10 ml methanol = 3.698 µmol/ml (nmol/µl)

Preparation of stock solution 2:

Add 54.08 µl stock solution 1 + 9945.92 µl Ringer/HEPES buffer

Concentration: 200 nmol/10 ml (20 nmol/ml = 20 pmol/µl)

E) Dehydroepiandrosterone

5-Androsten-3β-ol-17-one (MW 288.42): Paesel-Lorei GmbH

Preparation of stock solution 1:

1 mg = 0.003467 mmol = 3.467 µmol

Dissolve 10 mg DHEA in 10 ml methanol = 3.467 µmol/ml (nmol/µl)

Preparation of stock solution 2:

Add 57.68 µl stock solution 1 + 9942.32 µl Ringer/HEPES buffer

Concentration: 200 nmol/10 ml (20 nmol/ml = 20 pmol/µl)

F) Pregnenolone

5-Pregnen-3β-ol-20-one (MW 341.49): Paesel-Lorei GmbH

Preparation of stock solution 1:

1 mg = 0.002928 mmol = 2.928 µmol

Dissolve 10 mg P5 in 10 ml methanol = 2.928 µmol/ml (nmol/µl)

Preparation of stock solution 2:

Add 68.30 µl stock solution 1 + 9931.7 µl Ringer/HEPES buffer

Concentration: 200 nmol/10 ml (20 nmol/ml = 20 pmol/µl)

3.9.1.4.2 ^{3}H-Steroids

To determine the hydrolysis of sulfonated steroids (STS assay) or the sulfonation of free steroids (sulfotransferase assays), in addition to the respective unlabeled substrate approximately16000 cpm/tube of the respective ^{3}H-steroid were included. The ^{3}H-steroid working dilutions were prepared from the methanolic stock solutions (see chapter 3.9.1.2) as follows:

^{3}H-Estrone: 5.9 µl ^{3}H-E1 in 1 ml Ringer/HEPES buffer

^{3}H-DHEA: 7.6 µl ^{3}H-DHEA in 1 ml Ringer/HEPES buffer

^{3}H-Pregnenolone: 7.1 µl ^{3}H-P5 in 1 ml Ringer/HEPES buffer

^{3}H-Estrone sulfate: 5.3 µl ^{3}H-E1S in 1 ml Ringer/HEPES buffer

^{3}H-DHEA sulfate: 7.8 µl ^{3}H-DHEAS in 1 ml Ringer/HEPES buffer

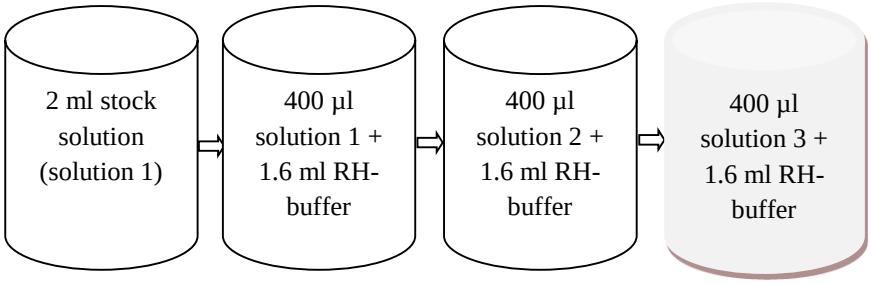
^{3}H-Pregnenolone sulfate: 7.8µl ^{3}H-P5S in 1 ml Ringer/HEPES buffer

3.9.1.4.3 3′-Phosphoadenosine-5′-phosphosulfate (PAPS)

Adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (Sigma-Aldrich Chemie GmbH, A1651, MW 507.26)

Dissolve 1 mg of PAPS in 4.0 ml Ringer/HEPES buffer (RH-buffer)

Make dilutions of 1/1:5/1:25 and 1:125



3.9.1.5 Solutions and buffers used for immunohistochemistry

10 mM Citrate buffer for antigen unmasking

Stock solution A:

Citric acid ($C_6H_8O_7$) × H_2O	10.505 g
ddH_2O	ad 500 ml

Stock solution B:

Sodium citrate ($C_6H_5O_7Na_{3)}$ × $2H_2O$	14.705 g
ddH_2O	ad 500 ml

Working solution:

Stock solution A	18 ml
Stock solution B	82 ml
ddH_2O	ad1000 ml

0.3% H_2O_2–Methanol (quenching of endogenous peroxidase activity)

Methanol	250 ml
30% H_2O_2	2.5 ml

Ethanol (96%)

Ethanol (100%)	96 ml
ddH_2O	4 ml

Ethanol (70%)

Ethanol (100%)	70 ml
ddH_2O	30 ml

ICC buffer PH 7.4

Na_2HPO_4	2.4 g
KH_2PO_4	0.4 g
KCl	0.4 g
NaCl	16.0 g
ddH_2O	ad 2000 ml

6 ml Triton X-100 was added after adjusting pH

3.9.2 Materials, kits and reagents

- AceGlow Ultrasensitive Chemiluminescence Substrate: PEQLAB Biotechnologie GmbH, Erlangen, Germany
- Acrylamide solution 30%: Roth GmbH & Co., Karlsruhe, Germany
- Adenosine 3´-phosphate-5´-phosphosulfate (PAPS) lithium salt hydrate: Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Aquasafe 300 Plus: biodegradable emulsifying scintillator for aqueous samples; Zinsser Analytic, Frankfurt, Germany.
- Ammonium persulfate (APS) 10%: Roth GmbH & Co., Karlsruhe, Germany
- BSA (Albumin from bovine serum): Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Calcium Chloride (147.01 g/mol): Merck KGaA, Darmstadt, Germany
- CHAPS (3-[(3-cholamidopropyl) dimethylammonio] – 1- propanesulfonate hydrate): Sigma-Aldrich Chemie GmbH, Steinheim, Germany.
- Dextran 60: Serva, Heidelberg, Germany
- Eppendorf tubes 1.5 ml colorless: Sarstedt, AG & Co., Nümbrecht, Germany
- Ethanol 99.6%: Roth GmbH & Co., Karlsruhe, Germany
- Formaldehyde solution 4%, buffered, pH 6.9: Merck KGaA, Darmstadt, Germany
- β-Glucuronidase/arylsulfatase (from Helix pomatia): Roche Diagnostics GmbH, Mannheim, Germany
- Glycerol for molecular biology, ≥99% (1.25 g/mL): Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Hand gloves UniGloves®: purchased from MAGV Laborbedarf & Laborgeräte, Rabenau-Londorf, Germany
- Histokit: Assistant, Osterode, Germany
- HEPES buffer solution (238 g/L HEPES in H_2O): Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Hydrochloric acid (hydrogen chloride solution, 1.19 g/cm³): Merck KGaA, Darmstadt, Germany
- Hydrogen peroxide 30%: Merck KGaA, Darmstadt, Germany
- Hematoxylin monohydrate (320.29 g/mol): Merck KGaA, Darmstadt, Germany
- Methanol (MW 32.04 g/mol): Merck KGaA, Darmstadt, Germany
- Microtome blade Leica DB 80L: Leica Microsystems GmbH, Nussloch, Germany

- Di-sodium hydrogen phosphate: Merck KGaA, Darmstadt, Germany
- Sodium azide: Serva, Heidelberg, Germany
- NovaRed Substrate Kit: Vector Laboratories, Burlingame, USA
- Pap Pen: G.Kisker Biotech, Steinfurt, Germany
- Pierce BCA protein assay: Fisher Scientific, Dreieich, Germany
- Potassium phosphate monobasic (MW 136.09): Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Potassium phosphate dibasic (MW 174.18): Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Potassium chloride (MW 74.55): Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Protease Inhibitor Cocktail tablet: Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany
- Wheaton reaction tubes, purchased from MAGV Laborbedarf, Rabenau-Londorf, Germany
- Sodium chloride (MW 58.44 g/mol): Merck KGaA, Darmstadt, Germany
- Sodium hydroxide solution (1.04 g/cm³): Merck KGaA, Darmstadt, Germany
- SuperFrost® Glass Slides, 24 mm × 40 mm, 24 mm × 50 mm and 24 mm × 60 mm: Menzel-Deckgläser, Gerhard Menzel GmbH, Braunschweig, Germany
- TEMED: Roth GmbH & Co., Karlsruhe, Germany
- Tert-butyl methyl ether (anhydrous, 99.8%): Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Toluene (MW 92.14 g/mol): Merck KGaA, Darmstadt, Germany
- Triton X-100: SERVA, Heidelberg, Germany
- VECTASTAIN ABC Kit (rabbit IgG), LINARIS BiologischeProdukte GmbH, Dossenheim, Germany
- Xylene (density: 0.86 g/cm³): Merck KGaA, Darmstadt, Germany

3.9.3 Equipment

- ß-Liquid scintillation counter: Beckman GmbH, Krefeld, Germany
- Electrophoresis machine: Gibco BRL, Life Technologies, Karlsruhe, Germany
- Fusion Solo Vilber Lourmat: PEQLAB Biotechnologie GmbH, Erlangen, Germany
- Gel holder cassette: BioRad Laboratories, Segrate, Italy

- Microscope Leitz DMRB with Digital Camera Leica DC300 and Leica IM-Software: Leica Microsystems GmbH, Wetzlar, Germany
- Microtome RM2125 RT: Leica Biosystem GmbH, Nussloch, Germany
- Pipettes: 10 µl, 20 µl, 100 µl and 1000 µl: Eppendorf AG, Hamburg, Germany
- pH-Meter: MAGV Laborbedarf + Laborgeräte, Rabenau-Londorf, Germany
- Scale: Mettler Toledo GmbH, Greifensee, Switzerland
- Sampler (10 µl, 100 µl, 1000 µl): Eppendorf AG, Hamburg, Germany
- Vacuum pump type No 035.1.2 AN. 18, Nr: 472678: KNF Neuber, Freiburg, Germany
- Vortexer Heidolph REAX control: purchased from MAGV Laborbedarf, Rabenau-Londorf, Germany
- Vortex-Evaporator Micro Dancer: Zinsser Analytic GmbH, Frankfurt, Germany
- Water bath TypeWB-24: Medax Nagel, KG Kiel, Germany

4. Results

4.1 Correlations between measurements of steroid concentrations (testosterone, estrone sulfate) obtained from radioimmunoassay vs. liquid chromatography-tandem mass spectrometry

In order to assess the consistency between the well-established radioimmunological methods for the measurements of T and E1S and the corresponding newly developed LC-MS-MS methods, measurements of the above-mentioned steroids were performed in parallel using either methods, and correlation analyses were performed using the data obtained from the secretory profiles from unstimulated boars (n=6) and from boars stimulated with hCG (protocols 1 and 2; n=7). For T, correlation analysis yielded a highly significant correlation ($p<0.0001$) between the two methods with a correlation coefficient $r=0.936$ (Fig. 10). By evaluating the profiles of individual animals, consistency between the two methods was especially very high when concentrations were below a concentration of approximately 5 ng/ml (Fig. 11). In case of higher concentrations, in part diverging results were obtained with generally higher concentrations measured with LC-MS-MS in comparison to RIA (Fig. 10 and 11).

Correlation between measurements using RIA or LC-MS-MS for E1S was even higher than obtained for T (Fig. 12; $r=0.982$), and no considerable difference in consistency was observed in animals exhibiting low or high E1S concentrations (Fig. 13).

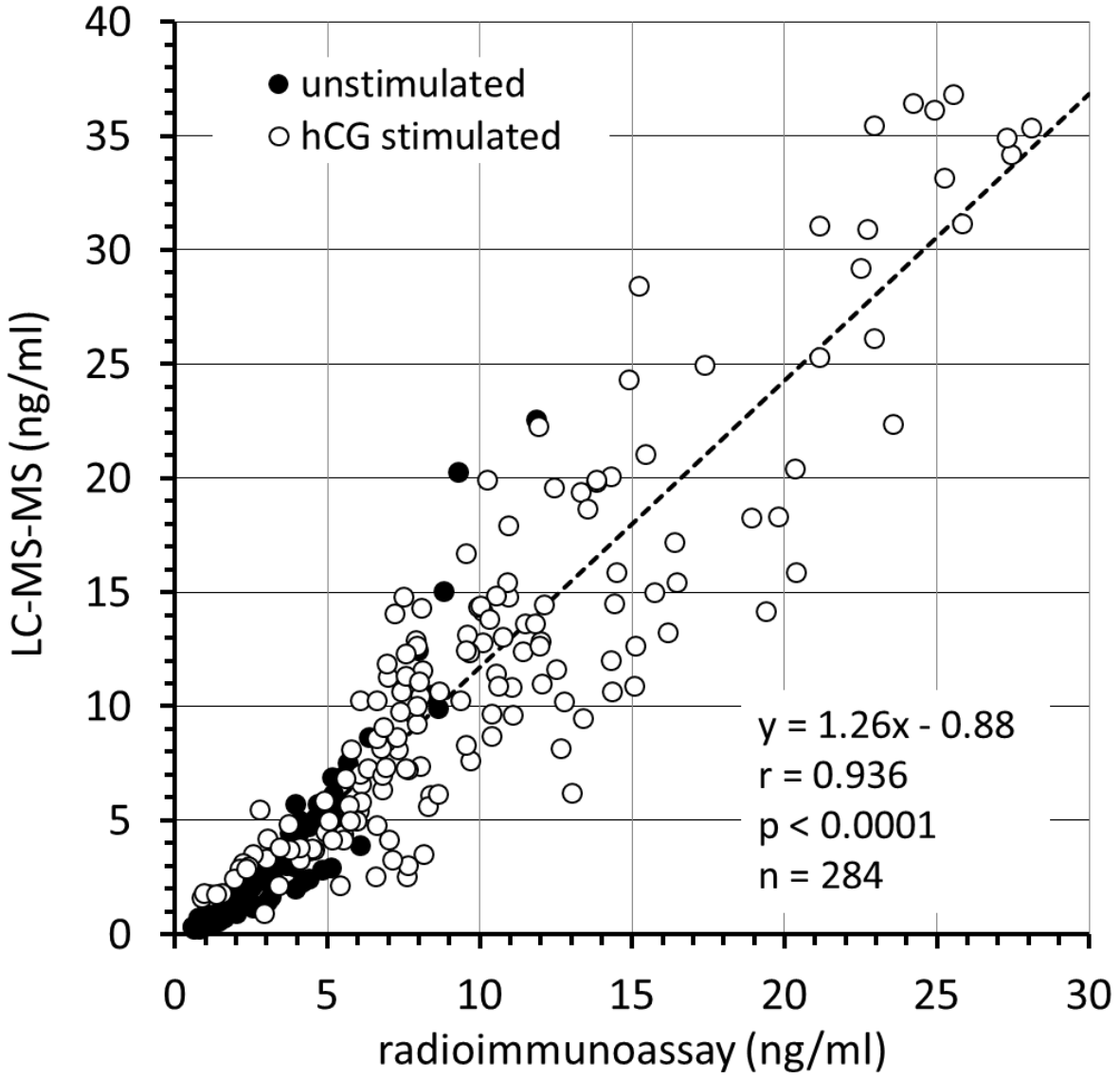


Figure 10. Comparative measurements of testosterone using radioimmunoassay and LC-MS-MS in unstimulated (closed symbols) and stimulated boars (open symbols): results from correlation analysis.

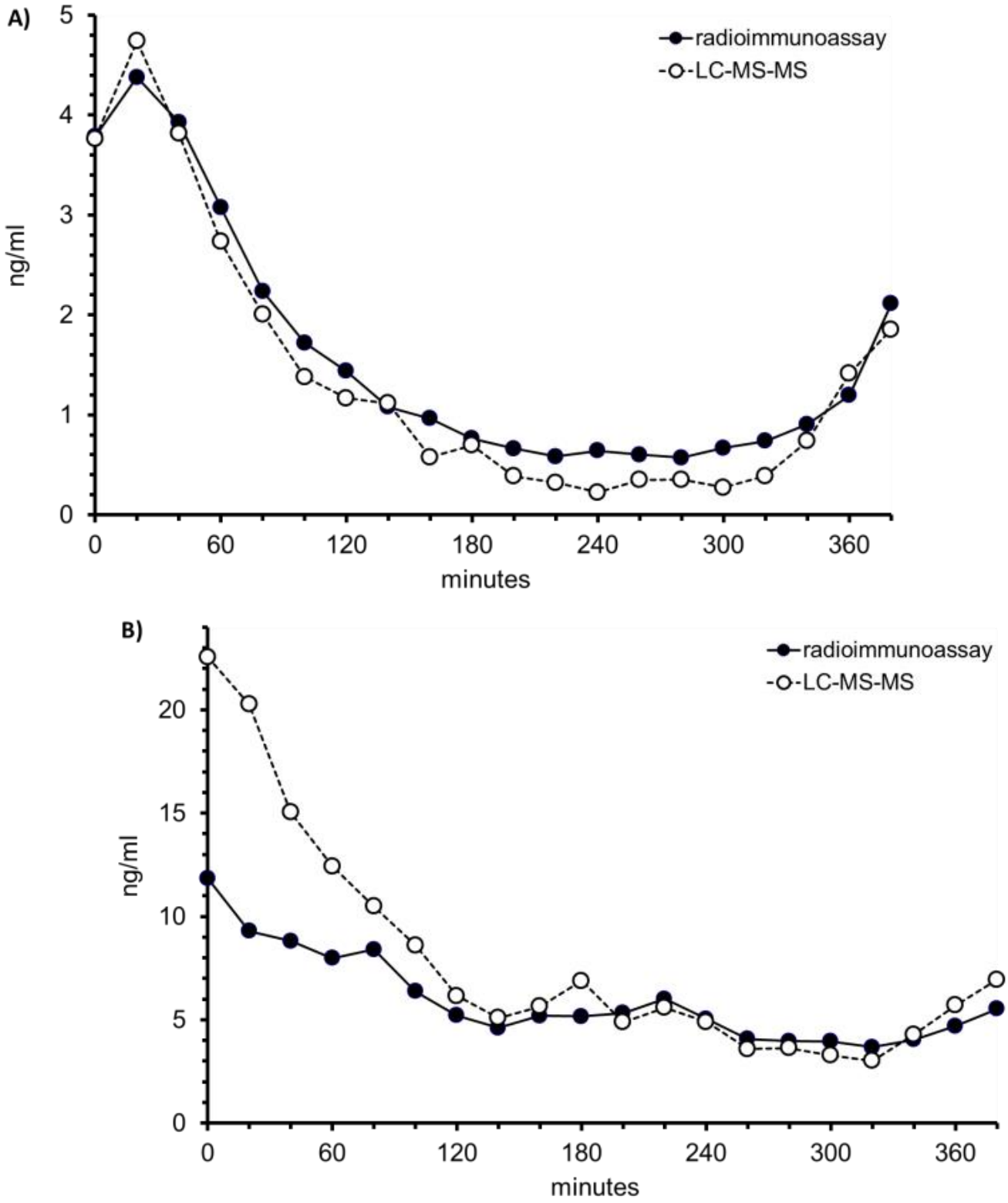


Figure 11. Testosterone concentrations measured in two unstimulated boars by radio-immunoassay and LC-MS-MS. A) Excellent consistency of the results in a boar exhibiting low testosterone concentrations. B) Diverging results in a boar in samples with high testosterone concentrations at the beginning of the sampling period.

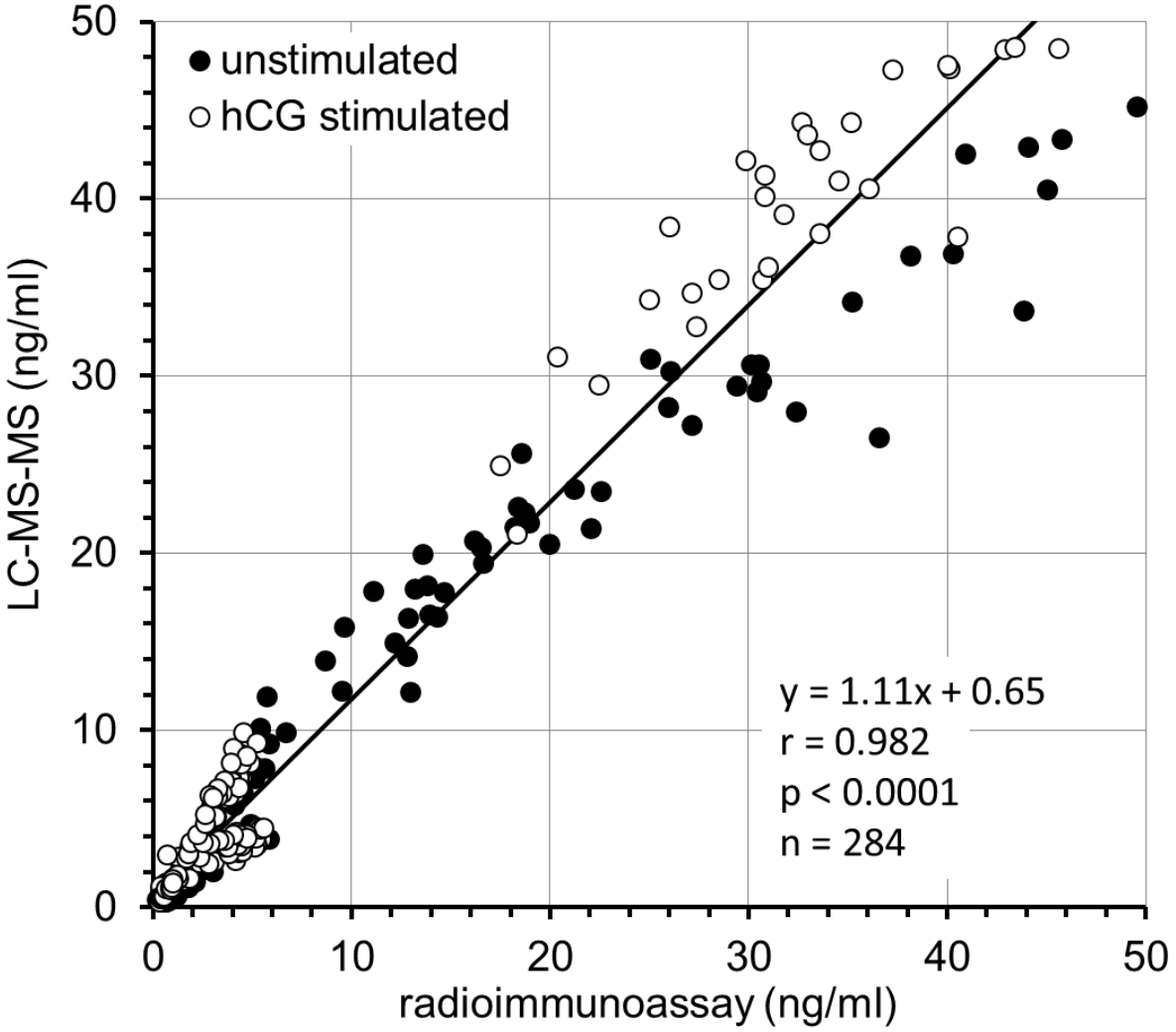


Figure 12. Comparative measurements of estrone sulfate using radioimmunoassay and LC-MS-MS in unstimulated (closed symbols) and stimulated boars (open symbols): results from correlation analysis.

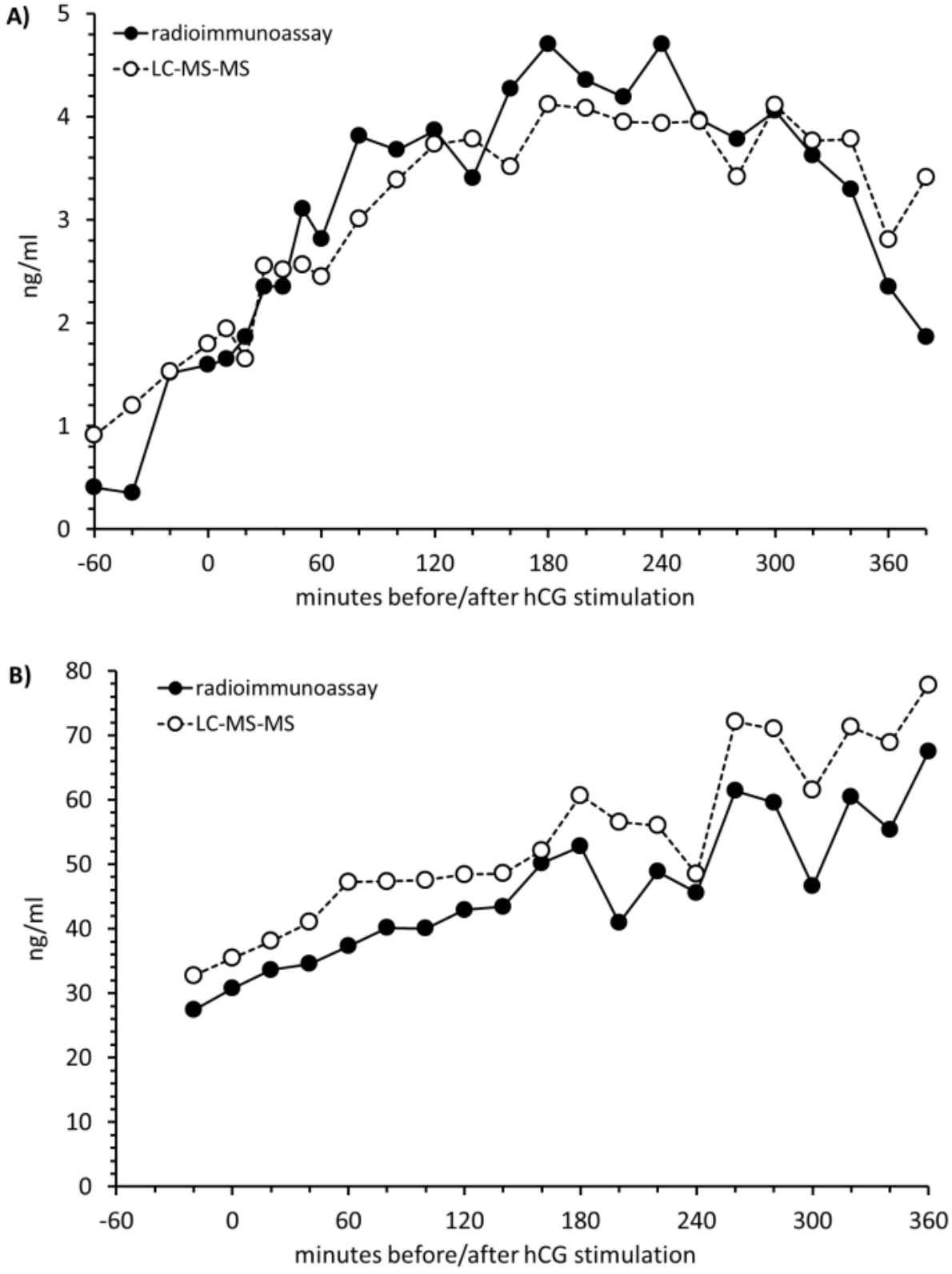


Figure 13. Estrone sulfate concentrations measured in two hCG-stimulated boars using radioimmunoassay and LC-MS-MS. As shown by these examples, consistent results were obtained in animals exhibiting low (A) or high (B) concentrations.

4.2 Secretory profiles of free and sulfonated steroids in unstimulated boars

Concentrations of all steroids assessed exhibited a high variability between individual animals (Fig. 14 [free steroids] and Fig. 15 [sulfonated steroids]). Highest concentrations were determined for DHEAS and E1S, ranging from 2.27-157.6 (median: 30.1) ng/ml and from 0.30-45.2 (median: 5.1) ng/ml, respectively. The concentrations measured varied between 0.12-9.24 (median: 1.21) ng/ml for P5S, 0.21-22.6 (median: 2.29) for T, 0.21-3.19 (median: 0.68) ng/ml for A4 and 0.030-0.525 (median: 0.080) ng/ml for E1. Concentrations of T and DHEAS showed a pronounced decrease during the sampling period, mostly followed by a weak increase at its end. A trend to a similar secretion pattern was also found for the other steroids assessed (E1S: five of six animals, 5/6; A4: 3/5; E1 and P5S: 3/6).

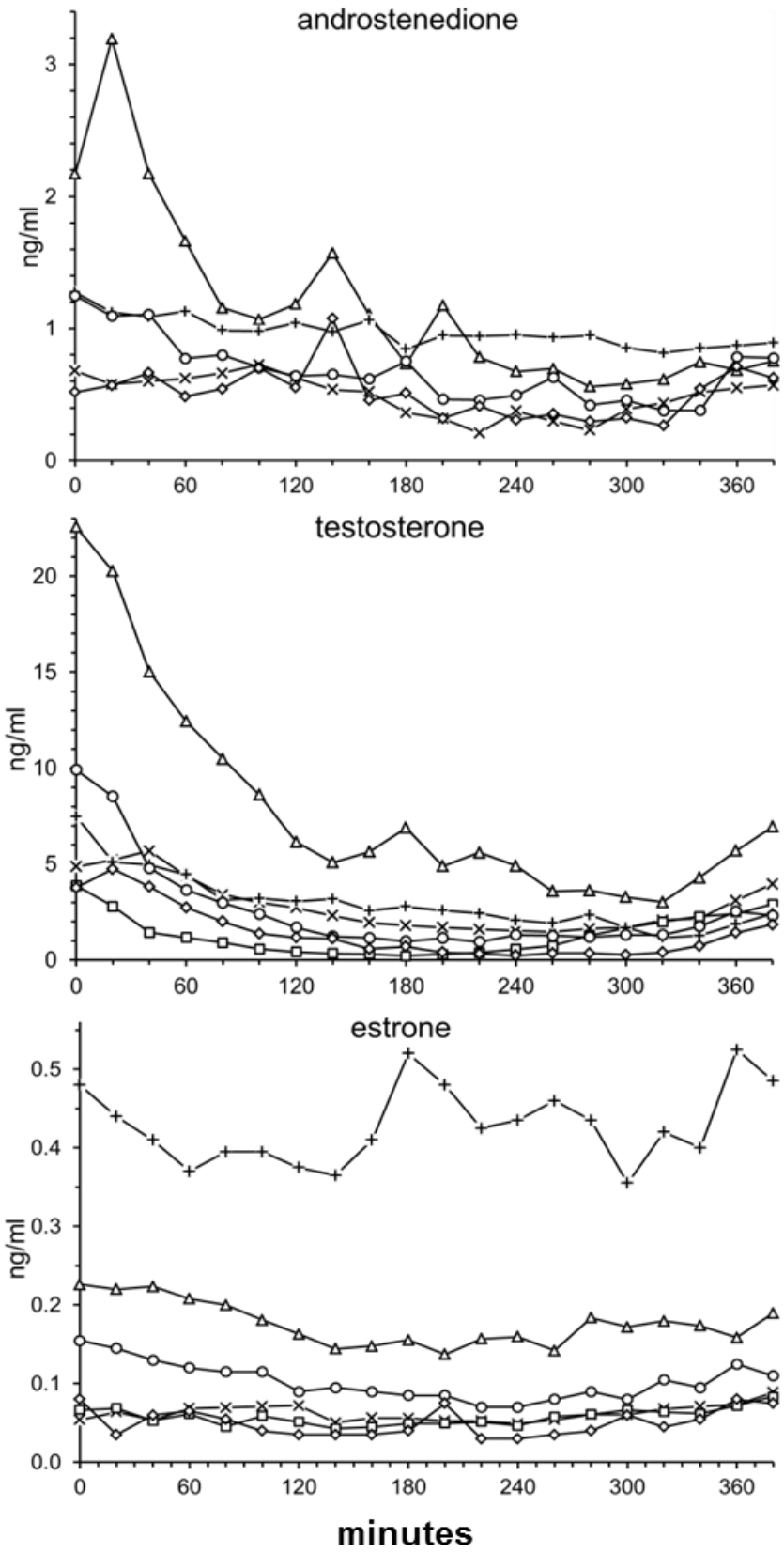


Figure 14. Concentrations of free steroids in six postpubertal 8-11 month old boars. Blood sampling in 20 min intervals from the jugular vein was started between 9:00-9:45 a.m. In the graphs, identical symbols are used for individual animals.

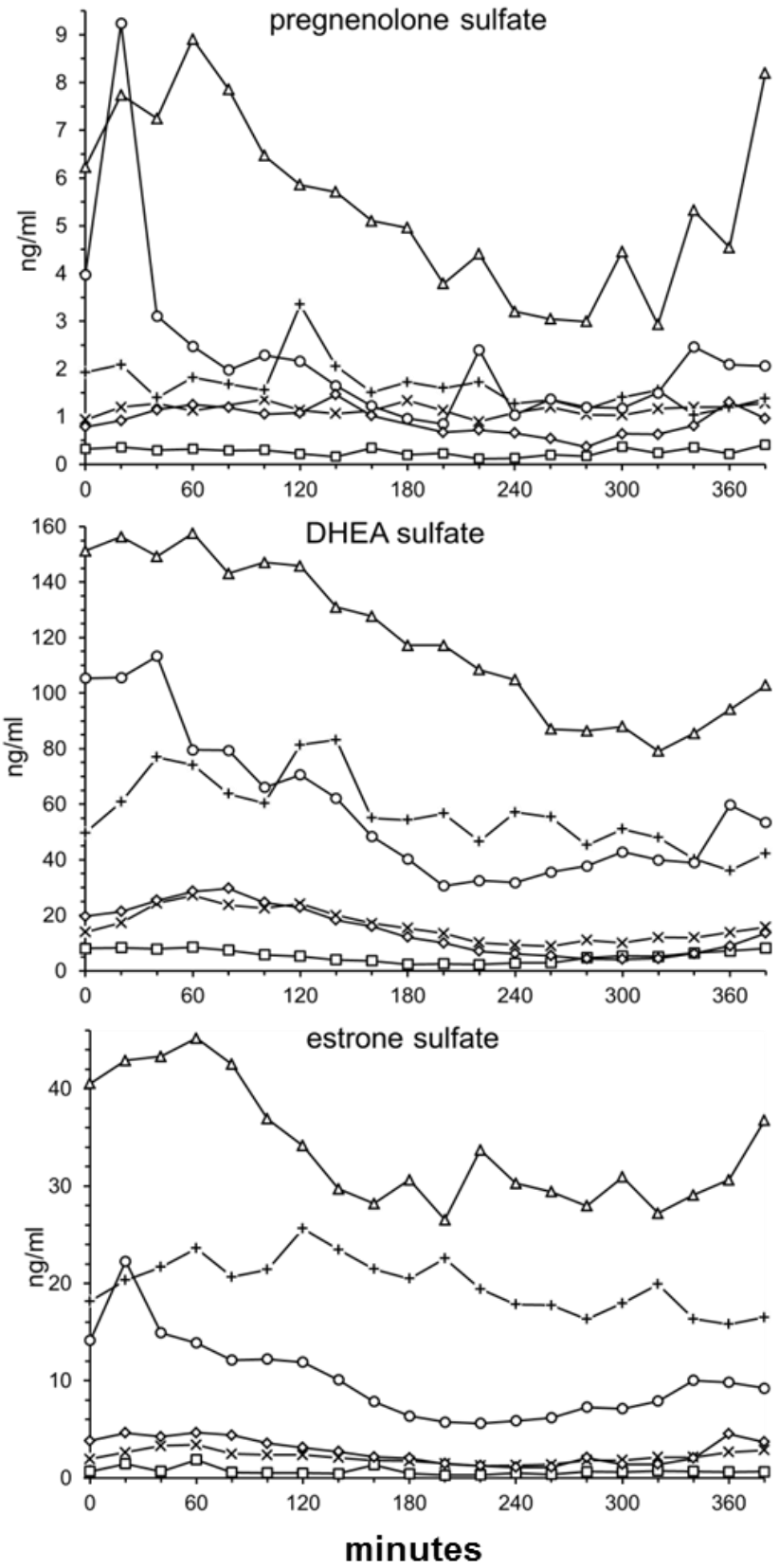
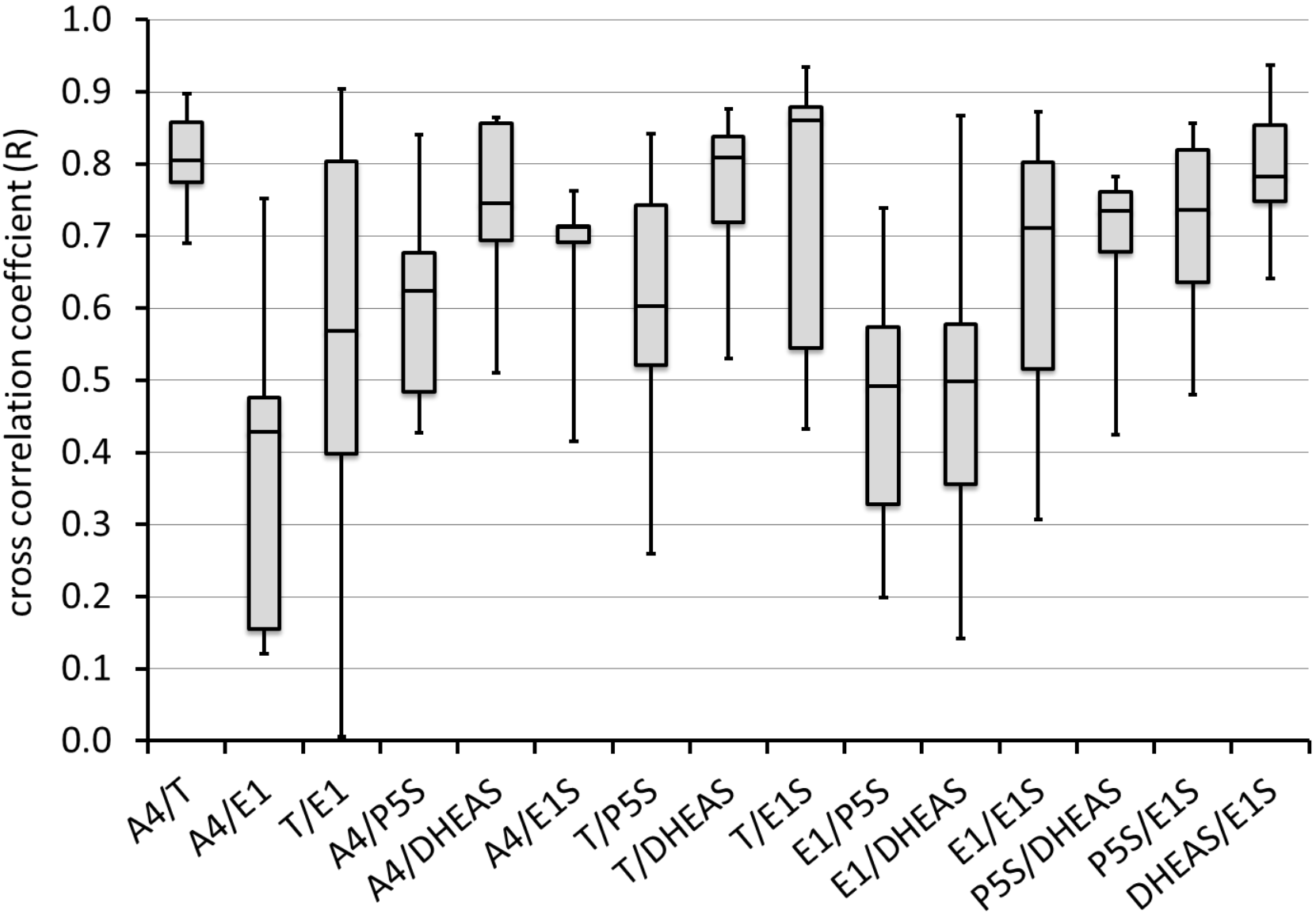


Figure 15. Concentrations of sulfonated steroids in six postpubertal 8-11 month old boars. Blood sampling in 20 min intervals from the jugular vein was started between 9:00-9:45 a.m. In the graphs, identical symbols are used for individual animals.

Correlation analysis yielded high coefficients of correlation (r) for all pairs of steroids tested but not in all animals, as indicated in Fig. 16. In all animals the level of significance ($p<0.05$) was reached for the following correlations: A4/T, A4/DHEAS, T/DHEAS, P5S/DHEAS, P5S/E1S and DHEAS/E1S. For the remaining correlations, the level of significance was missed in one or two animals. The mean coefficient of correlation (calculated as median) was relatively low for the correlations A4/E1 (0.428), E1/P5S (0.491) and E1/DHEAS (0.499) but was especially high between sulfonated steroids (P5S/DHEAS: 0.735, P5S/E1S: 0.735, DHEAS/E1S: 0.782) and for the correlations E1/E1S (0.711), T/A4 (0.804), T/DHEAS (0.809 and T/E1S (0.861). The variability of r was especially low for the correlations A4/T (0.690-0.897) and DHEAS/E1S (0.641-0.937).

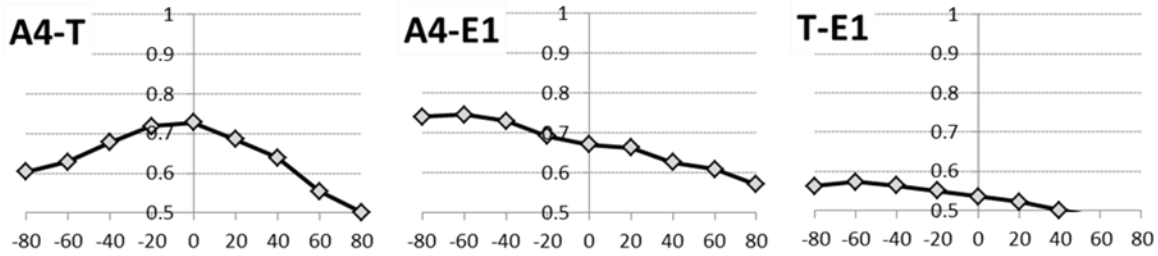
Figure 16 (next page). Results from pairwise correlation analyses (cross correlation between time series) for a linear correlation between the logarithms of the steroid concentrations assessed in six unstimulated boars (UB1-6), based on measurements in 20 blood samples collected in 20 min intervals. A4 = androstenedione, T = testosterone, E1 = estrone, E1S = estrone sulfate, DHEAS = dehydroepiandrosterone sulfate, P5S = pregnenolone sulfate.


cross correlation coeffcient (R)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A4/T
A4/E1
T/E1
A4/P5S
A4/DHEAS
A4/E1S
T/P5S
T/DHEAS
T/E1S
E1/P5S
E1/DHEAS
E1/E1S
P5S/DHEAS
P5S/E1S
DHEAS/E1S

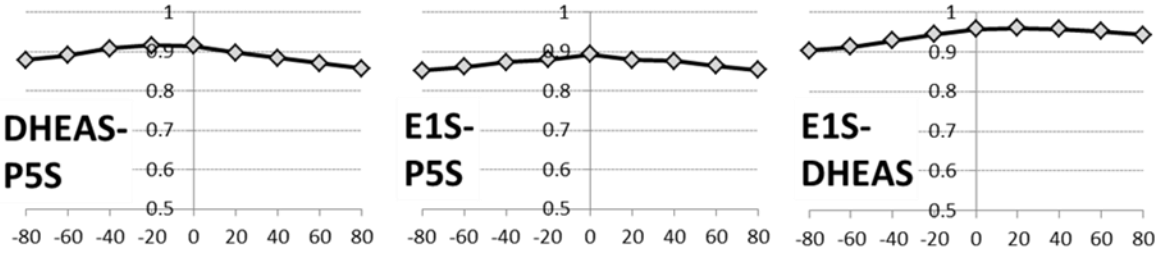
When shifting the secretory profiles pairwise relative to one another in both directions (from -80 min to +80 min) prior to regression analysis (biserial correlation function) using pooled data from all unstimulated animals, r clearly decreased for the correlations A4/T and A4/P5S, consistent with a simultaneous secretion for these pairs of steroids, respectively, based on a resolution in time of 20 min (Fig. 17). For the pairs A4/E1, T/E1, DHEAS/T and DHEAS/E1, r increased noticeably when the profiles where shifted relative to one another in one of the two directions. The direction of the shifting leading to an increase of r indicates that secretion of E1 precedes the secretion of A4 and T, while T and E1 precede the secretion of DHEAS. For the remaining pairs of steroids tested, only minor changes of r were observed. For biserial correlation functions calculated in individual unstimulated boars, see chapter 9.1.1 (appendix).

Figure 17 (next page). Biserial correlation functions calculated from pooled data of six unstimulated boars in order to demonstrate pairwise cross correlation in the presence of phase shiftings (time lag) between the steroid profiles. The graphs show the biserial correlation coefficient (r) (y-axis) as a function of the time shift (x-axis). A maximum at the point of origin indicates synchronous secretion, whereas a distinct maximum of r following shifting to either side suggests that secretion of one of the steroids determined is ahead of the other one (e.g. a maximum in the right side of the diagram of the steroid named fist would indicate that the profile of this steroid is ahead the other one; see text for more detailed information on the statistical evaluation procedure). A4 - androstenedione, T - testosterone, E1 - estrone, P5S - pregnenolone sulfate, DHEAS - dehydroepiandrosterone sulfate, E1S - estrone sulfate.

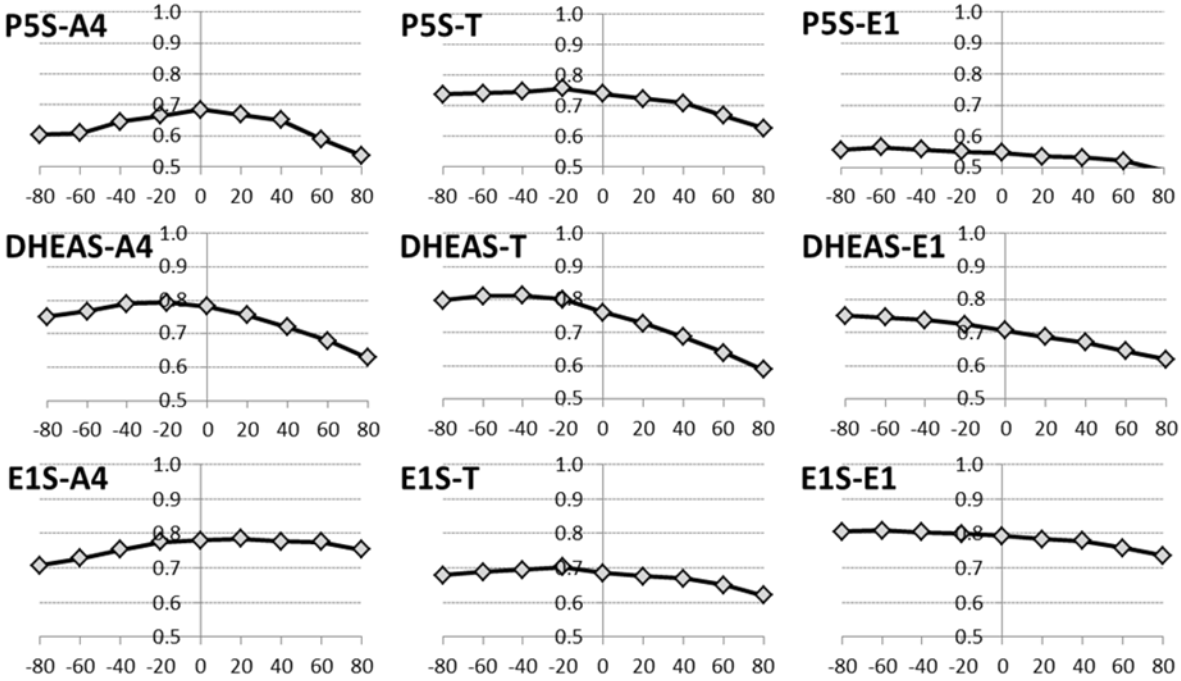
Correlations between free steroids



Correlations between sulfonated steroids



Correlations between sulfonated and free steroids

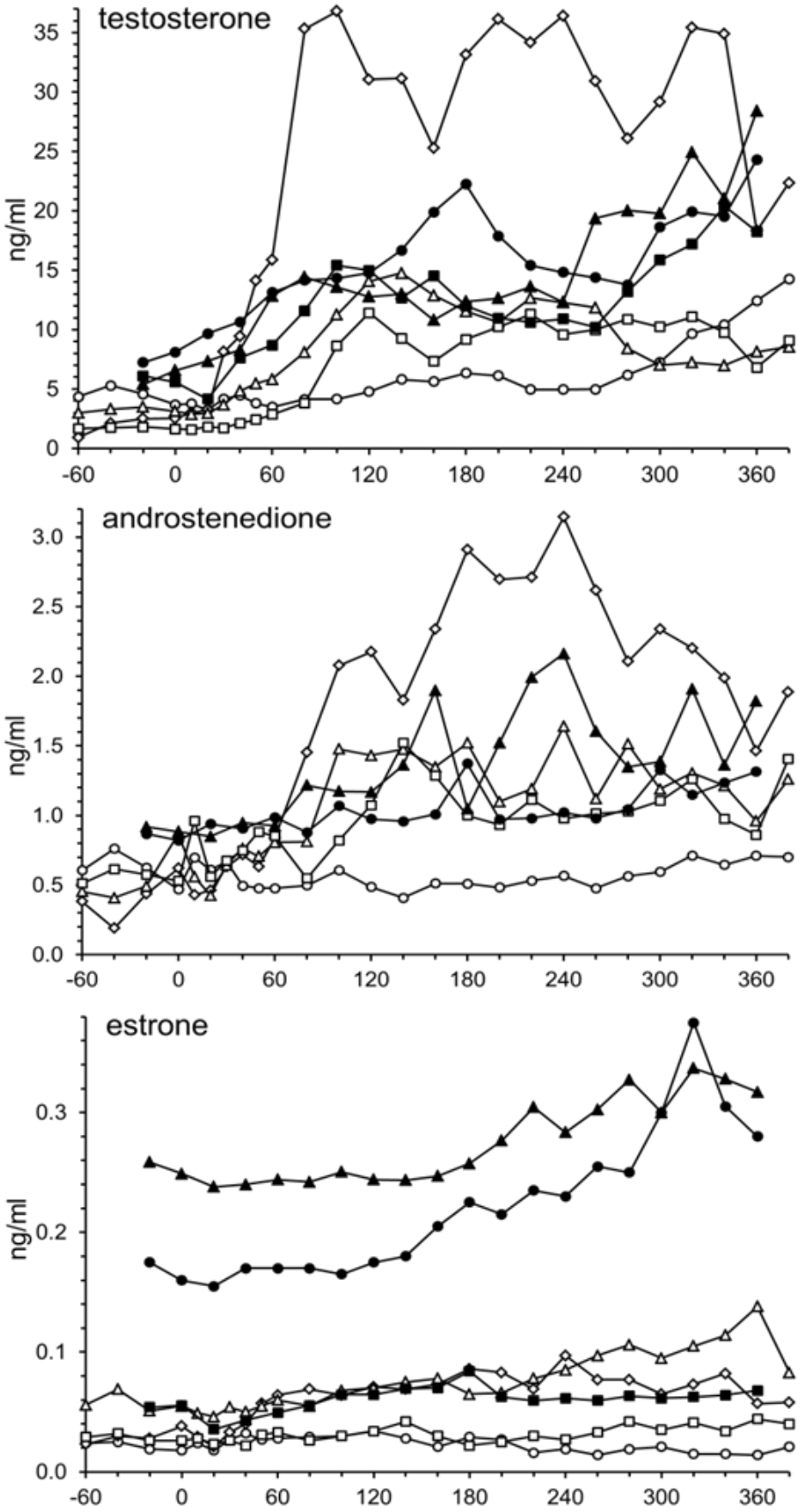


time lag (min) for the calculation of each biserial correlation coefficient

4.3 Secretory profiles of free and sulfonated steroids in stimulated boars

For all steroids measured, the prestimulatory levels and the profiles after hCG application exhibited a high variability between individual animals (Fig. 18 [free steroids] and Fig. 19 [sulfonated steroids]). Basically, application of 1500 I.U. hCG had a stimulatory effect on all steroids assessed but not in all animals. The maximum relative stimulatory effect – calculated as the maximum poststimulatory concentration relative to the mean of the prestimulatory values (expressed as percent) was highest for P5S (median: 518%; range: 256-1210%), followed by T (458%; 317-1823%), DHEAS (382%; 247-1245%), A4 (256%; 115-773%), E1S (244%, 157-613%) and E1 (158%; 133-326%). In the four animals sampled according to protocol 2 (SB4-7), arithmetic mean ($\overline{x}$) and standard deviation (SD) were calculated from the four prestimulatory samples, and a stimulatory effect of hCG application was defined as the occurrence of concentrations higher than $\overline{x}$+3xSD for at least one hour during the poststimulatory period. According to this definition, a poststimulatory increase was found in all four boars for P5S, DHEAS, E1S and T but only in three of four animals for A4 and E1. In animals with a detectable increase, the concentrations exceeded the threshold level at 30 min (median) for P5S, DHEAS and E1S (range: P5S: 30-80 min., DHEAS: 30-50 min., E1S: 30-100 min.). For A4, T and E1, the increase was observed at 80 (40-100), 40 (30-280) and 240 (50-320) min., respectively.

Figure 18 (next page). Concentrations of free steroids in seven postpubertal 9-15 month old boars before and after hCG application (1500 I.U.). In three animals (SB1-3) hCG was administered after two prestimulatory samples and sample collection was continued in 20 min intervals (protocol 1, closed symbols). In another four animals (SB4-7), four prestimulatory samples were taken, and during the first hour after hCG application the sampling interval was reduced to 10 min (protocol 2, open symbols). Blood sampling from the jugular vein was started between 9:00-9:45 a.m. In the graphs, identical symbols are used for individual animals


testosterone
ng/ml
androstenedione
ng/ml
estrone
ng/ml
minutes before/after stimulation with 1500 I.U. hCG

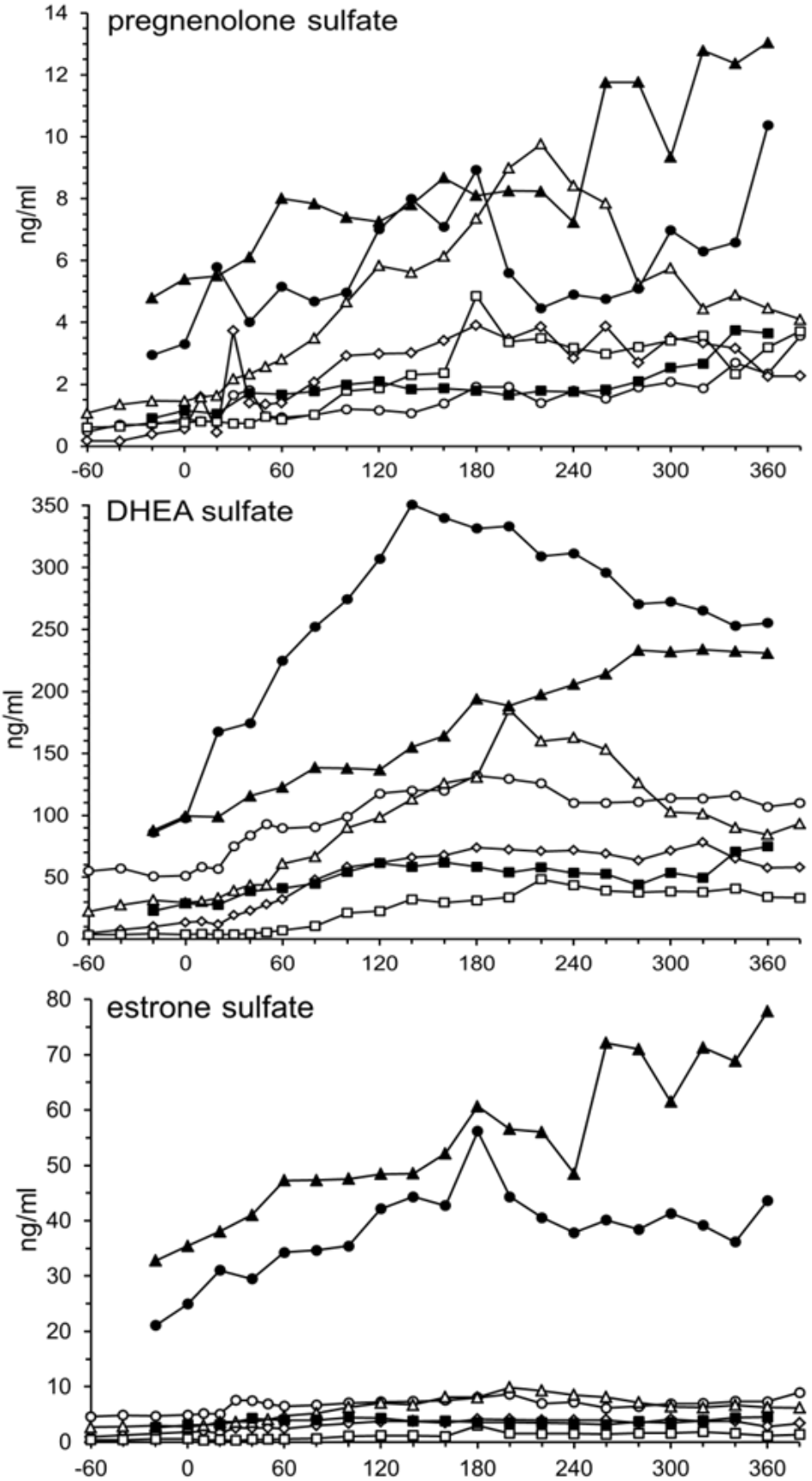
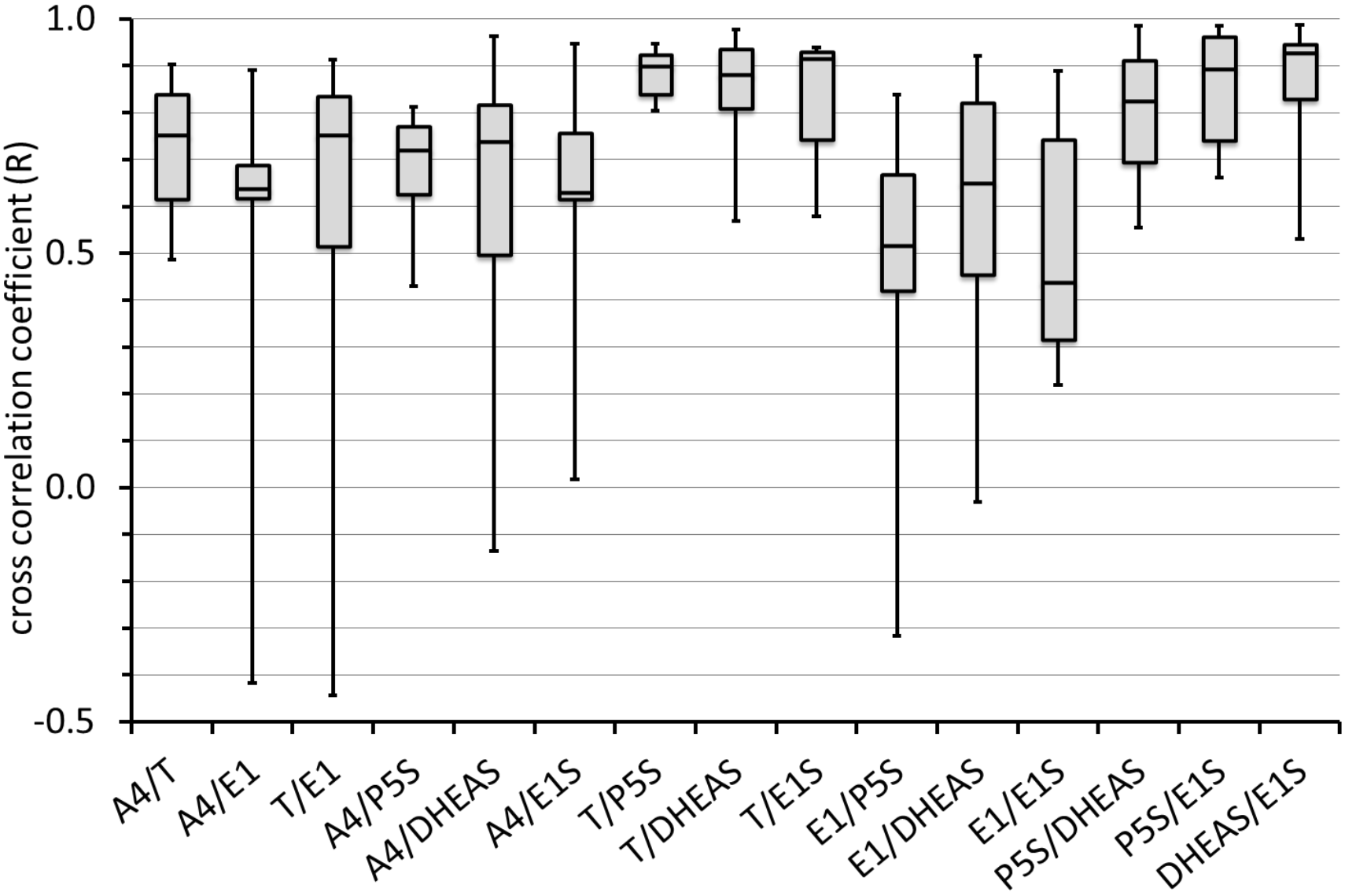

pregnenolone sulfate
ng/ml
0
2
4
6
8
10
12
14
-60
0
60
120
180
240
300
360
DHEA sulfate
ng/ml
0
50
100
150
200
250
300
350
-60
0
60
120
180
240
300
360
estrone sulfate
ng/ml
0
10
20
30
40
50
60
70
80
-60
0
60
120
180
240
300
360
minutes before/after stimulation with 1500 I.U. hCG

Figure 19 (previous page)**.** Concentrations of sulfonated steroids in seven postpubertal 9-15 month old boars before and after hCG application (1500 I.U.). In three animals hCG was administered after two prestimulatory samples and sample collection was continued in 20 min intervals (protocol 1, closed symbols). In another four animals, four prestimulatory samples were taken, and during the first hour after hCG application the sampling interval was reduced to 10 min (protocol 2, open symbols). Blood sampling from the jugular vein was started between 9:00-9:45 a.m. In the graphs, identical symbols are used for individual animals.

Results from correlation analyses applied to poststimulatory data from individual animals (Fig. 20) basically yielded similar results compared to unstimulated animals. Again, high mean r-values were obtained for the correlations A4/T (median: 0.750; range: 0.481-0.903, T/P5S (0.897; 0.808-0.947), T/DHEAS (0.880; 0.568-0.977), T/E1S (0.913; 0.578-0.939), P5S/DHEAS (0.822; 0.555-0.985), P5S/E1S (0.891; 0.662-0.985) and DHEAS/E1S (0.926; 0.530-0.988). However, different from unstimulated boars, in one stimulated boar significantly negative correlations were obtained for A4/E1 ($r=-0.417$, $p=0.048$) and T/E1 ($r=-0.444$, $p=0.034$)

Figure 20 (next page). Results from pairwise correlation analyses (cross correlation between time series) for a linear correlation between the logarithms of the steroid concentrations assessed in seven hCG-stimulated boars (SB1-7), based on measurements in poststimulatory samples. A4 - androstenedione, T - testosterone, E1 - estrone, P5S - pregnenolone sulfate, DHEAS - dehydroepiandrosterone sulfate, E1S - estrone sulfate.

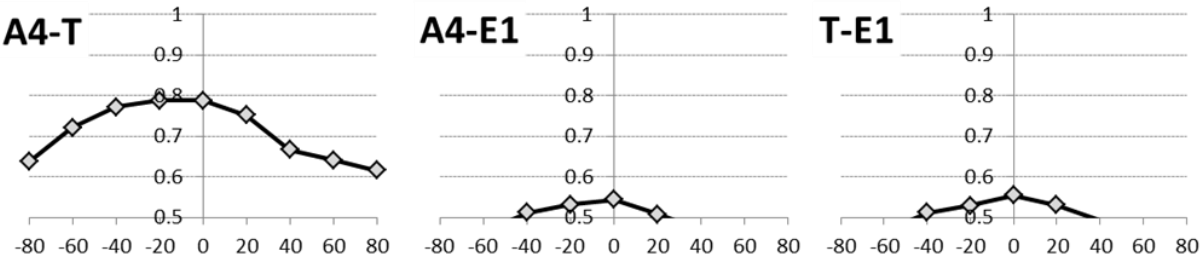
.


1.0
0.5
0.0
-0.5
cross correlation coefficient (R)
A4/T
A4/E1
T/E1
A4/P5S
A4/DHEAS
A4/E1S
T/P5S
T/DHEAS
T/E1S
E1/P5S
E1/DHEAS
E1/E1S
P5S/DHEAS
P5S/E1S
DHEAS/E1S

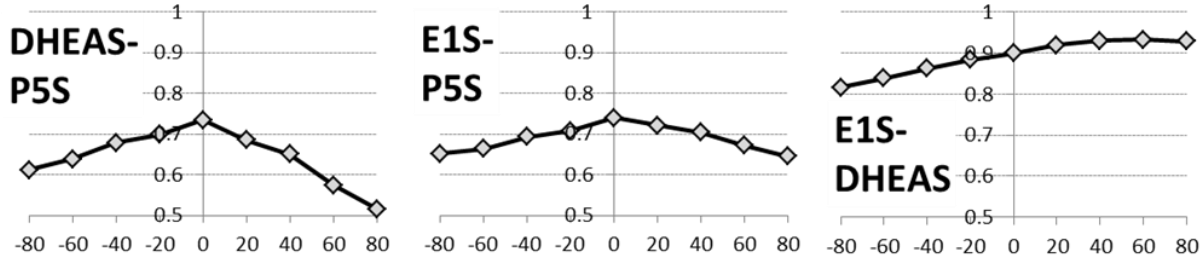
Stimulation with hCG abolished most of the above mentioned evidence for phase shifting obtained from unstimulated animals (see section 4.2) with the exception of the pair DHEAS/E1, where the biserial correlation function still indicates that secretion of E1 precedes the secretion of DHEAS. Moreover, the results obtained in stimulated boars for the pair E1S/DHEAS are consistent with the secretion of E1S preceding that of DHEAS. However, with the exception of the pairs P5S/E1 and E1S/E1, where r remained on a similar level over the range of phase shiftings tested, for the remaining pairs of steroids the results clearly point to a simultaneous secretion into the systemic circulation. For biserial correlation functions calculated in individual stimulated boars see chapter 9.1.2 (appendix).

Figure 21 (next page). Biserial correlation functions calculated from pooled data of seven hCG-stimulated boars in order to demonstrate pairwise cross correlation in the presence of phase shiftings (time lag) between the steroid profiles. The graphs show the biserial correlation coefficient (r) (y-axis) as a function of the time shift (x-axis). A maximum at the point of origin indicates synchronous secretion, whereas a distinct maximum of r following shifting to either side suggests that secretion of one of the steroids determined is ahead of the other one (e.g. a maximum in the right side of the diagram of the steroid named fist would indicate that the profile of this steroid is ahead the other one; see text for more detailed information on the statistical evaluation procedure). A4 - androstenedione, T - testosterone, E1 - estrone, P5S - pregnenolone sulfate, DHEAS – dehydroepiandrosterone sulfate, E1S - estrone sulfate.

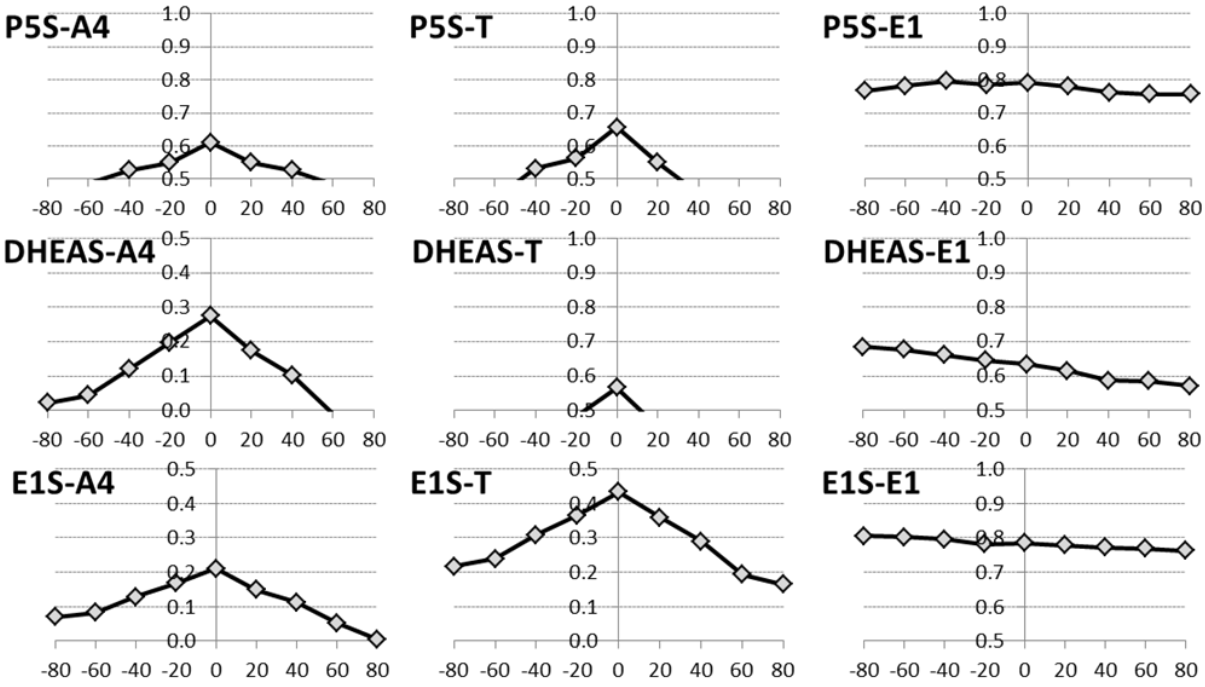
Correlations between free steroids



Correlations between sulfonated steroids



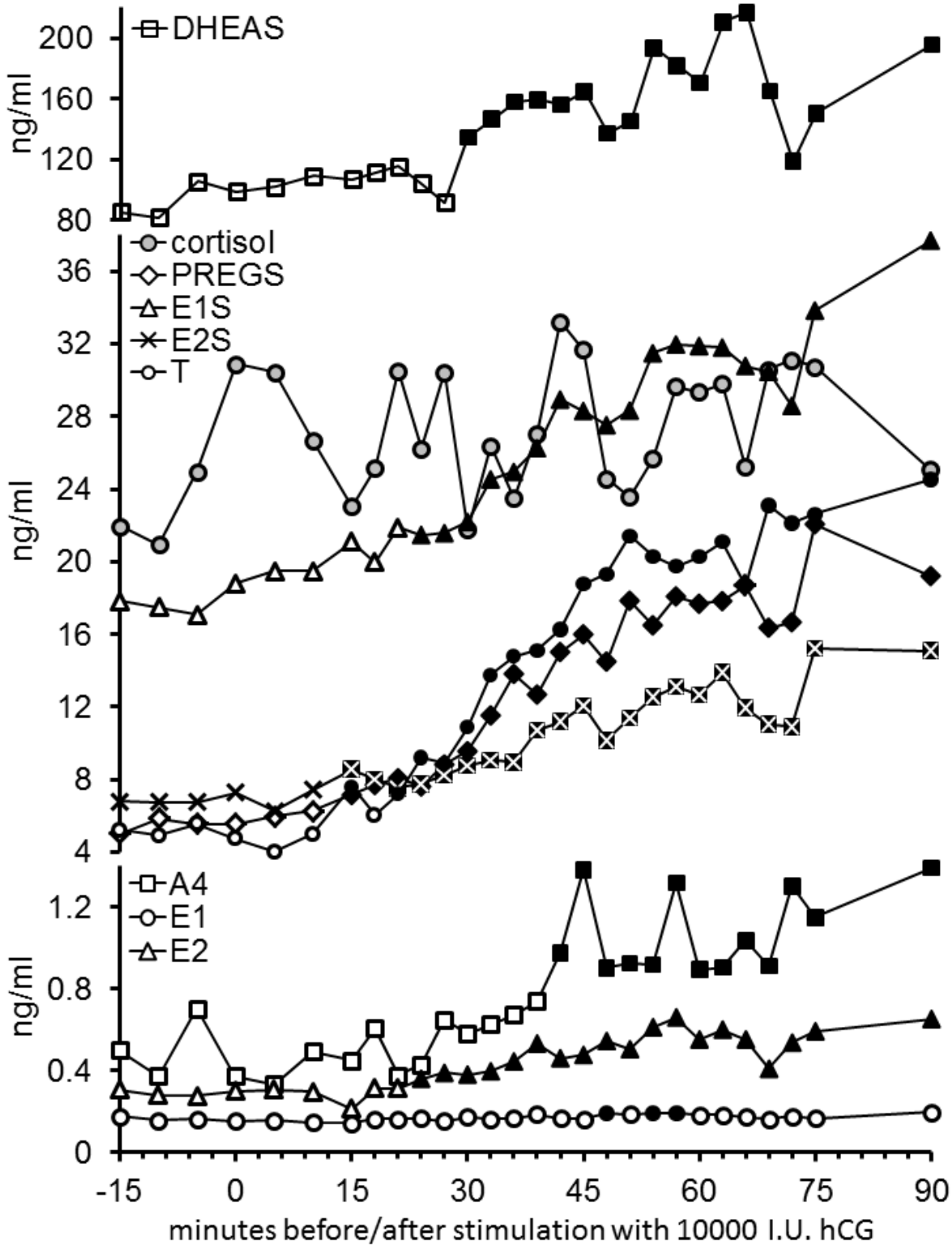
Correlations between sulfonated and free steroids



time lag (min) for the calculation of each biserial correlation coefficient

In order to exert maximum stimulation and to further increase the resolution in time, in a pilot experiment 10000 I.U. hCG were applied for stimulation and sampling was performed in 3-5 min. intervals during the poststimulatory period. Although the dosage of hCG was significantly higher in comparison to protocols 1 and 2 (1500 I.U.), the stimulatory effect of treatment on steroid concentrations during the sampling period was not noticeably different (Fig. 22). The maximum relative stimulatory effects in comparison to the mean prestimulatory level varied between 122% for E1 and 481% for T. The limit of $\bar{x}$+3SD of prestimulatory concentrations was first exceeded at 15 min for T, P5S, E1S and E2S, followed by E2 (24 min), DHEAS (30 min), A4 (42 min) and E1 (48 min).

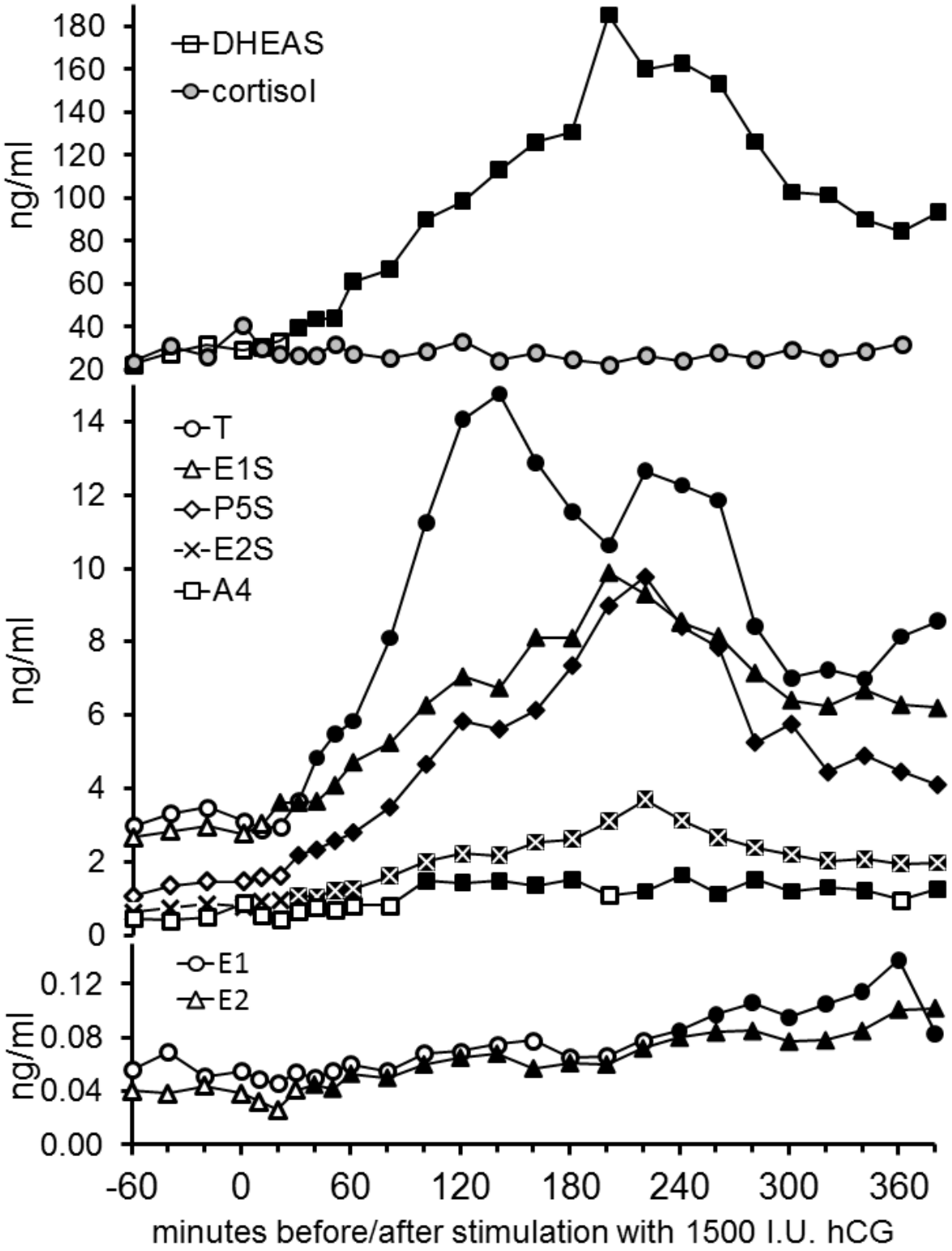
Figure 22 (next page). Concentrations of androstenedione (A4), estrone (E1), estradiol-17β (E2), testosterone (T), pregnenolone sulfate (P5S), dehydroepiandrosterone sulfate (DHEAS), estrone sulfate (E1S) and estradiol-17β-3-sulfate (E2S) in an 11.5 month old boar before and after stimulation with 10000 I.U. hCG. Sampling was in 5 min intervals from 15 minutes before until 15 min after hCG application, followed by sampling in 3 min intervals (protocol 3). Solid symbols indicate concentrations exceeding arithmetic mean plus three fold standard deviation calculated from the four prestimulatory samples. Cortisol was also measured to monitor adrenal activity during the sampling period.


DHEAS
ng/ml
200
160
120
80
cortisol
PREGS
E1S
E2S
T
ng/ml
36
32
28
24
20
16
12
8
4
A4
E1
E2
ng/ml
1.2
0.8
0.4
0
-15
0
15
30
45
60
75
90
minutes before/after stimulation with 10000 I.U. hCG

In boars SB6 (protocol 2, Fig. 23) and SB8 (protocol 3, Fig. 22) in addition to the set of steroids measured in all animals also E2 and E2S were measured. Prestimulatory concentrations of E2S were in the lower ng/ml range (SB6: 0.8±0.1, SB8: 6.9±0.2), and they were lower compared to E1S (ratio E1S/E2S: 3.5 and 2.6). E2S was clearly stimulated by hCG; the maximum relative stimulatory effect was 494% in SB6 and 221% in SB8. Mean prestimulatory estradiol-17ß concentrations were 40.0±2.5 pg/ml (SB6) and 290.0±12.2 pg/ml (SB8), the ratios E1/E2 were 0.6 and 1.4, respectively. In both animals hCG clearly stimulated the secretion of E2 (maximum relative stimulatory effect: 255% in SB6 and 223% in SB8). In both animals high correlation coefficients were found for E2/E1 (SB6: 0.90, SB8: 0.86), E2/E1S (0.73, 0.89), E2/E2S (0.75, 0.86) and E1S/E2S (0.99, 0.96).

Figure 23 (next page). Profiles of free and sulfonated steroids before and after hCG stimulation (1500 I.U.; protocol 2). In this animal (SB6) estradiol-17ß (E2) and estradiol-17ß-3-sulfate (E2S) were also measured. Solid symbols indicate concentrations exceeding arithmetic mean plus threefold standard deviation calculated from the four prestimulatory samples. Additionally, cortisol was included in the measurements to monitor adrenal activity during the sampling period.

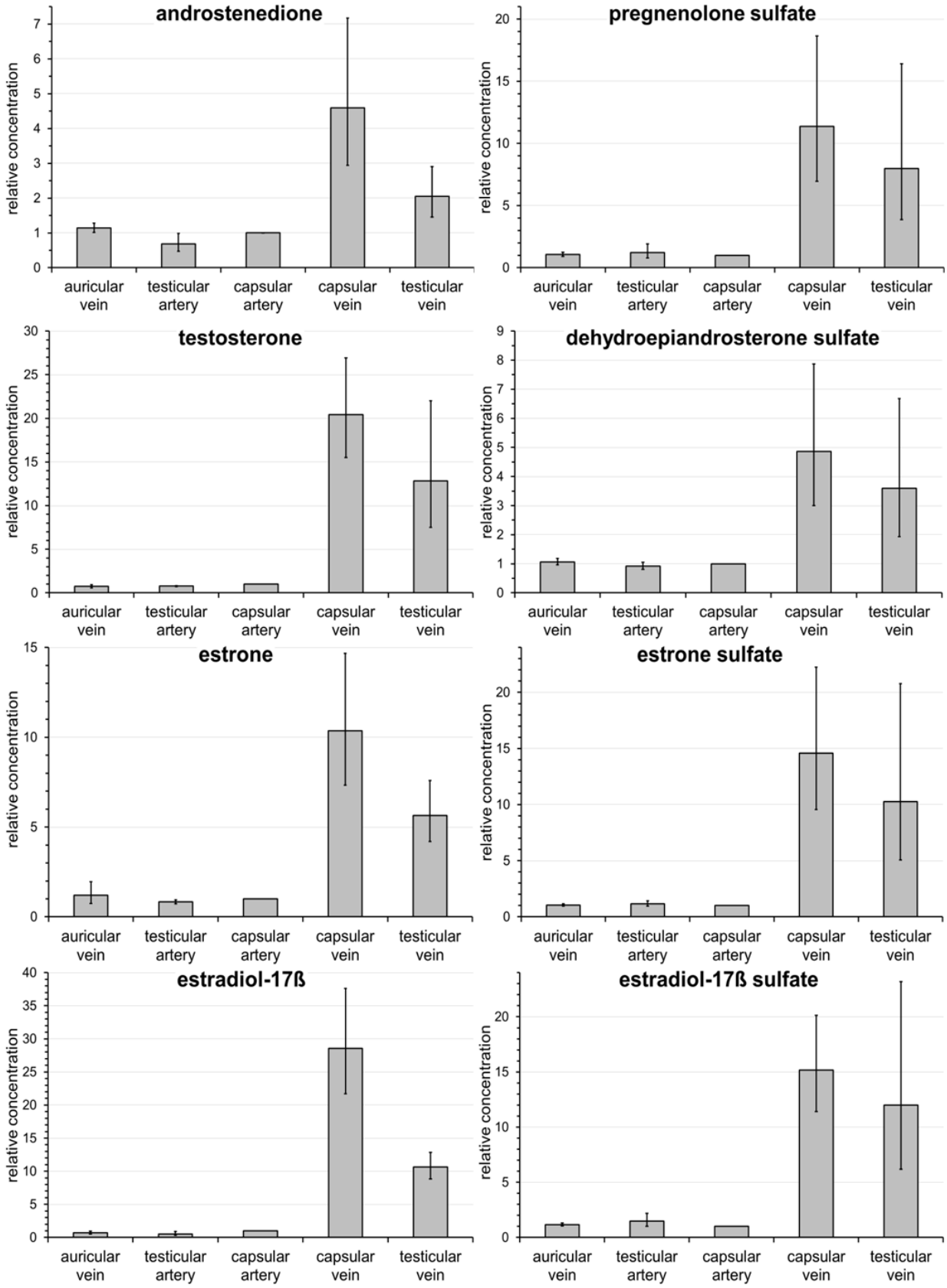
A4 - androstenedione, T - testosterone, E1 - estrone, P5S - pregnenolone sulfate, DHEAS - dehydroepiandrosterone sulfate, E1S - estrone sulfate.


DHEAS
cortisol
ng/ml
180
160
140
120
100
80
60
40
20
T
E1S
P5S
E2S
A4
ng/ml
14
12
10
8
6
4
2
0
E1
E2
ng/ml
0.12
0.08
0.04
0.00
-60
0
60
120
180
240
300
360
minutes before/after stimulation with 1500 I.U. hCG

4.4 Concentrations of free and sulfonated steroids in blood vessels of the testis and spermatic cord

Four animals were included in this part of the study. As with peripheral profiles, also the absolute concentrations in local blood vessels of the testicular-epididymal compartment showed a high variability between individual animals. However, for all steroids assessed including E2 and E2S the proportion of concentrations between the various blood vessels tested was rather similar (Fig. 24). The concentrations in the efferent blood vessels (capsular veins, testicular vein) were clearly higher compared to the afferent blood vessels (testicular artery, capsular artery) and the peripheral circulation (auricular vein), and for all steroids assessed there was a tendency of higher concentrations in the capsular veins compared to the testicular vein (Fig. 24). No substantial difference was found between steroid concentrations measured in the testicular artery and the capsular artery, which were similar to the levels observed in the auricular vein. Absolute concentrations (in ng/ml; geometric mean x dispersion factor$^{\pm 1}$) in the capsular artery were 1.11 x 1.72$^{\pm 1}$ for A4, 3.80 x 2.38$^{\pm 1}$ for T, 0.11 x 2.46$^{\pm 1}$ for E1, 0.13 x 2.39$^{\pm 1}$ for E2, 1.24 x 2.74$^{\pm 1}$ for P5S, 20.8 x 3.27$^{\pm 1}$ for DHEAS, 3.86 x 4.40$^{\pm 1}$ for E1S and 0.93 x 4.20$^{\pm 1}$ for E2S.

Figure 24 (next page). Concentrations of free and sulfonated steroids measured in the systemic circulation (auricular vein) and in local blood vessels of the testicular-epididymal compartment of four boars (for exact localizations of sampling see Fig. 6). The results are expressed as multiples of the value measured in the terminal branch of the testicular artery running on the testicular surface ("capsular artery") and are presented as geometric mean and scatter range.


androstenedione
pregnenolone sulfate
testosterone
dehydroepiandrosterone sulfate
estrone
estrone sulfate
estradiol-17ß
estradiol-17ß sulfate
relative concentration
auricular vein
testicular artery
capsular artery
capsular vein
testicular vein

4.5 Steroid sulfatase activity in the testicular-epididymal compartment

Among the various microsomal fractions prepared from tissues of the testicular-epididymal compartment from three postpubertal boars, a substantial hydrolysis of E1S was consistently found in the testis (conversion rates after 60 min: 44.9-59.1%; Fig. 25). In general, hydrolysis of E1S was also detectable in other tissue samples assessed but was clearly lower. However, in two of the three animals, a high conversion rate was also obtained in one of the epididymal samples, respectively (EH2: 26.3%, EB2: 42.0% after 60 min).

In comparative incubations of 200 µg testicular microsomal protein from three boars with 10 pmol of different sulfonated steroids as substrates - E1S, DHEAS and P5S – after an incubation time of 2 hours a substantial hydrolysis was only found for E1S (62.4±8.2%). The corresponding conversion rates for DHEAS (7.8±2.9%) and P5S (8.5±1.7%) were considerably lower.

To obtain basic information whether different steroid sulfates compete against one another for the same sulfatase enzyme, hydrolysis of 10 pmol of a certain steroid sulfate by 200 µg testicular microsomal protein from three boars was measured in the presence of increasing amounts of another steroid sulfate. Hydrolysis of DHEAS or P5S was significantly reduced in the presence of E1S in a dose-dependent manner ($P<0.001$; Fig.26). However, under the experimental conditions used, no significant effect on the hydrolysis of E1S was observed in the presence of DHEAS or P5S (Fig. 27).

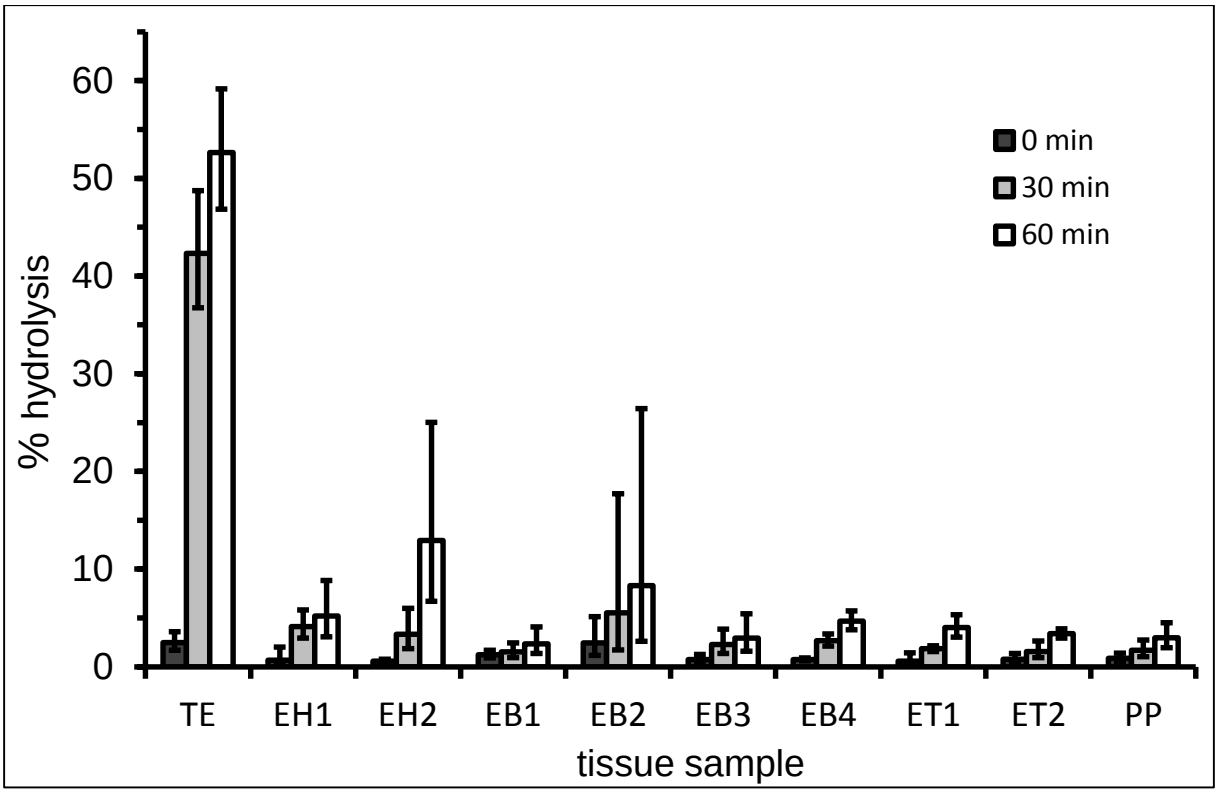


Figure 25. Hydrolysis of estrone sulfate by 200 µg microsomal protein prepared from different tissues of the testicular-epididymal compartment from three postpubertal boars. The results are presented as geometric mean x deviation factor$^{\pm 1}$. The amount of substrate was 10 pmol/tube.

TE: testis; EH1-2: proximal/distal segment of epididymal head; EB1-4: segments of the epididymal body (from proximal to distal); ET1-2: proximal/distal segment of epididymal tail; PP: pampiniform plexus.

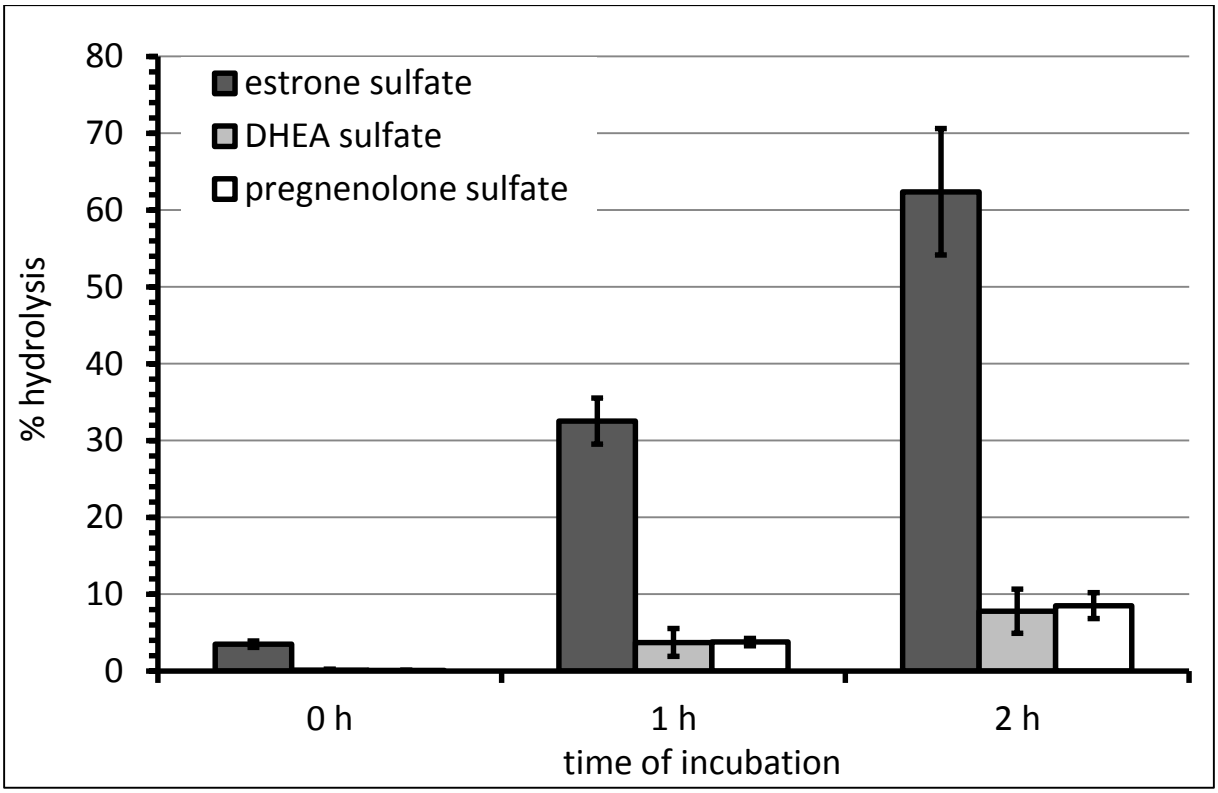


Figure 26. Hydrolysis of steroid sulfates by 200 µg microsomal protein prepared from the testes from three postpubertal boars. The results are presented as $\bar{x}$±SD. The amount of substrate was 10 pmol/tube.

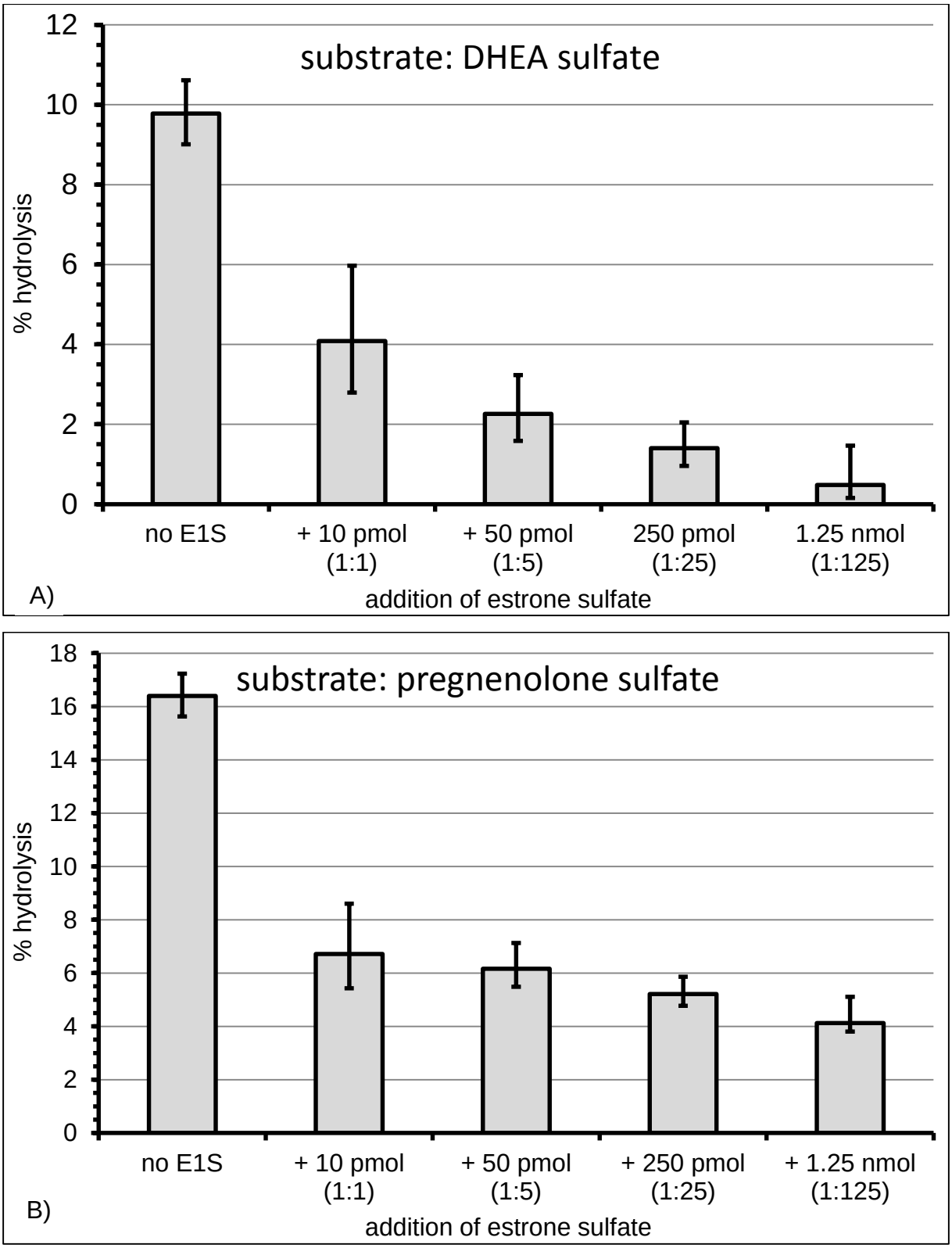


Figure 27. Hydrolysis of 10 pmol (A) DHEA sulfate or (B) pregnenolone sulfate by 200 µg microsomal protein from porcine testis (n=3) in the presence of increasing amounts of estrone sulfate. Time of incubation was 1 h.

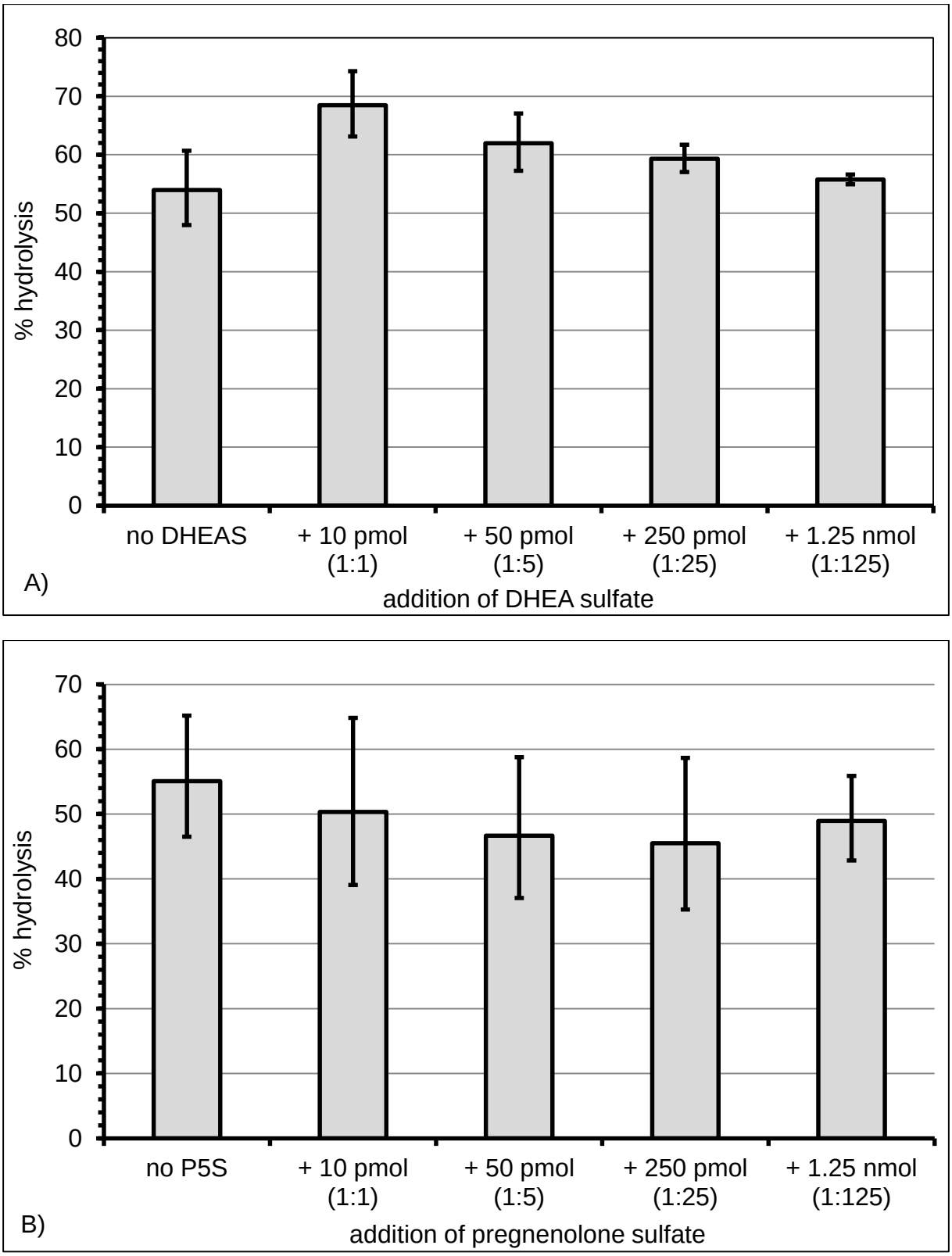


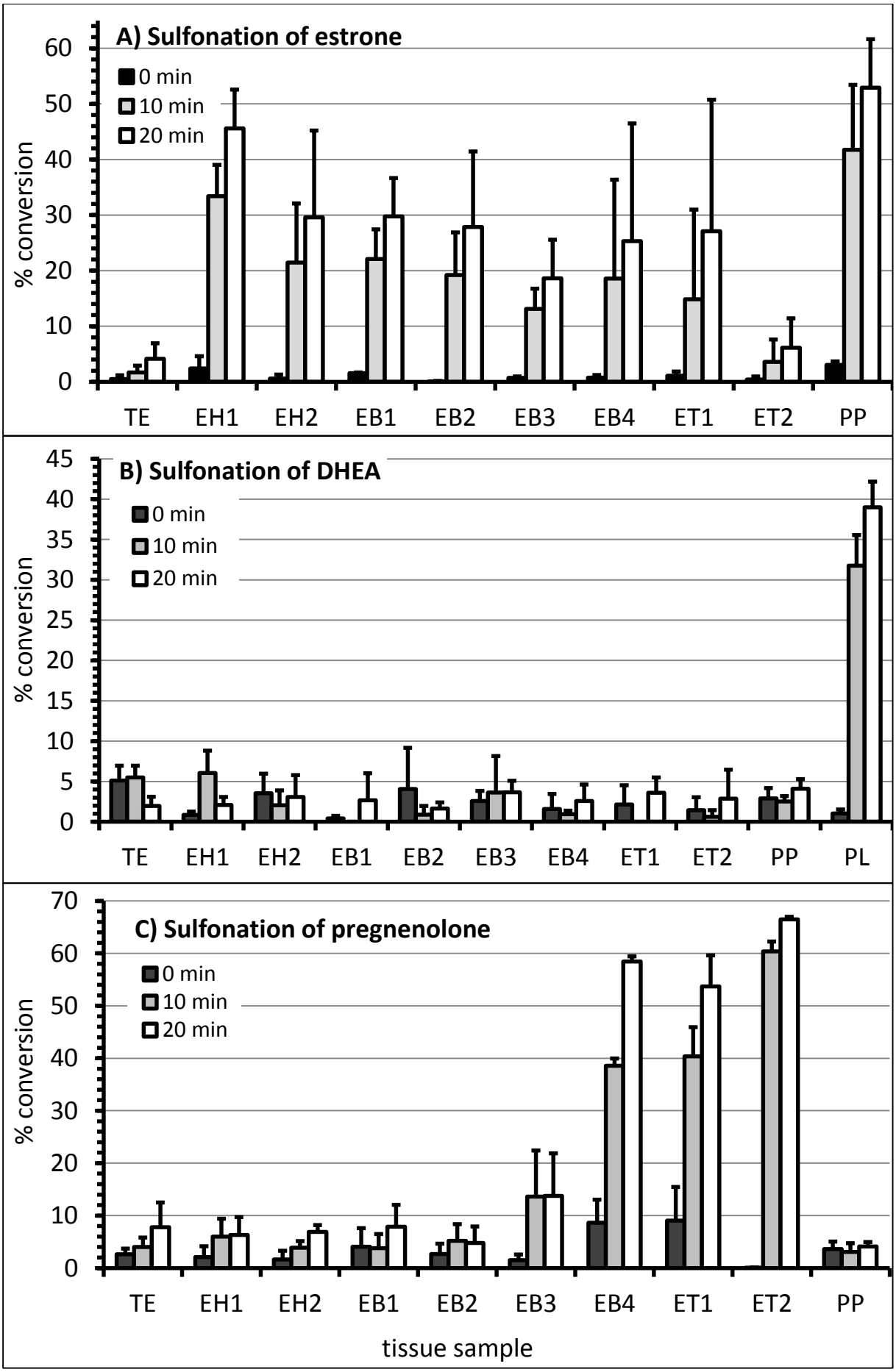
Figure 28. Hydrolysis of 10 pmol estrone sulfate by 200 µg microsomal protein from porcine testis (n=3) in the presence of increasing amounts of (A) DHEA sulfate or (B) pregnenolone sulfate. Time of incubation was 1 h.

4.6 Sulfonation of estrone, dehydroepiandrosterone and pregnenolone in the testicular-epididymal compartment

Among the tissues investigated, a considerable sulfonation of E1 was detected in the epididymal head, the proximal part of the epididymal body (EB 1 and 2) and the pampiniform plexus (mean conversion rates after 20 min: 27.8-52.9%). The conversion was low in ET2 (6.1±5.3%/20 min) and only marginal in testis (4.1±2.8%/20 min). A high variability was found in the distal part of the epididymis (EB4) and the proximal half of the epididymal tail (ET1), where estrogen sulfotransferase activity was high in two of the animals (23.6-57.7%/20 min) but virtually absent in the third. Sulfonation of DHEA was only low in all tissues investigated, ranging between 0-8.6% after 20 min and no time dependence of conversion rates was found. Sulfonation of P5 was low (< 15% after 20 min) in the testis and proximal epididymis (EH1-EB2), started to increase in EB3 and was maximal in ET2 (66.5±0.5%). No significant sulfonation of P5 was detected in the pampiniform plexus.

Figure 29 (next page). Sulfonation of (A) E1, (B) DHEA and (C) P5 by 200 µg cytosolic protein prepared from different tissues of the testicular-epididymal compartment from three postpubertal boars. The results are presented as $\overline{x}$±SD. The amount of substrate was 10 pmol/tube.

TE: testis; EH1-2: proximal/distal segment of epididymal head; EB1-4: segments of the epididymal body (from proximal to distal); ET1-2: proximal/distal segment of epididymal tail; PP: pampiniform plexus. When testing for sulfonation of DHEA, microsomal protein from human term placenta (PL) was included as a positive control.


A) Sulfonation of estrone
0 min
10 min
20 min
% conversion
60
50
40
30
20
10
0
TE
EH1
EH2
EB1
EB2
EB3
EB4
ET1
ET2
PP
B) Sulfonation of DHEA
0 min
10 min
20 min
% conversion
45
40
35
30
25
20
15
10
5
0
TE
EH1
EH2
EB1
EB2
EB3
EB4
ET1
ET2
PP
PL
C) Sulfonation of pregnenolone
0 min
10 min
20 min
% conversion
70
60
50
40
30
20
10
0
TE
EH1
EH2
EB1
EB2
EB3
EB4
ET1
ET2
PP
tissue sample

4.7 Expression of steroid sulfatase in the in the testicular-epididymal compartment

By means of immunohistochemistry, in testis STS was predominantly localized in Leydig cells (Fig. 31), which mainly exhibited a distinct to intense cytoplasmic staining. In Leydig cells showing cytoplasmic immunostaining of only weak to moderate intensity, occasionally staining associated with the nuclear membrane was visible (Fig. 32). Besides in Leydig cells, a moderate cytoplasmic immunostaining also occurred in the epithelium of the rete testis (Fig. 33). However, the overall staining intensity was clearly lower compared to Leydig cells. No certain immunostaining was found in the tubular compartment, the peritubular myoid cells and in the vascular system.

In the epididymis cytoplasmic immunostaining of mainly weak to moderate intensity was predominantly localized in the epithelium (Fig. 34), where the staining intensity tended to be higher in the head and the distal part of the tail compared to the segments located in between (Table 10). Throughout the epididymis, staining intensity was highest in protrusions from the epithelial surface into the lumen of the epididymal duct (Fig. 35). Only sporadically weak to moderate immunostaining was also found in the muscular layer and in the vascular system.

In the deferent duct, in two of the three boars assessed a weak to moderate staining was observed in the ductal epithelium, whereas in the third animal it was negative. In all three animals, a weak to moderate staining was consistently found in the vascular endothelium of the deferent duct and in the pampiniform plexus.

In the negative controls in the porcine testis (Fig. 30), epididymis and pampiniform plexus apart from the weak non-specific staining in the lumen of blood vessels, obviously associated with residual serum components, there were no other non-specific signals.

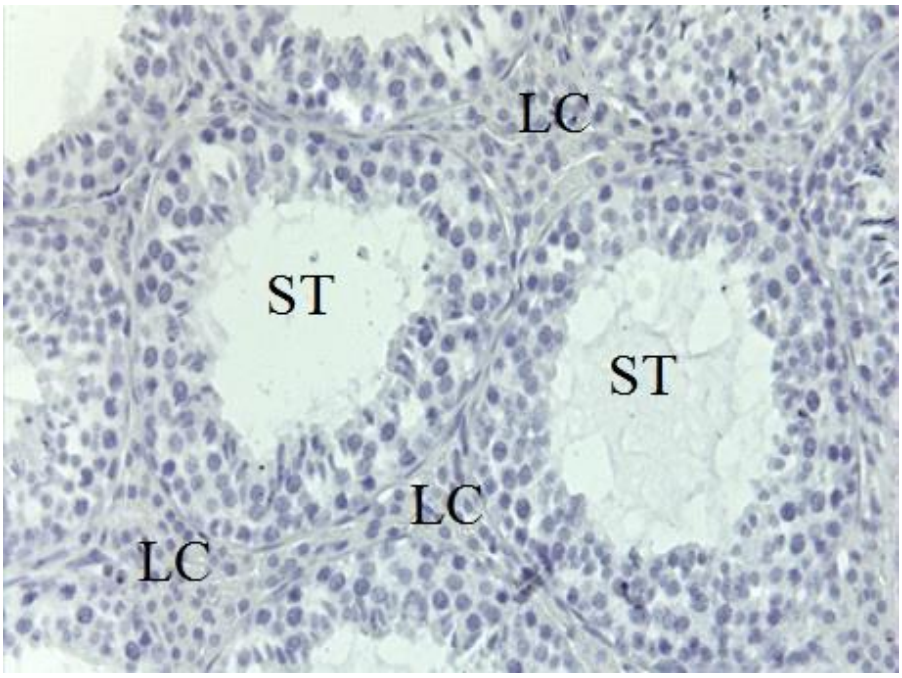


Figure 30. Immunohistochemical detection of steroid sulfatase (STS) in the porcine testicular-epididymal compartment: negative control section from a porcine testis in which the specific antiserum against STS was replaced by non-specific rabbit IgG. ST – Seminiferous tubules; LC – interstitial tissue with Leydig cells. Magnification: ×200.

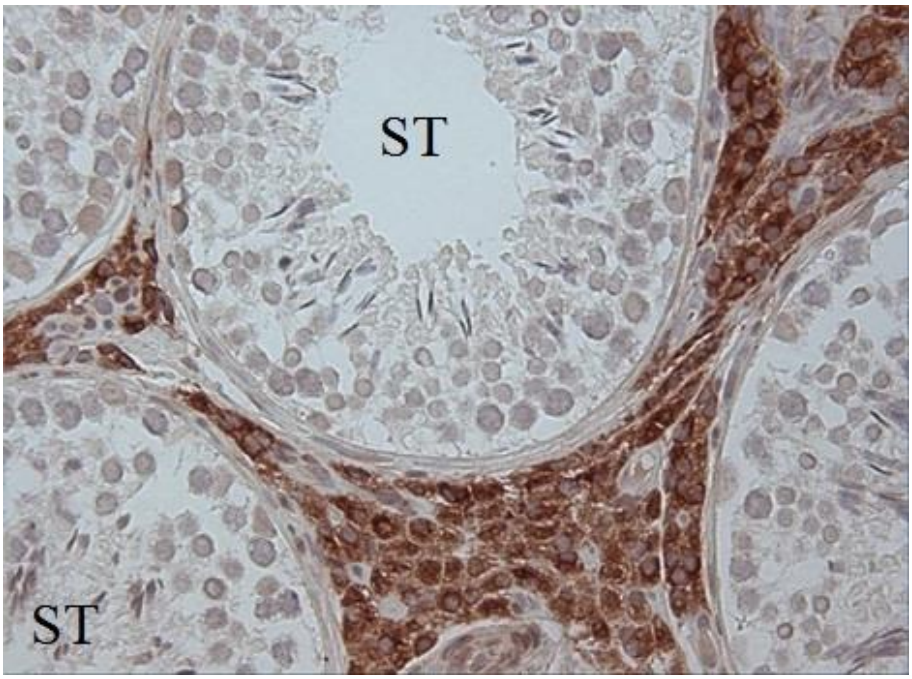


Figure 31. Immunostaining for steroid sulfatase in the porcine testis. Specific staining is confined to Leydig cells. ST – Seminiferous tubules. Magnification: ×200.

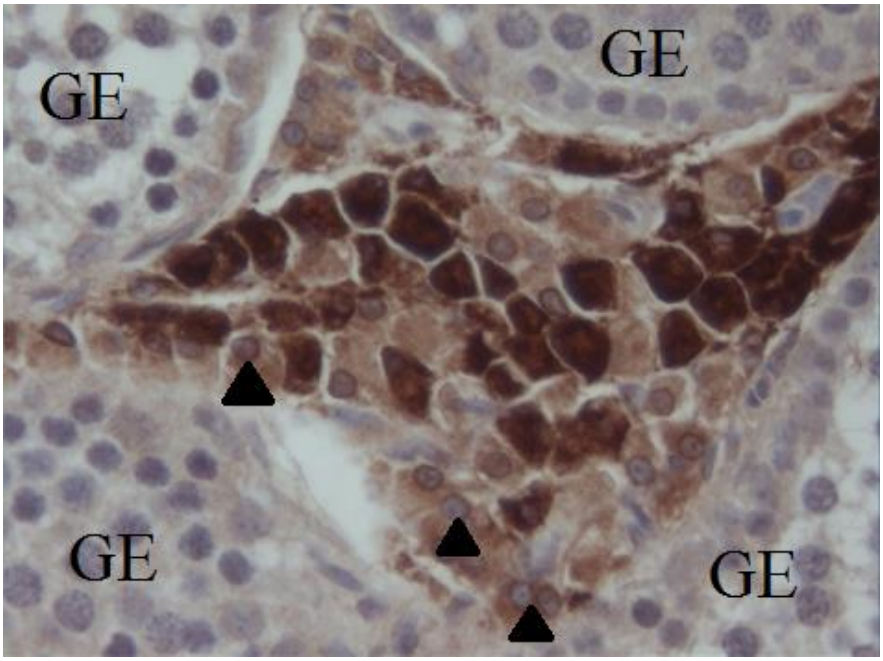


Figure 32. Immunostaining for steroid sulfatase in porcine Leydig cells. In cells exhibiting only a moderate cytoplasmic signal, in part a pronounced staining of the nuclear membrane is visible (▲). Magnification: ×400.

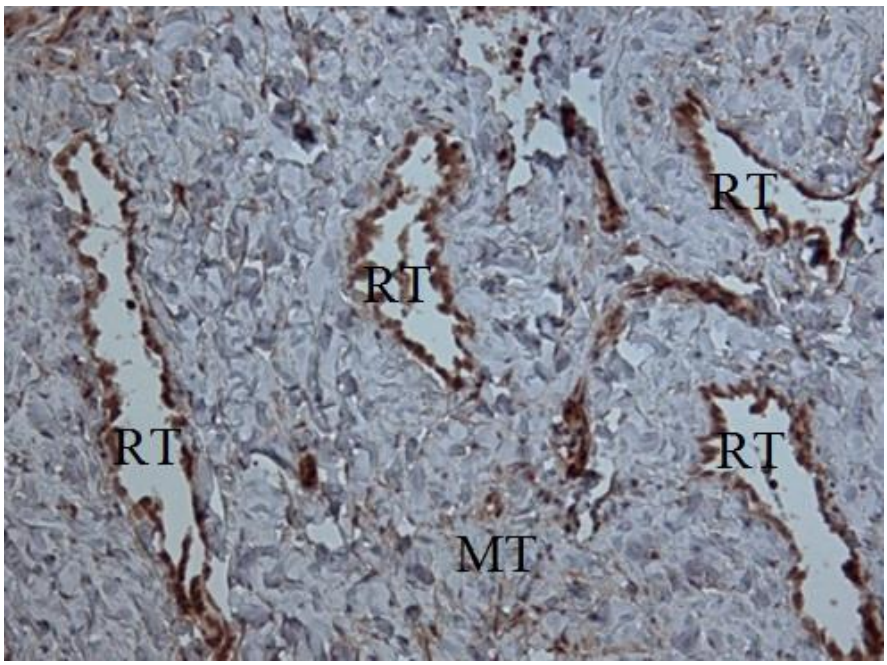


Figure 33. Immunostaining for steroid sulfatase in epithelial cells of the rete testis. RT – ductules of the rete testis; MT – connective tissue of mediastinum testis. Magnification: ×200.

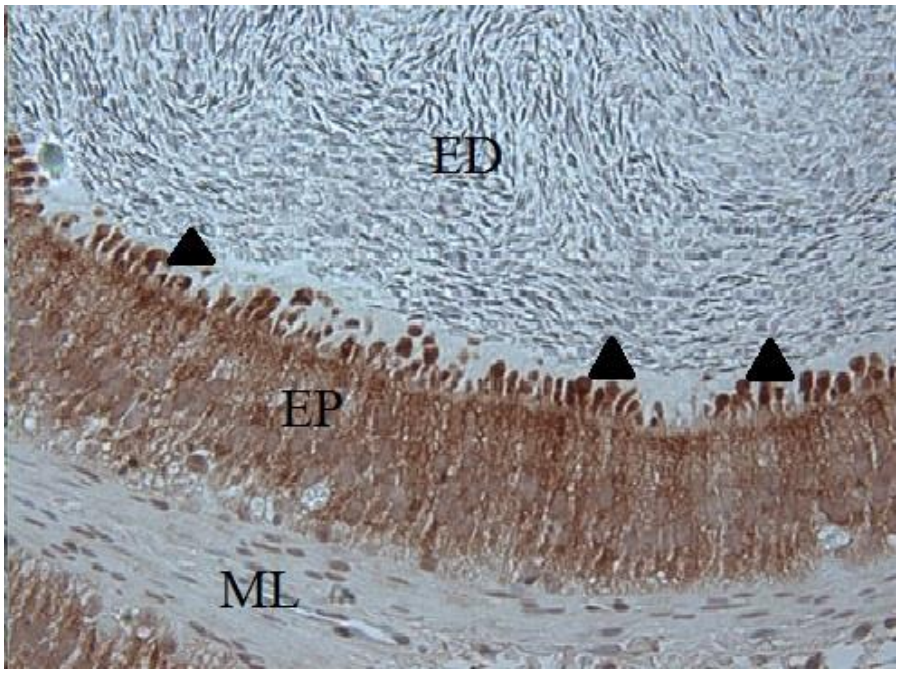


Figure 34. Immunolocalization of steroid sulfatase in the epididymal head (proximal part) showing a distinct cytoplasmic signal in the epithelium (EP). Staining intensity is highest in protrusions of its surface (▲). ML - muscular layer. ED – lumen of epididymal duct with spermatozoa. Magnification: ×200.

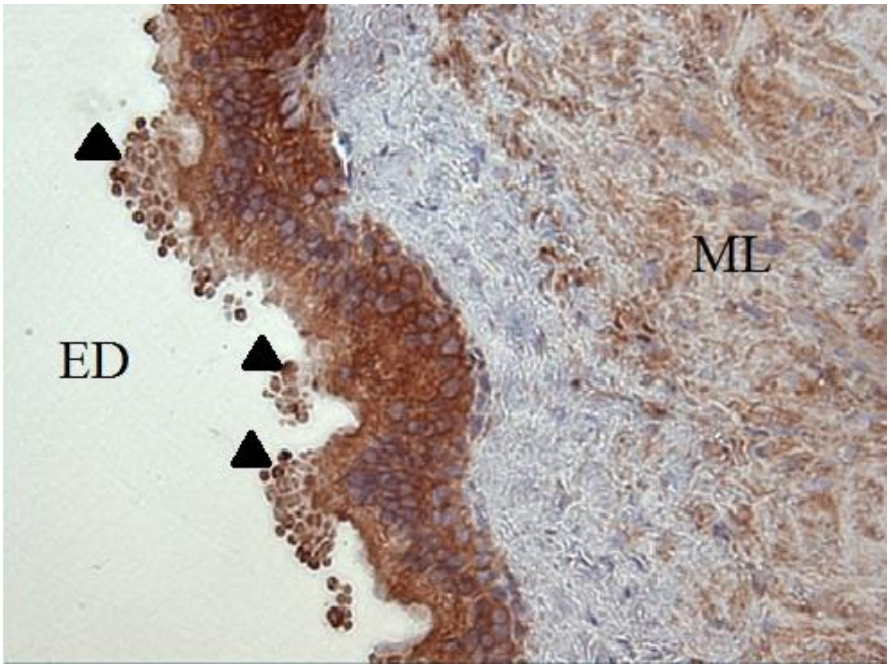


Figure 35. Immunolocalization of steroid sulfatase in the epididymal tail (distal part) showing a distinct cytoplasmic signal in the epithelium. Staining intensity is highest in protrusions of its surface (▲). Cytoplasmic staining of weaker intensity is also visible in the muscular layer (ML). ED – lumen of epididymal duct. Magnification: ×200.

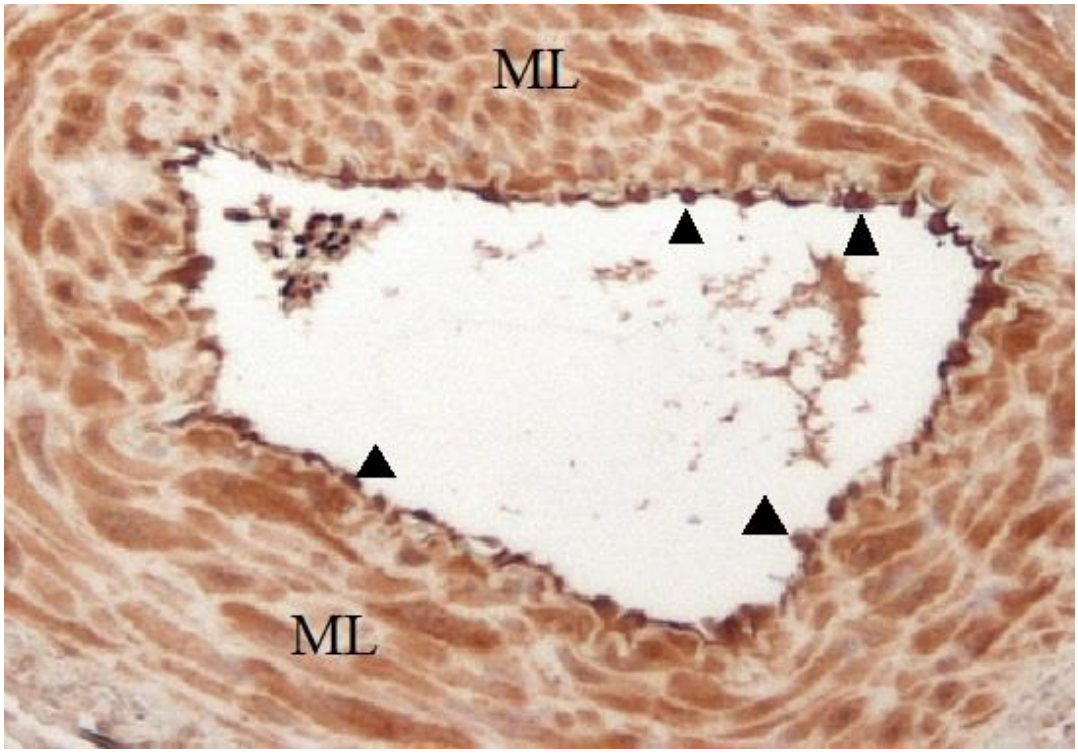


Figure 36. Immunolocalization of steroid sulfatase in a blood vessel of the pampiniform plexus with distinct staining of the vascular endothelium (▲). Cytoplasmic staining of weaker intensity is also visible in the muscular layer (ML). Magnification: ×200.

When analyzing microsomal fractions prepared from tissue samples of the testicular-epididymal compartment by means of Western blot, a distinct band of about 62 kDa, which is consistent with the molecular weight of steroid sulfatase, was found in testicular samples from all three boars investigated. In the epididymal samples, bands of an identical size but variable intensity were found (for example see Fig. 37).

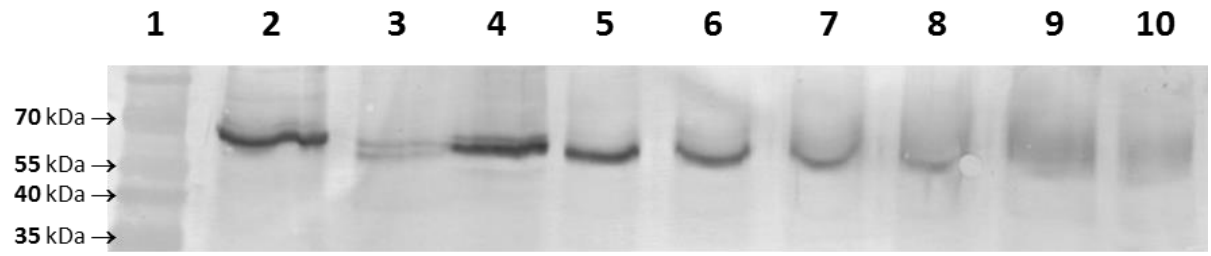


Figure 37. Western blot analysis of tissue samples collected from the testicular-epididymal compartment of a postpubertal boar for the expression of steroid sulfatase. For each sample 20 µg microsomal protein was loaded.

1 – molecular weight markers, 2 - TE, 3 - EH1, 4 - EH2, 5 - EB1, 6 - EB2, 7 - EB3, 8 - EB4, 9 - ET1, 10 - ET2.
TE: testis; EH1-2: proximal/distal segment of epididymal head; EB1-4: segments of the epididymal body (from proximal to distal); ET1-2: proximal/distal segment of epididymal tail.

Table 10. Results of the semi-quantitative evaluation of immunostaining for steroid sulfatase in the epididymis of three postpubertal boars (#1-3).

		EH1			EH2			EB1			EB2			EB3			EB4			ET1			ET2		
		#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
epithelium	superficial protrusions	++	++	++	+++	+++	+++	++	+++	++	++	++	+	++	+++	+	0	++	++	0	+	++	+++	+++	+++
	principal cells	++	++	++	+++	++	+++	++	+	++	+	++	+	+	++	++	+	++	++	++	++	++	++	++	++
	basal cells	++	++	++	+++	++	+++	++	++	++	++	++	++	+	++	++	+	++	++	++	++	++	++	++	++
muscular layer		-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
vascular endothelium		-	-	-	++	++	++	+	+	+	++	+	+	-	-	-	-	-	-	+	+	-	+	+	+

+++/++/+/-: intense/moderate/weak/no immunostaining

0: no superficial protrusions present in the section evaluated

EH1-2: proximal/distal segment of epididymal head

EB1-4: segments of the epididymal body (from proximal to distal)

ET1-2: proximal/distal segment of epididymal tail

4.8 Expression of SULT1E1 in the testicular-epididymal compartment

When screening the testicular-epididymal compartment for the expression of SULT1E1 by immunohistochemistry, in the testis weak cytoplasmic staining was found in some places in Leydig cells and occasionally in vascular endothelium (Fig. 38). No certain staining was found in the tubular compartment and in the peritubular myoid cells. In the epididymis, moderate to intense staining occurred in the samples EH1, EH2 and EB1, where SULT1E1 was predominantly localized in the epithelium, including the superficial epithelial protrusions (Table 11; Fig. 39, and Fig. 40). The epithelium in the remaining part of the epididymis was also immunopositive throughout. However, the mean staining intensity was clearly lower in comparison to the proximal part. Throughout the epididymis, variable immunostaining of generally weak to moderate intensity was observed in vascular endothelial cells. In the muscular layer only sporadically weak to moderate immunostaining was found. In the deferent duct, weak to moderate immunostaining occurred in the epithelium and the vascular endothelium. Also a proportion of the vascular endothelia in the pampiniform plexus showed a specific staining, which was of variable intensity (Fig. 41).

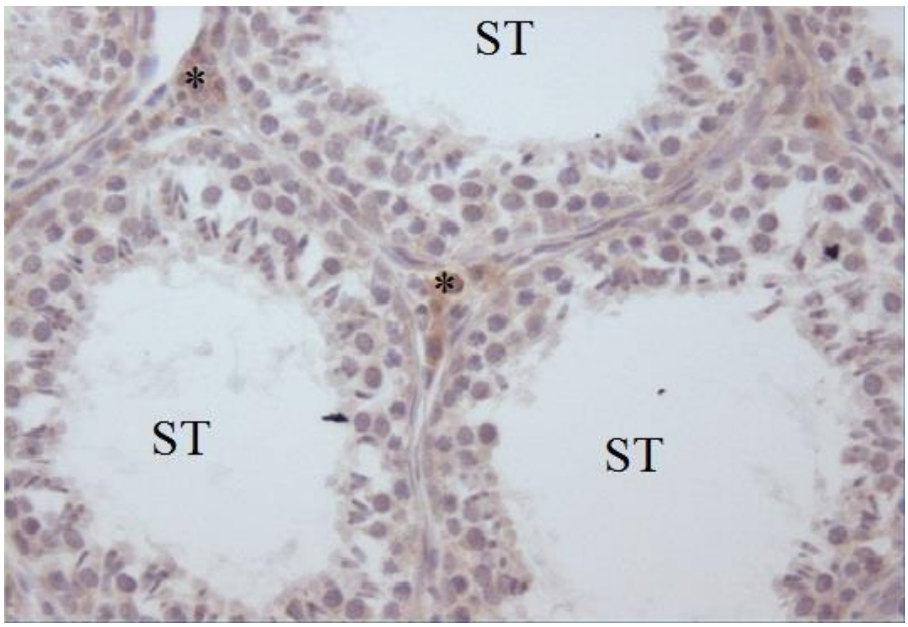


Figure 38. Immunostaining for SULT1E1 in the testis of a postpubertal boar. In this animal, a weak cytoplasmic staining is found in Leydig cells (*). ST – Seminiferous tubules. Magnification: ×200.

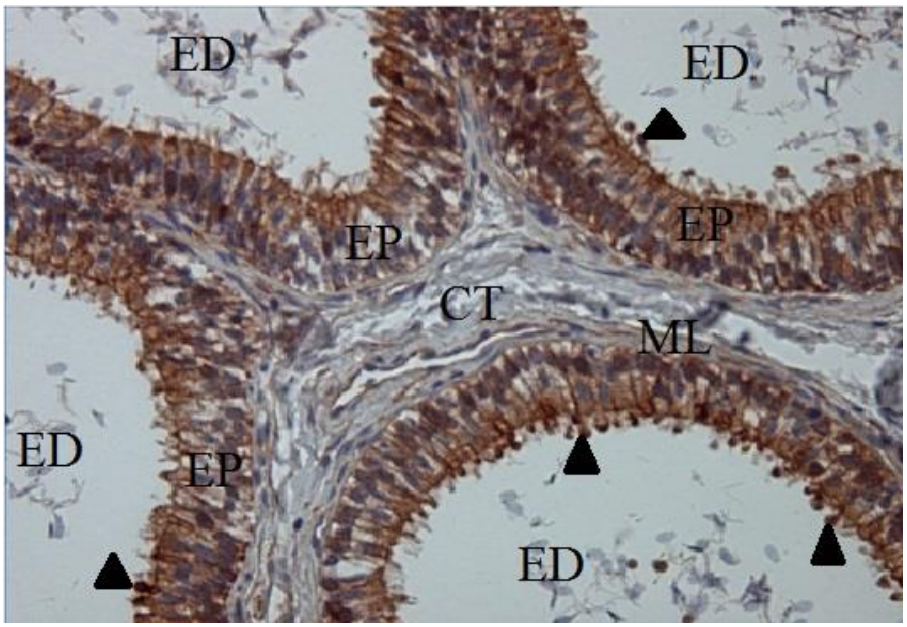


Figure 39. Immunostaining for SULT1E1 in the epididymal head (proximal part) of a postpubertal boar showing strong signals in the cytoplasm of epithelial cells (EP). Staining intensity is especially high in superficial protrusions (▲). ED – lumen of epididymal duct with spermatozoa; ML – muscular layer; CT – connective tissue. Magnification: ×200.

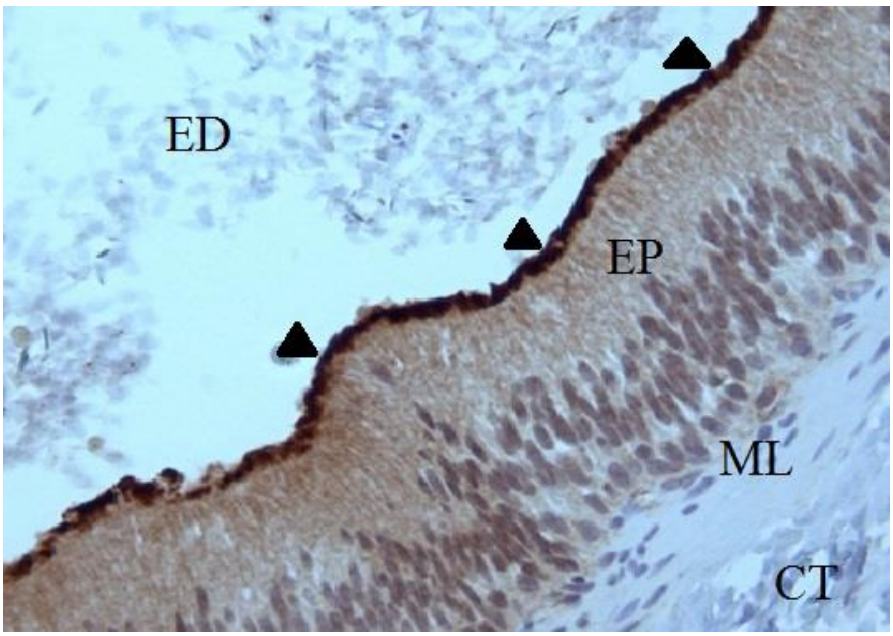


Figure 40. Immunostaining for SULT1E1 in the epididymal head (distal part) of a postpubertal boar showing distinct signals in the cytoplasm of epithelial cells (EP) and intense staining associated with their apical part (▲). ED – lumen of epididymal duct with spermatozoa; ML – muscular layer; CT – connective tissue. Magnification: ×200.

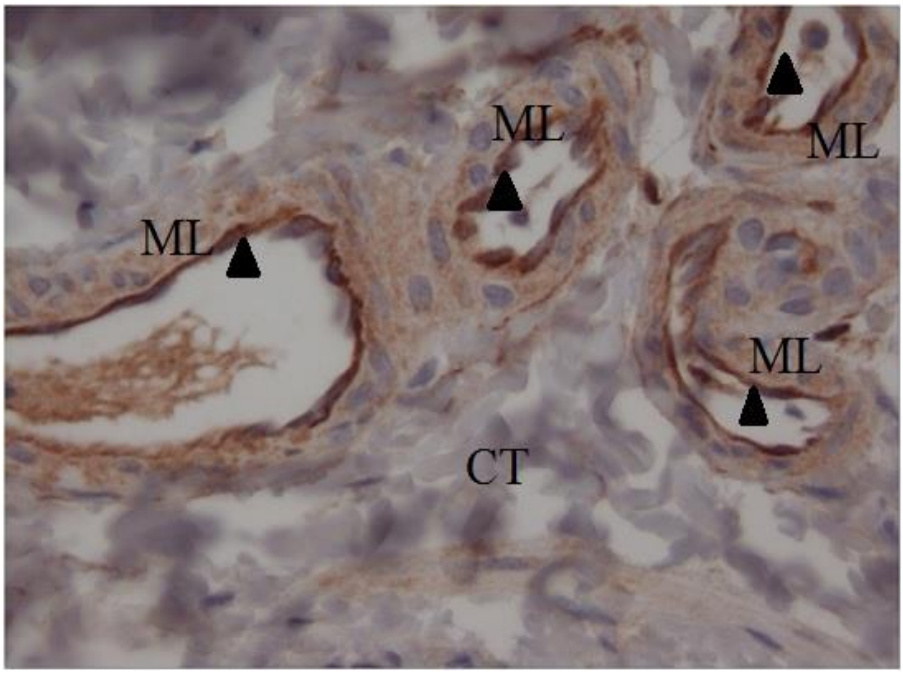


Figure 41. Immunostaining for SULT1E1 in the pampiniform plexus of a postpubertal boar. Intense staining is present in the endothelium of minor blood vessels (▲). Weaker cytoplasmic staining also occurs in their muscular layer (ML). CT – connective tissue. Magnification: ×200.

In Western blot, a band consistent with the molecular size of SULT1E1 (approx. 34 kDa) consistently occurred throughout the epididymis but was not observed in the testis (Fig. 42).

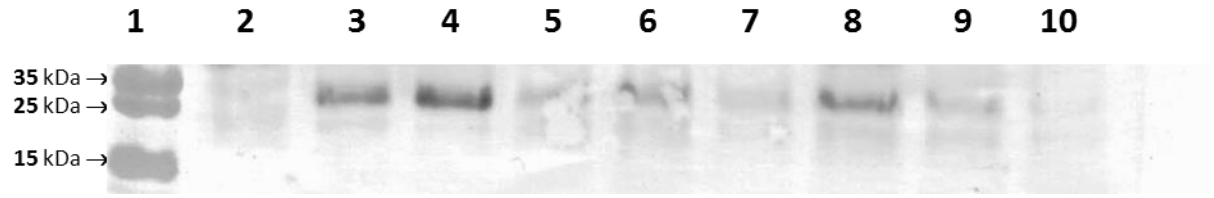


Figure 42. Western blot analysis of tissue samples collected from the testicular-epididymal compartment of a postpubertal boar for the expression of SULT1E1. For each sample 20 µg cytosolic protein was loaded.

1 – molecular weight markers, 2 - TE, 3 - EH1, 4 - EH2, 5 - EB1, 6 - EB2, 7 - EB3, 8 - EB4, 9 - ET1, 10 - ET2
TE: testis; EH1-2: proximal/distal segment of epididymal head; EB1-4: segments of the epididymal body (from proximal to distal); ET1-2: proximal/distal segment of epididymal tail.

Table 11. Results of the semi-quantitative evaluation of immunostaining for SULT1E1 in the epididymis of three postpubertal boars (#1-3).

		EH1			EH2			EB1			EB2			EB3			EB4			ET1			ET2		
		#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
epithelium	superficial protrusions	+++	++	+++	+++	0	+++	+++	++	++	-	+	+	+	+	+	+	0	++	++	-	++	++	++	++
	principal cells	+++	++	++	+++	++	+	++	++	++	+	+	+	++	+	++	++	+	+	+	+	++	+	++	++
	basal cells	+++	++	++	+++	++	+	++	++	++	+	+	+	++	+	+	++	+	+	+	-	++	+	++	++
muscular layer		+	-	-	-	-	-	+	-	-	-	-	-	+	-	++	+	-	-	-	-	-	-	-	+
vascular endothelium		-	+	++	-	-	+	+	+	+	++	-	++	+	++	++	+	-	+	-	+	+	+	++	++

+++/++/+/-: intense/moderate/weak/no immunostaining

0: no superficial protrusions present in the section evaluated

EH1-2: proximal/distal segment of epididymal head

EB1-4: segments of the epididymal body (from proximal to distal)

ET1-2: proximal/distal segment of epididymal tail

4.9 Expression of SULT2B1 in the testicular-epididymal compartment

In testis by means of immunohistochemistry in two animals specific staining was mainly found in spermatocytes and spermatids, which, however, was predominantly nuclear (Fig. 43). Additionally, a weak cytoplasmic staining was consistently observed in vascular endothelial cells, whereas in Leydig cells, Sertoli cells and the rete testis epithelium weak cytoplasmic signals only occurred in one or two of the three boars investigated. In one of three boars investigated, no staining at all occurred in the testis. In the epididymal epithelium cytoplasmic immunostaining was generally weak in the head, in the proximal half of the body and in the proximal half of the tail. In comparison, staining intensity was clearly higher in the distal part of the epididymal body, but the highest staining intensity was present in the distal half of the epididymal tail (Fig. 44; Table 12). Moreover, a moderate to intense staining also occurred in superficial protrusions of the epithelium in the head, the hind part of the body and the distal part of the tail. In addition to the weak cytoplasmic staining, in the epithelium of the epididymal head a distinct nuclear staining was found. In most of the samples, a weak to moderate signal was detected in vascular endothelial cells, whereas the muscular layer only sporadically exhibited weak staining with the exception of the distal epididymal tail, where staining intensity varied significantly between individual animals from weak to intense. Immunostaining in the deferent duct was similar to the staining pattern found in the distal epididymal tail. In the pampiniform plexus no specific staining was detected.

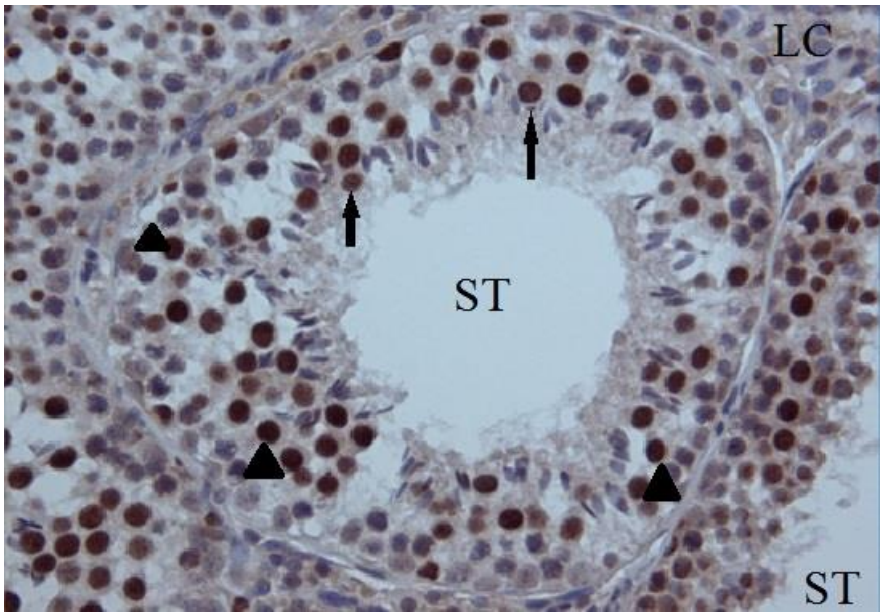


Figure 43. Immunolocalization of SULT2B1 in the testis of a postpubertal boar. Intense nuclear signals were predominantly found in spermatocytes (▲) and round spermatids (↑). ST – seminiferous tubules; LC – interstitial tissue with Leydig cells. Magnification: ×200.

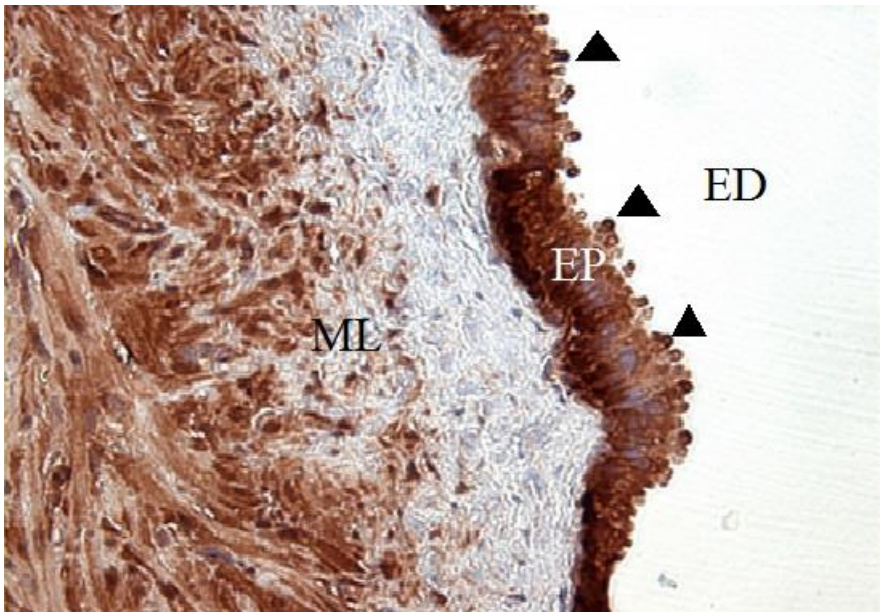


Figure 44. Immunolocalization of SULT2B1 in the distal epididymal tail (ET2) of a postpubertal boar. An intense cytoplasmic staining is present in epithelial cells (EP) and their superficial protrusions (▲). Distinct cytoplasmic staining is also found in the muscular layer (ML). ED – lumen of epididymal duct. Magnification: ×200

In Western blot, in first experiments using NovaRed (NovaRed substrate kit, Vector Laboratories, Burlingame, USA) as a substrate, no distinct band consistent with the molecular size of SULT2B1 (approx. 45 kDa) was detectable in any of the samples under investigation. When using a more sensitive chemiluminescence based method for detection (AceGlow Ultrasensitive Chemiluminescence Substrate, PEQLAB Biotechnologie GmbH, Erlangen, Germany), a 45 kDa band was clearly visible in the hind part of the epididymis, whereas it was undetectable or only of very weak intensity in the proximal part of the epididymis and in the testis (Fig. 45).

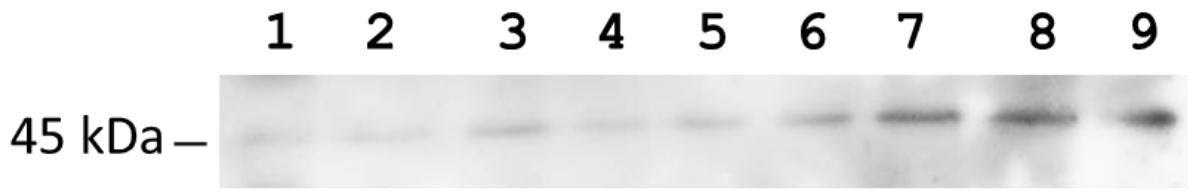


Figure 45. Western blot analysis of tissue samples collected from the testicular-epididymal compartment of a postpubertal boar for the expression of SULT2B1. For each sample 20 µg cytosolic protein was loaded.

1- TE, 2- EH1, 3- EH2, 4- EB1, 5- EB2, 6- EB3, 7- EB4, 8- ET1, 9- ET2
TE: testis; EH1-2: proximal/distal segment of epididymal head; EB1-4: segments of the epididymal body (from proximal to distal); ET1-2: proximal/distal segment of epididymal tail (see Fig. 7).

Table 12. Results of the semi-quantitative evaluation of immunostaining for SULT2B1 in the epididymis of three postpubertal boars (#1-3). In addition to the cytoplasmic staining indicated in the table, in the epithelium of the epididymal head a distinct nuclear staining was present.

		EH1			**EH2**			**EB1**			**EB2**			**EB3**			**EB4**			**ET1**			**ET2**		
		#1	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**	**#1**	**#2**	**#3**
epithelium	superficial protrusions	++	++	0	++	+	+	-	+	+	+	++	+	++	0	+	++	++	++	0	+	0	+++	+++	+++
	principal cells	+	+	+	+	+	+	+	+	+	++	++	++	++	+	++	++	++	++	+	+	+	+++	++	++
	basal cells	+	+	+	+	+	+	+	+	+	++	++	++	++	+	++	++	++	++	+	+	+	+++	++	+++
muscular layer		-	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-	+	+	-	-	-	+++	+	++
vascular endothelium		+	+	+	-	-	+	+	+	+	++	+	+	+	+	++	+	+	++	-	+	+	+	+	+

+++/++/+/-: intense/moderate/weak/no immunostaining

0: no superficial profusions present in the section evaluated

EH1-2: proximal/distal segment of epididymal head

EB1-4: segments of the epididymal body (from proximal to distal)

ET1-2: proximal/distal segment of epididymal tail

5. Discussion

5.1 Steroid measurements in blood

At the beginning of this study only RIA methods established at the endocrine laboratory of the Veterinary Clinic for Obstetrics, Gynecology and Andrology, Justus-Liebig-University, Giessen, were available for the measurement of various free steroids and E1S in porcine blood samples, which were used for the measurements of free estrogens and for preliminary measurements of T and E1S. Then LC-MS-MS methods newly developed in the framework of the DFG research group FOR1369 "Sulfated steroids in Reproduction" became successively available in the Laboratory for Translational Hormone Analytics in Pediatric Endocrinology, Steroid Research & Mass Spectrometry Unit, Division of Pediatric Endocrinology & Diabetology, Justus Liebig University, Giessen (head: Prof. Dr. S. Wudy) for the measurement of P5S, DHEAS, E1S, E2S, T and A4. The big advantage of LC-MS-MS consists of enabling the analysis of unconjugated and intact sulfonated steroids with currently highest specificity and good sensitivity, and without chemical modification of the compounds of interest (Galuska et al. 2013). Moreover, a significant number of analytes may be assessed in a single analytical procedure. Thus, for the final evaluation of results data from LC-MS-MS were used with the exception of free E1 and E2, which in boars occur in significantly lower concentrations in comparison to the sulfonated forms (Claus & Hoffmann 1980, Schwarzenberger et al. 1993) and therefore had to be measured by the more sensitive RIAs. With these highly reliable methods applied simultaneously in blood samples of boars it was for the first time possible to establish comprehensive profiles of free and sulfated steroids using a holistic approach and to test for correlations in secretory patterns in unstimulated and hCG stimulated animals. In combination with data from local blood vessels of the spermatic cord and the testicular surface, these data should allow further conclusions on the functionality of the sulfatase pathway in the porcine testicular-epididymal compartment. Key elements of such a pathway have already been described in the porcine testicular epididymal compartment such as the production of high amounts of sulfated estrogens (Claus & Hoffmann 1980, Raeside & Renaud 1983, Schwarzenberger et al. 1993, Zamaratskaia et al. 2004, Hoffmann et al. 2010), considerable expression and activity of STS in porcine Leydig cells and epididymis, as well as substantial estrogen sulfotransferase activity in the epididymis (Mutembei et al. 2009, Hoffmann et al. 2010, Zdunczyk et al. 2012). Moreover, various cell types of the porcine testis and epididymis exhibit a high expression of estrogen receptors (Mutembei et al. 2005, Lekhkota et al. 2006, Pearl et al. 2007). Consequently, the high levels of sulfonated estrogens may serve as substrates

for local sulfatase pathways in the porcine testicular-epididymal compartment. However, their definite role – as the roles of the many other sulfated steroids found in boars (Raeside 2006) – is still completely unclear. Thus, the analysis of data from frequent measurements over a significant period of time intended to obtain new information on metabolism and transport of sulfonated steroids in relation to the secretion of free steroids under in vivo conditions.

Concerning steroid levels in peripheral circulation both in stimulated and unstimulated boars a considerable variability was found between individual animals although with the exception of one boar (SB8, Large White) all animals used in these experiments were German Landrace x Pietrain crossbreds. As all methods applied were thoroughly validated, the highly variable steroid levels obtained in this part of the study clearly not result from methodological problems but reflect an intriguing biological variability.

In unstimulated animals, despite the high variability observed between individual boars the results suggest that with respect to the individual boars secretion of all steroids measured basically followed a similar pattern. High positive correlations were especially found between the profiles for T, P5S, DHEAS and E1S, for which in a considerable number of cases a more or less pronounced gradual decline of concentrations was observed, mostly followed by a weak increase at the end of the sampling period. This profile is consistent with previously published diurnal profiles for 5α-androstenone, T, DHEAS and total conjugated estrogens (Claus & Hoffmann 1980, Tan & Raeside 1980) pointing to episodic fluctuations or pulsatile secretion with a low frequency of 3-5 pulses/day. This pattern seems not be influenced to a noteworthy extent by a possible stress induced adrenal steroid secretion caused by sedation and sampling, as cortisol concentrations measured by RIA (37.8±17.1 ng/ml; range, 5.2–83.6 ng/ml) were in a similar range as previously reported for boars (Allrich et al. 1982, Bishop et al. 1999, Wise et al. 2000) and remained virtually unchanged during the sampling period or exhibited a profile clearly different from the steroids being in the focus of this study (see cortisol profiles presented in the appendix in section 9.2). The generally lower correlations of A4 and E1 with other steroids may in part result from the fact that their levels in a considerable proportion of samples were in the lower measuring range, where accuracy is inherently reduced. Nevertheless a consistently high correlation was observed between A4 and T indicating a sufficient accuracy of A4 measurements also in animals exhibiting low concentrations.

In addition to the characterization of spontaneous secretion, stimulation with 1500 I.U. hCG was performed to create a more dynamic situation for correlation analysis and to obtain evidence for differential transport as indicated by the time between hCG stimulation and detection of a stimulatory effect in the systemic circulation. The results obtained in one animal

after stimulation with 10000 I.U. hCG (Fig. 22) suggest that 1500 I.E. were sufficient to exert maximum stimulation. As with unstimulated animals, the results in stimulated boars exhibited a considerable variability between individual animals, which was observed both with respect to prestimulatory levels, the maximum stimulatory effect and the shape of the post-stimulatory profile. However, they give clear evidence that basically all steroids assessed are stimulated by hCG. Moreover, they show that increases of P5S, DHEAS, E1S and E2S may become obvious within 15-40 minutes after hCG application, which was not later than observed for free steroids. A sulfonation of significant amounts of testicular free steroids by epididymal sulfotransferases is very unlikely, as a transfer of free steroids to the epididymis via tubular fluid should be rather time consuming (diffusion through interstitial tissue, peritubular cells and the high germinal epithelium followed by a relatively slow transport via the tubular fluid). Thus, although there should be a transfer of free steroids via the tubular fluid to the epididymis, the rather simultaneous increases in free and sulfonated steroids after hCG-stimulation provide clear evidence that sulfonation of free testicular steroids in the epididymis is not the quantitatively major route for the synthesis of sulfonated steroids in the porcine testicular-epididymal compartment. From their measurements in blood and lymph of the spermatic cord, Setchell et al. (1983) concluded that testosterone and free estrogens preferentially leave the testis via the blood, whereas sulfonated estrogens and DHEAS are predominantly transported into the systemic circulation via the lymph. If so, the results from this work suggest that both systems accomplish the transport into the peripheral circulation at a similar pace and efficiency. As with unstimulated boars, cortisol concentrations (43.9±19.6 ng/ml; range, 16.6–103.5 ng/ml) remained fairly constant or exhibited a profile clearly different from other steroids (see cortisol profiles presented in the appendix in chapter 9.2). Thus, also in hCG-stimulated animals, a noteworthy interference of the profiles by steroids of adrenal origin is very unlikely.

In order to obtain evidence for differential distribution between the individual steroids assessed, biserial correlation functions were calculated to test pairwise for time shifts between their profiles in the systemic circulation. In fact the biserial correlation functions calculated from pooled data for the pairs A4/E1, T/E1, DHEAS/T and DHEAS/E1 in unstimulated boars (see Fig. 17) and for the pairs DHEAS/E1 and DHEAS/E1S in stimulated boars (see Fig. 21) showed their maxima outside of the point of origin when the respective profiles were shifted relative to one another. However, this effect was only moderate and mostly inconsistent between stimulated and unstimulated boars. Moreover, a high variability of biserial correlation functions was observed when analyzing data from individual animals (see chapter 9.1 in the appendix). Thus, taken together, these observations cannot be regarded as reliable indications for time

shifts. One could argue that the sampling interval of 20 min was too long to detect relevant time shifts. However, no evidence for phase shifts between free and sulfated steroids was obtained either when in a pilot experiment the sampling interval in the post-stimulatory period was reduced to 3 min. This further corroborates the concept that the production of sulfonated steroids is immediately linked to the production of T in porcine Leydig cells.

Further evidence for the predominant production of sulfonated steroids in testicular tissue comes from the measurements in local blood vessels. In the four animals assessed, the levels of all steroids measured were clearly higher in capsular veins compared to the capsular artery, whereas the levels in the capsular artery were virtually identical to the concentrations in the proximal part of the testicular artery and to systemic blood. These results are clearly in contrast to the concept that the pampiniform plexus and testicular artery not only form a counter-current exchanger for heat but also for hormones (Rerkamnuaychoke et al. 1991, Einer-Jensen & Hunter 2005). For all steroids determined, higher concentrations were found in the capsular veins compared to the testicular vein. This observations of a seeming "loss" of steroids during venous drainage can be explained by the fact that the capsular veins are directly originating from the highly steroidogenic testicular interstitial tissue, whereas the testicular vein - in addition to blood from the testicular parenchyma - gets influx from other parts of the testicular-epididymal system with no or only minimal steroidogenesis (see chapter 2.4). Thus, the lower concentrations in the testicular vein compared to the capsular veins is likely due to dilution.

Previous studies into conjugated estrogens in boars focused mainly on E1S or total conjugated estrogens; specific data on E2S are only sparsely available (Schwarzenberger et al. 1993). Consistent with this earlier report, E2S concentrations measured in two boars were somewhat lower in comparison to the corresponding concentrations in E1S but still sizeable, indicating that in boars either high amounts of the biologically highly active E2 are inactivated by sulfonation or that the boar has a substantial potential to form E2 by hydrolysis of E2S. Measurements in capsular artery and veins clearly show that E2S – as other steroid sulfates assessed - is predominantly an original product of the testis.

Data from parallel measurements of T and E1S using RIA and LC-MS-MS were analyzed for the consistency between the two methods. For E1S regression analysis yielded a high correlation between the two methods. A similarly good consistency between the RIA and LC-MS-MS was also found for the measurement of T for concentrations between approximately 0.1-5.0 ng/ml. At higher concentrations, values from measurements by LC-MS-MS were in a considerable proportion of cases clearly higher than those obtained from RIA. Observations from the comparison of the two methods indicate that the slope of the standard curve used in

the RIA in its upper concentration range (between approx. 0.5-0.74 ng/tube, corresponding to concentrations between 5-7.4 ng/ml at a sample volume of 0.1 ml) was not steep enough anymore to reliably identify concentrations above the highest point of the standard curve. As a consequence, the standard curve of the testosterone RIA applied should be limited to a range between 0.01-0.5 ng/ml, and correspondingly the dilution of samples or reduction of sample volume should be started at lower concentrations than previously performed.

5.2 Steroid sulfatase expression and activity in the porcine testicular-epididymal compartment

In previous studies using tissue homogenates as a source of the enzyme and E1S as substrate, STS activity was clearly detectable in the porcine testis (Hoffmann et al. 2010). By means of immunohistochemistry positive signals were confined to the Leydig cells (Mutembei et al. 2009). In the epididymis STS activity was also detectable but lower compared to the testis (Hoffmann et al. 2010). The predominant aim of this study concerning STS was 1) to characterize more closely enzyme activity in the epididymis using samples from defined sections of the organ, 2) to characterize epididymal STS expression on the cellular level and 3) to compare the activity of porcine testicular STS towards different sulfonated steroids (E1S, DHEAS, P5S).

In the two earlier studies on STS activity in porcine epididymis (Hoffmann et al. 2010, Zdunczyk et al. 2012), crude homogenates were us as a source of the enzyme, and no or only relatively little efforts were made to characterize STS activities in defined sections of the epididymis. Hoffmann et al. (2010) used pooled tissue from epididymal head and body for the preparation of epididymal homogenates. Zdunczyk et al. (2012) prepared homogenates from head, body and tail following the common anatomical classification. However, in order to cope with the obvious highly segmental functional organization of the epididymis, in this study for the preparation of microsomal fractions head and tail were each subdivided in two segments, whereas the body was subdivided in four segments of equal length to allow for a higher "functional resolution" (Turner et al. 2003).

Consistent with the earlier studies STS activity – measured as hydrolysis of E1S - was clearly detectable in all anatomical parts of the epididymis but in general considerably lower in comparison to the testis. In samples from the epididymal tail (ET1, ET2) only low conversion rates were found in all three animals investigated. In one animal similarly low conversion rates were found in all samples from the epididymal head and body (from EH1 to EB4). In the two other animals, a relatively high conversion rate was found in one sample of the head (EH2) or

of the body (B2), respectively. The reason of this variability of STS activity dependent on the individual animal and localization is unclear. However, observations in immunostained tissue sections (see below) suggest that STS is possibly released to a significant extent from the epithelial surface in exfoliating superficial protrusions (apocrine secretion leading to the formation of epididymosomes, see section 2.2.1.1.2). Thus the STS activity measured in a certain sample may not only dependent on the local STS expression but also on the activity in the epididymal fluid and on the content of epididymal fluid in the tissue samples analyzed.

STS is commonly considered as the relevant enzyme for the hydrolysis of sulfates of phenolic and neutral steroids. However, in the own experiments, in comparison to E1S porcine testicular STS exhibited only very low activities to P5S and DHEAS. The observation that E1S efficiently inhibited the hydrolysis of DHEAS and P5S is consistent with the above-mentioned concept that STS is the relevant enzyme for the hydrolysis of sulfonates of phenolic and neutral steroids. A physiological relevance of the inhibitory effect of E1S on the hydrolysis of DHEAS or P5S observed in vitro is currently unclear as it is unknown whether a considerable competition of the three substrates for the enzyme occurs in intact cells. Thus, investigations on STS expression on a subcellular level are necessary for further conclusions on the function of this enzyme.

By means of immunohistochemistry and Western blot expression of STS was detected in all samples investigated. In Western blot one prominent band of about 62 kDa occurred which gives evidence that the antiserum generated against human placental STS also detects specifically the porcine enzyme. Immunohistochemical investigations of testicular tissue confirmed previous observations by Mutembei et al. (2009) who localized a high STS expression in Leydig cells, whereas no staining was found in the tubular compartment. Although STS is generally considered as a membrane bound enzyme and has been described to be localized in various cellular membranes (Nardi et al. 2009, Chenet al. 2009), in many Leydig cells a distinct to intense cytoplasmic signal was found. However, in Leydig cells exhibiting only a moderate cytoplasmic immunostaining, frequently a staining associated with the nuclear membrane was visible. This observation again emphasizes the need to definitely identify the subcellular localization of STS as a prerequisite to understand its function in porcine Leydig cells. In addition to the Leydig cells, in the porcine testis a weaker immunostaining was observed in the ductal epithelium of the rete testis, which has not been reported in the previous study by Mutembei et al. (2009). Throughout the epididymis, the epithelium with its superficial protrusions exhibited the most intense immunostaining, which are presumably the morphological equivalent of apocrine secretion leading to the formation of epididymosomes (see chapter 2.2.1.1.2). The physiological role of STS in the lumen of the epididymal duct

remains unclear, all the more as also distinct SULT1E1 and SULT2B1 immunostaining has been observed in these superficial epithelial protrusions (see below).

5.3 Estrogen sulfotransferase activity and SULT1E1 expression in the porcine testicular-epididymal compartment

Measurements of EST activity using cytosol confirmed earlier studies with homogenates in that virtually no sulfonation of estrogen was detectable in the testis, whereas this reaction was readily measurable in the epididymis (Hoffmann et al. 2010, Zdunczyk et al. 2012). To allow a high sample throughput, as in these earlier studies, the analysis of the samples was based on the different distribution of free and sulfonated E1 between the aqueous and organic phase during extraction. As the measurement of enzyme activity was only based on the distribution of ^{3}H-activity between the fractions of free and conjugated steroids, no definite information was obtained by this method on the actual mechanisms leading to the shift of the ^{3}H-activity from fraction 1 (free steroids) to fraction 2 (conjugated steroids). However, preliminary results from a follow-up study using HPLC-based analysis are consistent with the formation of E1S as the underlying reaction (Klymiuk et al., unpublished data).

According to the virtual absence of EST activity, by means of immunohistochemistry using an antiserum against bovine SULT1E1 in the testis only occasionally weak signals were observed which occurred in Leydig cells and in vascular endothelial cells. Currently it is not definitely clear if this weak staining was actually SULT1E1 specific, as a cross-reactivity of the primary antiserum despite its high specificity for bovine SULT1E1 (Frenette et al. 2009, Polei et al. 2014) with other, structurally closely related porcine SULTs (Miller & Auchus 2011) cannot be ruled out completely. Further evidence for an extremely low or absent expression of SULT1E1 in the testis comes from Western blot experiments, where a specific band of 34 kDa was clearly visible in the epididymal samples, but not in the testis. Moreover, the immunostaining pattern for SULT1E1 in the testis and epididymis is basically consistent with data from a concomitant study using real-time RT-PCR, which showed that in the porcine testicular-epididymal compartment the epididymal head and the initial segment of the body by far exhibit the highest expression of SULT1E1-mRNA, whereas in the testis – at least from a quantitative point of view - it is negligible (Bingsohn et al. 2012).

As described above, there is now evidence from several lines that the epididymal head is the predominant site of SULT1E1 expression in the porcine testicular-epididymal compartment. However, when comparing SULT1E1 expression between porcine epididymal head and bovine placenta using Western blot in a follow-up study, it turned out that expression in the porcine

epididymal head was only low in comparison to the bovine tissue (Klymiuk et al., unpublished data). In the epididymal head and body, highest staining intensities were found in superficial epithelial protrusions, which are possibly the morphological equivalents of apocrine secretion. The apocrine secretion of SULT1E1 into the epididymal tubular lumen via its inclusion into epididymosomes has previously been described in bulls (Frenette et al. 2009). The role of the "high" SULT1E1 expression in the porcine epididymis and of its likely secretion into the lumen of the epididymal duct is unclear, especially in light of its co-localization with STS in the superficial epithelial protrusions (see chapter 4.7, Fig. 34). In a pilot experiment, tissue concentrations of free and sulfonated E1 measured in cytosols prepared from three boars were high in the testis (E1: equivalent to 4.4-7.8 ng/g wet tissue; E1S: 9.9-28.1 ng/g wet tissue) but were below the limit of detection in the epididymal head, body or tail (< 1 ng/g wet tissue). On the one hand, this observation suggests that despite its "high" SULT1E1 expression from a quantitative point of view the epididymis does not contribute significantly to systemic levels of sulfonated estrogens. On the other hand, despite their clearly lower concentrations in comparison to sulfonated estrogens, the levels of free estradiol-17ß and estrone circulating in adult boars must be considered as very potent in terms of bioactivity. Moreover, small but biologically relevant amounts of active free estrogens may be produced locally in the epididymis on the sulfatase pathway. In the epididymal head a high expression of both estrogen receptor isoforms has been described (Hess et al. 1997) indicating that this tissue is highly estrogen-responsive. Thus, the epididymis may be exposed to significant estrogenic activity, and SULT1E1 expression may serve the local control of the effects of estrogens entering the organ via blood and rete testis fluid.

The expression of SULT1E1 in the endothelium of blood vessels in the epididymis and the pampiniform plexus suggests that estrogens are inactivated when passing the wall of blood vessels in either direction. However, as also STS expression was detectable, in the vascular system the metabolism of estrogens may be more complex than just inactivation by sulfonation during passage of blood vessel walls.

The virtual absence of EST activity and the - if at all – very low expression of SULT1E1 in the testis are in striking contrast to the high efflux of sulfonated estrogens from the organ (see Fig. 24 in chapter 4.4) and to observations of a production of E1S in cultured porcine Leydig cells (Raeside & Renaud 1983). A sulfonation of estrogens in the testis by an enzyme different from SULT1E1 is very unlikely in the light of a virtually undetectable sulfonation of E1 in testicular cytosol preparations. A significant local transfer of sulfonated estrogens produced in the epididymal head into the testis is also very unlikely in the view of the results from steroid

measurements in local blood vessels (see Fig. 24 in chapter 4.4), and a morphological equivalent of a direct shunt of blood or lymphatic vessels between the epididymal head and the testis has not been described (see chapter 2.4). Thus the synthetic pathway for the production of testicular estrogen sulfates in the boar remains unclear.

5.4 Dehydroepiandrosterone sulfotransferase activity in the porcine testicular-epididymal compartment

Sulfonation of DHEA was measured in cytosolic fractions based on the decrease of ^{3}H-activity over the time of incubation in the fraction of free steroids (fraction 1). Measurement of the increase of ^{3}H-activity in the fraction of sulfonated steroids extracted after enzymatic hydrolysis (fraction 2) as applied for the assessment of estrogen sulfotransferase activity was not used due to methodological problems with the enzymatic hydrolysis of DHEAS. Anyhow, with the method applied, a significant sulfonation of DHEA should have been reliably detected. However, only marginal sulfonation rates were obtained with tissues of the testicular-epididymal compartment, which were considered nonspecific, as no time-dependency of the change in ^{3}H-activity over the period of incubation could be observed. In contrast, with human term placenta used as a positive control tissue, a time-dependent decrease of ^{3}H-activity in fraction 1 consistent with sulfonation of DHEA was readily detectable. In humans SULT2A1 is predominantly considered as the enzyme relevant for the sulfonation of DHEA. Using a primary antibody against the human enzyme (polyclonal antibody against full length human SULT2A1, SC-32941, Santa Cruz Biotechnology, Heidelberg, Germany), with immunohistochemistry in human term placenta distinct staining was found in decidua cells (not shown). When this method was applied to porcine testicular tissue, distinct staining was observed in round spermatids of individual tubules. Staining intensity in Leydig cells was inconsistent between animals but generally low. As in Western blot no specific band was obtained in porcine testicular tissue, specificity of the immunostaining observed in porcine testicular tissue was queried, and this experimental approach was discontinued. In a concomitant study, SULT2A1-mRNA was detected in all tissue samples collected from the porcine testicular-epididymal compartment investigated and the relative expression level was highest in the testis (Bingsohn et al. 2012). However, as only relative quantification of mRNA-expression was performed, no definite conclusions on protein expression levels and activity can be deduced from these data. Moreover, in pigs to date the SULT relevant for the sulfonation of DHEA under physiological conditions has not been identified yet.

The undetectable DHEA sulfotransferase activity in the testis is in contrast to the results from steroid measurements in local blood vessels giving convincing evidence for a significant efflux of DHEAS directly from the testis. Theoretically DHEA added as the substrate could have escaped from sulfonation by rapid competing conversions into metabolites unsuitable as substrates for sulfotransferases. With this regard, especially the conversion of DHEA into A4 by 3ß-hydroxysteroid dehydrogenase-$\Delta_{5/4}$ isomerase (HSD3B1) must be taken into account. Moreover, DHEA sulfotransferase activity could have been masked by the hydrolysis of DHEAS via STS. However, HSD3B1 activity was probably only low under the experimental conditions as it is a microsomal enzyme and the essential cofactor for this enzyme (NAD^+) was not supplemented in the incubation medium. Moreover, also STS is a microsomal enzyme and exhibits only a low activity for DHEAS (see Fig. 26 in chapter 4.5), and no significant sulfonation of DHEA was found even after supplementation of the incubation medium with the STS inhibitor STX64 or the HSD3B inhibitor trilostane in preliminary experiments (data not shown). Finally, in cytosol prepared from human term human placenta, which highly expresses HSD3B1 (Mason et al. 1993) and STS (Miki et al. 2002), DHEA sulfotransferase activity was readily detectable. Thus a masking of a significant DHEA sulfotransferase activity by competing metabolization is rather unlikely. A possible explanation for a high production of DHEAS in the absence of a relevant sulfotransferase activity could be its direct formation from sulfonated precursors, potentially starting from cholesterol sulfate ("sulfate pathway", see below). Individual steps of this sulfatase pathway have been described in different steroidogenic tissues from various species including the porcine testis (Booth 1975, Ruokonen 1978, Payne & Hales 2004).

5.5 Pregnenolone sulfotransferase activity and SULT2B1 expression in the porcine testicular-epididymal compartment

As with DHEA, sulfonation of P5 was measured based on the decrease of ^{3}H-activity in fraction 1 (free steroids) instead of an increase in fraction 2 (conjugated steroids), again due to methodological problems with the enzymatic hydrolysis of the sulfonated substrate before the second extraction. As the decrease of ^{3}H-activity in fraction 1 theoretically also could have been caused by other reactions than sulfonation of the substrate P5, the results of this part of the investigations should be interpreted with some reservation.

The results suggest that sulfonation of P5 was only very low or undetectable in the testis and in the epididymis between segments EH1 and EB2. However, it became readily detectable in the segment EB3 and was high in the remaining part of the epididymis. This pattern is consistent with results from Western blot analysis and data from the measurement of SULT2B1-mRNA expression performed in a parallel study (Bingsohn et al. 2012). The good consistency between results from the measurements of P5 sulfotransferase activity, relative SULT2B1-mRNA levels (Bingsohn et al. 2012) and Western blot analysis for SULT2B1 expression gives evidence that also in the pig SULT2B1 is the relevant enzyme for the sulfonation of P5.

Cytoplasmic immunostaining pattern for SULT2B1 basically exhibited a good consistency in the epididymis and testis with results from Western blot, measurement of P5 sulfotransferase activity and with the results from the determination of relative SULT2B1-mRNA levels by real-time RT-PCR performed in a parallel study (Bingsohn et al. 2012). However, in the epithelium of the epididymal head a distinct nuclear immunostaining occurred in addition to a weak to moderate staining of the cytoplasm and superficial protrusions. With immunohistochemistry especially debatable results have been obtained in the testis. Whereas in one of the three animals no significant staining was found at all in this organ, in the other two boars considerable nuclear staining occurred in various cell types including spermatocytes, round spermatids and Leydig cells. The significance of this observation is unclear as SULTs are commonly considered as cytosolic enzymes. However, a nuclear localization of SULT2B1 has previously been described (Falany & Rohn-Glowacki 2013). To date it is unclear if the nuclear signals in the testis and the epithelium of the epididymal head are actually related to SULT2B1, possibly corresponding to an enzymatically inactive form of SULT2B1, or result from a cross-reaction with a different SULT or an unrelated protein. In Western Blot the primary antiserum, which was the same as used in immunohistochemistry, specifically stained a band of approx. 45 kDa, which is consistent with the molecular weight of SULT2B1 and different from the molecular weight of other SULTs (Moe et al. 2007). However, consistent with results from the measurement of P5

sulfotransferase activity and SULT2B1-mRNA levels (Bingsohn et al. 2012), a clearly detectable 45 kDa band only occurred in the hind part of the epididymis, suggesting that the extensive nuclear immunostaining observed in the testis and epididymal head was due to an artifact.

Taken together the results from this part of the study suggest that SULT2B1 expression/P5 sulfotransferase activity is absent or extremely low in the testis and proximal part of the epididymis but continuously increases between the distal part of the epididymal body and the tail. The physiological role of the relatively high SULT2B1 expression and P5 sulfotransferase activity in the hind part of the epididymis as well as the relevant substrate in this tissue are unclear. The fact that besides pregnenolone also cholesterol is considered as a specific substrate for SULT2B1 and the possible secretion of SULT2B1 containing epididymosomes into the lumen of the epididymal duct may indicate that epididymal SULT2B1 expression could serve the production of cholesterol sulfate, an important component of sperm membrane (Girouard et al. 2011, Sullivan et al. 2007, Frenette et al. 2003, 2004).

Comparative measurements in veins directly emanating from the testicular parenchyma and in the terminal branch of the testicular artery (see chapter 4.4) provided clear evidence for a significant efflux of P5S from the testis, which is in contrast to the virtual absence of a P5 sulfotransferase activity in the testis. For the reasons mentioned in the discussion related to DHEAS production in the testis and the absence of a detectable DHEA sulfotransferase activity (see section 4.6), a masking of a significant P5 sulfotransferase activity by competing metabolization must be considered as rather unlikely. Thus, also concerning P5S a considerable production from a sulfonated precursor, i.e. cholesterol sulfate, must be taken into account.

5.6 Final conclusions

Despite the high variability observed between individual animals this study provides clear evidence that in boars all steroids which were in the focus of this study are predominantly produced in testicular tissue, most likely in the same cell type, the Leydig cell. Moreover, high correlations found between secretory profiles and similar short periods between hCG stimulation and detection of increases in peripheral blood suggests that they rapidly enter a common pool after synthesis, and there was no evidence for a differential transport between free and sulfonated steroids. The concept that all sulfonated steroids assessed are original products of the testis and the consistently high correlations between the individual sulfonated steroids point to a role of sulfonated steroids in boars as precursors/intermediates of

steroidogenesis in porcine Leydig cells rather than as inactivated end products. The direct production of sulfonated steroids from sulfonated precursors ("sulfate pathway"; Fig. 46) would be a plausible explanation for the high testicular output of sulfated steroids in the absence (estrogens) or debatable presence (DHEA, P5) of relevant steroid sulfotransferases. However, a significant sulfonation of free steroids in porcine Leydig cells cannot be ruled out completely even in the presence of only weak activities of steroid specific SULTs due to the high total mass of these cells in boars.

A substantial use of sulfonated precursors for the production of estrogens, androgens or steroidal pheromones would also provide an explanation for the intriguingly broad spectrum of sulfonated steroids found in boars (Ruokonen & Vihko 1974, Raeside et al. 2006) and would assign a crucial regulatory role to STS which is highly expressed porcine Leydig cells (Mutembei et al 2009 and this study). A survey of the literature shows that the metabolization of steroid sulfonates within steroidogenic organs was rarely addressed during the last 30 years. In fact earlier studies described the conversion of cholesterol sulfate or of P5S into products which still contain the sulfate group in various steroidogenic tissues like human, bovine and rat adrenal (Calvin et al. 1963, Roberts et al. 1964, Calvin & Lieberman 1964), human fetal testis (Lamont et al. 1970) and adult human and boar testis (Gasparini et al. 1976, Ruokonen 1978), respectively. From their study in rat adrenals, Hochberg et al. (1974) concluded that cholesterol sulfate is converted into P5S at a rate at least comparable to, and probably greater than that by which cholesterol is converted into C21 products. However, these studies do not provide definite information on the efficiency of the conversions of 3ß-hydroxy-5-ene steroid sulfates by CYP11A1 and CYP17A1 in comparison to their respective free counterparts, and to date no information is available on the physiological relevance of this "sulfate pathway" of steroidogenesis. Only one report was found in the literature describing the direct aromatization of DHEAS to E1S which was observed in human placenta (Oertel et al. 1967), but from this paper the efficiency of this conversion in relation to the aromatization of free androgens does not come clear. However, a direct conversion of DHEAS into E1S would be a convenient explanation for the otherwise inconsistent observations of a lack of estrogen sulfotransferase activity but a high E1S efflux from the porcine testis. Whereas on the still widely hypothetical sulfate pathway between cholesterol sulfate and DHEAS modifications of the molecule would occur at the D-ring of the sterane backbone, which is remote from the sulfonate group linked to the C3–atom of the A-ring, direct aromatization of a steroid-3-sulfate would have to take place in the immediate proximity of the large and highly polar sulfonate group, which, on the first view, seems very unlikely. Moreover, as indicated by low estrogen concentrations in cases of

STS deficiency, human placental estrogen synthesis obviously depends largely on the hydrolysis of sulfated C19 precursors (Sherwood & Rocks 1982). Thus, it is questionable whether a direct aromatization of DHEAS into E1S occurs to a significant extent in the human placenta but it could be a significant route of estrogen synthesis in boar testis. Interestingly, in pigs three aromatase isoforms (gonadal, preimplantation blastocyst, placental) encoded by different genes have been identified, and studies into catalytic activities of the placental vs. the gonadal isoform clearly showed distinct functional differences (Choi et al. 1997, Corbin et al. 1999). The physiological significance of these differences to the reproductive endocrinology of the pig is still unclear.

The porcine testis produces an intriguingly broad spectrum of various free and sulfonated steroids including androgens, estrogens and steroidal pheromones (Raeside et al. 2006). Different factors have been identified to influence the flow of substrates through the cascade of steroidogenic enzymes, one of them being the compartmentalization of steroidogenic enzymes. In steroidogenesis, the functional architecture of steroidogenic organs is commonly based on the specific expression of key enzymes within particular tissues or cells allowing for compartmental regulation, enabling a degree of independence that would otherwise be difficult to attain (Conley & Bird 1997, Conley et al. 2012). Typical examples for the compartmentalization of steroidogenic key enzymes to different cell types or tissues are the preovulatory follicle (provision of androgens by theca cells, which are aromatized in granulosa cells; Hillier et al. 1994), human placental estrogen synthesis (provision of sulfonated C19-precursors by fetal and maternal adrenals, hydrolysis and aromatization in the syncytiotrophoblast; Diczfalusy 1964) or bovine placental estrogen synthesis (expression of CYP17A1 in uninucleated trophoblast cells, expression of CYP19 in trophoblast giant cells; Schuler et al. 2006). However, as an exception to this principle of compartmentalization of steroidogenic key enzymes, in the porcine testis CYP11A1, HSD3B1, CYP17A1 and CYP19 are obviously co-localized in the Leydig cells, and despite many data on blood levels of various steroids the substrate flow in this cell type – at least from a quantitative point of view – is virtually unknown. Thus, based on the results of this and a concomitant study on the expression of steroidogenic enzymes (Bingsohn et al. 2012) the hypothesis was developed that in porcine Leydig cells steroid sulfonation and hydrolysis of steroid sulfonates could be engaged in the control of the substrate flow through the steroidogenic enzyme cascade to finally provide the requested levels of androgens, free and sulfonated estrogens and steroidal pheromones. In particular, the biological role of the suggested predominant or considerable use of sulfonated substrates during porcine testicular steroidogenesis could be a strategy to block a “preterm

leakage" of P5 or 17α-hydroxy-P5 into the Δ4-pathway via HSD3B1, which could lead to inappropriate levels of steroids needed for the maintenance of reproductive functions or to an impaired function of other steroidogenic tissues such as the adrenal cortex. Thus, for a better understanding of the complex steroidogenesis in the porcine testis the utilization of sulfonated substrates by porcine CYP11A1, CYP17 and CYP19 should be thoroughly characterized, and information on the substrate specificities of porcine SULTs relevant for steroids is needed. Finally, for a better understanding of the physiological role of STS in porcine Leydig cells and to get a more specific idea which of the many steroid sulfonates occurring in the porcine testis are actually exposed to the enzyme, definite knowledge on its subcellular localization is essential.

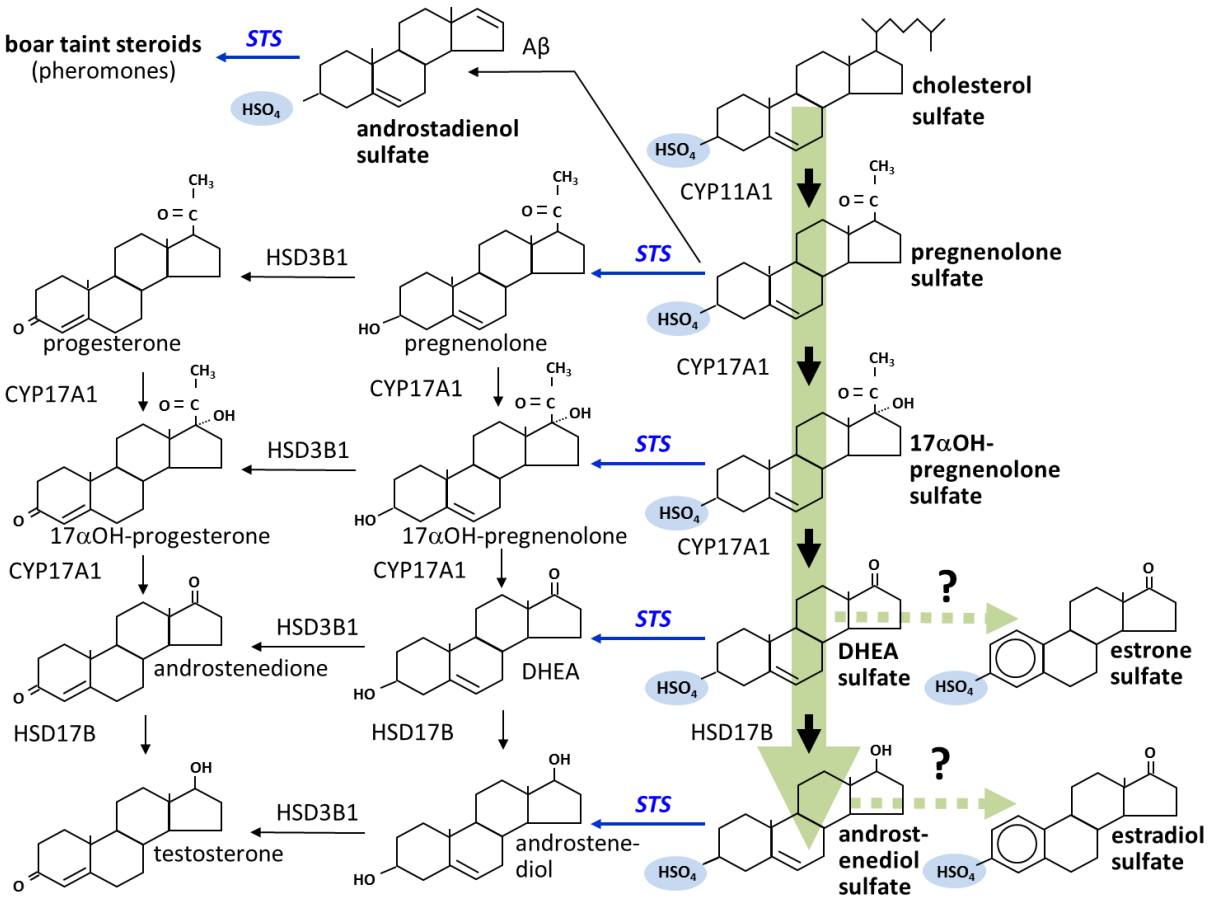


Figure 46: Hypothetical pathways for the synthesis of free and sulfonated steroids in porcine Leydig cells. More speculative steps of this concept are indicated by dotted lines and question marks.

CYP11A1: side-chain-cleavage enzyme; CYP17A1: 17α-hydroxylase-C17,20-lyase; HSD3B1: 3β-hydroxysteroid-dehydrogenase-Δ5/4-isomerase; CYP19: aromatase; SULT: cytosolic sulfotransferase; STS: steroid sulfatase. Steroidal pheromones (16-androstene steroids) diverge from pregnenolone or pregnenolone sulfate under the andien-β-synthase activity of CYP17A1 in the presence of adequate levels of cytochrome b5 (Meadus et al. 1993, Sinclair et al. 2005).

6. Summary

Sulfonated steroids have been traditionally regarded as inactive metabolites destined for excretion, as they are incapable of binding to classical nuclear steroid receptors. However, by the enzyme steroid sulfatase (STS) they may be converted into free steroids, which may be biologically active directly or after a few additional enzymatic reactions. Thus, as sulfonated steroids commonly circulate at relatively high concentrations, they may form an important pool of precursors for the local (intra-tissue) production of active free steroids. This so-called sulfatase pathway has received increased attention over recent years especially with respect to estrogen metabolism in human hormone-dependent breast cancer, where the intratumoral estrogen production from sulfonated precursors obviously has a much higher capacity in comparison to the de novo synthesis via free steroids. However, in contrast to this pathological situation, only very little information is available on the role of the sulfatase pathway in physiological settings. Thus, in this study on the metabolism and role of sulfonated steroids the porcine testicular-epididymal compartment was used as a model, as in previous work the presence of all components considered as essential for steroid effects via a sulfatase pathway have been demonstrated in close vicinity to one another in this organ system such as the production of high amounts of numerous steroid sulfates and the expression of STS, of different types of receptors for sex steroids and of the steroid sulfate transporter candidate SLC10A6. Despite a considerable number of excellent studies on testicular steroidogenesis in boars, there is still virtually no definite information on the synthetic pathways providing the intriguingly high amounts of sulfonated steroids and on their biological role(s).

This study is composed of two parts of which the first one addresses the secretory patterns of free and sulfonated steroids in vivo, whereas in the second part the expression of STS and of the steroid sulfotransferases SULT1E1 (estrogen specific) and SULT2B1 (specific for β-hydroxysteroids) was characterized in the testis and in different segments of the epididymis. Other subjects of the second part of this study were hydrolysis of steroid sulfates and the sulfonation of estrone (E1), dehydroepiandrosterone (DHEA) and pregnenolone (P5) in the tissues investigated.

Firstly, in order to establish long-term secretion profiles of free and sulfonated steroids, plasma samples were collected from six sexually mature boars over a period of six hours every 20 minutes from the jugular vein. To facilitate the analysis of correlations between the secretion patterns of individual steroids, in seven boars a more dynamic situation was induced by stimulation with human chorionic gonadotropin, followed by sampling for long term

measurements. Concentrations of androstenedione, testosterone, pregnenolone sulfate (P5S), dehydroepiandrosterone sulfate (DHEAS), estrone-3-sulfate (E1S)and 17β-estradiol-3-sulfate were performed in the Steroid Research & Mass Spectrometry Unit, Division of Pediatric Endocrinology & Diabetology, Center of Child and Adolescent Medicine, Justus-Liebig-University, Giessen (head: Prof. Dr. S. Wudy) applying liquid chromatography tandem mass spectrometry (LC-MS-MS). Moreover, 17β-estradiol (E2) and E1 were measured by inhouse radioimmunoassays to cope with the low concentrations of free estrogens in boars. Additionally, in order to directly characterize the testicular output, in four animals blood samples were collected from arterial and venous blood vessels of the testicular surface and the spermatic cord. Irrespective of a high variability between individuals, the results suggest that all steroids assessed are primarily produced in the testis, that they exhibit similar profiles pointing to a pulsatile secretion with low frequency (three to five pulses per day), and that after synthesis at least a major proportion is immediately released into peripheral circulation. The fact that all steroid sulfates assessed are original testicular products and their high correlations with one another suggest their role as being intermediates of testicular steroidogenesis rather than as being inactivated end products. No evidence was obtained for a significant involvement of the epididymis in the sulfonation of testicular free estrogens, which had been hypothesized based on the observations from a previous study that estrogen sulfotransferase activity was virtually absent in testicular homogenates, whereas it was readily detectable in the epididymis. In order to get new information on the sulfonation of free steroids and the hydrolysis of steroid sulfates in the porcine testicular-epididymal compartment, subcellular fractions were prepared from tissue samples collected from the testis and from defined sites of the epididymis (EH1, EH2: proximal/distal part of epididymal head; EB1-4: epididymal body, from proximal to distal; ET1, ET2: proximal/distal part of epididymal tail) using differential centrifugation. STS and steroid sulfotransferase activities were measured based on the differential distribution of free and sulfonated steroids between an aqueous phase and an organic solvent, tert butyl-methylether. STS activity was measured in microsomal fractions prepared from three boars using E1S as a substrate. Enzyme activity was clearly measurable in all segments of the epididymis but was generally lower compared to the testis. When comparing different substrates, hydrolysis of E1S by testicular microsomal protein was considerably higher compared to P5S or DHEAS. In cytosolic fractions prepared from three boars sulfonation of P5, DHEA and E1 was investigated in the presence of the universal sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Consistent with an earlier study using tissue homogenates, estrogen sulfotransferase activity was high in epididymal samples but virtually

undetectable in the testis. P5 sulfotransferase activity was not considerable different from background level in the testis, epididymal head and the proximal half of the epididymal body. It increased in the distal half of the epididymal body and was maximal in the distal part of the epididymal tail. Sulfonation of DHEA was below the sensitivity of the assay in all samples investigated.

The sulfonation of small biomolecules such as steroids is catalyzed by a large family of cytosolic sulfotransferases (SULTs), which may exhibit overlapping substrate specificities. In pigs, to date no definite information is available on the relevance of individual SULTs for steroids. However, SULT1E1 is generally considered as the relevant enzyme for the sulfonation of estrogens under physiological conditions. Thus, expression of this enzyme was investigated in the testis and epididymal tissue samples collected from three boars using Western blot (WB) and immunohistochemistry (IHC) applying a primary polyclonal antibody elicited against the bovine recombinant enzyme. With WB, a specific band of approx. 34 kDa consistent with the molecular weight of SULT1E1 was found in all epididymal samples but was absent in the testis. By means of IHC, in the epididymis specific staining was predominantly found in the epithelium with a trend to higher staining intensities in segments EH1, EH2 and EB1 compared to other parts of the organ. Staining was especially prominent in superficial epithelial protrusions possibly serving apocrine secretion. Occasional staining of weaker intensity was also found in the muscular layer and in vascular endothelium. In the testis, only occasionally a weak staining of questionable specificity was found, which occurred in Leydig cells and vascular endothelial cells.

Another SULT addressed was SULT2B1, which in humans is considered as the enzyme relevant for the sulfonation of cholesterol and P5. In WB and IHC, a polyclonal antiserum against the human enzyme was applied. In WB, a specific band of the expected molecular size of 45 kDa was clearly detectable in the hind part of the epididymis (segments EB4 to ET2). In the more proximal segments of the epididymis intensity was clearly weaker, whereas in the testis the specific band was barely recognizable. In IHC, in two of three animals investigated surprisingly a distinct nuclear signal was found in different cell types of the testis and in the epithelium of the epididymal head. No such staining occurred in the third animal. Although a nuclear localization has been previously described for SULT2B1 in certain human cell types, the specificity of the nuclear signal observed in the porcine testis and epididymal head is highly questionable in light of the results obtained with WB. In all animals, cytoplasmic staining was found predominantly in the epididymal epithelium with a trend to higher staining intensity in the hind part of the epididymis compared to the head and proximal segments of the body. As

with SULT1E1, staining was especially prominent in superficial epithelial protrusions, and occasional cytoplasmic staining was also observed in the epididymal muscular layer and vascular endothelium.

In a previous immunohistochemical study applying the same primary antiserum against human STS, in the porcine testis STS expression was exclusively localized in Leydig cells. In this study including three boars, in addition to Leydig cells STS specific cytoplasmic staining was also observed in epithelial cells of the rete testis. In the epididymis specific cytoplasmic staining predominantly occurred in the epithelium, with a trend for higher staining intensities in EH2, EB1 and ET2 compared to other segments. As with SULTs 1E1 and 2B1, immunostaining was especially prominent in superficial epithelial protrusions. Sporadic staining of weaker intensity was also found in the muscular layer and in the vascular endothelium. With WB, a specific band of the expected molecular size (approx. 61 kDa) was found in the testis and all segments of the epididymis.

In conclusion, these results show that STS is widely expressed in the porcine testicular-epididymal compartment, indicating a high potential for sulfatase pathways especially in Leydig cells and the epithelial cells of the rete testis and epididymis. The co-expression of STS with SULTs 1E1 and 2B1 in the epididymal epithelium and especially their colocalization in superficial protrusions are very intriguing. In the epididymal duct, apocrine secretion has been described to give rise to the formation of epididymosomes, small vesicles which are considered as vehicles for the transfer of certain molecules to the maturing sperm cells. Other intriguing findings are the virtual absence of a sulfonation of E1, DHEA and P5 in testicular cytosols as well as the absent or questionable detection of SULTs 1E1 and 2B1 in light of the high efflux of various steroid sulfates from the testis. A plausible explanation could be a significant use of sulfonated steroids as precursors/intermediates in porcine testicular steroidogenesis starting from cholesterol sulfate. The concept of a “sulfate pathway” of steroidogenesis would not only provide an explanation for the production of high amounts of steroid sulfates in the virtual absence of relevant steroid sulfotransferase activities but also for the high STS expression in Leydig cells. According to this concept, STS could play a crucial role in the control of the substrate flow through the steroidogenic enzyme cascade by mediating the transition of sulfonated precursors into the pool of free steroids, with the exact subcellular localization being of importance for the step of the enzyme cascade at which this transition(s) may occur. Thus, in order to corroborate this concept investigations into the utilization of sulfonated substrates by steroidogenic enzymes and on the subcellular localization of STS are necessary.

7. Zusammenfassung

Da sulfonierte Steroide nicht an klassische nukleäre Steroidrezeptoren binden, wurden sie lange Zeit als inaktive Ausscheidungsformen angesehen. Durch das Enzym Steroidsulfatase (STS) können sie jedoch in freie Steroide umgewandelt werden, welche direkt oder nach wenigen weiteren Umwandlungsschritten biologische Aktivitäten entfalten können. Da sulfonierte Steroide häufig in relativ hohen Konzentrationen zirkulieren, können sie somit einen wichtigen Pool von Präkursoren für die lokale Synthese aktiver freier Steroide in bestimmten Zielzellen darstellen. Dieser sogenannte Sulfatase-Weg der Steroidsynthese hat in den vergangenen Jahren ein zunehmendes Interesse im Zusammenhang mit dem Östrogenstoffwechsel in menschlichem Brustkrebsgewebe erfahren. Hier konnte gezeigt werden, dass der Sulfatase-Weg im Vergleich zur de novo-Synthese von Östrogenen aus freien Steroiden weitaus ergiebiger ist. Im Gegensatz zu diesem pathologischen Umfeld liegen zur Bedeutung des Sulfatase-Wegs unter physiologischen Verhältnissen bisher noch kaum Informationen vor. Für eigene Untersuchungen zum Metabolismus und zur biologischen Bedeutung von Steroidsulfaten wurde als Modell das Hoden-Nebenhodensystem des Ebers gewählt, da dort in vorangegangenen Untersuchungen auf engem Raum alle wesentlichen Komponenten nachgewiesen wurden, die für lokale Steroidwirkungen über den Sulfatase-Weg als erforderlich erachtet werden: die Produktion großer Mengen an zahlreichen verschiedenen Steroidsulfaten sowie die Expression der STS, verschiedener Rezeptoren für Sexualsteroide und des Steroidsulfattransporter-Kandidaten SLC10A6. Trotz zahlreicher hervorragender Untersuchungen zur Steroidsynthese im Eberhoden lagen zu den der Produktion von Steroidsulfaten zugrunde liegenden Synthesewegen sowie zur biologischen Bedeutung der Steroidsulfate beim Eber bisher keine konkreten Informationen vor.

Die durchgeführten Untersuchungen gliedern sich in zwei Teile. Gegenstand des ersten Teils war die Charakterisierung der Sekretion verschiedener freier und sulfonierter Steroide in vivo. Im zweiten Teil wurde die Expression der STS sowie der beiden steroidspezifischen Sulfotransferasen SULT1E1 (östrogenspezifisch) und SULT2B1 (spezifisch für 3β-Hydroxysteroide) im Hoden und verschiedenen Abschnitten des Nebenhodens untersucht. Weiterer Gegenstand des zweiten Untersuchungsabschnittes waren die Hydrolyse von Steroidsulfaten sowie die Sulfonierung von Estron (E1), Dehydroepiandrosteron (DHEA) und von Pregnenolon (P5) in den Zielgeweben.

Zur Erstellung von Langzeitprofilen für verschiedene freie und sulfonierte Steroide wurden von sechs geschlechtsreifen Ebern in 20-minütigen Intervallen Blutproben über einen Zeitraum von

sechs Stunden gewonnen. Um die Beziehungen zwischen den Profilen einzelner Steroide besser analysieren zu können, wurden bei sieben Ebern durch eine Stimulation mit humanem Choriongonadotropin ausgeprägte Konzentrationsveränderungen induziert und ebenfalls Langzeitprofile erstellt. Die Messung von Androstendion, Testosteron, Pregnenolonsulfat (P5S), Dehydroepiandrosteronsulfat (DHEAS), Estron-3-sulfat (E1S) und 17β-Estradiol-3-sulfat erfolgen im Steroidlabor des Zentrums für Kinderheilkunde, Universitätsklinikum Gießen (Leiter: Prof. Dr. Stefan Wudy) mittels Flüssigkeitschromatographie-Tandemmassenspektrometrie (LC-MS-MS). E1 und 17β-Estradiol (E2) wurden wegen der beim Eber niedrigen Konzentrationen mittels im eigenen Labor etablierter Radioimmunoassays gemessen. Zur näheren Charakterisierung der testikulären Steroidsekretion wurden bei vier Tieren Messungen in arteriellen und venösen Blutgefäßen der Hodenoberfläche sowie im Bereich des Samenstranges durchgeführt. Trotz einer außerordentlich hohen tierspezifischen Variabilität der gemessenen Konzentrationen und erstellten Hormonprofile lassen die Ergebnisse dieser Untersuchungen erkennen, dass alle erfassten Steroide primär im Hoden gebildet werden. Bezogen auf das einzelne Tier wiesen die erstellten Hormonprofile meist eine große Ähnlichkeit untereinander auf und deuteten auf eine pulsatile Sekretion mit niedriger Frequenz hin (ca. 3-5 Pulse/Tag). Die Ergebnisse lassen weiterhin darauf schließen, dass die erfassten Steroide zumindest zu einem erheblichen Teil unmittelbar nach ihrer Produktion im Hoden in die periphere Zirkulation abgegeben werden. Da die Profile aller erfassten Steroidsulfate besonders eng miteinander korrelierten ist zu vermuten, dass sie eher Vor- oder Zwischenstufen der testikulären Steroidsynthese als inaktivierte Endprodukte darstellen. Es ergaben sich keine Hinweise auf eine erhebliche Sulfonierung freier testikulärer Östrogene im Nebenhoden. Dies war im Vorfeld dieser Untersuchungen vermutet worden, da in früheren Untersuchungen an Gewebehomogenaten eine hohe Östrogensulfotransferaseaktivität im Nebenhoden, nicht jedoch im Hoden nachweisbar war.

Um neue Informationen über die Sulfonierung freier Steroide und die Hydrolyse von Steroidsulfaten im Hoden-Nebenhodensystem zu erhalten, wurden mittels Differentialzentrifugation subzelluläre Fraktionen aus Hodengewebe und definierten Anteilen des Nebenhodens hergestellt (EH1, EH2: proximaler/distaler Teil des Nebenhodenkopfs; EB1-EB4: Nebenhodenkopf, von proximal nach distal; ET1, ET2: proximaler/distaler Teil des Nebenhodenschwanzes). Basierend auf der unterschiedlichen Verteilung von freien und sulfonierten Steroiden zwischen einer wässrigen bzw. organischen Phase (Tert-Butyl-Methylether) wurden Messungen der STS- bzw. Steroidsulfotransferaseaktivitäten durchgeführt. Die Messung der STS-Aktivität erfolgte in den Mikrosomenfraktionen von drei

Ebern unter Verwendung von E1S als Substrat. Im Nebenhoden war STS-Aktivität in allen Abschnitten nachweisbar. Im Vergleich zum Hoden war sie im Allgemeinen deutlich niedriger. Ein Substratvergleich ergab, dass E1S durch testikuläre Mikrosomenfraktionen deutlich stärker hydrolysiert wurde als P5S oder DHEAS. In Zytosolen von drei Ebern wurde die Sulfonierung von E1, DHEA und P5 in Anwesenheit des universellen Sulfat-Donors 3´-Phosphoadenosin-5´-phosphosulfat (PAPS) untersucht. In Übereinstimmung mit früheren Untersuchungen an Gewebehomogenaten fanden sich in den Zytosolen aus allen Nebenhodenabschnitten hohe Östrogensulfotransferaseaktivitäten, während diese in Proben aus Hodengewebe praktisch nicht nachweisbar war. In Hoden, Nebenhodenkopf und der proximalen Hälfte des Nebenhodenkörpers war eine Sulfonierung von P5 nicht eindeutig nachweisbar. Sie stieg jedoch in der zweiten Hälfte des Nebenhodenkörpers deutlich an; die höchsten Aktivitäten wurden im distalen Nebenhodenschwanz gemessen. In keiner der untersuchten Proben war eine Sulfonierung von DHEA zu verzeichnen.

Die Sulfonierung kleiner Biomoleküle wie der Steroide wird von Mitgliedern der großen Familie der zytosolischen Sulfotransferasen (SULTs) katalysiert, welche ein überlappendes Substratspektrum aufweisen können. Über die beim Schwein für die Sulfonierung von Steroiden relevanten SULTs liegen bisher keine spezifischen Informationen vor. Allgemein wird jedoch die SULT1E1 als hochspezifisch für die Sulfonierung von Östrogenen angesehen. Daher wurde die Expression dieses Enzyms bei drei Ebern im Hoden und den verschiedenen Nebenhodenabschnitten mittels Western Blot (WB) und Immunhistologie (IHC) unter Verwendung eines primären Antiserums gegen das bovine rekombinante Enzym untersucht. In allen Nebenhodenabschnitten, nicht jedoch im Hoden zeigte sich im WB eine spezifische Bande von ca. 34 kDa, was dem Molekulargewicht der SULT1E1 entspricht. Mittels IHC war im Nebenhoden ein spezifisches zytosolisches Signal vorwiegend in den Epithelzellen nachweisbar, wobei die Färbeintensität in den Abschnitten EH1, EH2 und EB1 tendenziell stärker war als in den restlichen Nebenhodenabschnitten. Ein besonders intensives Signal fand sich in oberflächlichen Protrusionen, welche vermutlich das morphologische Äquivalent für eine apokrine Sekretion dieser Zellen darstellen. Gelegentlich waren schwächere Anfärbungen in der Muskelschicht und in Gefäßendothelien vorhanden. Im Hoden traten stellenweise leichte Anfärbungen der Leydigzellen auf. Schwache Signale waren gelegentlich auch in Gefäßendothelien zu beobachten. Die Spezifität der im Hoden beobachteten Anfärbungen ist jedoch als zweifelhaft zu beurteilen.

Beim Menschen wird die SULT2B1 als spezifisch für Cholesterin und P5 angesehen, daher wurde die Expression dieses Enzyms mit in die eigenen Untersuchungen einbezogen. Für den

Nachweis im WB und in der IHC wurde ein polyklonales Antiserum gegen die menschliche SULT2B1 eingesetzt. Im WB war eine spezifische Bande mit dem erwarteten Molekulargewicht von 45 kDa in den Proben aus dem distalen Bereich des Nebenhodens eindeutig nachweisbar (Abschnitte EB4 bis ET2). In den weiter proximal gelegenen Nebenhodenabschnitten war die Intensität dieser Bande deutlich geringer, während sie im Hoden – wenn überhaupt – nur sehr schwach erkennbar war. Mittels der IHC waren bei zwei von drei untersuchten Tieren überraschenderweise in verschiedenen Zelltypen des Hodens sowie im Epithel des Nebenhodenkopfes deutliche nukleäre Signale vorhanden. Eine nukleäre Lokalisation der SULT2B1 wurde zwar bereits für bestimmte menschliche Zelltypen beschrieben, angesichts der Ergebnisse aus dem WB ist die Spezifität der beobachteten nukleären Signale jedoch sehr zweifelhaft. Bei allen untersuchten Tieren (n=3) fanden sich zytosolische Anfärbungen vorwiegend im Nebenhodenepithel, wobei die Intensität in den distalen Nebenhodenabschnitten tendenziell stärker war als im Nebenhodenkopf und der proximalen Hälfte des Nebenhodenkörpers. Wie im Fall der SULT1E1 waren die stärksten Signale in den oberflächlichen Protrusionen der Epithelzellen zu beobachten. Gelegentlich waren auch schwächere zytoplasmatische Anfärbungen in der Muskelschicht sowie in Gefäßendothelien zu beobachten.

In einer vorangegangenen Arbeit war die STS im Hoden ausschließlich in den Leydigzellen lokalisiert worden. In den eigenen Untersuchungen an drei Ebern unter Verwendung desselben Primärantikörpers gegen die menschliche STS fanden sich jedoch zusätzlich zytoplasmatische Signale auch in den Epithelzellen des Rete testis. Im Nebenhoden waren spezifische zytoplasmatische Signale überwiegend in den Epithelzellennachweisbar, wobei die Intensität der Immunfärbung in den Abschnitten EH2, EB1 und ET2 tendenziell höher war als in den restlichen Nebenhodenabschnitten. Wie im Fall der SULTs 1E1 und 2B1 trat die stärkste Färbeintensität in den oberflächlichen epithelialen Protrusionen auf. Gelegentlich waren schwächere zytoplasmatische Anfärbungen auch in der Muskelschicht und in den Gefäßendothelien vorhanden. Im WB war in allen untersuchten Proben eine spezifische Bande mit dem erwarteten Molekulargewicht von ca. 61 kDa nachweisbar.

Aus der weit verbreiteten Expression der STS ergeben sich in Hoden und Nebenhoden des Ebers vielfältige Möglichkeiten für das Vorliegen eines Sulfatase-Weges, insbesondere in den Leydigzellen und den Epithelzellen des Rete testis und des Nebenhodens. Die Co-Lokalisation der STS mit den Sulfotransferasen 1E1 und 2B1 im Nebenhodenepithel, insbesondere in dessen oberflächlichen Protrusionen, ist jedoch sehr erstaunlich. Im Nebenhodengang wurde beschrieben, dass aus der apokrinen Sekretion Epididymosomen, kleine membranumhüllte

Vesikel, hervorgehen. Diese stellen vermutlich eine besondere Transportform dar, um bestimmte vom Nebenhoden produzierte Moleküle in die reifenden Samenzellen zu integrieren. In den unmittelbar aus dem Hoden ein- bzw. austretenden Blutgefäßen wurden hohe arterio-venöse Differenzen für alle gemessenen Steroidsulfate gefunden. Angesichts dieser Beobachtung ist es ein weiterer erstaunlicher Befund, dass in Zytosolen aus Hodengewebe eine Sulfonierung von E1, DHEA und P5 praktisch nicht messbar war und dass mittels IHC und WB eine Expression der SULTs 1E1 und 2B1 nicht bzw. kaum nachweisbar war. Eine Erklärung für diese widersprüchlichen Befunde könnte sein, dass in der Steroidsynthese des Eberhodens ausgehend von Cholesterinsulfat in erheblichem Umfang sulfonierte Vorstufen bzw. Zwischenprodukte verwendet werden. Dieses Konzept eines „Sulfat-Weges“ der Steroidsynthese würde auch eine Erklärung für die hohe Expression der STS in den Leydigzellen bieten. Da die STS den Übergang von sulfonierten Steroiden in freie Formen vermittelt, würde der STS in diesem Konzept eine wichtige Funktion in der Steuerung des Substratflusses durch die Kaskade der steroidogenen Enzyme zukommen, wobei auch die exakte subzelluläre Lokalisation der STS eine entscheidende Rolle spielen würde. Zur weiteren Untermauerung dieses Konzepts müssten demnach Untersuchungen zur direkten Umwandlung von Steroidsulfaten durch steroidogene Enzyme sowie zur subzellulären Lokalisation der STS in Leydigzellen durchgeführt werden.

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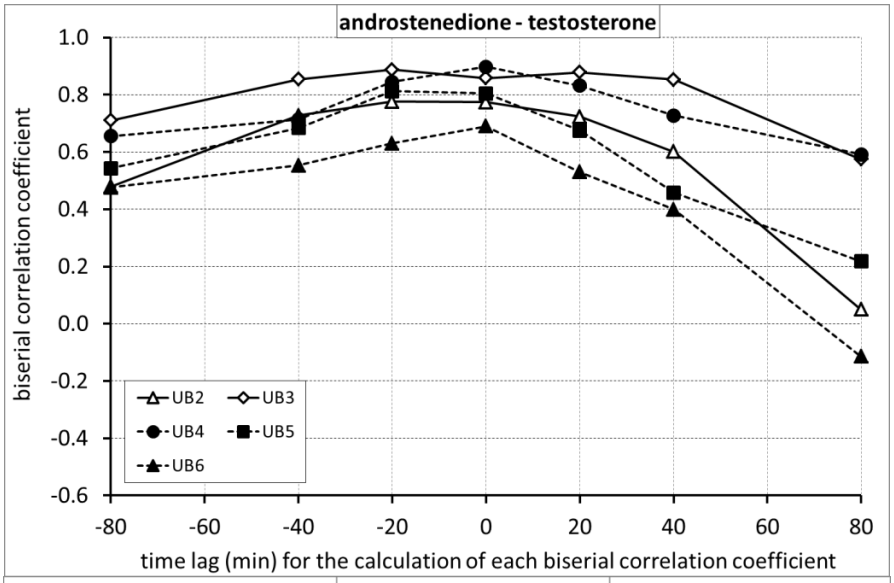
9. Appendix

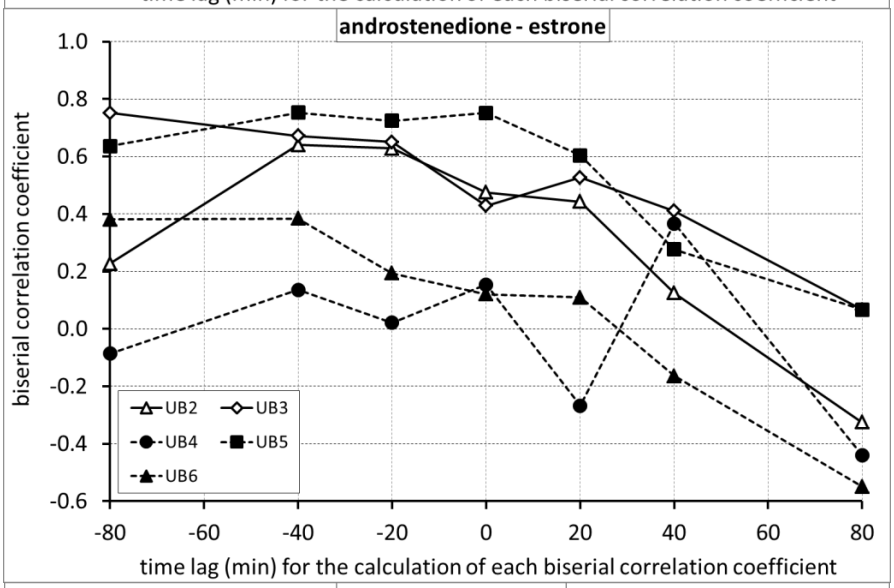
9.1 Results from the calculation of biserial correlation functions in individual animals

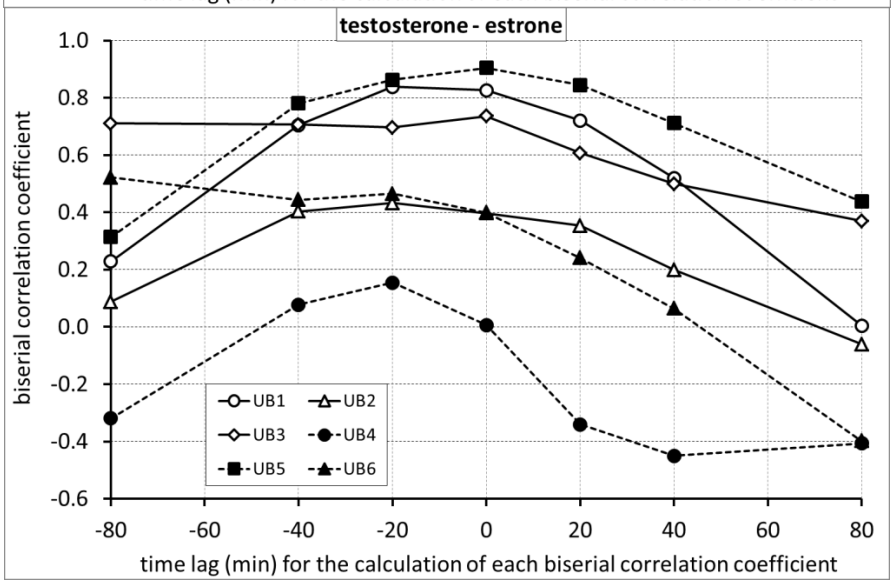
In order to obtain evidence for differential transport or distribution between the individual steroids assessed, biserial correlation functions were calculated to test pairwise for time shifts between their profiles. In section 4 results from the evaluation of pooled data from unstimulated (boars UB1-6; section 4.2) and stimulated animals (boars SB1-7; section 4.3) are presented, respectively. In the following part results from the calculation of biserial functions in individual animals are documented. The graphs show the biserial correlation coefficient (r) (y-axis) as a function of the time shift (x-axis). A maximum at the point of origin indicates synchronous secretion, whereas a distinct maximum of r following shifting to either side suggests that secretion of one of the steroids determined is ahead of the other one (e.g. a maximum of the steroid named first in the diagram on the right side would indicate that this steroid is ahead the other one; see section 3.8.2 for more detailed information on the statistical evaluation procedure).

9.1.1 Biserial correlation functions in individual unstimulated animals

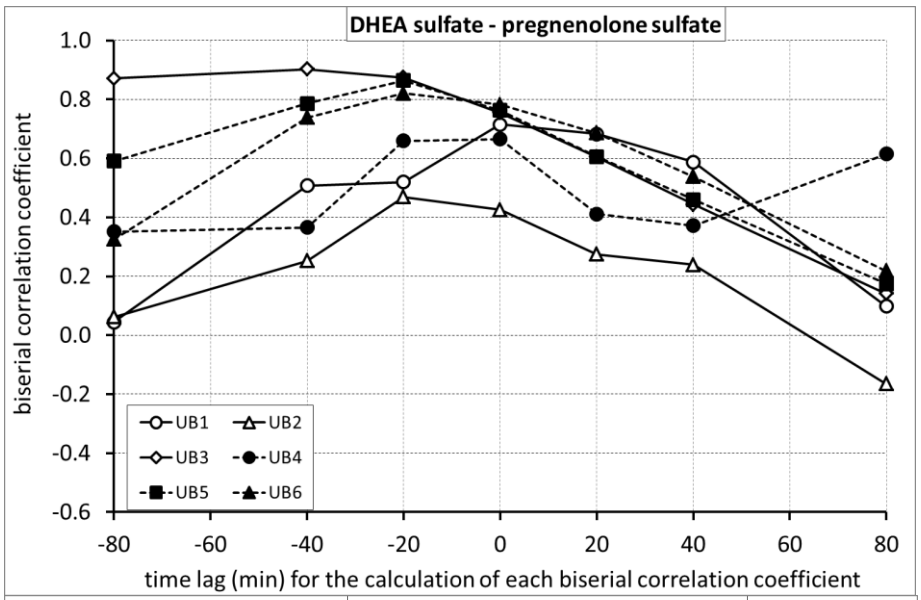
9.1.1.1 Biserial correlation functions between free steroids

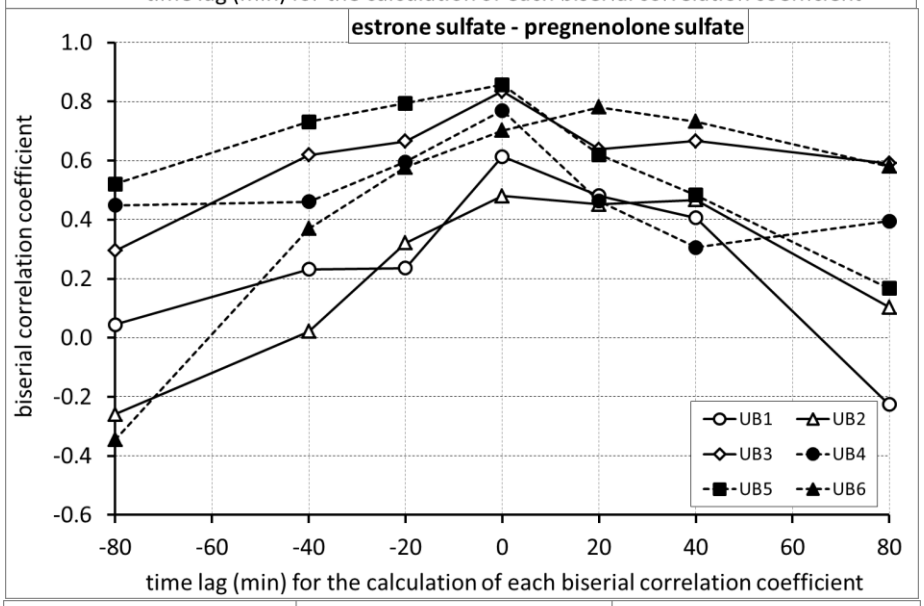


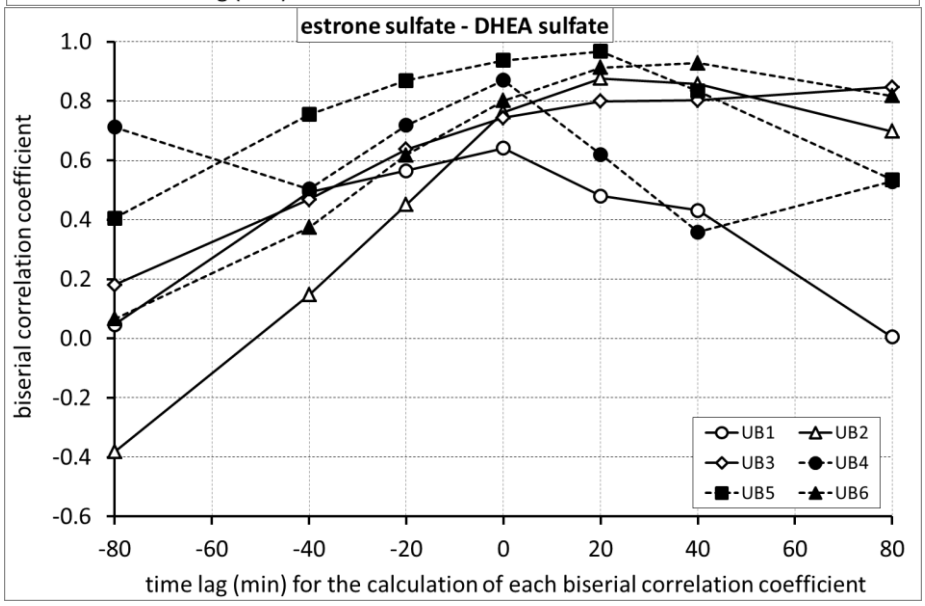




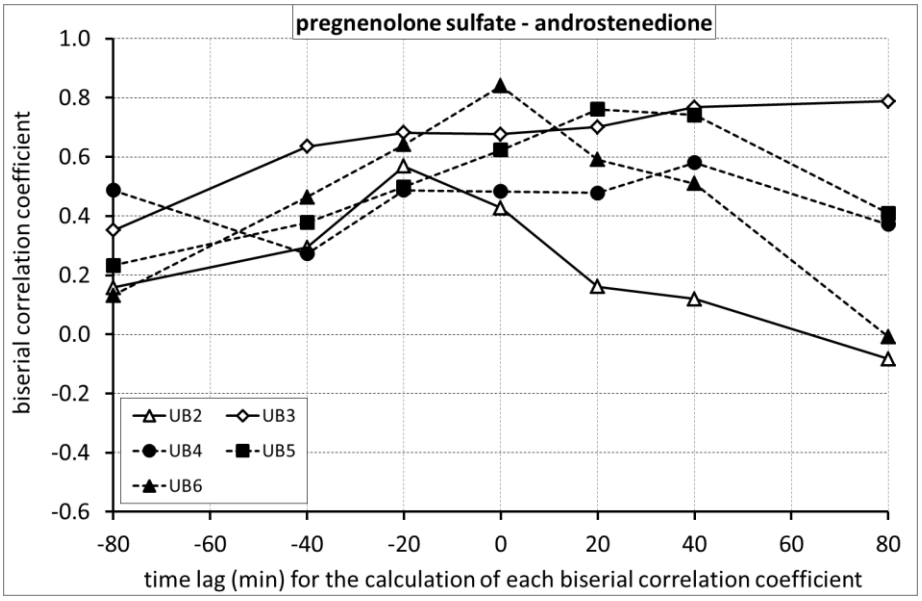
9.1.1.2 Biserial correlation functions between sulfonated steroids

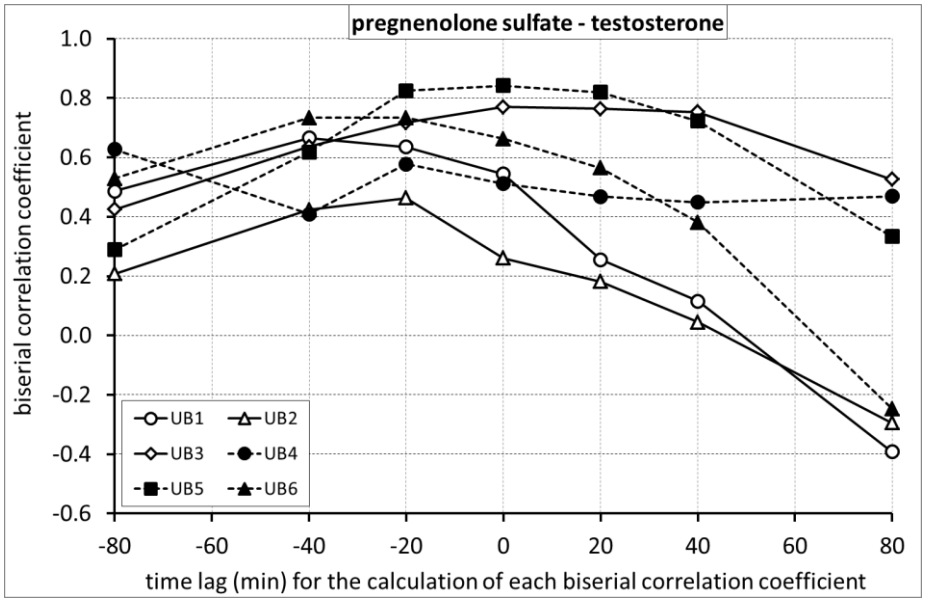


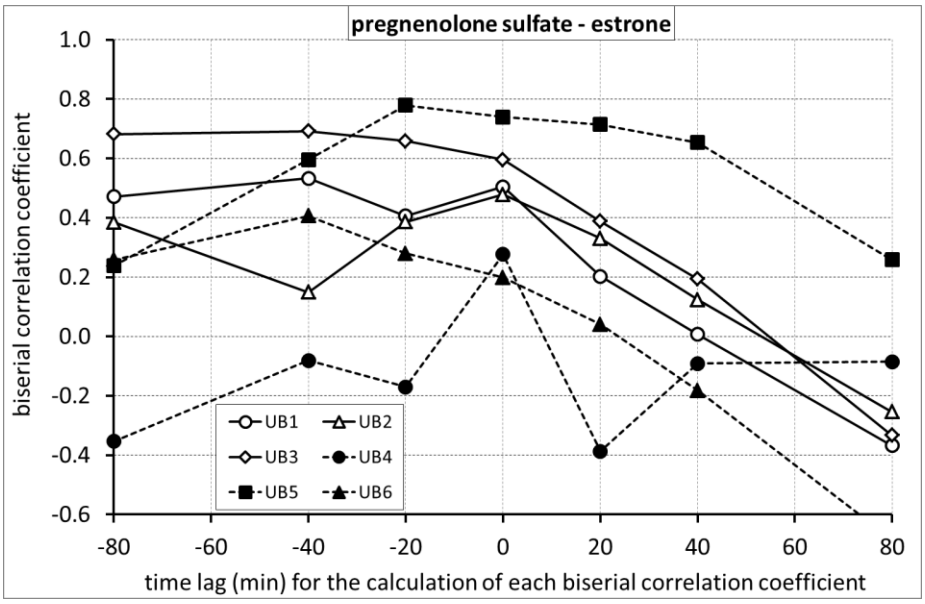


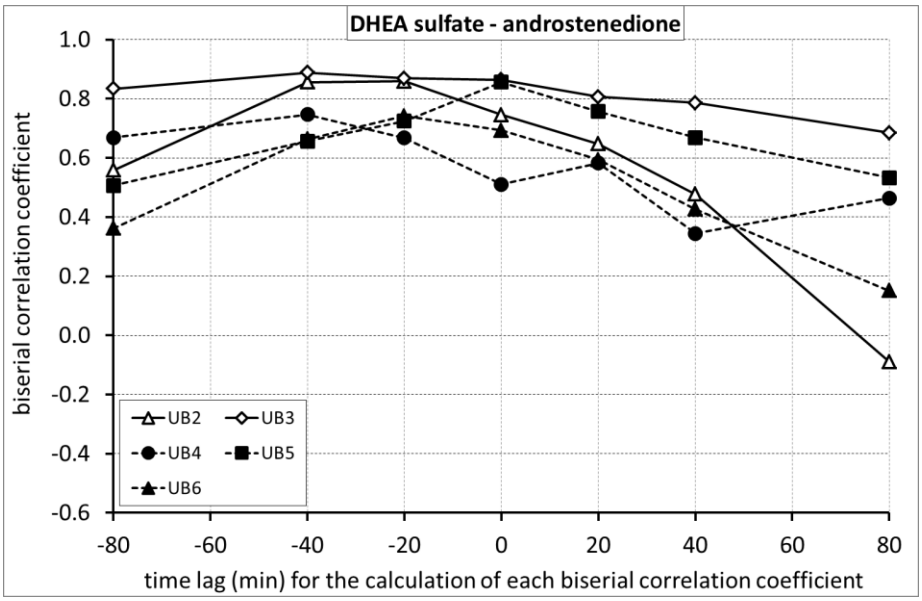


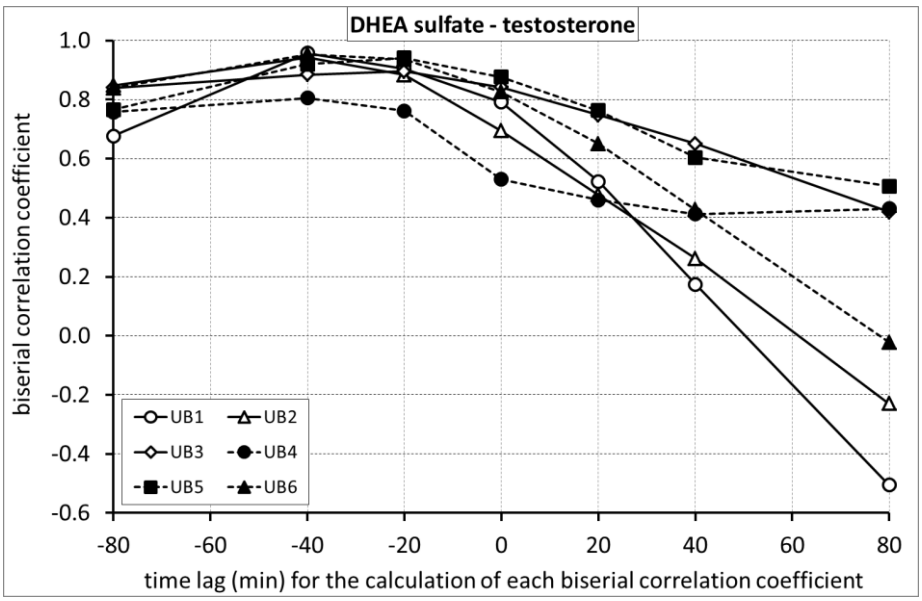
9.1.1.3 Biserial correlation functions between sulfonated and free steroids

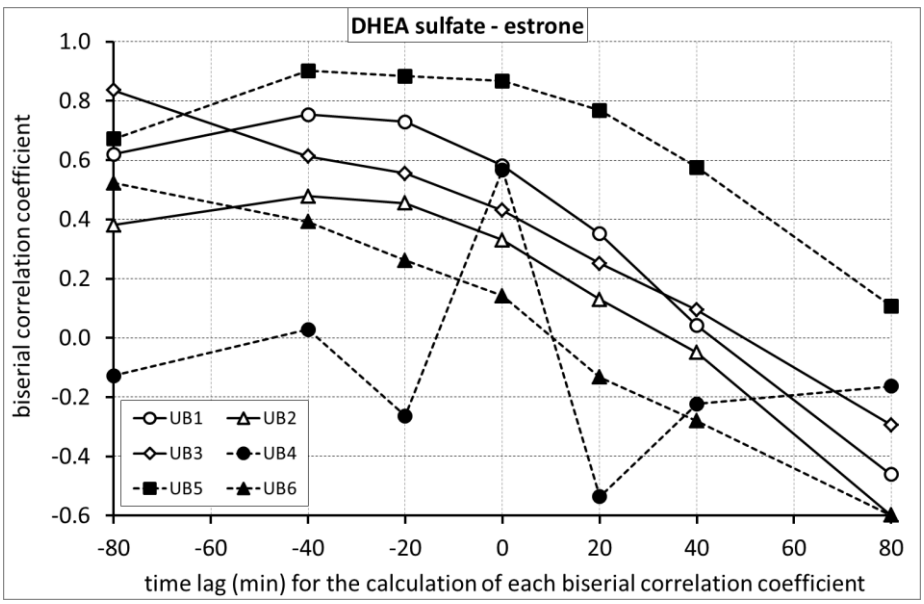







DHEA sulfate - androstenedione
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-80
-60
-40
-20
0
20
40
60
80
UB2
UB3
UB4
UB5
UB6
time lag (min) for the calculation of each biserial correlation coefficient


DHEA sulfate - testosterone
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-80
-60
-40
-20
0
20
40
60
80
UB1
UB2
UB3
UB4
UB5
UB6
time lag (min) for the calculation of each biserial correlation coefficient


DHEA sulfate - estrone
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-80
-60
-40
-20
0
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40
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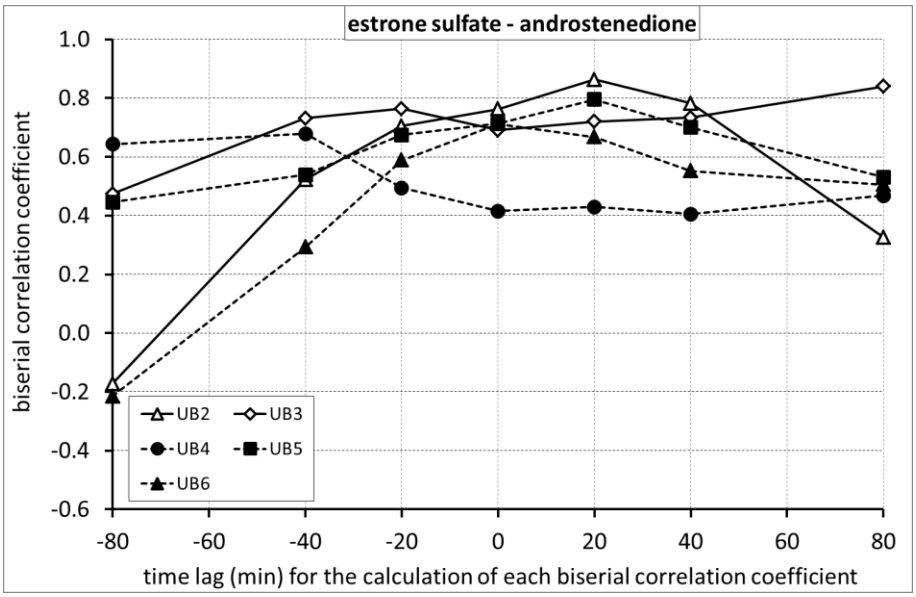
80
UB1
UB2
UB3
UB4
UB5
UB6
time lag (min) for the calculation of each biserial correlation coefficient


estrone sulfate - androstenedione
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-80
-60
-40
-20
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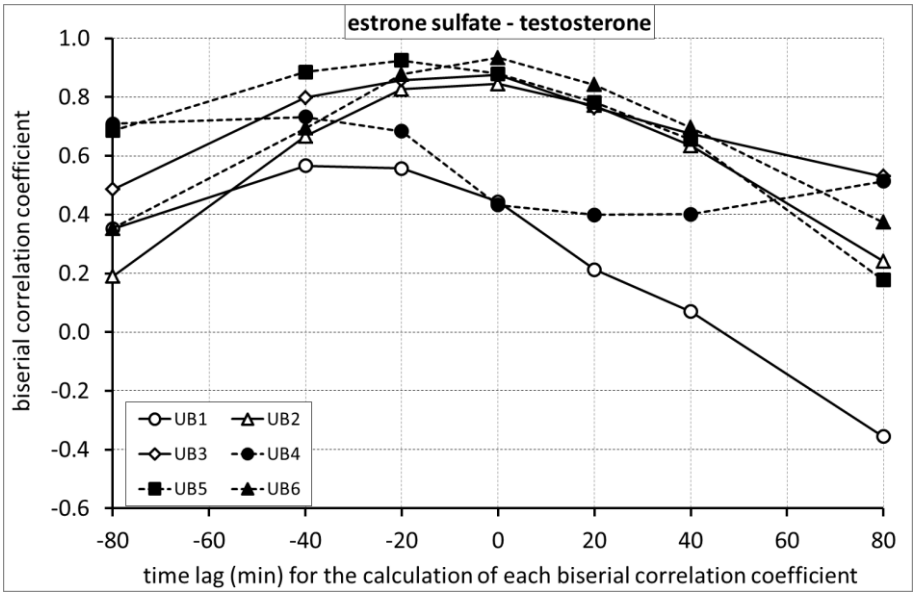
UB2
UB3
UB4
UB5
UB6
time lag (min) for the calculation of each biserial correlation coefficient


estrone sulfate - testosterone
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-80
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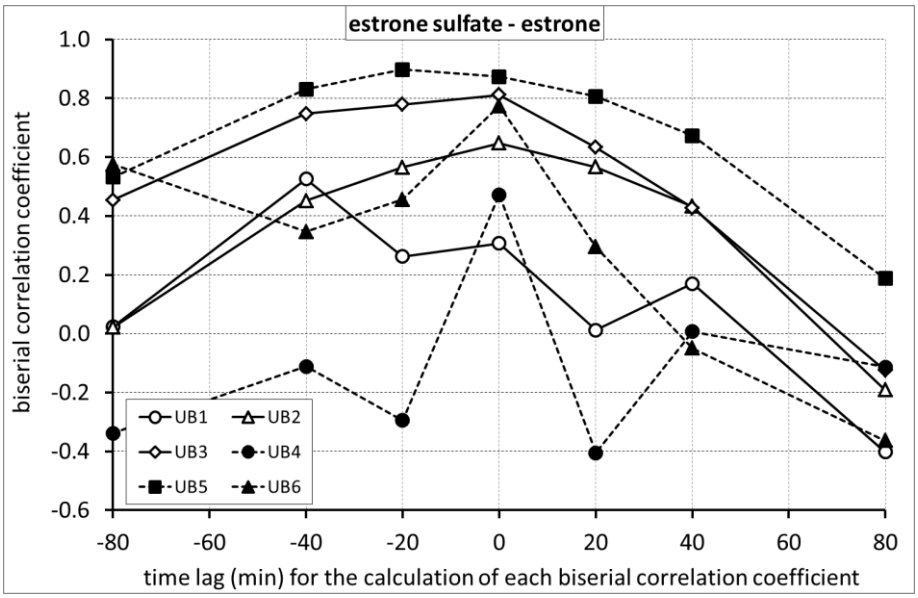
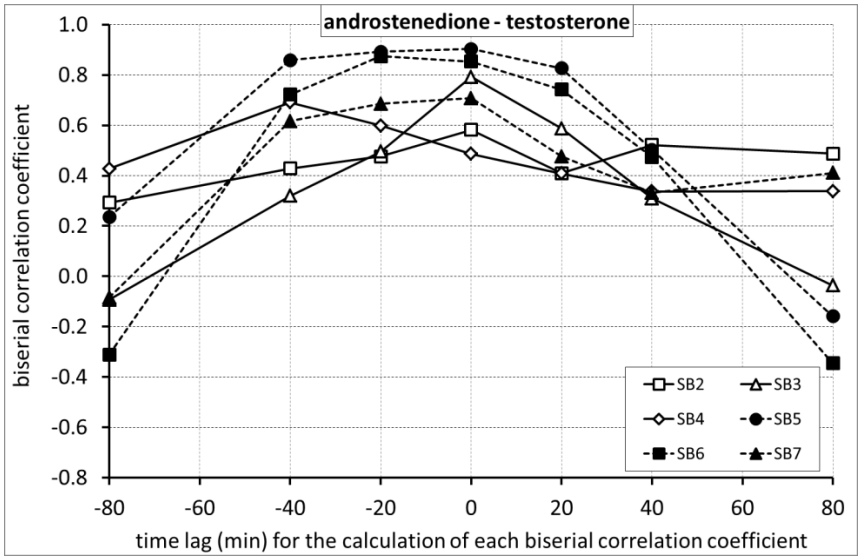
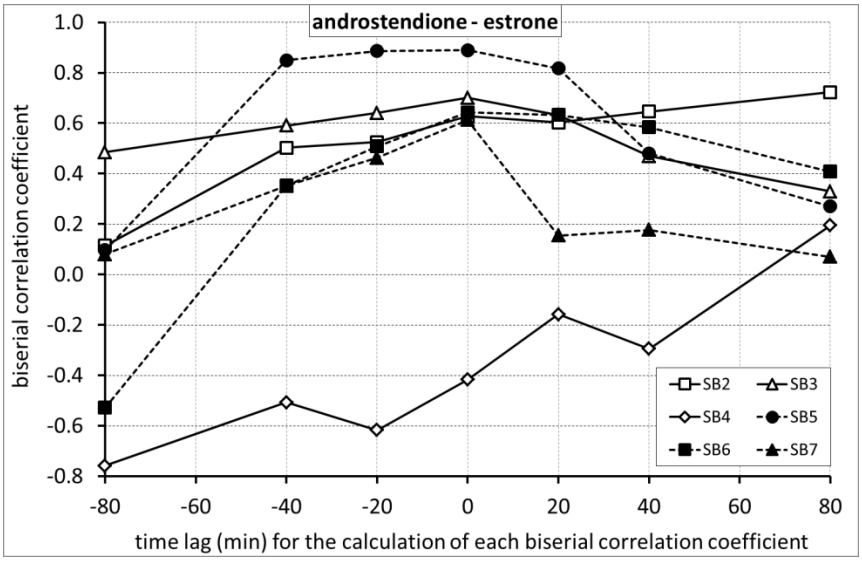
time lag (min) for the calculation of each biserial correlation coefficient

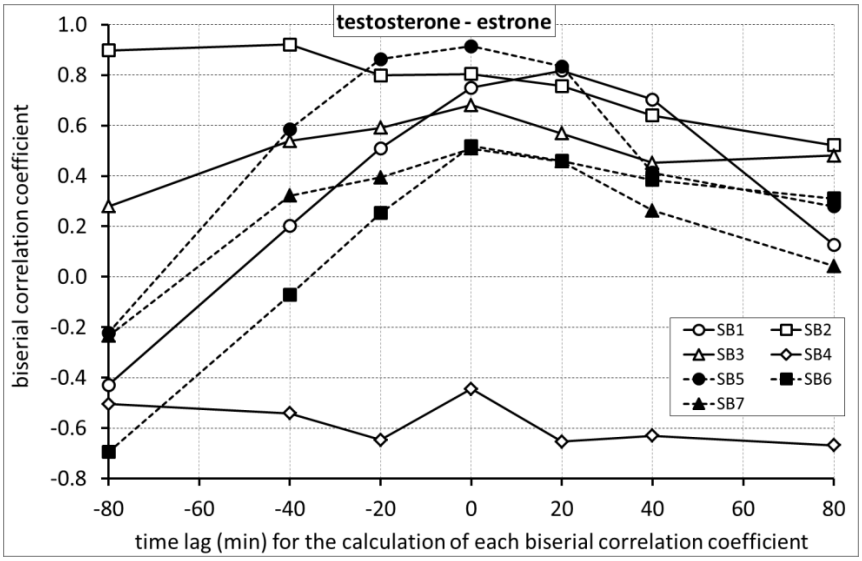

estrone sulfate - estrone
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-80
-60
-40
-20
0
20
40
60
80
UB1
UB2
UB3
UB4
UB5
UB6
time lag (min) for the calculation of each biserial correlation coefficient

9.1.2 Biserial correlation functions in individual stimulated animals

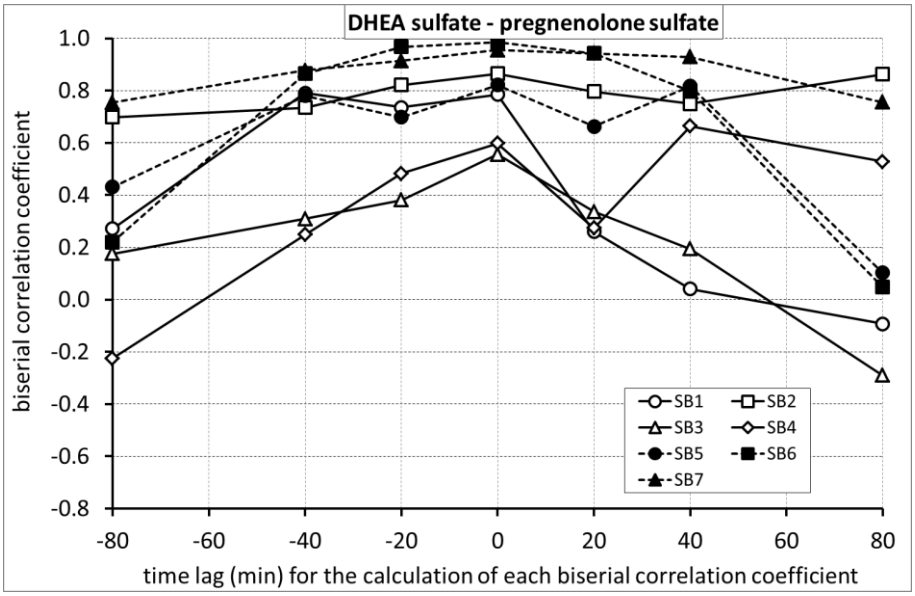
9.1.2.1 Biserial correlation functions between free steroids

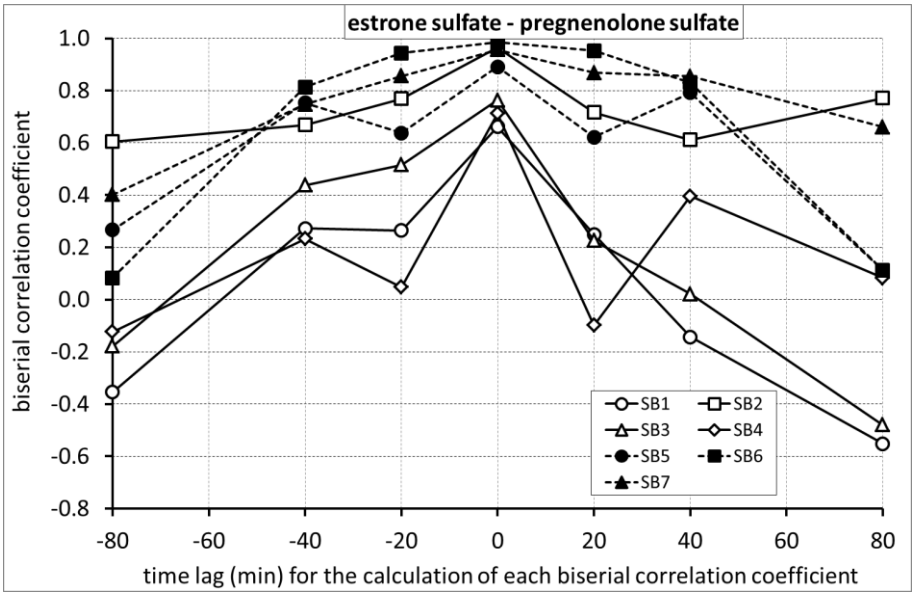


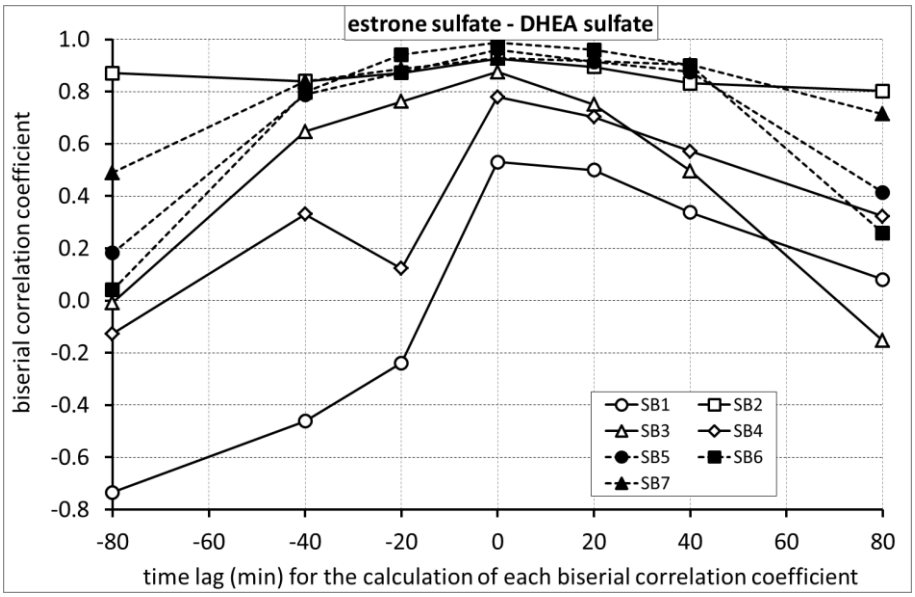




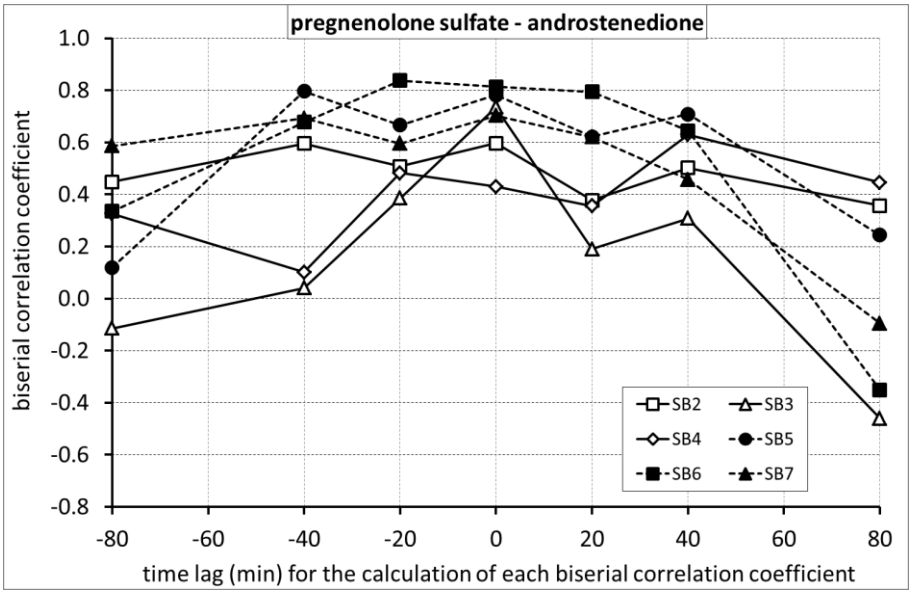
9.1.2.2 Biserial correlation functions between sulfonated steroids

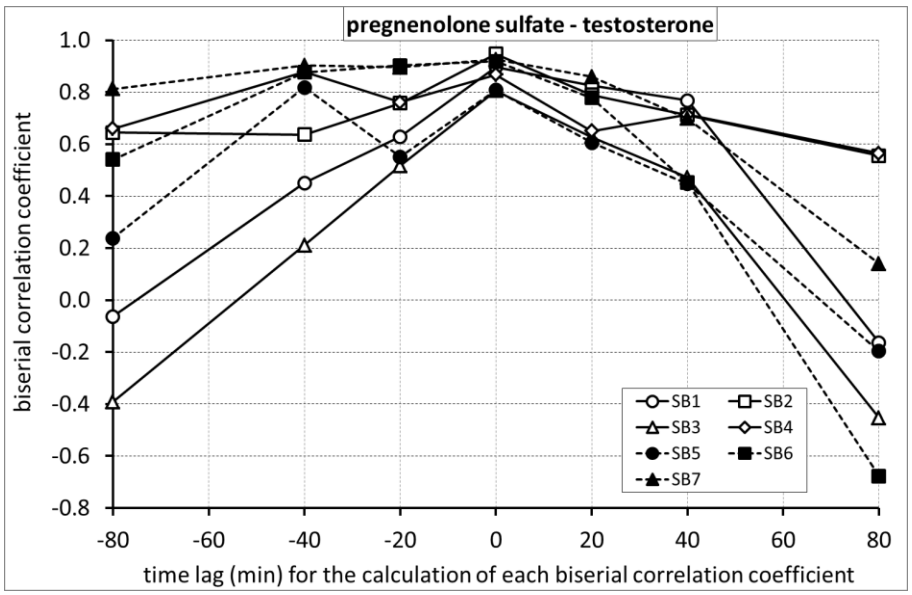


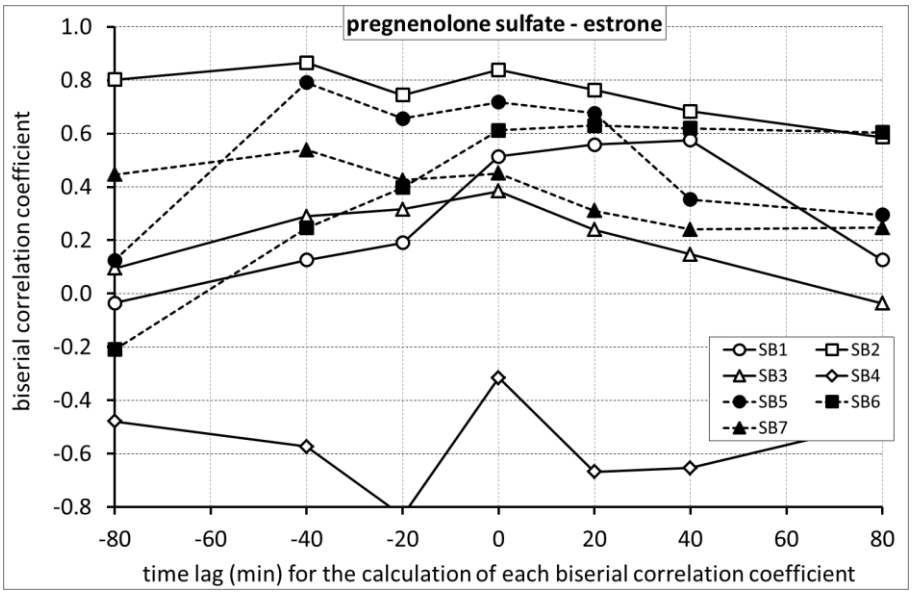


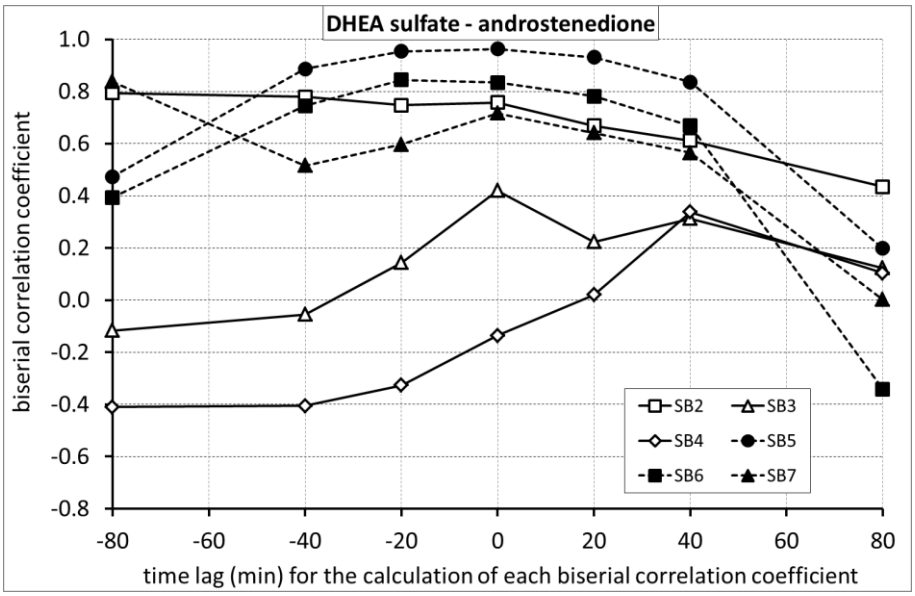


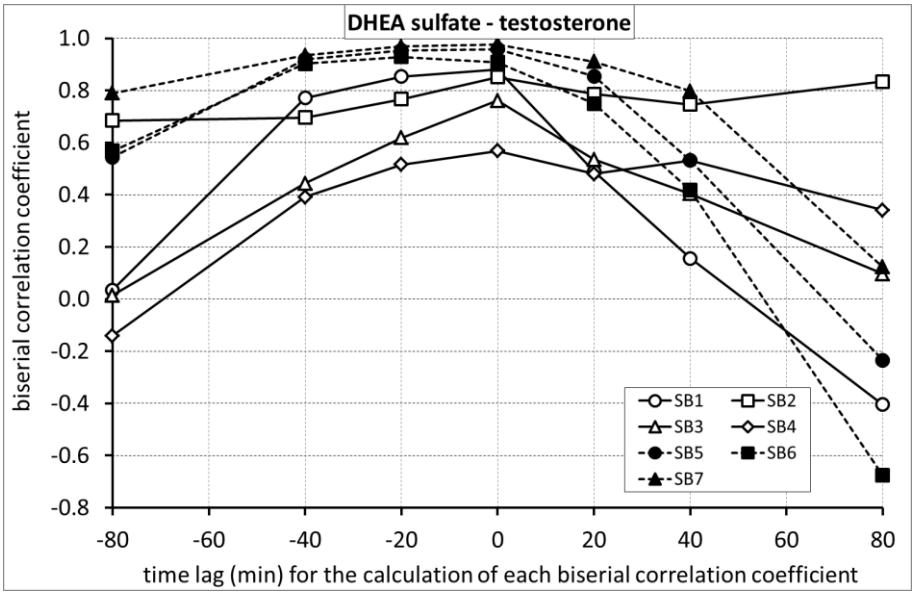
9.1.2.3 Biserial correlation functions between sulfonated and free steroids

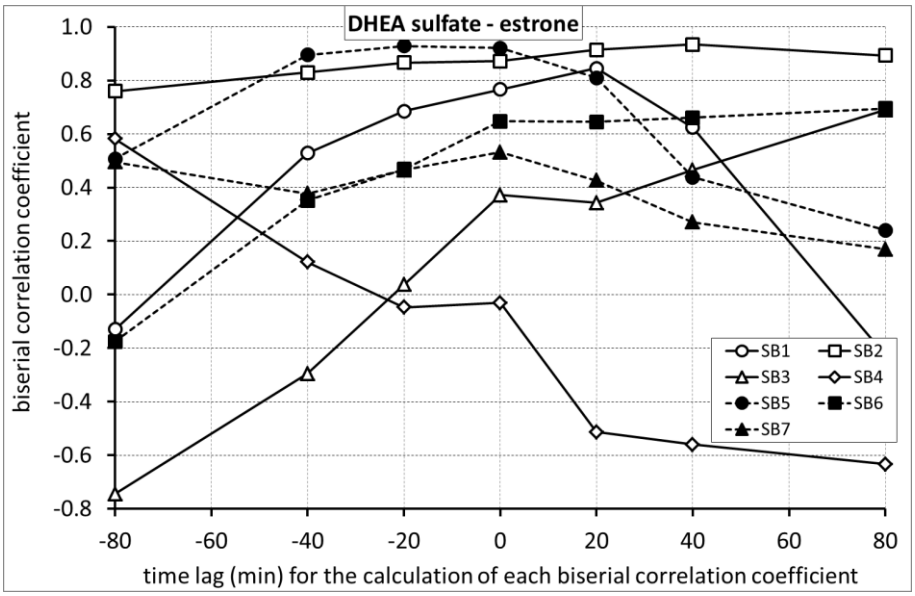


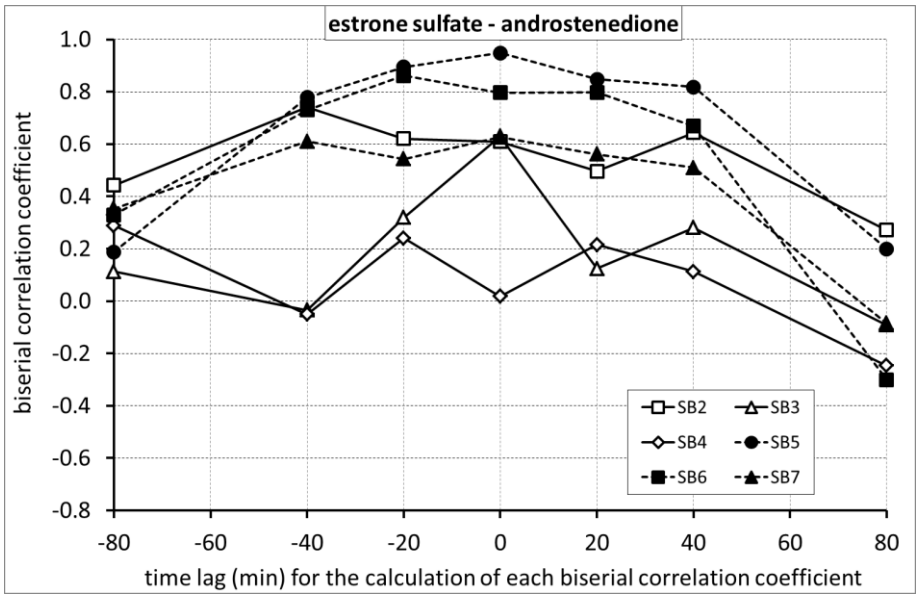


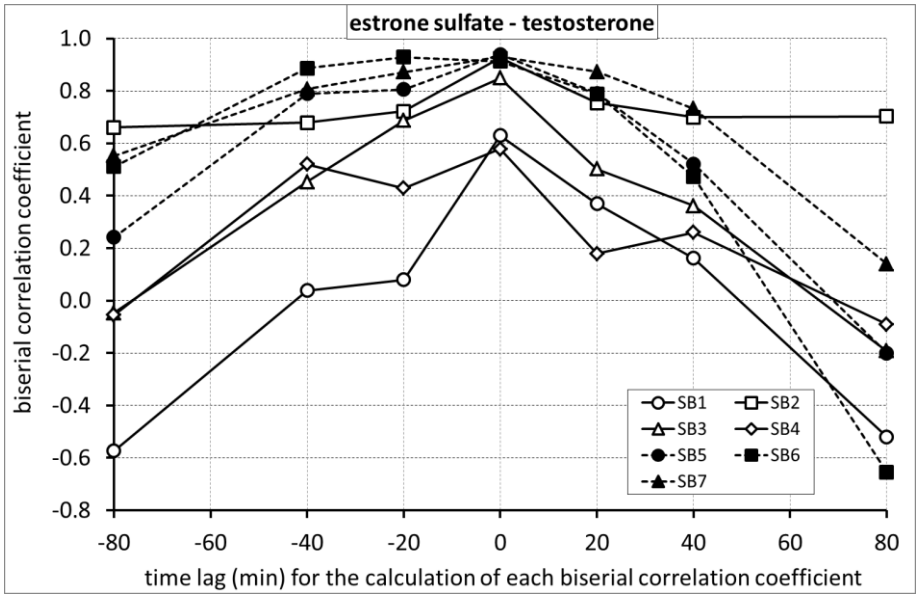


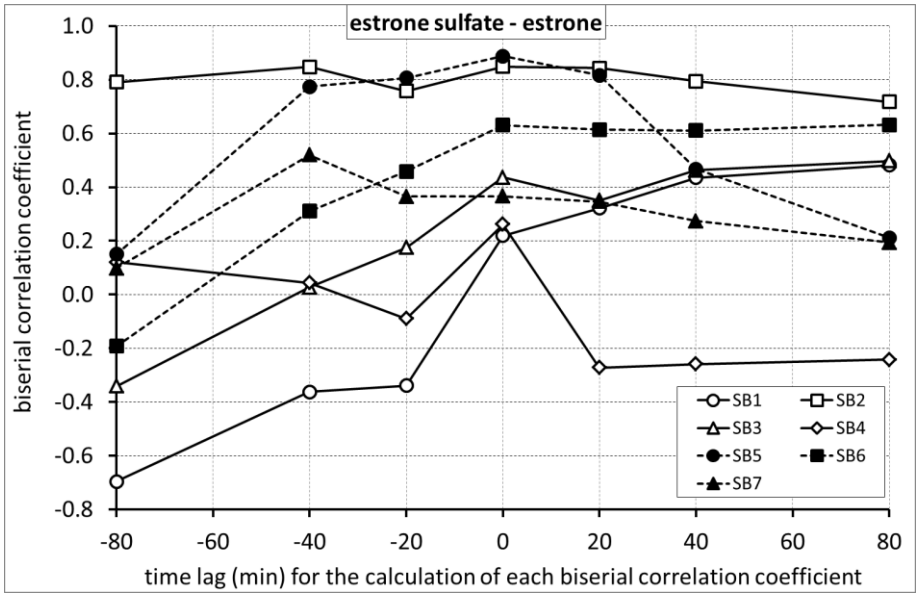

DHEA sulfate - androstenedione
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-80
-60
-40
-20
0
20
40
60
80
time lag (min) for the calculation of each biserial correlation coefficient
SB2
SB3
SB4
SB5
SB6
SB7


DHEA sulfate - testosterone
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-80
-60
-40
-20
0
20
40
60
80
time lag (min) for the calculation of each biserial correlation coefficient
SB1
SB2
SB3
SB4
SB5
SB6
SB7


DHEA sulfate - estrone
biserial correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-80
-60
-40
-20
0
20
40
60
80
time lag (min) for the calculation of each biserial correlation coefficient
SB1
SB2
SB3
SB4
SB5
SB6
SB7


estrone sulfate - androstenedione
biserial correlation coefficient
time lag (min) for the calculation of each biserial correlation coefficient
SB2
SB3
SB4
SB5
SB6
SB7

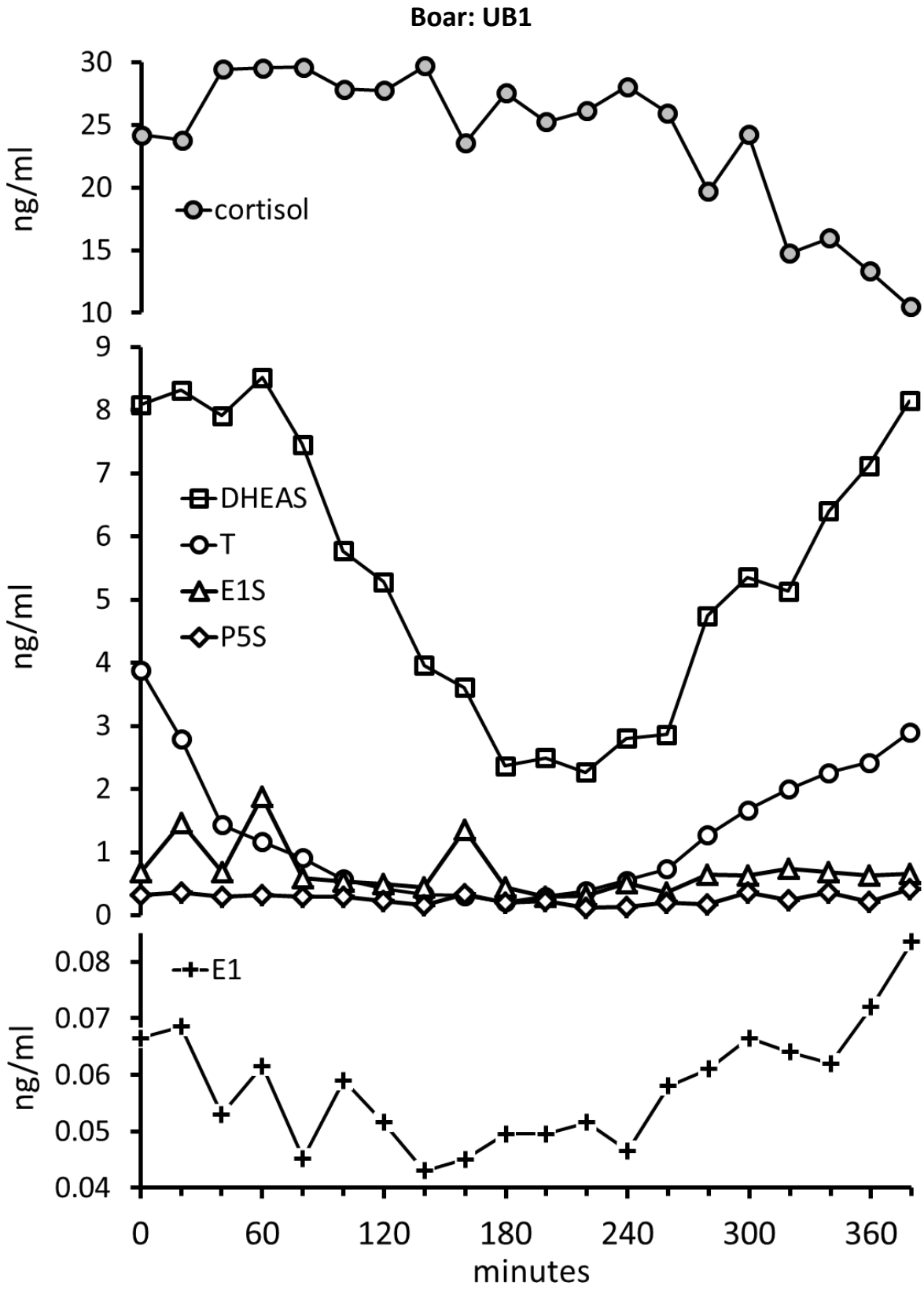

estrone sulfate - testosterone
biserial correlation coefficient
time lag (min) for the calculation of each biserial correlation coefficient
SB1
SB2
SB3
SB4
SB5
SB6
SB7


estrone sulfate - estrone
biserial correlation coefficient
time lag (min) for the calculation of each biserial correlation coefficient
SB1
SB2
SB3
SB4
SB5
SB6
SB7

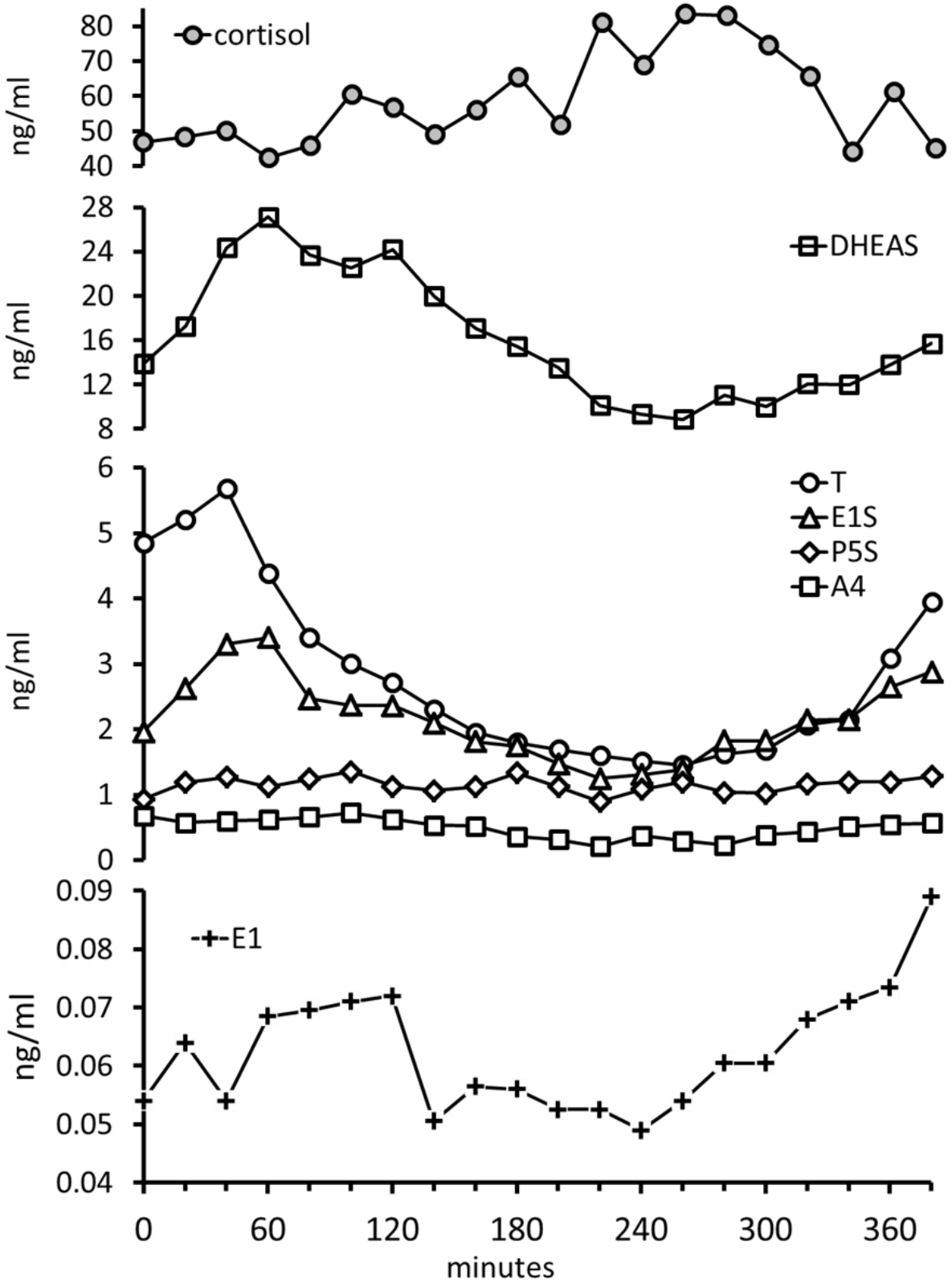
9.2 Steroid profiles presented according to the individual animals

In section 4.1 steroid profiles are presented sorted by the individual steroids being in the focus of this study, which however, exhibited a high variability between individual animals. In order to demonstrate the mostly high similarity between profiles for the various steroids found in individual animals, which is also obvious from the generally high correlation coefficients from pairwise correlation analyses (see Fig. 16 and 17 in chapter 4.2), here their profiles are shown as sorted by the individual boars. Cortisol profiles are also included to provide evidence that adrenal activity had no significant influence on the section pattern of steroids considered predominantly of testicular origin.

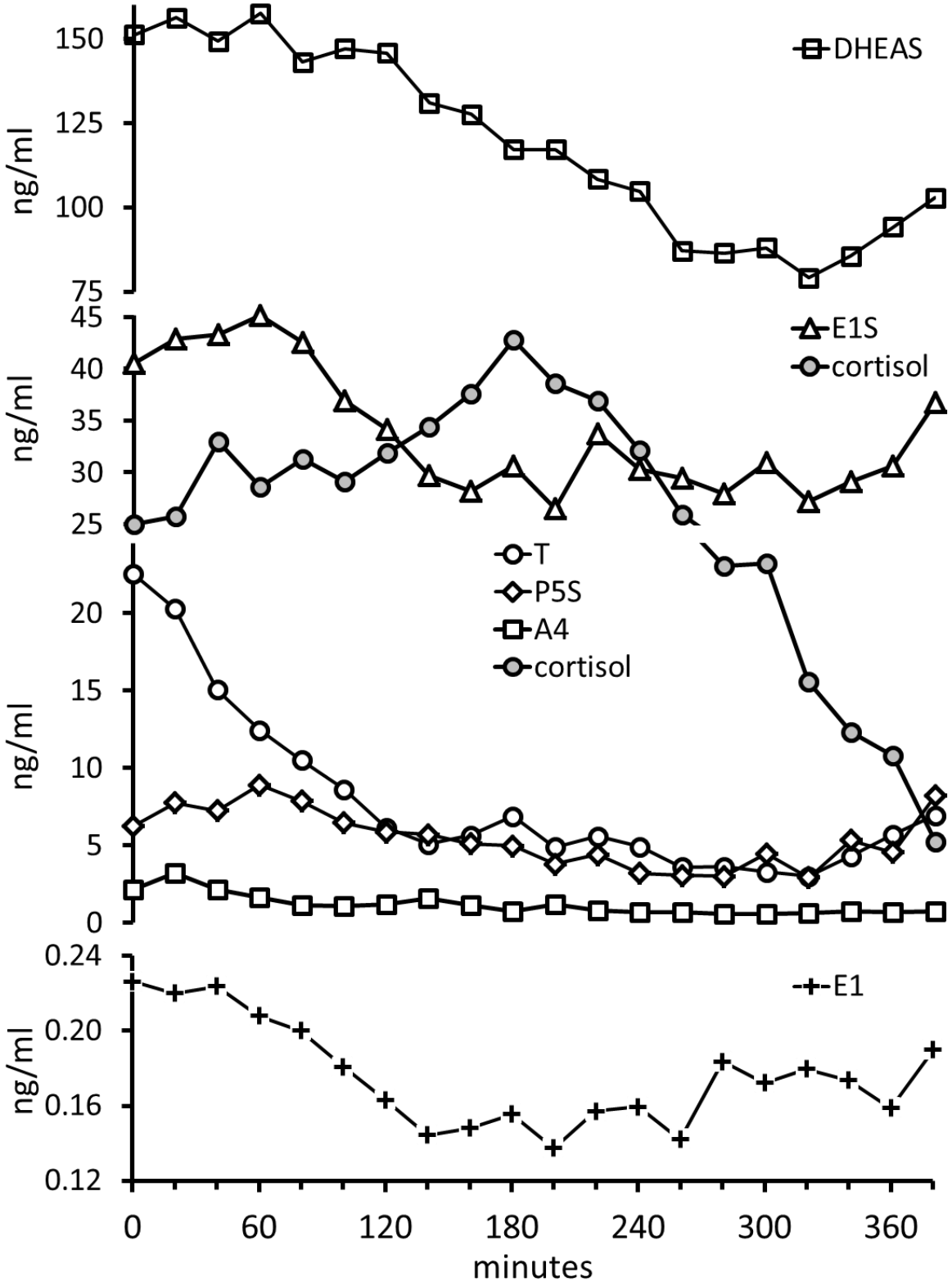
9.2.1 Steroid profiles in unstimulated boars

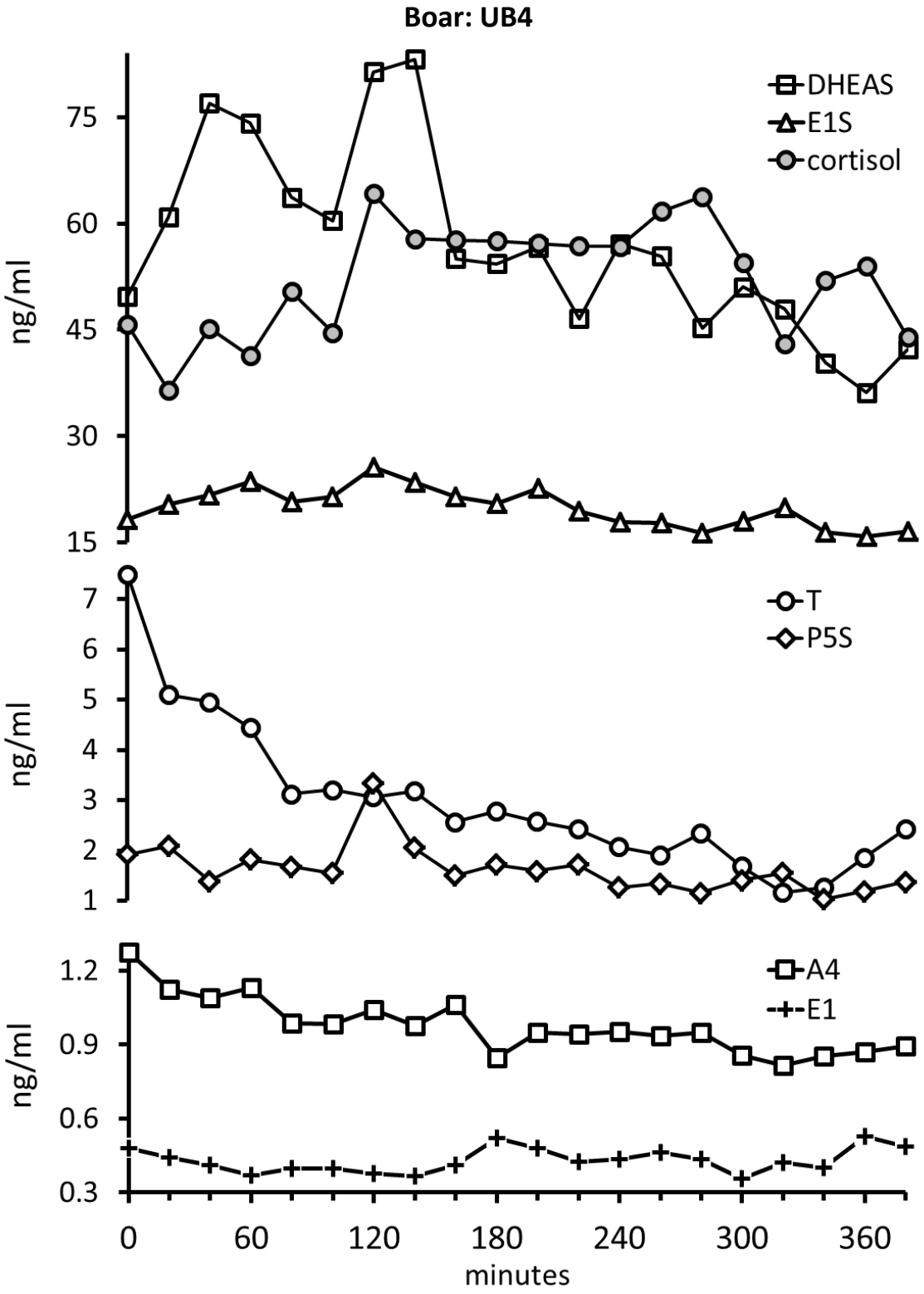


Boar: UB2

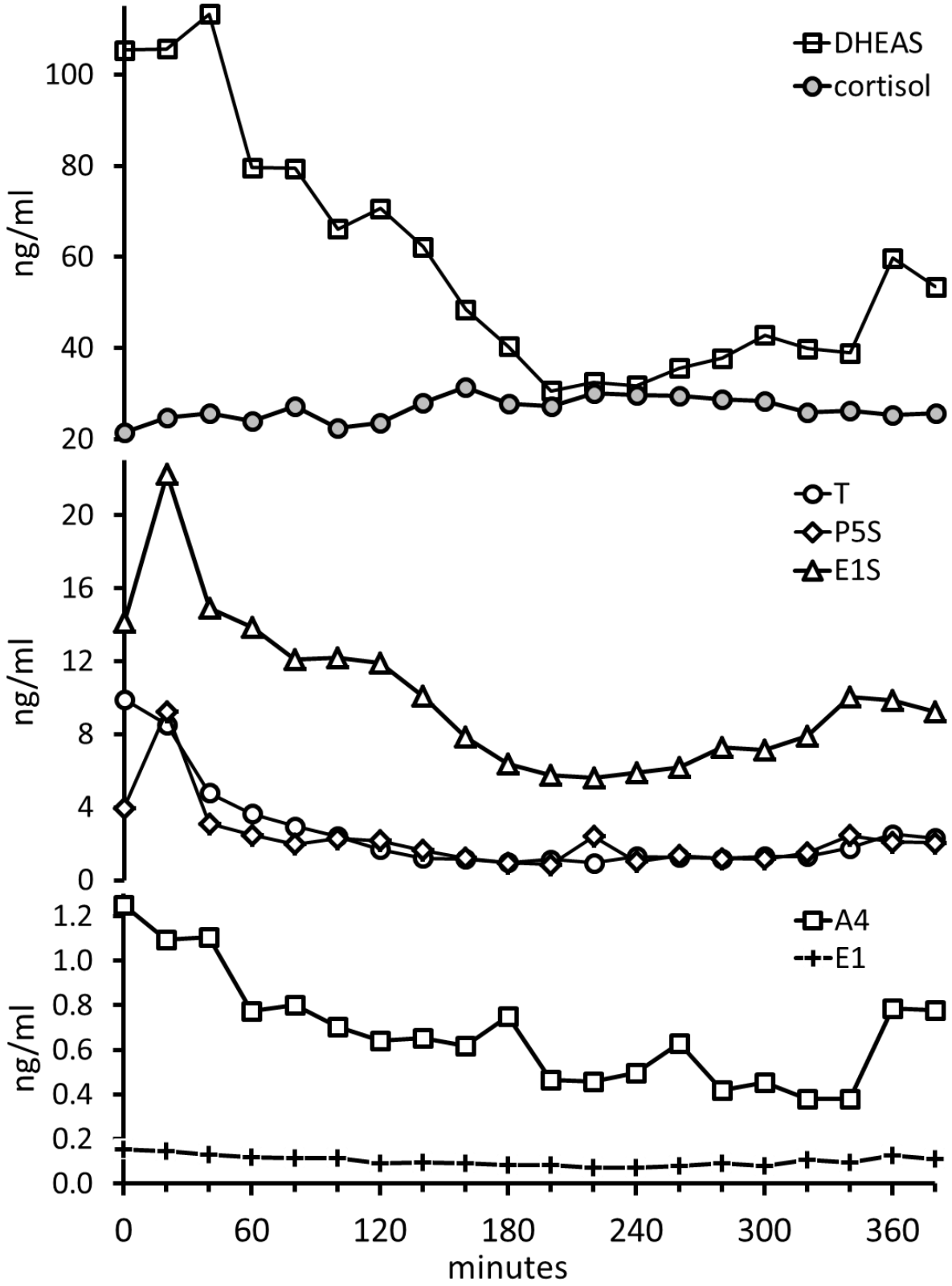

cortisol
ng/ml
80
70
60
50
40
DHEAS
28
24
20
16
12
8
T
E1S
P5S
A4
6
5
4
3
2
1
0
E1
0.09
0.08
0.07
0.06
0.05
0.04
0
60
120
180
240
300
360
minutes

Boar: UB3

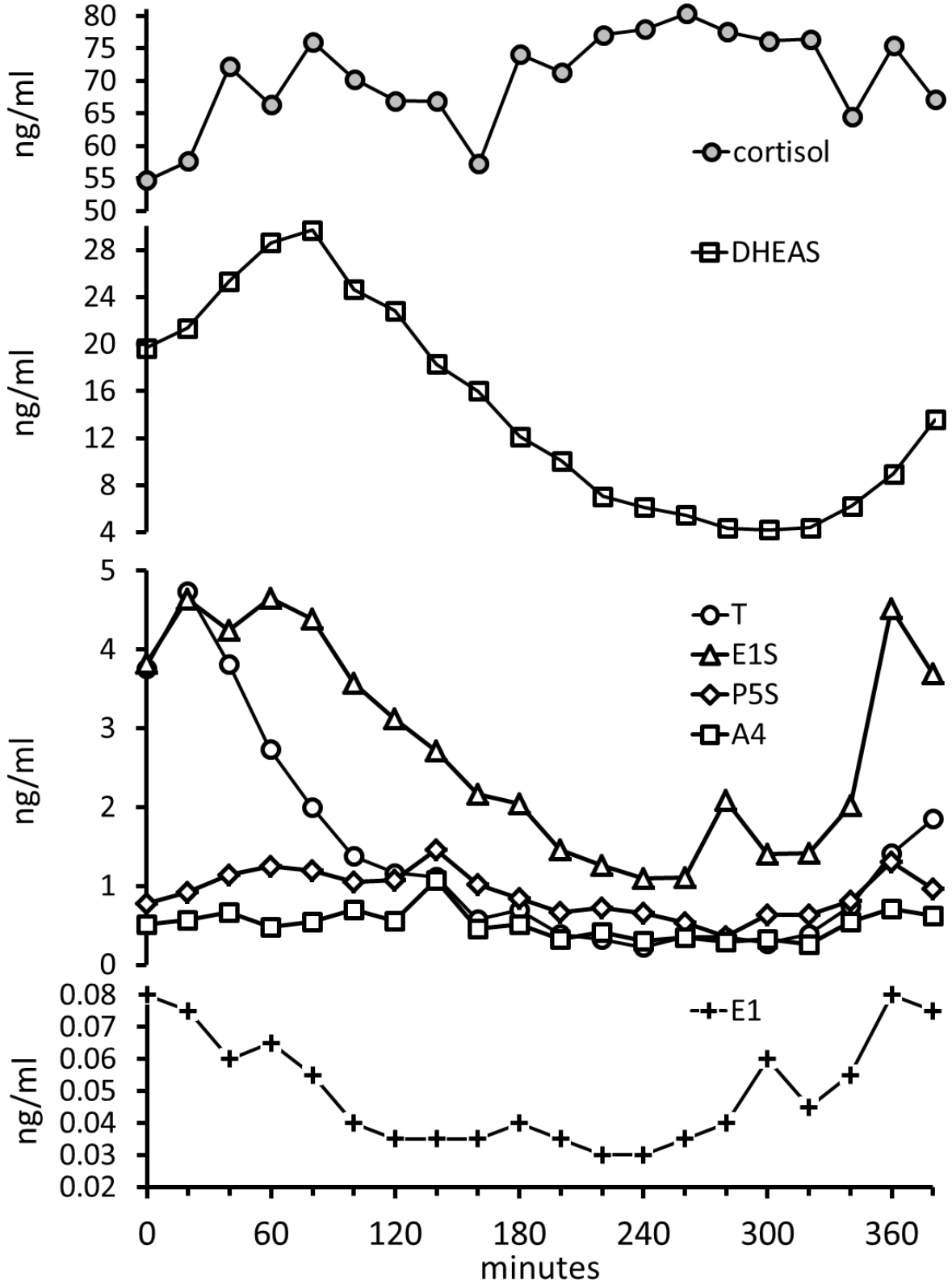

DHEAS
ng/ml
150
125
100
75
E1S
cortisol
45
40
35
30
25
T
P5S
A4
cortisol
20
15
10
5
0
E1
0.24
0.20
0.16
0.12
0
60
120
180
240
300
360
minutes


Boar: UB4
DHEAS
E1S
cortisol
ng/ml
75
60
45
30
15
T
P5S
ng/ml
7
6
5
4
3
2
1
A4
E1
ng/ml
1.2
0.9
0.6
0.3
0
60
120
180
240
300
360
minutes

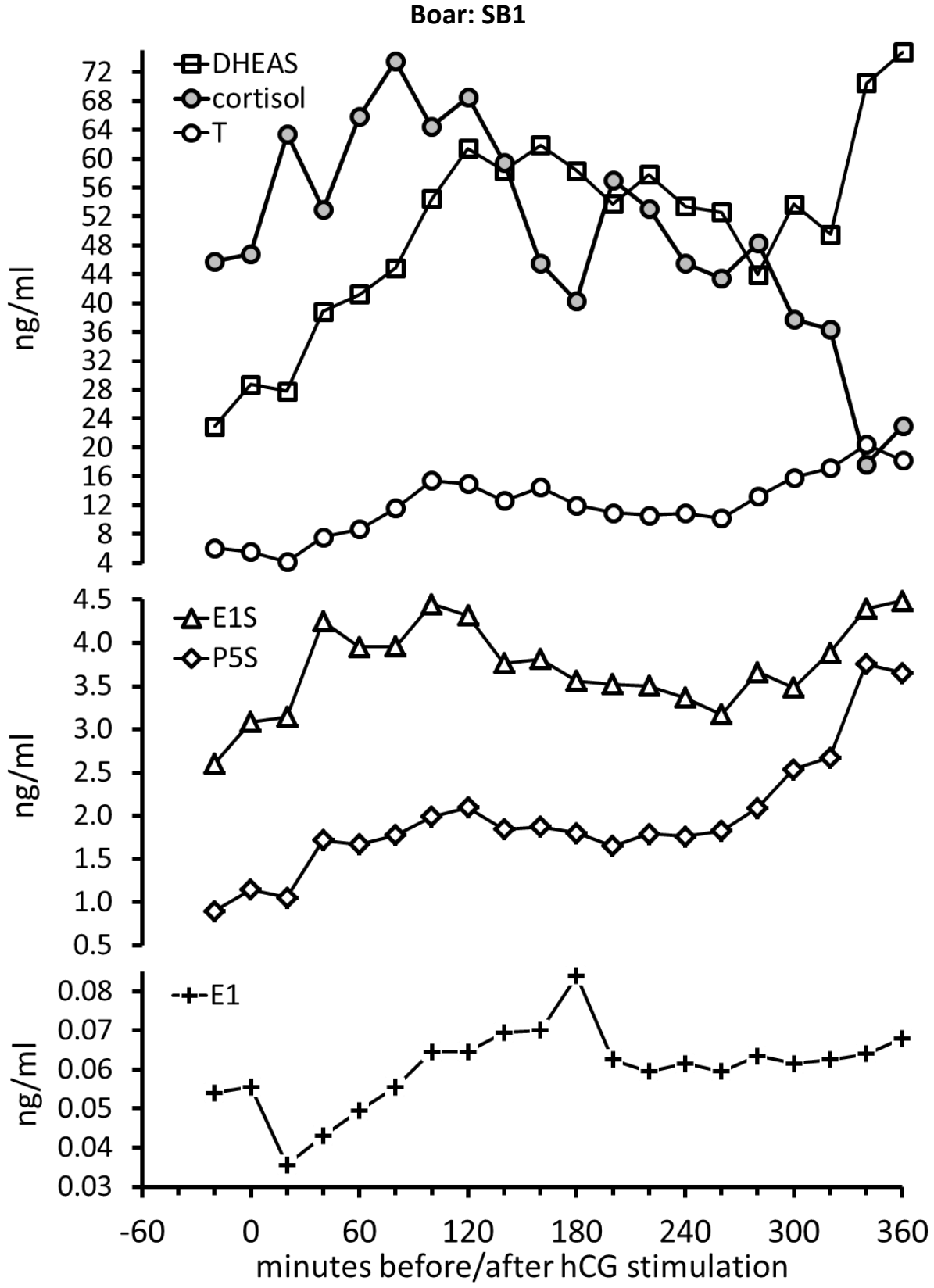
Boar: UB5


DHEAS
cortisol
ng/ml
100
80
60
40
20
T
P5S
E1S
ng/ml
20
16
12
8
4
0
A4
E1
ng/ml
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
60
120
180
240
300
360
minutes

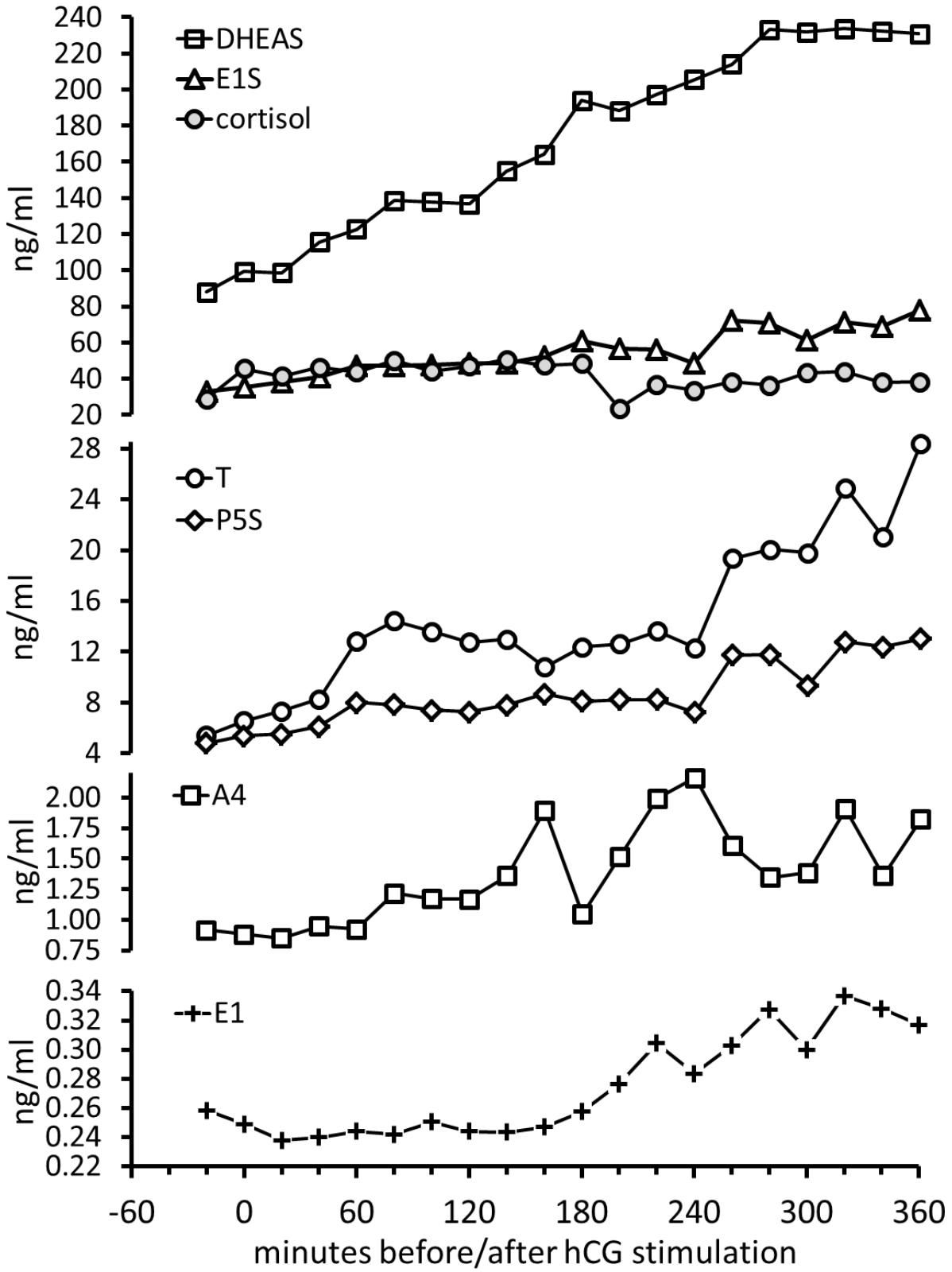
Boar: UB6

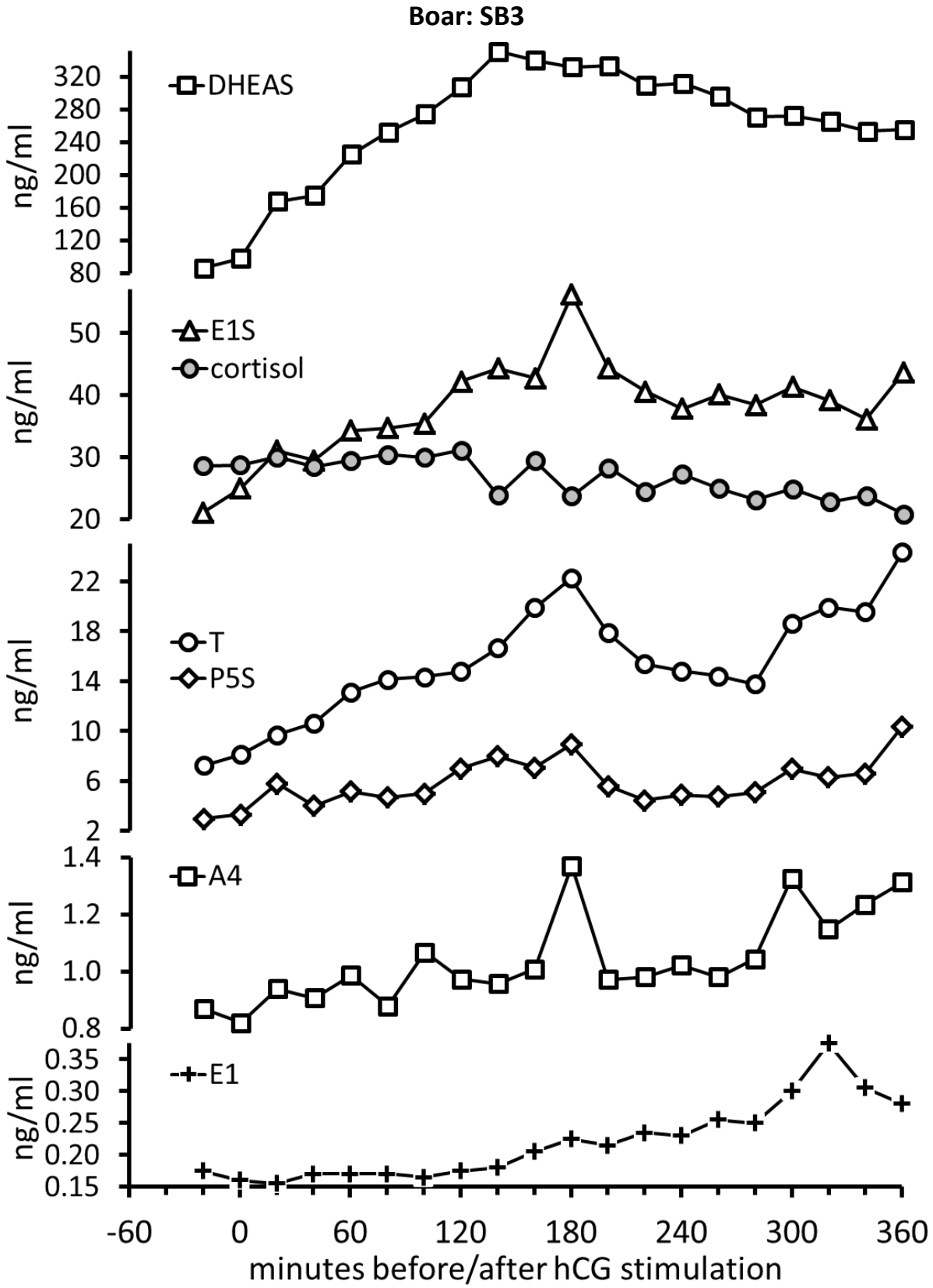

ng/ml
80
75
70
65
60
55
50
cortisol
ng/ml
28
24
20
16
12
8
4
DHEAS
ng/ml
5
4
3
2
1
0
T
E1S
P5S
A4
ng/ml
0.08
0.07
0.06
0.05
0.04
0.03
0.02
E1
0
60
120
180
240
300
360
minutes

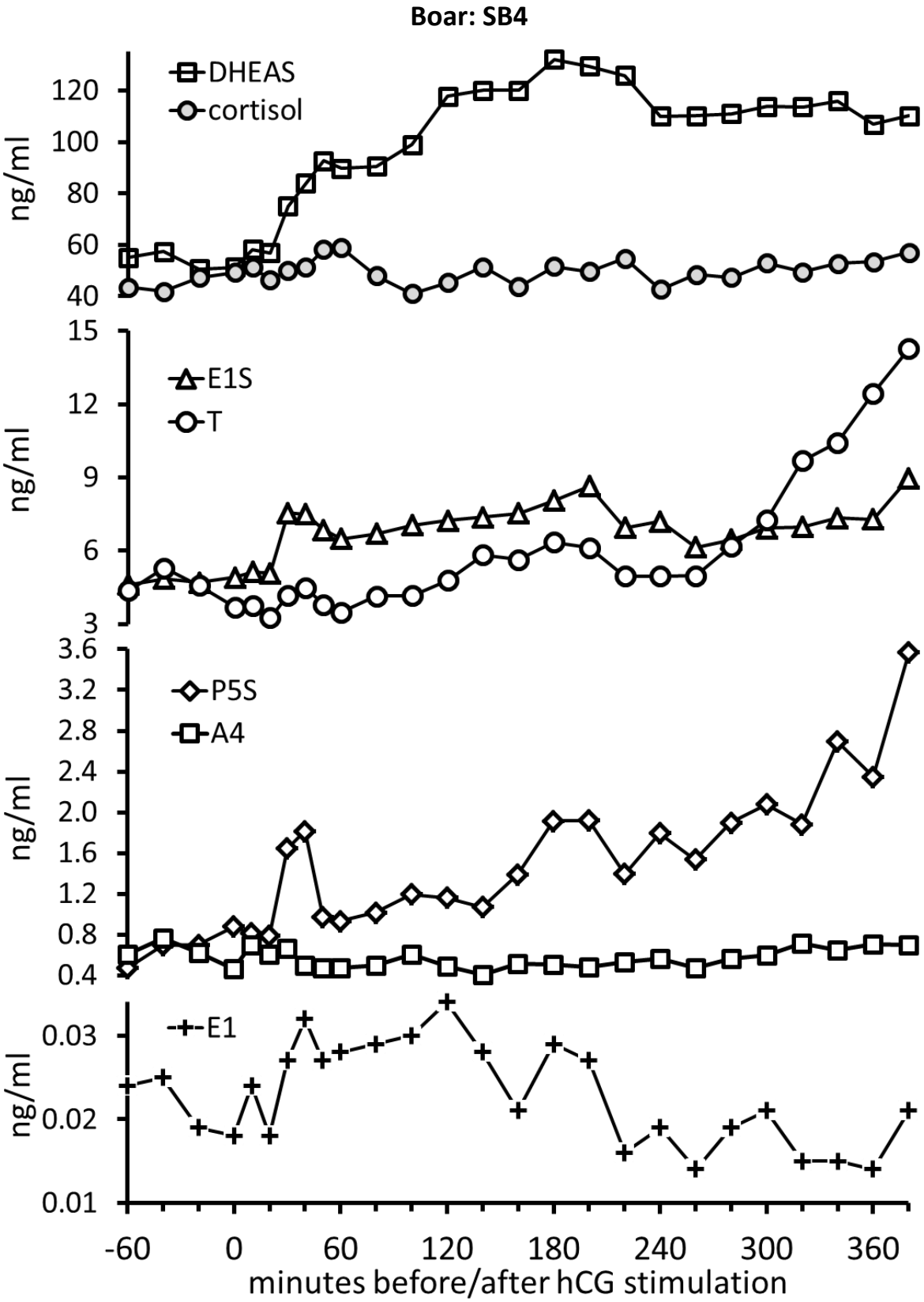
9.2.2 Steroid profiles in hCG-stimulated boars



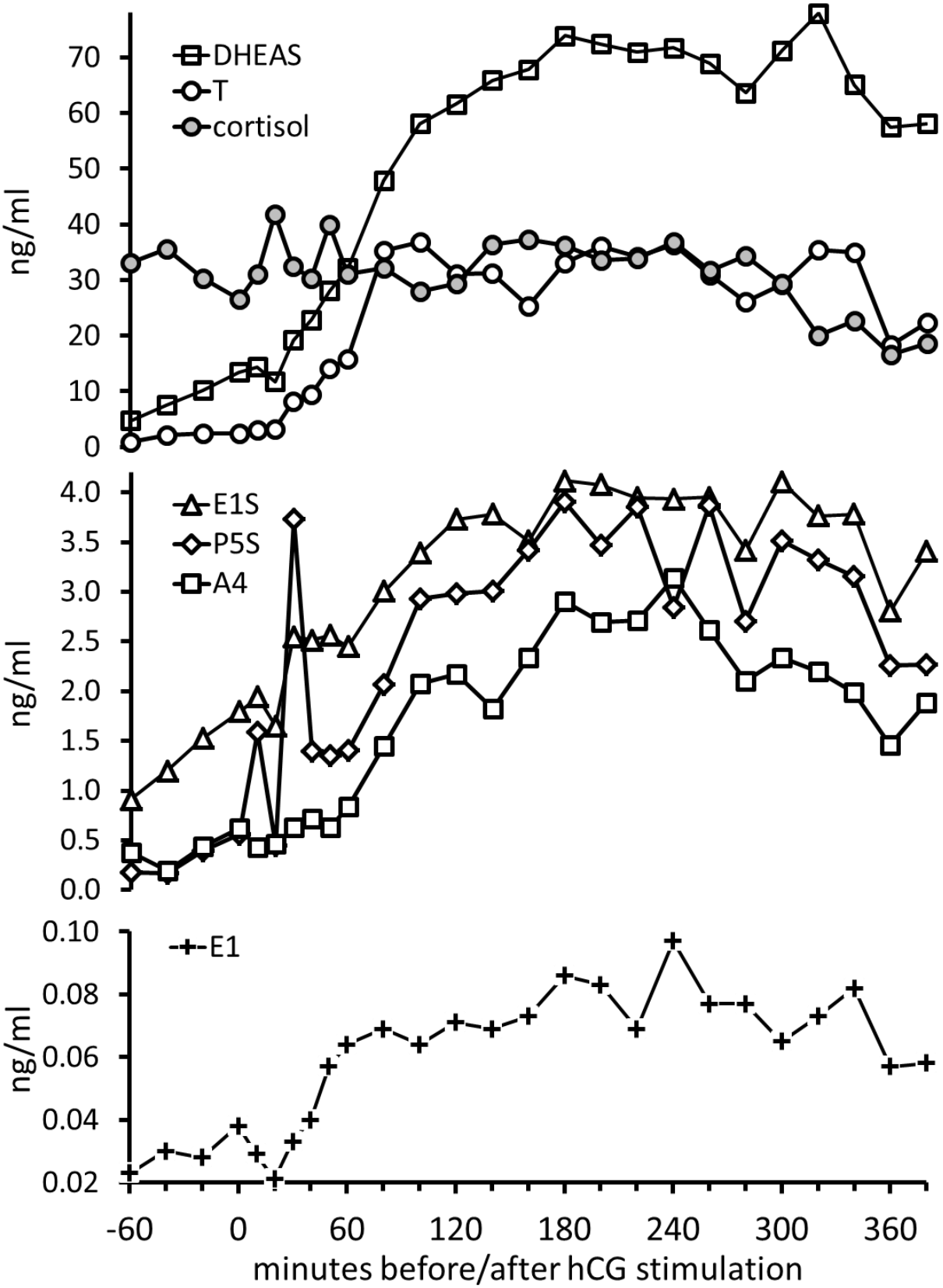
Boar: SB2


DHEAS
E1S
cortisol
ng/ml
240
220
200
180
160
140
120
100
80
60
40
20
T
P5S
28
24
20
16
12
8
4
A4
2.00
1.75
1.50
1.25
1.00
0.75
E1
0.34
0.32
0.30
0.28
0.26
0.24
0.22
-60
0
60
120
180
240
300
360
minutes before/after hCG stimulation

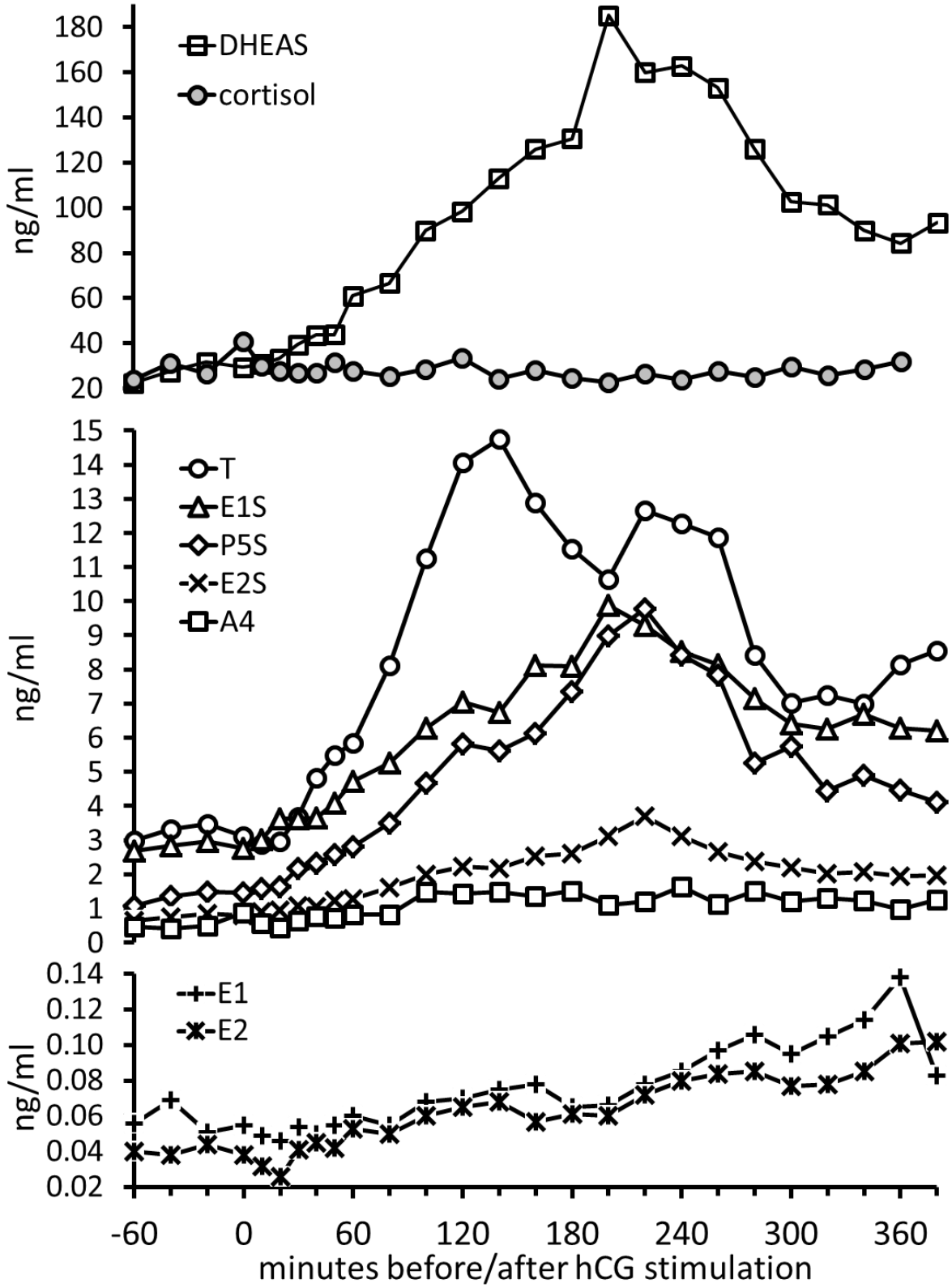

Boar: SB3
DHEAS
E1S
cortisol
T
P5S
A4
E1
ng/ml
320
280
240
200
160
120
80
50
40
30
20
22
18
14
10
6
2
1.4
1.2
1.0
0.8
0.35
0.30
0.25
0.20
0.15
-60
0
60
120
180
240
300
360
minutes before/after hCG stimulation


Boar: SB4
DHEAS
cortisol
ng/ml
120
100
80
60
40
E1S
T
15
12
9
6
3
P5S
A4
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
E1
0.03
0.02
0.01
-60
0
60
120
180
240
300
360
minutes before/after hCG stimulation

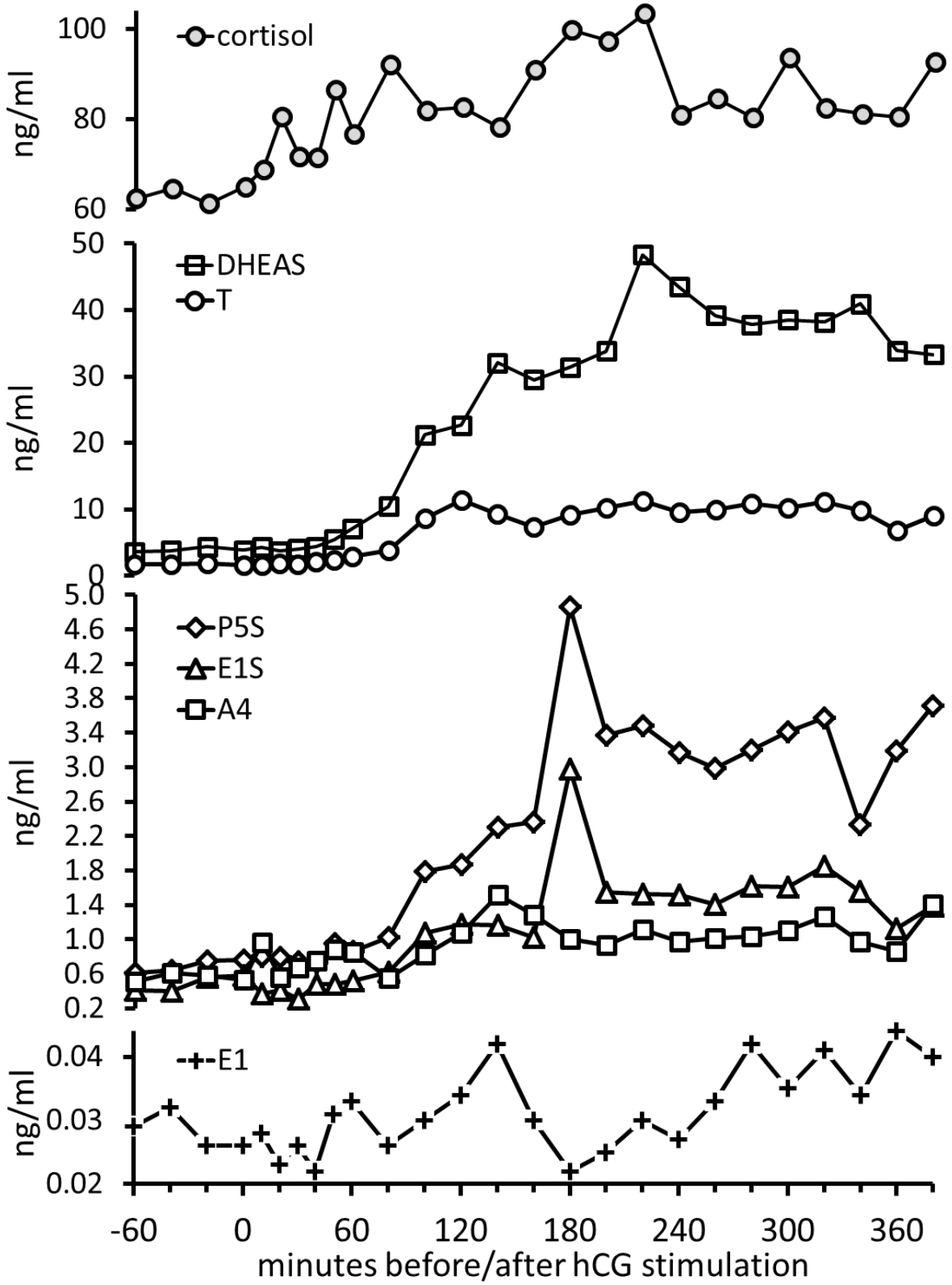
Boar: SB5


DHEAS
T
cortisol
ng/ml
70
60
50
40
30
20
10
0
E1S
P5S
A4
ng/ml
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
E1
ng/ml
0.10
0.08
0.06
0.04
0.02
-60
0
60
120
180
240
300
360
minutes before/after hCG stimulation

Boar: SB6


DHEAS
cortisol
ng/ml
T
E1S
P5S
E2S
A4
ng/ml
E1
E2
ng/ml
minutes before/after hCG stimulation

Boar: SB7


cortisol
ng/ml
100
80
60
DHEAS
T
ng/ml
50
40
30
20
10
0
P5S
E1S
A4
ng/ml
5.0
4.6
4.2
3.8
3.4
3.0
2.6
2.2
1.8
1.4
1.0
0.6
0.2
E1
ng/ml
0.04
0.03
0.02
-60
0
60
120
180
240
300
360
minutes before/after hCG stimulation

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