NON-INVASIVE MONITORING OF GONADAL ACTIVITY IN TWO CARNIVORES, THE SPOTTED HYENA (*Crocuta crocuta*) AND THE CHEETAH (*Acinonyx jubatus*)

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1. Supervisor:	Prof. Dr. Heribert Hofer
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Eigenständigkeitserklärung

Hiermit erkläre ich, die Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze keinen entsprechenden Doktorgrad. Ich erkläre, dass ich die Dissertation oder Teile davon nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht habe und dass sie dort weder angenommen, noch abgelehnt wurde.

Susanne Pribbenow

Ort, Datum

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Contents

Summary

The development of enzyme immunoassays (EIAs) to measure faecal testosterone metabolites (fTM) facilitates a non-invasive study design to objectify an animal's gonadal status without taking blood samples. Despite the wide use of these techniques, EIAs need to be carefully validated for each species and hormone. This dissertation was designed to establish and validate EIAs to monitor gonadal activity non-invasively based on faecal hormone analyses in two carnivore species, the spotted hyena (*Crocuta crocuta*) and the cheetah (*Acinonyx jubatus*).

In ***Article 1***, I detail the development and validation of an epiandrosterone EIA for the measurement of fTM in spotted hyenas. We performed radiometabolism studies to characterise fTM excreted in a captive male and female spotted hyena, respectively and demonstrated that the epiandrosterone EIA detected immunoreactive metabolites corresponding to radiolabelled metabolites in both sexes. The physiological and biological relevance of the epiandrosterone EIA was validated by demonstrating (1) a significant increase in fTM concentrations after a testosterone challenge test, (2) a lack of cross-reactivities with faecal glucocorticoid metabolites (fGM), and (3) significant differences in fTM levels in juvenile males and adult males of a free-ranging population.

In ***Article 2***, we validated an epiandrosterone EIA for male cheetahs by performing a testosterone radiometabolism study followed by high-performance liquid chromatography (HPLC) analyses and excluding possible cross-reactivities with fGM. The physiological and biological relevance of the epiandrosterone EIA was validated by demonstrating (1) a significant increase in fTM concentrations after a testosterone injection, (2) a significant increase in fTM concentrations after a gonadotropin-releasing hormone (GnRH) injection, and (3) significant differences in fTM concentrations in adult male, adult female and juvenile male cheetahs of a free-ranging population.

In ***Article 3***, we highlight the challenge of undesired cross-reactivities of testosterone EIAs to unknown fGM. For this purpose, we compared the ability of four different testosterone EIAs to monitor gonadal activity in spotted hyenas non-invasively based on fTM analyses. Our study demonstrates that a careful methodological and biological validation is requested to ensure that fTM concentrations quantitatively reflect gonadal activity. Even for an EIA passed the biological validation based on a challenge experiment, it is

highly recommended to ensure that stress-related increases in fGM are not monitored when applying an assay for fTM quantification.

Our studies describe the first EIAs for the non-invasive monitoring of testosterone and its underlying endocrine mechanisms, thereby providing the necessary tools for long-term investigations of hormone-behaviour relationships in both carnivore species.

Zusammenfassung

Die Entwicklung von Enzymimmunoassays (EIAs) zur Bestimmung fäkaler Testosteronmetaboliten (fTM) ermöglicht ein nicht-invasives Studiendesign zur Beurteilung des gonadalen Staus eines Tieres ohne die Notwendigkeit der Blutentnahme. Trotz der weitverbreiteten Anwendung dieser Techniken müssen EIAs sorgfältig für jede Spezies und jedes Hormon validiert werden.

Ziel der vorliegenden Dissertation war die Etablierung und Validierung von EIAs zur Beurteilung der Gonadenaktivität in zwei Raubtierspezies, der Tüpfelhyäne (*Crocuta crocuta*) und dem Geparden (*Acinonyx jubatus*), anhand fäkaler Hormonanalysen.

In ***Artikel 1*** beschreibe ich die Entwicklung und Validierung eines Epiandrosteron-EIAs für die Messung von fTM in Tüpfelhyänen. Wir haben Radiometabolismusstudien in jeweils einem gefangen-gehaltenen Männchen und Weibchen durchgeführt, um die ausgeschiedenen fTM zu charakterisieren. Für beide Geschlechter wurde gezeigt, dass der Epiandrosteron-EIA immunreaktive Testosteronmetaboliten detektiert, die mit den Elutionspositionen radioaktiv-markierter Metaboliten korrespondieren. Die physiologische und biologische Bedeutung des Epiandrosteron-EIAs konnte durch (1) einen signifikanten Anstieg in fTM Konzentrationen nach Testosteroninjektion, (2) fehlender Kreuzreaktivitäten mit fäkalen Glucocorticoidmetaboliten (fGM), und (3) signifikanten Unterschieden in fTM Konzentrationen juveniler und adulter Männchen einer freilebenden Population nachgewiesen werden.

In ***Artikel 2*** haben wir einen Epiandrosteron-EIA für männliche Geparden validiert. Dazu haben wir eine Testosteronradiometabolismusstudie und anschließende Hochleistungsflüssigkeitschromatographie (HPLC)-Analysen durchgeführt und konnten mögliche Kreuzreaktivitäten mit fGM ausschließen. Die physiologische und biologische Bedeutung des Epiandrosteron-EIAs konnte durch (1) einen signifikanten Anstieg der fTM Konzentrationen nach Testosteroninjektion, (2) einen signifikanten Anstieg der fTM Konzentrationen nach Gonadotropin-Releasing-Hormon (GnRH)-Injektion, und (3) signifikanten Unterschieden in fTM Konzentrationen adulter und juveniler Geparden einer freilebenden Population nachgewiesen werden.

In ***Artikel 3*** beleuchten wir die Problematik ungewünschter Kreuzreaktivitäten von Testosteron-EIAs mit unbekannten fGM. Dazu wurde die Eignung von vier Testosteron-EIAs zum nicht-invasiven Monitoring der Gonadenaktivität in Tüpfelhyänen verglichen.

Unsere Studie zeigt, dass eine sorgfältige methodische und biologische Validierung nötig ist, um sicherzustellen, dass fTM Konzentrationen quantitativ die Gonadenaktivität widerspiegeln. Auch wenn ein EIA die biologische Validierung besteht, ist es ratsam sicherzustellen, dass stressbedingte Anstiege in fGM nicht von einem Assay für die fTM Quantifizierung mitgemessen werden.

Unsere Studien beschreiben die ersten EIAs für das nicht-invasive Testosteronmonitoring und der zu Grunde liegenden endokrinen Mechanismen, und stellen damit die notwendigen Instrumente für Langzeituntersuchungen von Hormon-Verhaltensbeziehungen in beiden Raubtierspezies dar.

Part I.

General Introduction

Reproduction is essential for the survival of all known life. As most complex physiological and metabolic processes, reproduction is regulated by the endocrine system, depending on the hormones that comprise it. As gonadal hormones are the key components in regulating reproductive physiology, fundamental knowledge of the reproductive endocrinology of animals and the understanding of factors that may influence it, is essentially required for their effective management and conservation. Traditionally, studies of reproductive endocrinology relied on repeated blood draws to analyse circulating reproductive hormones. However, evident disadvantages due to the invasive nature of blood sampling led to the development of alternative techniques to evaluate concentrations of hormones and their metabolites in non-invasively collected biological samples, such as faeces, urine, saliva, and hair.

This dissertation aims to provide the necessary tools to monitor gonadal activity non-invasively based on faecal testosterone metabolite measurements in spotted hyenas (*Crocuta crocuta*) and male cheetahs (*Acinonyx jubatus*) to further investigate hormone-behaviour relationships and the impact of intrinsic, social and environmental factors on testosterone output.

In this part I will present the current understanding of the mammalian reproductive hormone testosterone; discuss the feasibility of non-invasive methods to monitor gonadal activity and review our current knowledge of testosterone secretion in both spotted hyenas and cheetahs.

1. Testosterone

Testosterone is an androgenic steroid hormone found in humans and other vertebrates and invertebrates (Janer et al., 2005). As shown in Figure 1.1, testosterone ($C_{19}H_{28}O_2$) and all other steroids are lipophilic molecules with a common 4-ring structure composed of three cyclohexane (C_6) and one cyclopentane ring (C_5), designated as A, B, C, D, respectively (Pineda, 2003; Walters, 2007). Testosterone, the major natural androgenic sex hormone, mainly originates from gonadal tissues; it is primarily secreted by male testicular Leydig cells but also to a lesser extent by female ovarian theca cells (Mooradian et al., 1987; Shahidi, 2001). In both sexes, small amounts (< 5%) of circulating testosterone are of adrenocortical origin (Behre and Keck, 2002). Testosterone has a tremendous impact on an individual's phenotype, physiology and psychology as it exerts a variety of androgenic and anabolic effects on both reproductive and non-reproductive tissues (Shahidi, 2001; Nieschlag and Nieschlag, 2014).

In male mammals, testosterone has a key role in reproductive physiology and is required throughout the organism's life- from the prenatal heterogametic sexual differentiation and development of internal and external reproductive organs through secondary sexual development during puberty to the establishment and maintenance of adult sexual function, including the stimulation and maintenance of spermatogenesis and thus male fertility (Wu, 1997; Hiller-Sturmhöfel and Bartke, 1998; Stoker and Zorilla, 2010). Although often referred to as a "male" hormone, testosterone is also crucial for the maintenance of female reproductive function as it serves as an immediate precursor for oestrogens, the "female" sex hormones. In humans, testosterone is also thought to have an important impact on female sexual arousal, motivation and receptivity (Traish et al., 2002; Bolour and Braunstein, 2005). However, depending on the specificity of target tissues, testosterone may act either as hormone itself or as circulating precursor for its biologically active metabolites dihydrotestosterone (DHT) and oestradiol (Wu, 1997). In reproductive tissues testosterone is primarily converted to DHT via the enzyme 5α-reductase, whereas the enzyme aromatase converts testosterone to oestradiol in ovarian theca and granulosa cells, distinct brain nuclei and adipose tissues (Brodie, 1979; Mooradian et al., 1987).

In addition to the reproductive function, testosterone has numerous essential functions throughout the body that vary between the sexes and change with age (Hiller-Sturmhöfel and Bartke, 1998). Testosterone exerts organisational and activational effects on skeletal

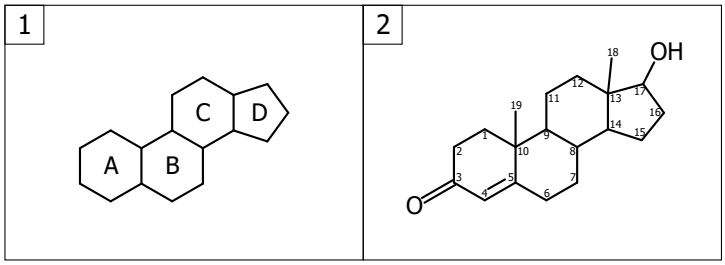

Figure 1.1. Illustration of the general ring structure of steroid hormones (1) and testosterone (2) with numbered C-atoms.

and cardiac muscles and bones as a consequence of increased protein synthesis (Mooradian et al., 1987; Celotti and Negri-Cesi, 1992). It stimulates the haematopoietic system including the stimulation of erythropoietin release and bone marrow activity (Shahani et al., 2009). Furthermore, testosterone may act as neuromodulator thereby shaping behaviour, preparing organisms to interact optimally with the environment, and thus contributing to the general health and well-being (Crockett and Fehr, 2013).

1.1. Regulation of Testosterone Secretion

Hierarchically organised endocrine axes consisting of hypothalamus, pituitary, and peripheral target organs integrate and control fundamental life processes such as development, cell differentiation, and reproduction as well as various metabolic processes (Köhrle et al., 2014). The production and secretion of testosterone throughout an individual's lifespan are controlled by a complex series of hormonal interactions and feedback mechanisms regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Ray and Choudhuri, 2011).

Synthesis and secretion of testosterone are orchestrated by the hypothalamus that serves as a link between the central nervous system (CNS) and the endocrine system. The hypothalamus contains interconnected groups of secretory neurons, mainly located in the *Nuclei praeopticus* and *Eminentia mediana*, that synthesise and secrete the primary regulatory neuropeptide gonadotropin-releasing hormone (GnRH) in discrete pulses (Ray and Choudhuri, 2011; Kleine and Rossmanith, 2014). The pulse frequency of GnRH is the result of a signal generator, the GnRH pulse generator, in the CNS which is located in the *Nuclei arcuatus*. These neuronal centres incorporate and process external and internal stimuli such as food supply, day length, "stress" as indicated by elevated glucocorticoid levels and social status and in turn secrete neuromodulators to regulate GnRH secretion (Aurich and Töpfer-Petersen, 2010). GnRH is released in discrete pulses via the hypophyseal portal system into the anterior pituitary lobe (Gore, 2002). There it binds to

GnRH receptors and stimulates the synthesis and pulsatile secretion of gonadotropins, namely luteinising hormone (LH) and follicle-stimulating hormone (FSH), into systemic circulation (Gore, 2002). These two hormones bind to LH and FSH receptors on testicular cells to stimulate the production and secretion of testosterone and inhibins (Meethal and Atwood, 2005). In male mammals, LH stimulates testicular Leydig cells to synthesise and secrete testosterone (Aurich and Töpfer-Petersen, 2010; Stoker and Zorilla, 2010). Within the testis, FSH and testosterone also synergistically act on Sertoli cells to facilitate spermatogenesis and to enhance production of steroidogenic enzymes (Stoker and Zorilla, 2010). In ovarian theca cells of females, LH stimulates the production of testosterone which is then, within granulosa cells, converted by aromatase to oestradiol (Kleine and Rossmanith, 2014).

The production and secretion of each of these hormones are regulated by complex feedback regulatory loops: Testosterone and inhibins provide negative feedback to the pituitary and the hypothalamus, resulting in a decrease in gonadotropin release (Meethal and Atwood, 2005). High circulating levels of testosterone inhibit hypothalamic GnRH and pituitary LH release, whereas pituitary FSH release is inhibited by inhibins (Hiller-Sturmhöfel and Bartke, 1998). It is also thought that a dysfunction of pulsatile GnRH secretion, i.e. constantly high or absent GnRH levels, inhibits the release of gonadotropins by internalisation of GnRH receptors, a translocation process by which receptors are engulfed by the cell membrane and drawn into the cell (Kleine and Rossmanith, 2014). Reproductive function and behaviour can further be inhibited by the mammal gonadotropin-inhibitory hormone (GnIH) homologue RFRP (RF amide-related peptides) (Ubuka et al., 2008). Although GnIH was originally identified in quail, RFRP and its receptors have also been identified in mammals (Ubuka et al., 2006; Ubuka et al., 2008). As GnIH/RFRP expression has been shown in the CNS and gonads, the inhibitory effect of GnIH/RFRP may be accomplished by decreasing activities of GnRH neurons via blocking or by acting directly on the gonads (Ubuka et al., 2008; Kleine and Rossmanith, 2014).

1.2. Testosterone Biosynthesis

Steroid hormones are synthesised from cholesterol, produced either by *de novo* synthesis or by conversion of acetyl coenzyme A, through a series of enzymatic conversions (Walters, 2007).

Figure 1.2 depicts the pathways of testosterone steroidogenesis. In males, testosterone biosynthesis primarily occurs in the Leydig cells of testis initiated by the binding of LH to its specific receptors on the cell surface (Payne and Youngblood, 1995). This interaction

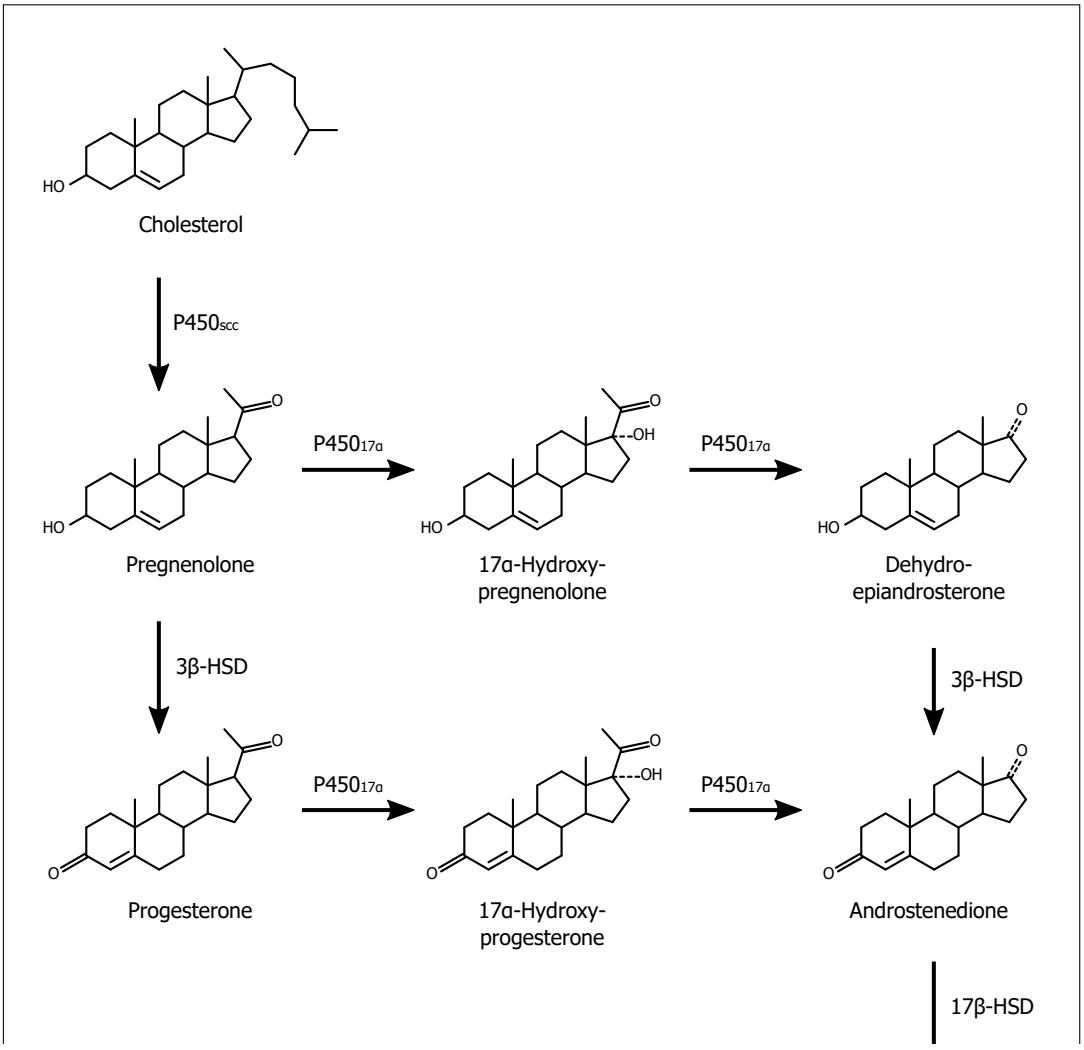

Figure 1.2. Biosynthetic pathway of testosterone in Leydig cells. The initial step in testosterone biosynthesis is the conversion of cholesterol to pregnenolone. Further on, testosterone is synthesised either via the Δ^5 (17α-hydroxypregnenolone, dehydroepiandrosterone, androstenedione) or the Δ^4 (progesterone, 17α-hydroxyprogesterone, androstenedione) pathway. $P450_{scc}$: cytochrome P450 side-chain cleavage; $P450_{17\alpha}$: cytochrome P450 17α-hydroxylase/17,20-lyase; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase. According to Tsutsui (2011).

induces the adenylate cyclase system in Leydig cell plasma membranes resulting in an increased conversion of ATP to cyclic AMP (cAMP) and expression of the steroidogenic acute regulatory protein (StAR) (Payne and Youngblood, 1995; Pineda, 2003). StAR is a transport protein that regulates the cholesterol transport from the outer to inner membranes of the mitochondria, the rate-limiting step in testosterone biosynthesis (Shi et al., 2007; Walters, 2007). The initial enzymatic step within mitochondria is the conversion of cholesterol to pregnenolone by enzymatic side-chain cleavage catalysed by cytochrome $P450_{scc}$ (Payne and Youngblood, 1995; Payne and Hales, 2004). Pregnenolone diffuses into the smooth endoplasmatic reticulum where three steroidogenic enzymes are located, namely 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hydroxylase/17,20-lyase ($P450_{17\alpha}$) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Payne and Youngblood, 1995; Walters, 2007; Ye et al., 2011). Depending on the species, either the Δ^4 or Δ^5 pathways predominate (Ye et al., 2011): In the course of the Δ^4 pathway the steroid intermediates progesterone, 17α-hydroxyprogesterone and androstenedione are formed, whereas 17α-hydroxypregnenolone, dehydroepiandrosterone and androstenedione are produced during the Δ^5 pathway (Payne and Youngblood, 1995; Walters, 2007; Ye et al., 2011). The final reaction is the reduction of androstenedione catalysed by 17β-HSD resulting in the formation of testosterone (Payne and Youngblood, 1995; Shi et al., 2007; Walters, 2007). Testosterone then is released into systemic blood circulation and transported bound to carrier proteins, called sex hormone-binding globulins (SHBG), to target tissues.

1.3. Mechanisms of Testosterone Action

At the target tissues testosterone dissociates from SHBG and diffuses into the cells. Depending on the tissue specificity of androgen action, testosterone acts either directly or in form of its specific metabolites, including DHT and oestradiol (Wu, 1997). In either case, the general mechanisms of steroidogenic signal transduction are similar and exemplarily described for testosterone in this paragraph.

The biological actions of testosterone on target cells can be mediated by two different pathways, defined as classical and non-classical mechanism, functioning independently or synergistically to initiate steroid responses, as shown in Figure 1.3 (Foradori et al., 2008; Walker, 2009). The classical and predominant mechanism of testosterone signalling is transduced via specific nuclear receptors, androgen receptors (AR), which function as ligand-activated transcription factors (Heinlein and Chang, 2002; Foradori et al., 2008; Walker, 2009). Inactivated ARs are expressed in the cytoplasm of target tissue cells bound to heat shock proteins. The first step in testosterone signalling is its diffusion through the

plasma membrane and binding to AR (Simoncini and Genazzani, 2003; Foradori et al., 2008). This interaction induces conformational changes in the receptor and in turn separates it from the heat shock proteins (Walker, 2009). Liganded ARs are then translocated into the nucleus where they bind to specific DNA-binding sites in target gene promoters, called androgen responsive elements (AREs), causing activation of transcription and subsequent protein synthesis (Simoncini and Genazzani, 2003; Foradori et al., 2008; Walker, 2009). This mechanism requires at least 30 – 40 minutes to alter gene expression, and several hours to produce significant levels of nascent proteins (Walker, 2009).

There is evidence that testosterone affects cellular processes in a non-genomic fashion, also termed non-classical mechanisms, as DNA-binding of the ARs and RNA synthesis are not required (Simoncini and Genazzani, 2003; Foradori et al., 2008). These non-genomic pathways are thought to be mediated by activation of an AR population located at the plasma membrane. Several studies showed that these testosterone-bound ARs are involved in the regulation of G-protein-coupled receptors, cell membrane ion channels and phosphorylation of protein kinase signalling pathways in a variety of cell types such as macrophages, neuroblastoma cells, cardiac myocytes and Sertoli cells, resulting in a rapid regulation of multiple cellular functions (within seconds to minutes) and also in the modulation of behaviour and longer-term processes in terms of gene expression, protein synthesis and cell proliferation (reviewed by Heinlein and Chang, 2002; Foradori et al., 2008; Walker, 2009, 2010).

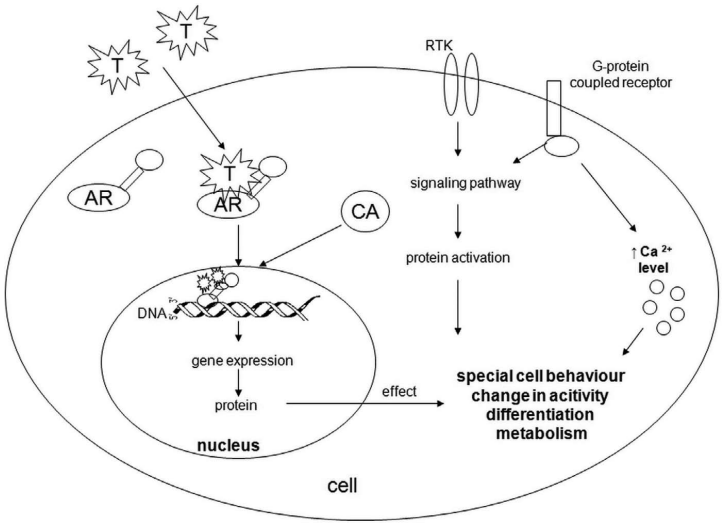

Figure 1.3. Genomic and non-genomic effects of testosterone. Right: Unbound biologically active testosterone (T) interacts with the cytoplasmatic androgen receptor (AR). Ligand binding induces conformational changes of the receptor. T-AR complex forms dimers and acts as a functional transcription factor. Activated AR recognises the androgen response element (ARE) in the nucleus due to its specific structure. Coactivators (CA) and RNA polymerase II are recruited for transcription initiation. Gene expression produces a pool of specific proteins that can affect cell characteristics, metabolism and activity. Left: The non-genomic response is mediated via receptor-tyrosine-kinases (RTK) or G-protein coupled receptors. Subsequently, downstream signalling cascades are activated, that can result in genomic effect (activation of various transcription factors, protein activation or new protein synthesis). G-protein coupled receptors can activate phospholipase C and cause an increase of intracellular Ca^{2+}. All these processes are linked with changes in cell activity (Durdiakova et al., 2011).

2. Non-Invasive Monitoring of Gonadal Activity

Sex hormones are normally tightly bound to carrier proteins (i.e. albumin and SHBG) and only the free forms (10 – 15% of the total) are biologically active (Rosner, 1990; Sheriff et al., 2010). According to the free-hormone hypothesis, it is thought that only free sex hormones are available to be translocated into the cell, where they initiate a biological response and their concentrations constitute reliable indicators of hormonal effects (Mendel, 1989; Rosner, 1990). Analyses of blood samples can provide information on both the total sex hormone concentrations and the amount that is free by using radio-immunoassays (RIA) or enzyme immunoassays (EIA) (Sheriff et al., 2010). Thus, the most common approach to evaluate hormone concentrations in livestock, domestic animals and laboratory species is blood sampling. This is a difficult method to apply to wildlife: Drawing blood requires handling, capture and anaesthesia of an animal which may induce a stress response and thereby alter physiological hormone values (Touma and Palme, 2005; Palme, 2012). As steroid hormones are secreted in a pulsatile and circadian manner, single plasma hormone levels only represent an animal's endocrine status at a specific point in time (Möstl and Palme, 2002; Schwarzenberger, 2007). Thus, serial blood samples are necessary to accurately investigate reproductive-endocrine relationships (Schwarzenberger et al., 1996; Goymann, 2005). Furthermore, restricted access especially to threatened and endangered species may limit blood sampling and thus, makes it difficult to gain knowledge on the fundamental reproductive-endocrine physiology of the given species (Schwarzenberger et al., 1996).

To avoid these problems, non-invasive sample collection methods are needed to accurately assess gonadal activity in animals by which direct animal contact can nearly be avoided thereby providing the opportunity to monitor endocrine parameters without disturbing an animal's natural behaviour or modifying its environment even for repeated measurements in longitudinal studies (Möstl and Palme, 2002; Goymann, 2005; Touma and Palme, 2005; Heistermann, 2010). These alternative approaches are based on the principle that hormones and their metabolites can also be measured in other biological matrices than blood such as faeces, urine, saliva or hair (Möstl and Palme, 2002; Heistermann, 2010; Palme, 2012). The choice of which sampling is used depends on factors including the feasibility of repeated sample collection, the type of information required and species-specific or sex-specific differences in hormone metabolism and route of

excretion (Palme et al., 2005; Heistermann, 2010). Additionally, non-invasive sampling methods do not conflict with ethical considerations and thus do not require special permits (Goymann, 2005). Urine and saliva are constantly produced and depending on the given species, represent sources to measure endocrine parameters as steroid hormones are excreted into urine following extensive metabolism by the liver, or can be found as native forms in saliva (Heistermann, 2010; Kersey and Dehnhard, 2014). However, the collection of both need some manipulation or training of the animal and can only be used to a limited extent in free-ranging individuals (Möstl and Palme, 2002; Kersey and Dehnhard, 2014). Steroid hormones are also able to diffuse into hair follicle cells and subsequently become accumulated in hair shafts during the growing phase over weeks and months, thereby providing a source for evaluating long-term hormonal changes (Koren et al., 2002; Terwissen et al., 2013; Kersey and Dehnhard, 2014). As animals may have to be shaved to obtain samples which at least require handling or even restraint, this method may not completely non-invasive (Heistermann, 2010; Kersey and Dehnhard, 2014). In addition, measurements of hairy hormone concentrations can be influenced by local synthesis of steroid hormones in hair follicles and thus may not accurately reflect averaged HPG axis activity (Keckeis et al., 2012).

In contrast, faecal hormone metabolite analyses represent a suitable alternative to plasma hormone measurement and have been commonly used and accepted as an important tool to study fundamental reproductive endocrinology in domestic and laboratory species as well as in captive and free-ranging animals (reviewed by Schwarzenberger, 2007). It is assumed that only free steroid hormones are metabolised by the liver prior to excretion, whereas protein-bound steroid hormones pass through the liver (Sheriff et al., 2010). Thus, faecal samples provide an integrated measure of the physiological activity of a specific hormone (Palme et al., 2005; Sheriff et al., 2010). One major important advantage is that faeces can be easily identified and collected, thus offering a comprehensive characterisation of reproductive events for an individual, a population or species (Brown et al., 1996a; Touma and Palme, 2005; Schwarzenberger, 2007). Furthermore, depending on an animal's defaecation rate steroid metabolites represent pooled values of excreted hormones which are less affected by short-time fluctuations, thereby providing an integrated measure over a certain time rather than a single point-in-time given by a plasma sample (Brown et al., 1996a; Goymann, 2005). In the last two decades, faecal hormone metabolite measurements have been successfully applied to assess reproductive status, characterise seasonal reproductive activity, diagnose pregnancy or pseudo-pregnancy and to determine relationships between hormonal data and reproductive behaviour in numerous mammals (Brown et al., 1996a; Velloso et al., 1998; Brown et al., 2002; Walker et al., 2002; Jewgenow et al., 2006; Dehnhard et al., 2008; Finkenwirth et al., 2010).

2.1. Testosterone Metabolism and Methodological Issue

The measurement of faecal hormone metabolites includes several challenges, as parent hormones are barely present in faeces (Palme, 2005). Steroid hormones are extensively metabolised by the liver and subsequently excreted in free or conjugated form into the gut via the bile ducts (Taylor, 1971; Lindner, 1972; Möstl and Palme, 2002). Conjugation reactions coupling the hormone or its metabolites to glucuronic acid or sulphate make them water soluble to support the elimination from the body via urine or faeces, as shown in Figure 2.1 (Schänzer, 1996). Reabsorption into enterohepatic circulation and additional metabolism by intestinal bacteria can affect chemical structures and characteristics of the produced metabolites prior to excretion, thereby producing a vast number of different faecal metabolites (Palme et al., 2005; Schwarzenberger, 2007; Sheriff et al., 2011).

Figure 2.2 depicts possible pathways for testosterone metabolism. The main abundant natural testosterone metabolites are produced by oxidoreductive reactions at the A- and D-ring of the molecule at positions C-3, C-4, C-5 and C-17 (Schänzer, 1996). The initial step in testosterone metabolism is the reduction of the double bond at the A-ring at positions C4-5 catalysed by the enzymes 5α-reductase or 5β-reductase, which are located mainly in the liver (Celotti and Negri-Cesi, 1992; Schänzer, 1996). These transformations are irreversible and result in the formation of two isoforms with either a 5α-HSD (dihydrotestosterone) or 5β-configuration. Following reduction of the double bond, the 3-keto group of both 5α- and 5β-derivates is rapidly reduced by either 3α- or 3β-HSD (Celotti and Negri-Cesi, 1992; Schänzer, 1996). These enzymatic transformations result in the formation of 5α,3α-hydroxy, 5α,3β-hydroxy and 5β,3α-hydroxy metabolites — the possible formation of 5β,3β-hydroxy metabolites has not been reported yet (Schänzer, 1996). The main and well-known excreted metabolites of testosterone are 17-keto metabolites (D-ring metabolism) including the formation of androsterone, epiandrosterone, etiocholanolone and androstenedione. 17-keto metabolites are formed by enzymatic oxidation catalysed by 17β-HSD and can be converted back to 17β-hydroxy metabolites by the same enzyme (Schänzer, 1996). A-ring metabolism of testosterone can also result in the formation of oestrogenic molecules. The enzyme aromatase catalyses the irreversible reaction from testosterone to oestradiol (Celotti and Negri-Cesi, 1992).

Determination and characterisation of metabolic endproducts can be carried out by injecting a small amount of radiolabelled steroid hormones and collecting faecal samples followed by high-performance liquid chromatography (HPLC) analyses after extracting the target compounds from the biological matrix (Schwarzenberger et al., 1996; Palme et al., 2005). In a comparative study of three anthropoid primate species, Möhle et al. (2002) found multiple radiolabelled testosterone metabolites with a characteristic pattern for

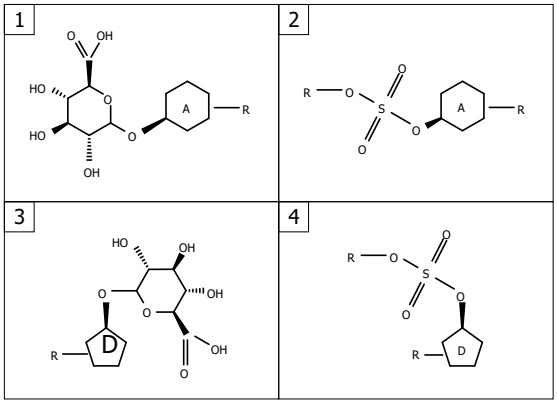

Figure 2.1. Illustration of possible conjugation sites of testosterone metabolites. Conjugation to glucuronides and sulphates can occur at A-ring at position C-3 (1, 2) or D-ring at position C-17 (3, 4). A: A-ring, D: D-ring, R: rest. According to Schänzer (1996).

each species. Goymann (2005) even demonstrated sex-specific differences in the excretion pattern of ^{3}H-testosterone in European stonechats (*Saxicola rubicola*), which can also vary between seasons. The excretion of polar, conjugated testosterone metabolites (presumably glucuronides or sulphates) has been reported in several species [domestic cat, *Felis catus* (Brown et al., 1996a); Eurasian and Iberian lynx, *Lynx lynx* and *Lynx pardinus* (Jewgenow et al., 2006); common marmoset, *Callithrix jacchus* (Möhle et al., 2002); chinchilla, *Chinchilla lanigera* (Busso et al., 2005) and laboratory and field mouse, *Mus musculus* and *Peromyscus maniculatus* (Billitti et al., 1998)].

2.2. Validation of Enzyme-Immunoassays

Quantification of faecal hormone metabolites can be performed with enzyme immunoassays (EIAs) (Touma and Palme, 2005). However, commercially available EIA kits often use antibodies that are primarily produced to measure the parent hormones in blood and thus might have some deficiencies in detecting steroid hormone metabolites (Touma and Palme, 2005). To negotiate these problems, the use of group-specific antibodies that recognise an array of structurally similar metabolites is a preferred alternative (Touma and Palme, 2005; Kersey and Dehnhard, 2014).

The development of a reliable EIA requires an appropriate methodological and biological validation to confirm that the assay actually measures the hormone or hormone-related metabolites it is supposed to and that measured changes in concentrations are a consequence of physiological responses.

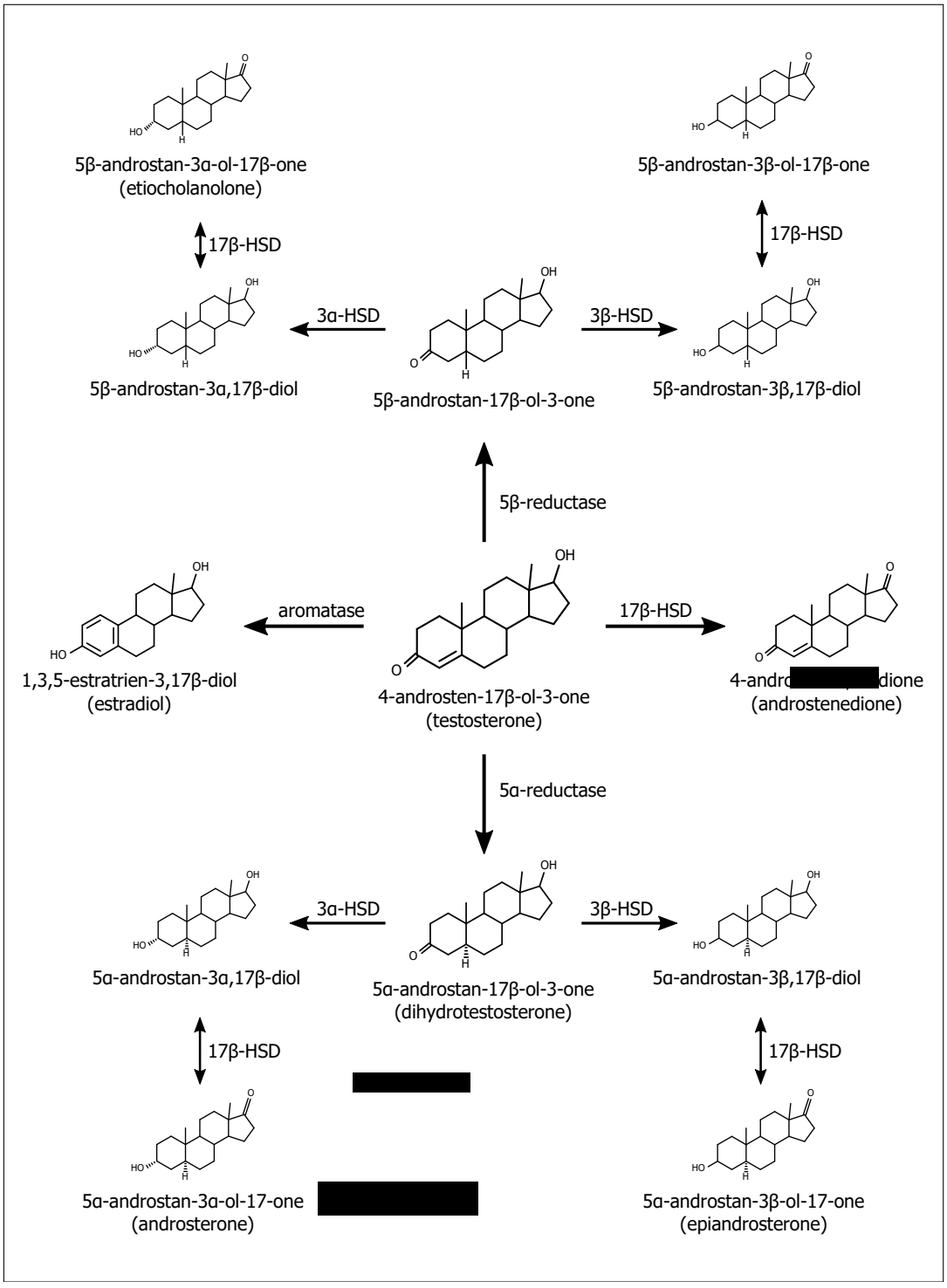

Figure 2.2. Illustration of metabolic testosterone pathways. Arrows indicate the possible metabolic pathways of testosterone. The occurrence of 5β-androstane-3β,17β-diol and 5β-androstan-3β-ol-17β-one is possible but has not been reported yet. 3α-HSD: 3α-hydroxysteroid dehydrogenase; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase. According to Schänzer (1996).

2.2.1. Methodological Validation

Major faecal hormone metabolites can be determined by performing radiometabolism studies followed by reversed phase high-performance liquid chromatography (RP-HPLC) analyses. These analytical methods clarify whether and which metabolites derived from the hormone of interest are measured by the respective EIA. RP-HPLC analyses using an unpolar column allow the separation and elution of excreted hormone metabolites according to their polarity. Individually collected fractions can be analysed for radioactivity and for immunoreactivities detected by the respective EIA. A comparison of elution fractions of radiolabelled and immunoreactive hormone metabolites indicates the degree to which metabolites cross-react and bind to the utilised antibody (Kersey and Dehnhard, 2014). Thus, the co-elution of radiolabelled and immunoreactive substances demonstrates that the respective EIA is capable of detecting faecal metabolites derived from the parent hormone of interest (Touma and Palme, 2005; Kersey and Dehnhard, 2014). In contrast, radiolabelled peaks not supported by immunoreactive ones indicate that these metabolites are not detected by the EIA (Touma and Palme, 2005; Kersey and Dehnhard, 2014). Moreover, if immunoreactivities are detected without accompanying radioactivity, then this suggests that the EIA seems to cross-react with metabolites not derived from the parent hormone of interest (Kersey and Dehnhard, 2014). Methodological validation represents a crucial step as metabolism of other steroid hormones may lead to structurally similar metabolites excreted via the faecal route. The majority of faecal androgen metabolites and several faecal glucocorticoid metabolites (fGM) possess similar androstane-based structures (C-19 androstanes) which may only differ at single C-atom positions as shown for the typical metabolites of testosterone and cortisol, etiocholanolone and 11-oxoetiocholanolone, respectively (Ganswindt et al., 2003; Palme et al., 2005). Hence, antibodies directed against C-19-testosterone metabolites may cross-react with glucocorticoid metabolites (Ganswindt et al., 2003; Palme et al., 2005; Kersey and Dehnhard, 2014). These undesired cross-reactivities may invalidate faecal testosterone metabolite (fTM) measurements and thus lead to wrong results (Ganswindt et al., 2003).

In species where testosterone metabolites are predominantly excreted in their conjugated form, hydrolysis using enzyme preparations from a snail (*Helix pomatia*) that contain glucuronidase and/or sulphatase activity following extraction of faecal samples may improve results. Enzymatic hydrolysis prior to EIA and RP-HPLC analyses may be essential, as testosterone metabolites can be liberated from their conjugates, thereby enhancing the sensitivity of the applied EIA.

2.2.2. Physiological and Biological Validation

The terms "physiological" and "biological" validation are defined by Touma and Palme (2005) to mean either (1) to pharmacologically induce physiological changes in circulating steroid hormones and evaluate whether these changes are reflected in measured concentrations of faecal hormone metabolites afterwards, or (2) to assess changes in metabolite concentrations according to life history parameters (e.g. reproductive status). These approaches require a strict sampling regime of faeces collected from known individuals: Serial faecal samples have to be collected before and after injection or the occurrence of an expected event, and the total time period during which the samples are collected should be adjusted to the defaecation rate of the species concerned (Touma and Palme, 2005).

The most widely used experiment to physiologically validate a testosterone EIA are "GnRH-challenge" tests. The injection of a pharmacological GnRH stimulates HPG axis activity, resulting in an increased plasma testosterone concentration. This increase should be clearly reflected in faecal testosterone metabolite (fTM) concentrations after a species-specific time lag (Touma and Palme, 2005). As the excretion pattern of fTM may vary between the sexes it is recommended to perform experiments at least in one male and female per species (Palme, 2012; Kersey and Dehnhard, 2014). However, a physiological validation might not be possible in each species due to its conservation status, limited access or ethical considerations. Nevertheless, at least a biological validation must be performed before a testosterone EIA can be considered to be reliable, as it would demonstrate that changes in faecal hormone metabolite concentrations truly reflect biological changes (Goymann, 2005; Palme et al., 2005; Touma and Palme, 2005; Kersey and Dehnhard, 2014). The biological relevance of an EIA measuring gonadal activity based on fTM levels can be determined by comparing faecal samples from males of different reproductive statuses (mature vs. immature, castrated vs. intact, reproductively active vs. non-active) and age (subadult vs. adult) or by demonstrating the ability to differentiate male and female samples (Ganswindt et al., 2002; Dloniak et al., 2004; Barelli and Heistermann, 2012).

3. Measurement of Testosterone in Spotted Hyenas and Cheetahs

3.1. Testosterone Measurement in Spotted Hyenas (*Crocuta crocuta*)

Among carnivores, spotted hyenas (*Crocuta crocuta*) exhibit unique social and morphological features: They live in large groups, called clans, which represent highly structured, female-dominated social units (Kruuk, 1972). A spotted hyena clan consists of several matrilines of related females which remain in their natal clan to breed, whereas males usually disperse and join other clans where they become reproductively active (Frank, 1986). Within a spotted hyena society females and males have separate linear dominance hierarchies in which each individual can be ranked, and priority access to food varies with social status (Kruuk, 1972; Frank, 1986; East and Hofer, 1991). Dominance relations among clan females are characterised by a stable order in which daughters usually obtain a rank position just below their mother (Frank, 1986; East et al., 2009).

Within the male social hierarchy, non-dispersing (natal) males are dominant over immigrant males (East and Hofer, 2001). Non-dispersing males benefit from the fact that they are usually being treated by the rest of the clan as sons to their mothers, implying that they will have the same rank as their mothers — and thus higher than any immigrant male. Immigrant males queue for high social status amongst males, starting at the very bottom of the male hierarchy and moving up in rank with the duration of their tenure in a clan (East and Hofer, 1991, 2001). High social status is not correlated with size or fight ability, and physical contests among males are rare (East and Hofer, 1991; East et al., 1993; Hofer and East, 2003). While queuing, males develop close relationships to more dominant females which are essential for a male's reproductive success owing to the unusual reproductive anatomy of females. Female spotted hyenas exhibit masculinised external genitalia with a penile clitoris and pseudo-scrotum and access to the vagina through the opening at the tip of the clitoris, thus successful copulation requires complete cooperation of females (Kruuk, 1972; East et al., 1993).

It has been suggested that testosterone plays an important role during the evolution and ontogenetic development of female dominance and masculinised genitalia (Goymann

et al., 2001). Endocrine studies based on plasma testosterone concentrations produced inconsistent findings: Some studies reported unusual high testosterone concentrations in females, resulting in difficulties to differentiate between sexes on the basis of the hormone alone (Racey and Skinner, 1979; Lindeque et al., 1986; van Jaarsveld and Skinner, 1991b). In contrast, other studies found significant sex differences in adult spotted hyenas with males exhibiting significantly higher testosterone levels than females (Glickman et al., 1987; Van Jaarsveld and Skinner, 1991a; Glickman et al., 1992). As revealed by Goymann et al. (2001), differing laboratory protocols, sampling individuals without paying attention to reproductive or social status or cross-reactivities of the used testosterone antibody with other androgens might have led to these conflicting results. To avoid cross-reactivities of the respective androgen antibody, Goymann et al. (2001) analysed circulating testosterone, DHT and androstenedione levels following chromatographic extraction and were able to demonstrate that spotted hyenas followed the typical mammalian pattern of sex-specific circulating testosterone concentrations, with males having significantly higher plasma testosterone concentrations than females.

Non-invasive monitoring of testosterone based on fTM analyses allows studying the underlying endocrine mechanisms in this female-dominated species and may contribute to the long-running debate about female androgenisation. So far, no androgen EIA has been validated that exclusively detects fTM in spotted hyenas. Although Dloniak et al. (2004) validated a testosterone EIA to evaluate faecal androgen metabolites (fAM), this assay does not allow an estimation of single androgen's impact on measured fAM concentrations as it detects the sum of excreted immunoreactive faecal testosterone, DHT and androstenedione metabolites.

3.2. Testosterone Measurement in Cheetahs (*Acinonyx jubatus*)

The known free-living cheetah population comprises approximately 7,000 individuals with a regional stronghold in southern Africa (Durant et al., 2015). Cheetahs (*Acinonyx jubatus*) are particularly vulnerable to habitat loss and fragmentation as both sexes have very large home ranges with an average of approximately 1,600 km^2 (Durant et al., 2015). As the free-living cheetah population is declining, captive breeding increases in importance for the conservation of the species. However, the zoo population has never been self-sustaining (Marker-Kraus and Grisham, 1993). In contrast to their free-living counterparts, captive cheetahs show poor reproductive success, with low conception rates, low fertility and low juvenile survival (Lindburg et al., 1993; Marker-Kraus and Grisham, 1993; Thalwitzer et al., 2010; Wachter et al., 2011).

In comparison to other felids, male cheetahs secrete low amounts of testosterone and exhibit poor ejaculate qualities with low spermatozoal concentrations and numerous pleiomorphic forms (Wildt et al., 1983; Wildt et al., 1988). Husbandry conditions and the captive environment have been suggested as stressful and thus as contributing factors to the poor reproductive performance of captive cheetahs (Terio et al., 2004). However, reproductive physiology of free-ranging and captive male cheetahs seems to be unrelated to differences in apparent reproduction deficiencies, as wild-caught and captive-held cheetahs did not differ in their reproductive physiological characteristics (Crosier et al., 2007). Comparative studies also demonstrated similar mean plasma testosterone concentrations for free-ranging and captive individuals as well as similar testicular and seminal traits of proven and non-proven breeders (Wildt et al., 1987; Wildt et al., 1993).

Endocrine studies to evaluate testicular activity have been almost exclusively based on plasma testosterone analyses. Bertschinger et al. (2008) demonstrated a distinct diurnal rhythm of plasma testosterone secretion with 4 – 5 peaks per day. Thus, data on circulating testosterone might be biased by diurnal fluctuations. A reliable androgen EIA to monitor fTM would represent a powerful tool for the long-term evaluation of gonadal status of males, thereby providing critical information for appropriate captive management and the improvement of husbandry conditions and an increased understanding as to how environmental changes affect reproductive activity.

4. Aims of this Thesis

Monitoring gonadal activity by measuring testosterone over a period of days, weeks or months would provide an indicator of reproductive activity and fertility in male individuals. A better understanding of the reproductive biology of a species is beneficial for *in-situ* and *ex-situ* conservation by providing essential information for optimising husbandry management and animal welfare to facilitate assisted reproductive techniques and captive breeding. The overall aim of the present dissertation was to develop and validate non-invasive techniques to assess gonadal activity in spotted hyenas (***Article 1***) and male cheetahs (***Article 2***) based on fTM analyses, since the feasibility of using fTM measurements in both species has not yet been established. In order to develop reliable testosterone EIAs in both species, we aimed to obtain basic data on testosterone metabolism and excretion using radiometabolism studies. The validity of the used EIAs should be demonstrated by performing comprehensive methodological and physiological validation procedures. The biological relevance of the used EIAs should then be verified by comparing fTM levels of free-ranging, individually known animals. Within this framework, we also aimed to highlight the major difficulty in using EIAs, the cross-reactivities with unrelated hormone metabolites. For this purpose, we compared different testosterone EIAs to verify their ability to monitor gonadal activity without reflecting changes in adrenocortical activity (***Article 3***).

Part II.

Research Articles

In this part, I will present the results of the experimental studies in spotted hyenas and cheetahs conducted within the framework of the current dissertation. At first, I will highlight the main objectives and results of each study by stating a summary, respectively. Afterwards, I will detail the development and validation of EIAs to monitor faecal testosterone metabolites in spotted hyenas and male cheetahs as published (***Article 1*** and ***Article 2***) and submitted for publication (***Article 3***).

5. Summary of Research Articles

5.1. Article 1: Measuring Faecal Epi-Androsterone as an Indicator of Gonadal Activity in Spotted Hyenas (*Crocuta crocuta*), Pribbenow et al. 2015, *PLoS ONE*

The aim of the study was to present the first comprehensive validation of an "in-house" epiandrosterone EIA to monitor gonadal activity in spotted hyenas non-invasively as indicated by faecal testosterone metabolites. To ensure that the epiandrosterone EIA is able to detect testosterone-related metabolites and that measured changes in fTM concentrations are a consequence of genuine physiological changes, we performed methodological and biological validation procedures. To methodologically validate the epiandrosterone EIA we performed a radiometabolism study in one captive male and female spotted hyena, respectively. We compared the elution positions of radiolabelled fTM and immunoreactive fTM detected by the EIA following HPLC analyses. We also assessed whether enzymatic hydrolysis with β-glucuronidase extracted from either *Helix pomatia* or *Escherichia coli* influenced our fTM measurements. Furthermore, we aimed to identify the excreted fTM by performing gas chromatography-mass spectrometry (GC-MS) analyses. The potential impact of fGM on fTM measurements was assessed by analysing faecal samples from an ACTH challenge test in one captive male spotted hyena. To physiologically and biologically validate the epiandrosterone EIA we determined its efficiency to detect an increase in fTM concentrations following a testosterone injection in a captive female spotted hyena, and finally to discriminate between free-ranging males with different reproductive status by comparing fTM in juvenile and adult male spotted hyenas.

Results of the study:

1. HPLC analyses of radiolabelled fTM in captive spotted hyenas demonstrated that testosterone metabolites are excreted almost exclusively as highly polar, enzyme-hydrolysable conjugated faecal metabolites, mainly epiandrosterone conjugates. Hydrolysis using β-glucuronidase from *Helix pomatia* and *Escherichia coli* liberated epiandrosterone from its polar conjugates.

2. HPLC analyses also demonstrated that the epiandrosterone EIA detects immunoreactive fTM corresponding to radiolabelled fTM in both sexes. Following hydrolysis, HPLC profiles of captive and free-ranging spotted hyenas showed a unified elution pattern, thereby confirming that testosterone is mainly processed via the epiandrosterone pathway.

3. Epiandrosterone was identified by GC-MS as the major testosterone metabolite in hydrolysed faecal samples.

4. The testosterone injection resulted in a rapid increase in plasma testosterone concentrations, which was clearly reflected in fTM concentrations 15.5 h post-injection, irrespective of the hydrolysis method used.

5. The epiandrosterone EIA showed no biological responsiveness to ACTH, thus fGM had no impact on the measured fTM levels.

6. In free-ranging spotted hyenas, fTM levels of adult immigrant males were significantly higher than in juvenile natal males.

Conclusion:

The study demonstrated that monitoring fTM concentrations with the epiandrosterone EIA is a reliable indicator of gonadal activity in spotted hyenas.

5.2. Article 2: Validation of an Enzyme-Immunoassay for the Non-Invasive Monitoring of Faecal Testosterone Metabolites in Male Cheetahs (*Acinonyx jubatus*), Pribbenow et al. 2016, *General and Comparative Endocrinology*

This study aimed to validate an “in-house” epiandrosterone EIA for the non-invasive monitoring of testicular activity in male cheetahs based on fTM analyses. We performed a testosterone radiometabolism study followed by HPLC analyses in three captive male cheetahs from a German zoo and Namibian farmland to characterise radiolabelled and immunoreactive testosterone metabolites excreted in faeces. Faecal samples from both a GnRH and testosterone challenge experiment conducted in a captive male cheetah were analysed to physiologically validate the epiandrosterone EIA. Furthermore, we determined whether an injection itself triggers an increase in fTM concentrations by analysing faecal samples prior to and following a placebo injection of NaCl. In order to exclude potential cross-reactivities with metabolites derived from glucocorticoid degradation,

faecal samples from an ACTH challenge test were analysed with the epiandrosterone EIA. The biological relevance of the epiandrosterone EIA was validated by comparing fTM concentrations in free-ranging adult male and female as well as juvenile male cheetahs. Finally, we determined whether environmental conditions have an impact on the measured fTM concentrations.

Results of the study:

1. HPLC analysis of radiolabelled fTM demonstrated a cluster of highly polar, enzyme-hydrolysable metabolites, mainly epiandrosterone conjugates. Following hydrolysis epiandrosterone was liberated from its conjugates.

2. HPLC immunograms confirmed that the epiandrosterone EIA detects immunoreactive fTM corresponding to radiolabelled ones. FTM of captive male cheetahs from a German zoo and Namibian farmland showed the same excretion pattern.

3. fTM concentrations increased significantly within one day in response to both a testosterone and GnRH challenge experiment. In contrast, a placebo injection of NaCl did not result in elevated fTM concentrations.

4. In both sexes, the epiandrosterone EIA showed no cross-reactivities with fGM, as fTM concentrations remained stable following an ACTH injection.

5. fTM concentrations differed significantly between free-ranging adult male, adult female and juvenile male cheetahs, with adult males exhibiting significantly higher fTM concentrations than adult females or juvenile males, and juvenile males showing significantly higher fTM concentrations than adult females.

6. fTM concentrations in faecal samples collected in a German zoo and on a Namibian farmland remained stable over a time period of 48 h and 72 h, respectively.

Conclusion:

This study demonstrates that variations in testicular activity in male cheetahs can be reliably monitored non-invasively using the epiandrosterone EIA.

5.3. Article 3: Measuring Faecal Testosterone Metabolites in Spotted Hyenas: Choosing the Wrong Assay may Lead to Erroneous Results, Pribbenow et al. (*under review*)

The goal of this study was to indicate and highlight the major challenge in using EIAs for non-invasive hormone monitoring based on faecal samples: undesirable cross-reactivities with metabolites not derived from the hormone of interest. For this purpose, we compared the ability of four different testosterone EIAs directed against testosterone-3-CMO, -6-CMO, -11-HS and -17-HS to monitor gonadal activity in spotted hyenas without reflecting adrenocortical activity. The first aim was to investigate the efficiency of the testosterone EIAs to detect increases in fTM concentrations by analysing faecal samples from a testosterone challenge test. The second aim was to determine the potential of the testosterone EIAs to cross-react with metabolites derived from glucocorticoid degradation by analysing faecal samples from an ACTH challenge test. Finally, in order to characterise the immunoreactive metabolites detected by the respective EIAs we performed HPLC analyses of faecal samples following a testosterone injection.

Results of the study:

1. Comparing the four testosterone EIAs resulted in widely differing results in response to a testosterone challenge. Only the testosterone-3-CMO and -6-CMO seemed to potentially able to detect physiological changes in fTM concentrations, as they demonstrated the expected increase following testosterone injection. The testosterone-11-HS EIA showed a pronounced but late increase.

2. The testosterone-3-CMO, -6-CMO and -11-HS EIAs detected pronounced increases following an ACTH challenge test, indicating high cross-reactivities with metabolites derived from glucocorticoid degradation.

3. HPLC immunograms conducted with the testosterone-3-CMO, -6-CMO and -11-HS EIAs did not explain the cross-reactivity with fGM, as their major immunoreactivities were not associated with the elution range of known glucocorticoids.

4. In contrast, the testosterone-17-HS EIA showed no biological responsiveness to the testosterone nor the ACTH challenge test. The HPLC profile demonstrated a major non-polar metabolite of unknown origin.

Conclusion:

None of the four tested testosterone EIAs seem appropriate to measure fTM in spotted hyenas, as all of them showed substantial cross-reactivities with fGM and unknown metabolites not derived from testosterone metabolism.

6. Article 1

Measuring faecal epi-androsterone as an indicator of gonadal activity in spotted hyenas (*Crocuta crocuta*)

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Contribution of each author to the manuscript: The experiments of this study were conceived and designed by SP, MLE, AG, ASWT, HH and MD. MD, AG and ASWT performed the challenge experiments in South Africa. The endocrine laboratory analyses were performed by SP. The data were analysed by SP, HH, MLE and MD. MD, SP, AG, ASWT, HH and MLE contributed the reagents, materials and analysis tools. The paper was drafted and written by SP; MD, AG, ASWT, MLE and HH revised the manuscript.

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Abstract

Enzyme immunoassays (EIA) that measure faecal testosterone metabolites (fTM) are useful tools to monitor gonadal activity. The aim of this study was to validate an "in-house" epiandrosterone EIA to monitor fTM in spotted hyenas. FTM were characterised in a male and a female hyena that each received an injection of ^{3}H-testosterone. High-performance liquid chromatography (HPLC) analyses revealed a cluster of highly polar enzyme-hydrolysable hormone metabolite conjugates. We performed hydrolysis using β-glucuronidase to deconjugate metabolites and improve sensitivity of the assay. Because

β-glucuronidase from *Helix pomatia* has been reported to bias testosterone measurements in some species, we compared the enzymatic activity of the commonly used β-glucuronidase extracted from *H. pomatia* with the same enzyme from *Escherichia coli*. Our results showed that β-glucuronidases from both sources produced similar results from spotted hyena faeces. We therefore hydrolysed samples with *H. pomatia* enzymes. HPLC analyses also demonstrated that following hydrolysis the epiandrosterone EIA measured significant amounts of immunoreactive metabolites corresponding to radiolabelled metabolites in both sexes. Additionally, HPLC and GC-MS analyses confirmed the presence of epiandrosterone in faeces of spotted hyenas. The biological relevance of the epiandrosterone EIA was validated by demonstrating (1) a significant increase in fTM levels in response to a testosterone injection within 16 h, (2) no biological responsiveness to an adrenocorticotropic hormone (ACTH) injection and (3) significant differences in fTM levels between juvenile males and adult immigrant males in a free-ranging wild population. Our results clearly demonstrate that the epiandrosterone EIA is a reliable non-invasive method to monitor gonadal activity in spotted hyenas.

6.1. Introduction

In mammals, androgens are involved in shaping phenotypic and life history traits [1, 2]. Androgens influence gene expression and the development of foetuses, secondary sexual characteristics and sexually dimorphic behaviour [3, 4]. Contest competition among males for access to mating partners can be associated with elevate androgen concentrations [e.g. 5, 6 – 8], as predicted by the 'challenge hypothesis' [9]. Similarly, in some species contests among males for social dominance can results in dominant males having higher circulating concentrations of androgens than subordinate males (e.g. African wild dog, *Lycaon pictus*, [10]; golden lion tamarin, *Leontopithecus rosalia*, [7]), but this is not the case in other species (e.g. common brown lemur, *Eulemur fulvus rufus*, [11]; bonobo, *Pan paniscus*, [8]).

Routinely, blood samples are used to measure circulating hormone concentrations to evaluate an animal's endocrine status. However, restricted access to animals may limit blood sampling, particularly in wildlife species [12]. Furthermore, handling procedures including capture, restraint and anaesthesia, which are necessary for blood sampling, may alter circulating hormone concentrations, which is especially problematic when an individual is handled repeatedly to obtain multiple samples [13 – 15]. The measurement of faecal hormone metabolite concentrations using enzyme immunoassays (EIA) provides a non-invasive alternative method to the use of blood samples. Faecal samples can be

collected easily and without disturbing the animal, thereby allowing repeated sampling, even over long periods [12, 15]. Furthermore, faecal hormone metabolite concentrations are integrated over a period of time and hence are less affected by the pulsatory hormone secretion pattern present in blood samples. Hence the application of EIAs has many advantages — but their application presents several challenges.

Circulating steroid metabolites are extensively metabolised by the liver and intestinal bacteria, thus faecal steroid metabolites are a mixture of several metabolites [16]. Therefore, EIAs used to monitor faecal hormone metabolites and extraction procedures for steroids from faeces should be carefully validated for each species and type of hormone [16 – 18]. Antibodies used for EIAs are group-specific and rely on cross-reactivities to hormone related metabolites with similar functional groups present in the native steroid hormone [15]. Steroid hormones, such as androgens, may be converted to polar water-soluble glucuronide and/or sulphate conjugates prior to excretion [15], which have been demonstrated by radiometabolism studies to occur in the faeces of a taxonomically diverse range of mammalian species (e.g. primates [19]; maned wolf, *Chrysocyon brachyurus* [20]; Eurasian lynx, *Lynx lynx*, and Iberian lynx, *Lynx pardinus* [21]; spotted hyena, *Crocuta crocuta* [22]). Hydrolysis can liberate steroids from their conjugates, thereby increasing steroid detection by antibodies and thus the sensitivity of EIAs. The enzyme β-glucuronidase from *Helix pomatia* is commonly used to deconjugate steroids, but its application may transform androgens and produce the artefact of an increase in the "measured" testosterone levels [23 – 26]. One known major challenge to the development of an EIA for measuring faecal androgen metabolites (fAM) is that the majority of faecal androgens and some of the faecal glucocorticoid metabolites (fGM) have similar androstane-based structures, hence antibodies directed against C19-cortisol metabolites may cross-react with androgen metabolites [15, 27].

Our study aimed to validate an "in house" EIA to monitor faecal testosterone metabolites (fTM) in the spotted hyena, suitable for monitoring gonadal activity in wild populations. The spotted hyena is a highly social carnivore (for a review of the species biology see [28]). Spotted hyenas live in groups termed clans [29] containing natal females, their juvenile offspring and reproductively active adult males that are mostly but not exclusively immigrants from other clans [30, 31]. One noticeable feature of spotted hyena society is the low frequency and low intensity of physical aggression among immigrant males [30, 32]. At immigration, males join the male dominance hierarchy at the lowest rank and increase in dominance as males of higher status die or leave the clan [30]. Hence the immigrant male hierarchy functions as a social queue because males mostly adhere to a strict queuing convention [30]. The rank of an immigrant male is therefore chiefly determined by his length of tenure [30].

All female clan members breed and births occur throughout the year [33, 34], yet high-ranking males do not monopolise the paternity of offspring produced in a clan [35]. Rather, females exercise strong mate-choice [31, 35] because the occurrence and position of their peniform clitoris [36, 37] ensures that copulation cannot occur without the complete cooperation of the female [29, 32]. Immigrant males employ various mating tactics [30, 35, 38]. One tactic used mostly by immigrant males above median rank is to prevent lower-ranking immigrant males interacting with the female they have chosen to "defend" [30]. In accordance with the 'challenge hypothesis' [9] immigrant males defending females have higher testosterone concentrations than those not defending females [5]. Furthermore, because of the typically low frequency and intensity of aggression among immigrant males there is no significant correlation between an immigrant male's rank and his testosterone concentration [5, 39]. In general, testosterone concentrations in young pre-dispersal natal males are lower than those of immigrant males, and normally remain low for approximately 12 months after dispersal and immigration into another clan [5, 40].

In this study we: (1) investigated the efficiency of an EIA with an antibody directed against epiandrosterone (epi-A) to monitor the expected increase in fTM following the injection of 20 mg testosterone in a female captive hyena; (2) characterised faecal testosterone metabolites by performing a radiometabolism study and evaluating the specificity of the epi-A antibody by comparing immunoreactivities towards radiolabelled peaks originating from testosterone degradation using HPLC analyses; (3) identified the major fTM by gas chromatography-mass spectrometry (GC-MS) analyses; (4) assessed whether enzymatic hydrolysis with β-glucuronidase extracted from either *H. pomatia* or *E. coli* influenced our measurements of fTM concentrations; (5) determined whether our EIA was influenced by fGM of adrenocortical origin by comparing levels of fGM and fTM in samples of an ACTH challenge test and (6) biologically validated the epi-A EIA by measuring fTM concentrations of free-ranging juvenile natal male and adult immigrant male spotted hyenas, with the expectation that juveniles would have lower fTM levels than adult immigrant males.

6.2. Materials & Methods

6.2.1. Ethics Statement

The treatment of the captive spotted hyenas was performed when the animals were anaesthetised by zoo veterinarians using a combination of medetomidine, midazolam and butorphanol at the National Zoological Gardens (NZG) of South Africa and ketamine hydrochloride and xylazine at Leipzig Zoo. All methods applied, and the study design

were approved and in agreement with the animal ethics and welfare committee at the Leibniz Institute for Zoo and Wildlife Research (IZW), the Leipzig Zoo and NZG (permit numbers: 2012-03-01, Leipzig Zoo, Germany; 2012-08-31, NZG of South Africa). The field research and collection of faecal samples of free-ranging spotted hyenas in Tanzania was approved by the Tanzanian Commission for Science and Technology (COSTECH) and the Tanzania Wildlife Research Institute (TAWIRI). The permission to work in the Serengeti National Park was granted by Tanzanian National Parks Authority (TANAPA).

6.2.2. Study Animals and Processing of Faecal Samples

Sample collection in captivity. One adult female and two adult male spotted hyenas were studied in captivity. The female was kept in the National Zoological Gardens of South Africa, Pretoria, whereas the males were kept in Leipzig Zoo (Germany) and Dvur Králové Zoo (Czech Republic), respectively. All faecal samples were collected by animal keepers within the animal management schedule of each zoo. The samples were taken from the centre of a dropping to avoid cross-contamination with urine. The samples were mixed by hand and a 5 – 10 g portion was placed in individual collection vials and frozen immediately upon collection at −20 °C.

Sample collection in the wild. A total of 32 faecal samples were collected immediately after deposition from individually known free-ranging spotted hyenas in the Serengeti National Park in northern Tanzania (adult immigrant male hyenas, n = 15; adult females, n = 1; juvenile males n = 15). To avoid the potential impact of twin litter sibling aggression [41 – 43] on fTM levels we only analysed faecal samples from singleton male litters. Faeces were stored in the field in a cold box for less than 3 hours after collection. Faeces were mechanically mixed and an aliquot of faeces was stored in either liquid nitrogen or frozen at approximately −10 °C in Tanzania until transported frozen to Germany where they were stored at −80 °C until analyses.

The animals were members of three clans which are part of a long-term research programme [35]. Animals were categorised as juveniles when less than 24 months of age and as adults thereafter [44, 45]. All samples from adult males were from immigrant males with at least 12 months of tenure. We recognised individuals by their distinctive spot patterns, ear notches, scars and bald patches [42, 46]. The age of juveniles was estimated in days when first observed at the birth den or the communal den using pelage characteristics, whether their ears were flattened or upright, and their balance and coordination during locomotion [47]. Estimates of age were accurate to within 7 days. We used the dimorphic glans morphology of the erect phallus to determine sex [48].

Extraction of faecal samples. For captive spotted hyenas, an aliquot of 0.5 g wet faeces were extracted with 4.5 ml 90% methanol for 30 min using a universal shaker (SM-30, Edmund Buhler GmbH, Hechingen, Germany). After centrifugation (15 min, 1000 g) the supernatant was transferred to a new tube and diluted 1:1 with water. Aliquots of faecal extracts were subjected either to HPLC analyses, or directly to the in-house epiandrosterone EIA.

Faecal samples of free-ranging spotted hyena may vary considerably in their degree of humidity. To avoid a possible diluting effect, faecal samples were dried for 22 h in a freeze-drier (EPSILON1-4, LSC plus, Martin Christ GmbH, Osterode, Germany). After powdering the dried faeces, 0.1 g of well-mixed powder were extracted with 0.9 ml 90% methanol and homogenised on a shaker for 30 min. After centrifugation (3000 rpm, 15 min) the supernatant was transferred to a new tube. For EIA analyses aliquots were diluted 1:2 with water and 20 µl were subjected to the androgen EIA.

6.2.3. Radiometabolism Study

To characterise fTM, we performed two radiometabolism studies on one captive adult female and on one captive adult male hyena, respectively.

The radiometabolism study in the female was conducted in combination with a testosterone challenge experiment (see section Testosterone challenge) in the National Zoological Gardens of South Africa (NZG), Pretoria, in 2012. The design of this study followed a standard procedure previously described for hyenas and used for the evaluation of faecal glucocorticoid metabolite measurements [45]. The hyena was immobilized in its enclosure via remote injection (DanInject darting system), using a combination of medetomidine (0.03 mg/kg) + midazolam (0.15 mg/kg) + butorphanol (0.2 mg/kg). After the collection of a blood sample (5 ml in an EDTA tube) taken from the jugular vein, a solution (0.5 ml) containing 20 mg unlabelled and 250 µCi [^{3}H] testosterone (70 – 105 Ci/mmol, TRK921, Amersham Bioscience, UK) in ethanol was added to 2.5 ml of a sterile 0.9% NaCl solution, vigorously vortexed, and injected i.m. Faecal samples were collected from 4 days prior to steroid administration until 196 h post-injection.

The radiometabolism study in the male hyena was conducted at Leipzig Zoo (Germany) in 2008. 2.5 ml of a sterile 0.9% NaCl solution was added to a solution (0.25 ml) containing ~250 µCi 1,2,6,7-[^{3}H] testosterone (70 – 105 Ci/mmol, TRK402, Amersham Bioscience, UK) in ethanol and injected into the cephalic vein following anaesthesia using a combination of medetomidine (0.03 mg/kg) + midazolam (0.15 mg/kg) + butorphanol (0.2 mg/kg). Faecal samples were collected from 2 days prior to steroid administration until 2 days post-injection.

Aliquots of each sample were extracted for testosterone metabolite determination and radioactivity counting. All radioactive counting was conducted in a Packard TRI-CARB 1900 TR liquid scintillation counter (Canberra-Packard GmbH, Germany). Samples used for HPLC analyses were in the female, the first sample collected 15.5 h following injection, and in the male the sample collected 47.25 h following injection, as they contained the highest amount of radioactivity (see section HPLC analysis), respectively.

Prior to HPLC, both faecal extracts were subjected to enzyme hydrolysis (see below) in order to test whether faecal testosterone metabolites were conjugated to glucuronides.

6.2.4. Hydrolysis

For hydrolysis, 100 µl of faecal extracts were hydrolysed with 900 µl 0.05 M acetate buffer (pH 4.8) containing 4 µl β-glucuronidase and arylsulfatase from *H. pomatia* (*Hp*) (Roche Diagnostics GmbH), hereafter termed *Hp*-enzymes, for 2 h at 37 °C. Steroids were then extracted 3 times with 2.5 ml tert methylbutylether/petroleum ether (30:70, v/v) for 30 min using a universal shaker. Phase separation was achieved by freezing for 15 min at −80 °C. The extracts were combined, evaporated in a sample concentrator (Dri Block DB3, Techne, Staffordshire, UK) under nitrogen at 55 °C, dissolved in 40% methanol and stored at −20 °C until HPLC analyses.

Hydrolysis with β-glucuronidase from *E. coli* (*Ec*), hereafter termed *Ec*-enzyme, was modified from [23]. Lyophilized β-glucuronidase Type VII-A from *E. coli* (5000U, Sigma-Aldrich, Inc. USA) was dissolved in 1.4 ml water. 200 µl of faecal extracts were diluted in 800 µl 0.25 M phosphate buffer (pH 6.9) and then hydrolysed adding 40 µl enzyme for 22 h at 37 °C. Steroids were extracted twice with 3 ml tert methylbutylether/petroleum ether (30:70, v/v), for 30 min using a universal shaker. Phase separation was achieved by freezing for 15 min at −80 °C. The extracts were combined, and processed as described above.

To compare the enzymatic activity of β-glucuronidase from *Hp* and *Ec*, faecal extracts from the testosterone challenge were hydrolysed with both enzymes. Because both enzymes produced highly congruent results, hydrolysis of all further samples was performed using β-glucuronidase from *Hp*.

6.2.5. HPLC Analysis of Faecal Testosterone Metabolites

Aliquots of 150 µl faecal extracts of non-hydrolysed and hydrolysed samples from one female and one male hyena containing the highest amount of radioactivity, respectively and two hydrolysed samples containing high endogenous concentrations of epiandrosterone from one free-ranging male and one free-ranging female hyena, respectively, were

selected for HPLC analyses. Sample preparation on C18 columns and chromatographic separation using an Ultra Sep ES-RP 18/6 µm HPLC column (250x4 mm, Sepserv, Berlin, Germany) were carried out as previously described [49]. The elution positions of authentic cortisol, corticosterone, testosterone, epiandrosterone and dihydrotestosterone (DHT) on this column were determined in separate HPLC runs.

6.2.6. Testosterone Challenge

The testosterone challenge experiment was conducted in combination with a radiometabolism study (see section Radiometabolism study) in the female hyena in 2012. The hyena received 20 mg unlabelled testosterone. In addition to faecal samples, one blood sample prior to and after the injection was taken to prove the increase in plasma testosterone. Following extraction, faecal samples were subjected to enzymatic hydrolysis and stored at −20 °C until analyses.

6.2.7. ACTH Challenge

The ACTH challenge was conducted in Dvur Králové Zoo (Czech Republic) in 1998 on a male captive adult spotted hyena. Detailed descriptions of treatment and sample collection procedures were previously published [45, 50].

6.2.8. Enzyme Immunoassays

Methanol-extracted faecal samples were analysed with an "in-house" epiandrosterone (epi-A) EIA (endocrinology laboratory of the IZW). The antibody was polyclonal and was raised in rabbit against the 3-hemisuccinat (HS)-steroid coupled with bovine serum albumin (BSA). The corresponding epiandrosterone-3-HS-peroxidase was used as label. The antibody and label were used in a 1:20.000 and 1:2.000 dilution, respectively. The cross-reactivities of the antibody were determined as follows: 100% epiandrosterone (5α-androstan-3β-ol-17-one), 88% epiandrosterone glucuronide (5α-androstan-3β-ol-17-one glucosiduronate), 60% androsterone (5α-androstan-3α-ol-17-one), 60% androstenedione (4-androsten-3, 17-dione), 24% androsterone sulphate (5α-androstan-3α-ol-17-one sulphate), 19.2% dehydroandrosterone (5-androsten-3α-ol-17-one), 17% dehydroepiandrosterone (5-androsten-3β-ol-17-one), and 0% for cortisol and corticosterone. The assay was validated by demonstrating parallelism of faecal extracts to the epiandrosterone standard curve. The intra-assay and inter-assay coefficients of variation were determined by using faecal extracts containing known concentrations of epi-A. The inter-assay coefficients were 8.6% (n = 10) for extracts containing low and 9.3% (n = 10) for extracts

containing high concentrations of epi-A. The intra-assay coefficients were 12.7% (n = 8) for extracts containing low and 6.7% (n = 8) for faecal extracts containing high concentrations of epi-A.

Testosterone measurements in blood plasma (see section Testosterone challenge) were carried out using an antibody directed against testosterone-11-HS-BSA as previously described for blood plasma [51]. For comparative measurements, faecal samples from the ACTH challenge were also analysed using an antibody directed against cortisol-3-CMO-BSA, previously validated to assess changes in adrenocortical activity in spotted hyenas based on fGM analyses [45], and an antibody directed against testosterone-11-HS-BSA, previously validated for fTM analyses in lynx [21]. Enzyme immunoassays were performed as previously described [49].

6.2.9. Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analyses were carried out as previously described [49] following derivatisation of samples and standards with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA, CS-Chromatographie Service, Langerwehe, Germany), trimethylsilyl iodide (TMSI), and dithioerythritol (DTE, both from Sigma Chemie GmbH, Deisenhofen, Germany) according to Magnisali et al. (2008) allowing quantification of the resulting trimethylsilyl derivatives by GC-MS. Sample analysis was performed on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Böblingen, Germany) equipped with an Agilent HP-5MS (5% phenyl-, 95% methylsiloxane) fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 μm) interfaced with an Agilent 5975C mass selective detector.

The MS was operated in the EI mode with the electron voltage set to autotune value. MS acquisition was performed in selected ion monitoring mode (SIM) by monitoring the ions m/z 434 and 419 for epiandrosterone. The Agilent MSD chemstation data chemstation software was used for peak integration and library searches.

6.2.10. Data Analysis

Calculation of baseline values was performed using an iterative process excluding all values greater than the mean +2SD [52]. Significant increases were defined as peaks with values exceeding the baseline +2SD. Statistical analyses were performed with the NSM3 package version 1.1 in R version 3.0.2 [53] and StatXact version 10 (Cytel Software Inc., Cambridge, Massachusetts, USA). The level of significance was fixed at 5% and all tests were two-tailed. For biological validation of the epiandrosterone EIA we compared fTM levels between juvenile male and adult immigrant male spotted hyenas. Since the concentrations in both groups differed in dispersion, as documented by Conover's squared ranks test

[54] (T = 2559, exact p = 0.0019), the usual procedure of applying a Mann-Whitney U-test would have been inappropriate. Instead, we resorted to two robust tests which do not make assumptions about equality of dispersion, the Fligner-Policelli test [55] and the permutation test [56] in the version of the maximin efficiency robust test approach of Gastwirth [57], as implemented in StatXact.

6.3. Results

6.3.1. Radiometabolism Study and HPLC Analyses

HPLC elution positions of the reference steroid standards cortisol, corticosterone, testosterone, dihydrotestosterone and epiandrosterone obtained when applying the corresponding steroid hormone specific assays are shown in Fig. 6.1. HPLC profiles of radiolabelled testosterone metabolites in non-hydrolysed and hydrolysed spotted hyena faeces are shown in Fig. 6.2A & B, respectively. Using reversed-phase HPLC, metabolites were separated according to their polarity and the more polar metabolites were eluted first. In non-hydrolysed extracts of one captive adult male (Fig. 6.2A) and captive adult female (Fig. 6.2B) hyena, only one polar peak was detected in fractions 1 – 5, probably indicating a cluster of conjugated radiolabelled metabolites. Minor variations in the composition of radiolabelled metabolites in both sexes might result of individual or sex-specific differences in testosterone metabolism. Enzymatic hydrolysis of faecal extracts changed the elution patterns of polar radiolabelled metabolites in extracts from both sexes. In the male (Fig. 6.2A) as well as in the female (Fig. 6.2B) polar radiolabelled conjugates disappeared and were substituted by a major radiolabelled peak in fractions 39 – 41, co-eluting with the epi-A standard. In the male an additional minor proportion of radiolabelled metabolites occurred in fractions 44 – 46. In both sexes no radiolabelled metabolites were detectable at the elution position corresponding to the testosterone standard, thus the circulating hormone itself is not present in faeces. Additionally, no radiolabelled metabolites were detected at the elution positions of authentic cortisol, corticosterone and DHT.

Before hydrolysis, HPLC profiles revealed polar radiolabelled testosterone metabolites (Fig. 6.2) that were not apparent in the HPLC immunograms of the captive male (Fig. 6.3A), the free-ranging adult male (Fig. 6.4A) and the free-ranging adult female (Fig. 6.4B), probably due to the inability of the polar radiolabelled metabolites to cross-react with the antibody. In contrast, before hydrolysis the polar radiolabelled metabolites from the captive female (Fig. 6.2B) fitted with corresponding polar immunoreactivities (Fig. 6.3B). Nevertheless, hydrolysis with the *Hp*-enzymes standardizes the patterns of immunoreactivities in both the captive male and female (Fig. 6.3), liberating a major metabolite from its

conjugate corresponding to the elution position of epi-A in fraction 40 and a minor one at fraction 25 (Fig. 6.3). Similarly, in the hydrolysed extracts from an adult free-ranging male (Fig. 6.4A) and an adult free-ranging female (Fig. 6.4B), the epi-A EIA demonstrated a major peak of immunoreactive metabolites in fractions 40, co-eluting with the epi-A standard, and two minor peaks in fractions 25 and 43, respectively. As a consequence of these results, all further analyses were carried out in hydrolysed extracts. To exclude crossreactivities of the epiandrosterone antibody with other possible faecal steroid metabolites, we analysed the HPLC elution fractions from the captive female with a cortisol-21, corticosterone-21, testosterone and dihydrotestosterone (DHT) EIA, respectively. In comparison to the epi-A EIA, none of the EIAs detected significant amounts of immunoreactivities (supplement, Fig. 12.1). This suggests that additional steroid metabolite levels did not falsify the measurement of immunoreactive epiandrosterone metabolites.

6.3.2. GC-MS Analyses

Prior to analyses of faecal extracts, the retention times of the silylated standard epiandrosterone were established. Following derivatisation of faecal extracts we expected to detect epiandrosterone following hydrolysis with *Hp*-enzymes in samples from the testosterone challenge (see below) by characteristic mass-to-charge ratios (m/z 434 and m/z 419). Applying the SIM modus, we confirmed the presence of epiandrosterone based on its retention time and its m/z ratios of 434 and 419 (434 minus two silylations, 2 x 72, reveals a molecular weight of 290, that of epi-A, data not shown).

6.3.3. Testosterone Challenge

Administration of testosterone results in a rapid artificial induced increase in plasma testosterone levels and should be clearly reflected in the concentrations of fTM after a species-specific time lag. In the female spotted hyena an increase in concentrations of plasma testosterone from 11 ng/g to 238 ng/g was detected 15 min after injection. To validate the ability of the epi-A EIA to detect the expected increase in fTM caused by the testosterone challenge, we analysed the faecal extracts following hydrolysis with *Hp*-enzymes. A significant increase in fTM levels (Fig. 6.5) was detected 15.5 h post-injection, with a maximum concentration 4-fold above baseline (0.72 ± 0.24 µg/g wet faeces). FTM returned to baseline levels within 24 h following injection. This course is congruent with the course of total amounts of radiolabel excreted with faeces. However, a peak of almost a similar intensity was detected 3 days before testosterone treatment. *Ec*-hydrolysed extracts revealed a similar profile of fTM concentrations in response to the testosterone challenge (Fig. 6.6). A linear regression of *Ec*-hydrolysis fTM levels on *Hp*-hydrolysis fTM levels

produced a high degree of congruence ($r^2 = 0.83$; $F = 81.36$; $df = 1, 16$; $p < 0.0001$, with the regression equation being fTM concentration (*Hp*) = 0.06 + 1.32 x fTM concentration (*Ec*), and the intercept not being significantly different from 0, $t = 0.792$, $p = 0.44$). Therefore, all further analyses were carried out in *Hp*-hydrolysed faecal extracts.

6.3.4. ACTH Challenge Test

To exclude the possibility that the epi-A EIA tracks androgenic steroids from cortisol metabolism, faecal samples from an ACTH challenge experiment were assayed for fGM in hyenas with antibodies directed against cortisol-3-CMO and epi-A EIA (Fig. 6.7), respectively. The cortisol EIA detected a large percentage increase in fGM in response to ACTH, with a maximum concentration 16-fold above pre-treatment levels. No similarly large percentage corresponding increase was obtained when applying the epi-A EIA. This result suggests that androgenic glucocorticoid metabolites do not contribute to the measurement of immunoreactive testosterone metabolites present in faeces of spotted hyenas. In contrast, the use of an EIA with an antibody directed against testosterone-11-HS-BSA revealed that this assay was responsible for substantial cross-reactivities with fGM in faeces from spotted hyenas.

6.3.5. Comparing Levels of fTM in Male Cubs and Adult Males

To confirm the physiological importance of faecal testosterone metabolite analyses, we compared fTM levels between adult immigrant male and juvenile male spotted hyenas (Fig. 6.8). Adult immigrant male spotted hyenas possessed significantly higher fTM levels than male juveniles (Fligner-Policelli test, $U = 2.3208$, exact p using 10,000 Monte Carlo simulations = 0.0294; permutation test, test statistic = 8.349, $p = 0.0087$). In juvenile males, fTM levels ranged from 272.7 to 1295.05 ng/g wet faeces and in adult immigrant males from 179.3 to 4259.75 ng/g wet faeces, indicating high inter-individual variation.

6.4. Discussion

In this study we demonstrated that an EIA based on an antibody against epi-A is a useful tool for monitoring gonadal activity in spotted hyenas. We detected a significant increase in fTM concentrations following a testosterone challenge experiment. The results of our radiometabolism studies confirmed that the epi-A EIA detected immunoreactive, radiolabelled fTM in both an adult male and female. EIAs using antibodies directed against epi-A have also been validated for monitoring the gonadal status of other mammals, including the African and Asian elephant, *Loxodonta africana* and *Elephas maximus*

[58, 59] and the sun bear, *Helarctos malayanus* [60]. Epi-A is also a major testosterone metabolite in faeces of macaques [19, 61]. This suggests our assay may be useful for monitoring fTM across a broader range of mammalian species.

Usually the pattern of radiolabelled metabolites in an HPLC analysis does not coincide with the pattern of immunoreactive hormone metabolites. Part of the reason may be that the antibody used cross-reacts with metabolites other than the targeted radiolabelled metabolites. When interpreting an HPLC immunogram it is extremely important to consider that an immunoreactive peak does not reflect the quantity of faecal hormone metabolites but rather represents the percentage cross-reactivity of the antibody together with the amount of metabolite in a particular HPLC fraction. By contrast, in an HPLC analysis of radiolabelled metabolites, the radioactivity measured in a given elution fraction directly reflects the quantitative amount of the metabolites, even though it cannot be related to any chemical structure [62, 63].

In one captive female, radiolabelled metabolites mainly consisted of polar conjugated metabolites (Fig. 6.2B). Hydrolysis released epi-A from these polar conjugated metabolites regardless of the origin of the glucuronidase enzyme applied (e.g. either from *Hp* or *Ec*). Interestingly in one captive and one free-ranging male and one free-ranging female our epi-A did not detect polar labelled conjugates suggesting possible individual differences in the testosterone metabolism (Figs. 6.3A and 6.4). Individual differences in steroid metabolisms and those associated with factors such as sex, reproductive status [15] and diet [64] have been documented. Nevertheless, following hydrolysis, profiles of radiolabelled and immunoreactive metabolites were almost identical across the individuals examined (Figs. 6.3 and 6.4), thereby confirming that testosterone metabolites in spotted hyena faeces are mostly processed via the epi-A pathway and therefore are specifically detected by our epi-A EIA. The antibody might also bind to metabolites not necessarily derived from testosterone degradation, explaining immunoreactivities that are not correlated to radiolabelled metabolites. This might explain the appearance of a minor immunoreactive peak in fraction 25. The presence of epi-A was also clearly confirmed by GC-MS based on its molecular ion of 434 and its retention time on the GC-MS column. In general, our profiles of immunoreactivities differed distinctly from those previously described for fAM (sum of immunoreactive faecal testosterone, DHT and androstenedione metabolites) in spotted hyenas where at least six immunoreactivities covering a wide range of polarities were detected [22]. However, a direct comparison is impossible as solvents of different elution strengths as well as different antibodies were applied.

The results of our radiometabolism studies and comparative HPLC analyses confirmed the presence of epi-A glucuronide as a major conjugate in spotted hyena faeces, with an 88% cross-reactivity with our antibody (see section enzyme immunoassay). The applica-

tion of hydrolysis increased the accuracy in which our epi-A EIA measured fTM in samples from captive animals (Fig. 6.3), but had little effect on fTM measures in samples from free-ranging animals (Fig. 6.4). Such increased sensitivity is desirable even when only subtle differences in immunoreactivities occur following hydrolysis, because in analyses involving larger samples this will improve the power of statistical comparisons between age or sex categories.

Hp-enzymes are commonly used for the total deconjugation of steroids and include both β-glucuronidase and arylsulfatase activities which can cleave both steroid glucuronides and sulphates. However, several studies have demonstrated that arylsulfatase in *Hp*-enzymes cause steroid conversion that increases the measured hormone metabolite concentrations [23, 24]. This unwanted artefact is unlikely to occur with *Ec*-enzymes which do not contain arylsulfatase [24, 25]. Our measurements of immunoreactive fTM following both methods revealed no evidence of this artefact as our *Ec*-hydrolysis produced a similar fTM profile to that following *Hp*-hydrolysis (Fig. 6.6). Furthermore, our HPLC immunograms showed that native testosterone was not present in faeces of spotted hyenas, excluding the possibility that testosterone may have been generated from other metabolites that might have served as precursors for other *Hp*-enzymes [65]. Given that both hydrolysis methods liberated epi-A from its conjugates equally well, our results indicate that in the spotted hyena, the less time consuming *Hp*-hydrolysis can be applied, thereby overcoming the need to implement the complex protocol for *Ec*-hydrolysis [23]. In contrast to *Ec*-hydrolysis, *Hp*-treatment permits a 1:10 dilution with hydrolysis buffer of the faecal epi-A concentrations to reduce the methanol proportion prior to enzyme treatment. Drying down aliquots of faecal extracts and dissolving them in hydrolysis buffer as described earlier in the lynx [21] is not necessary for spotted hyenas.

Structural similarities between androgen and glucocorticoid metabolites [27], can result in cross-reactivities of antibodies directed against androgen metabolites with glucocorticoid metabolites. We examined whether the results of our epi-A EIA included such cross-reactivities using faecal extracts from an ACTH challenge experiment. Glucocorticoid metabolites had no influence on the measurement of fTM (Fig. 6.7), and our HPLC immunograms only reveal significant amount of immunoreactivities which were correlated with radiolabelled fTM. Thus, our results indicate that the epi-A EIA only detected metabolites which derived from testosterone degradation. In contrast, the use of an EIA with an antibody directed against testosterone-11-HS-BSA with spotted hyena faeces, previously validated in two lynx species to detect changes in testicular activity [21], revealed an increase in putative "fTM" almost identical to that measured by the antibody (Fig. 6.7) previously validated to measure fGM in the spotted hyena [45]. Thus, this testosterone antibody is inappropriate to analyse fTM in spotted hyenas because of its substantial

cross-reactivities with unknown fGM. This underlines the necessity and importance of validating EIAs for each species and hormone. One minor problem with our EIA were minor immunoreactive peaks between fraction 20 and 28 in captive (Fig. 6.3) and free-ranging (Fig. 6.4) animals which might be caused by non-testosterone metabolites. Even so, this unwanted cross-reactivity is minor compared to the substantial cross reactivities associated with other EIAs (Fig. 6.7).

The first step in our physiological validation of our epi-A EIA revealed a significant increase in fTM levels 15.5 h following injection of unlabelled testosterone in one female spotted hyena (Fig. 6.5). This coincided with the time course of excreted radiolabelled fTM as the first sample collected 15.5 h following ^{3}H-testosterone injection contained the highest amount of radioactivity (Fig. 6.2). In spotted hyenas, little is known about the food transit time: it is thought to take approximately 24 h but may increase as the food volume ingested increases [50]. Dloniak et al., 2004 observed a delay of 24 – 72 h in detecting fAM in response to the injection of luteinising hormone-releasing-hormone (LHRH) [22]. In comparison, an increase in fGM concentrations was detected 16 – 96 h following an ACTH challenge experiment [45]. Taken together the studies imply that the time lag of faecal testosterone metabolite excretion detected by the epiandrosterone EIA corresponds to the gut passage time.

The peak in fTM levels detected 72 h pre-treatment cannot be attributed to any known event in the area around the animal's enclosure (Fig. 6.5) but may be associated with the movement of the animal from its normal enclosure to an enclosure at the NZG hospital during the experiment. Alternatively, androgens are known precursors of oestrogens [66] and hence in female mammals, a rise in fTM levels could be of ovarian origin. For example, elevated fTM concentrations are a reliable indicator of the follicular phase in female sun bears [60]. In female domestic dogs, fTM peaks and serum testosterone peaks are positively correlated with the day of the pre-ovulatory surge of LH [67, 68], and in female domestic pigs (*Sus scrofa*) plasma testosterone concentrations showed a significant increase 2 days before the LH peak [69]. Thus we cannot exclude the possibility that the pre-treatment fTM peak in the female spotted hyena (Fig. 6.5) was of ovarian origin and related to oestrus.

The second step in the physiological validation of our EIA was to test our expectation that wild adult immigrant males in spotted hyena clans would show higher fTM concentrations than juvenile natal males. Our results confirmed this prediction as fTM concentrations in adult immigrant males were significantly higher than those in juvenile males (Fig. 6.8). We currently cannot exclude the possibility that juvenile males and immigrant, adult males metabolise testosterone differently, but we are unaware of any study indicating that this is likely to be the case. Hence, we assume that the detected differences between

cubs and adults indicate an increase in testosterone concentrations in reproductively active immigrant males. Furthermore, as expected we found considerable variation in fTM concentrations in both juvenile natal males and adult immigrant males. We suspect this variation reflects the differences between individuals in the frequency with which they "win" social interactions with other clan members as suggested by the "challenge hypothesis" [9]. Further studies will have to determine to what extent individual differences in fTM are related to the context and outcomes of behavioural interactions, reproductive status, social status, age or season, or possibly other behavioural or environmental factors. Our epi-A EIA provides the required tool to rigorously test these ideas.

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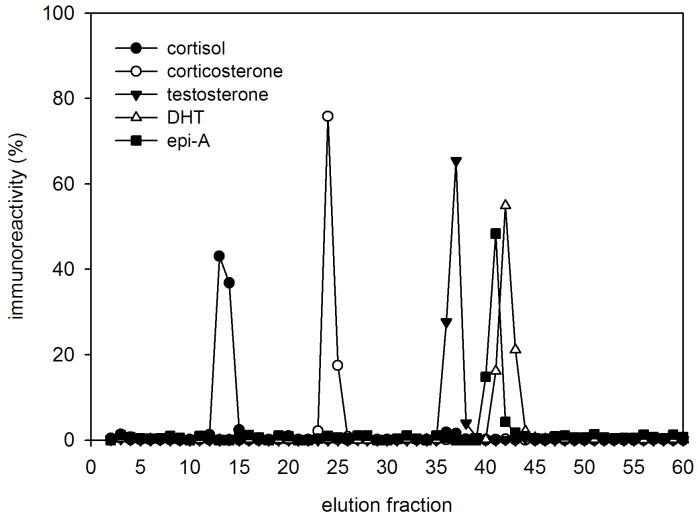

Figure 6.1. HPLC profiles of steroid standards. Elution positions of authentic cortisol, corticosterone, testosterone, epiandrosterone and dihydrotestosterone (fractions 12, 23, 36, 40, and 41 respectively) obtained by applying the corresponding steroid hormone specific assays. For comparison results are presented as percentage of overall eluted steroid concentration.

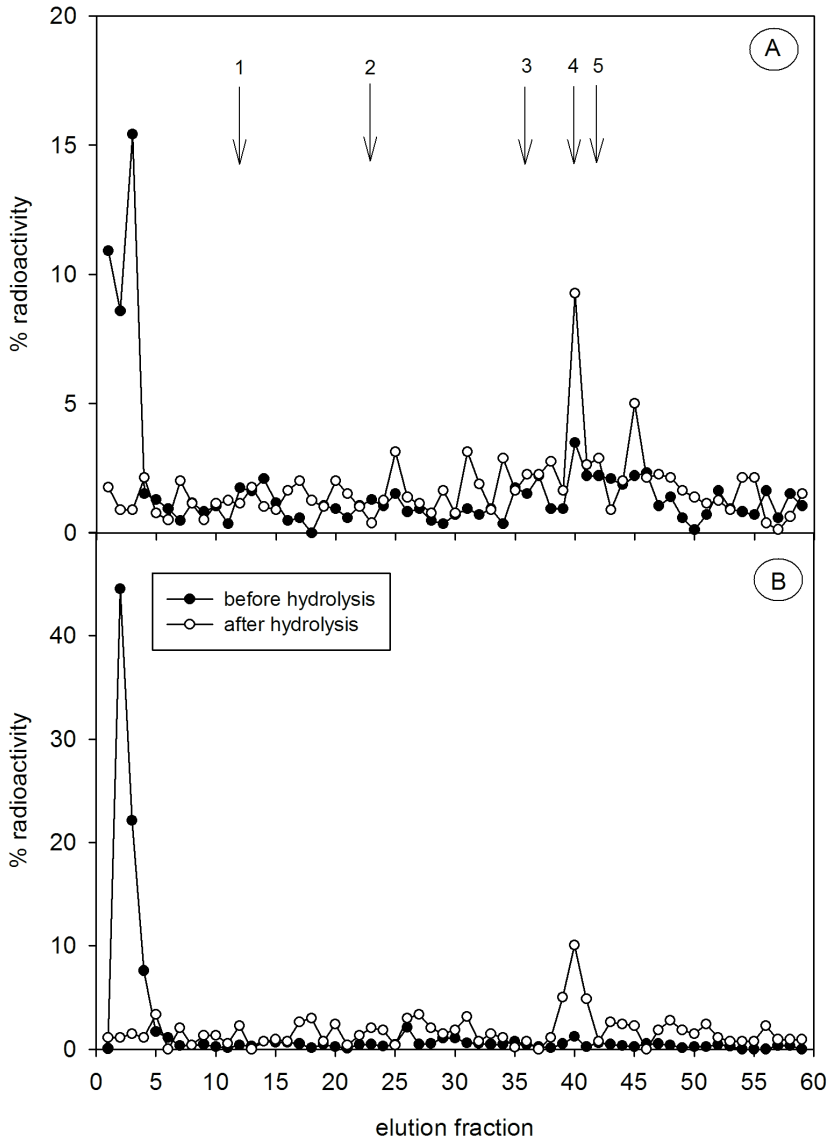

Figure 6.2. HPLC profiles of ^{3}H-testosterone metabolites. ^{3}H-testosterone metabolites were analysed in non-hydrolysed (black circles) and hydrolysed (white circles) faecal extracts of one captive male (A) and one captive female (B) spotted hyena. Extracts were separated by RP-HPLC and then radioactivity of each fraction was analysed. Radioactivity is presented as a percentage of the overall eluted activity. The arrows represent the elution positions of reference standards cortisol (1), corticosterone (2), testosterone (3), epiandrosterone (4) and dihydrotestosterone (5), as detailed in Fig. 6.1.

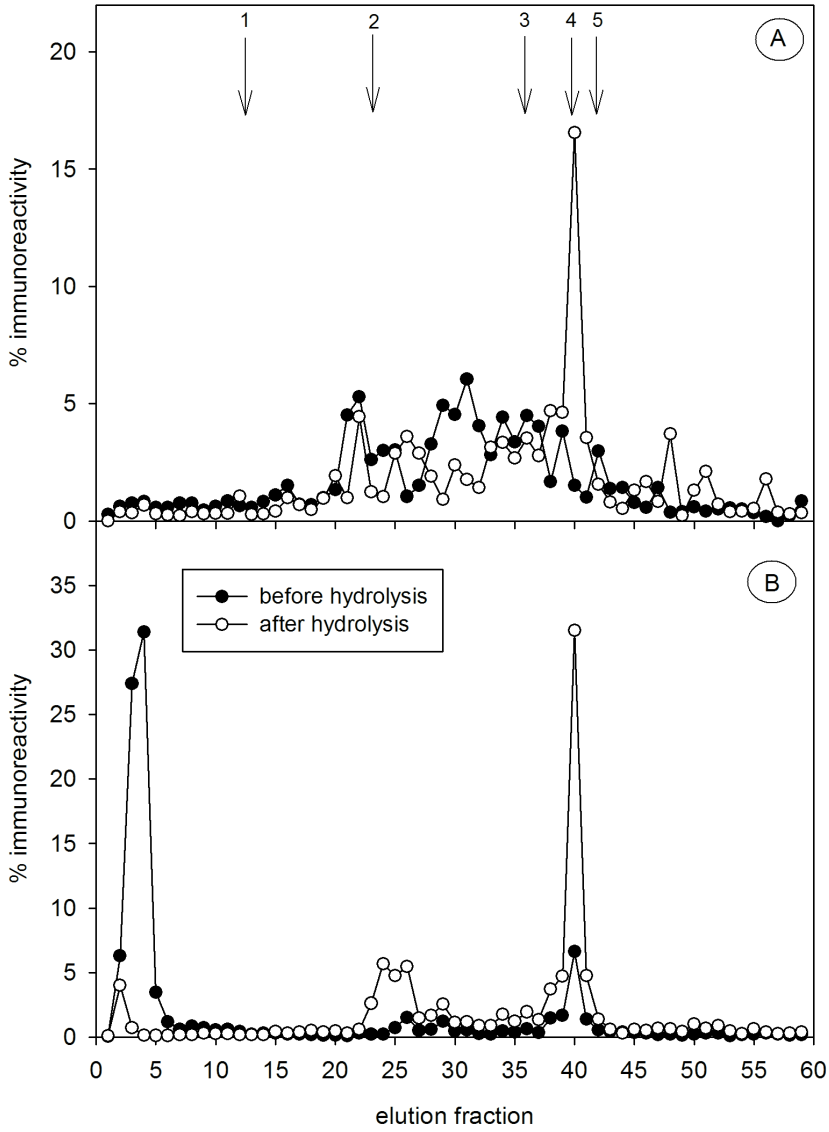

Figure 6.3. HPLC profiles of immunoreactive testosterone metabolites in captive hyenas. Testosterone immunoreactivity of faecal extracts were analysed in faecal extracts of one captive adult male (A) and one captive adult female (B) spotted hyena. Immunoreactivity was determined in the epiandrosterone EIA and is presented as a percentage of overall eluted activity. Lines with black circles represent immunoreactivity in each fraction. Lines with white circles show immunoreactivity in the fractions of the same extract after hydrolysis. The arrows represent the elution positions of reference standards cortisol (1), corticosterone (2), testosterone (3), epiandrosterone (4) and dihydrotestosterone (5).

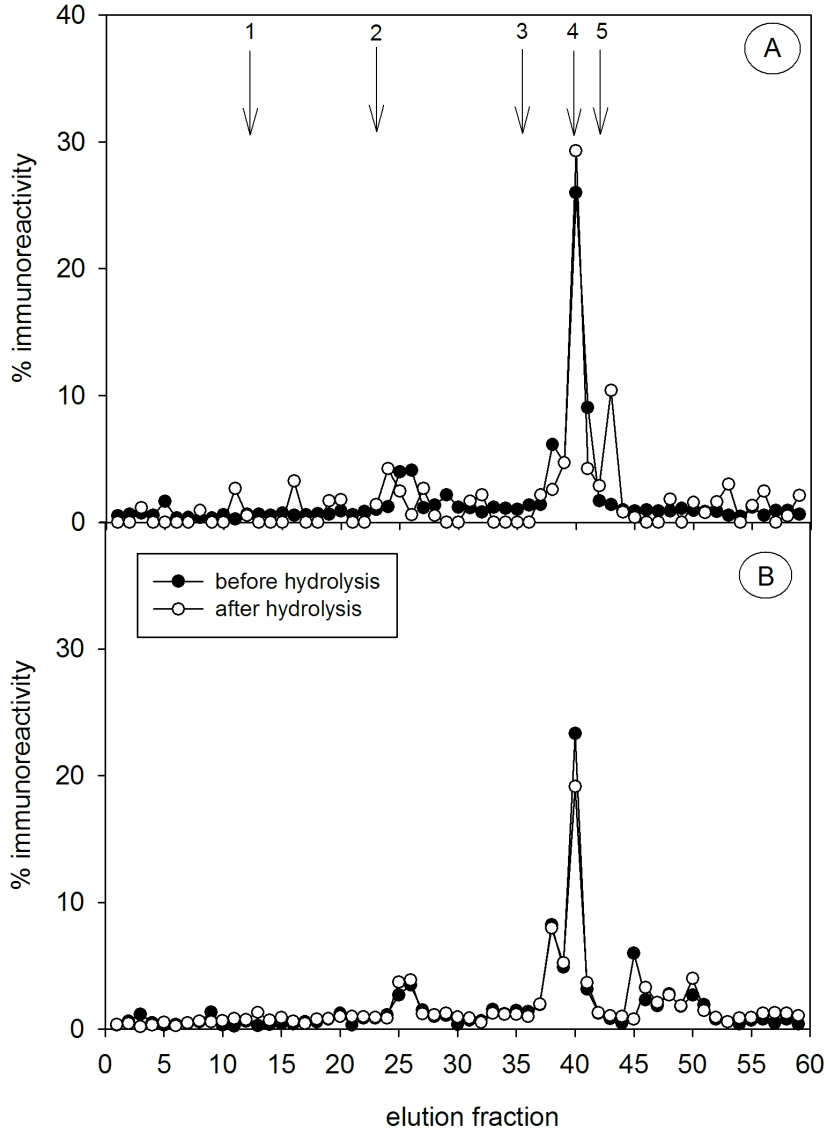

Figure 6.4. HPLC profiles of immunoreactive testosterone metabolites in free-ranging hyenas. Testosterone immunoreactivity was analysed in faecal extracts of one free-ranging adult male (A) and one free-ranging adult female (B) hyena. Immunoreactivity was determined in the epiandrosterone EIA and is presented in percentage of overall eluted activity. Lines with black circles represent immunoreactivity in each fraction. Lines with white circles show immunoreactivity in the fractions of the same extract after hydrolysis. The arrows represent the elution positions of reference standards cortisol (1), corticosterone (2), testosterone (3), epiandrosterone (4) and dihydrotestosterone (5).

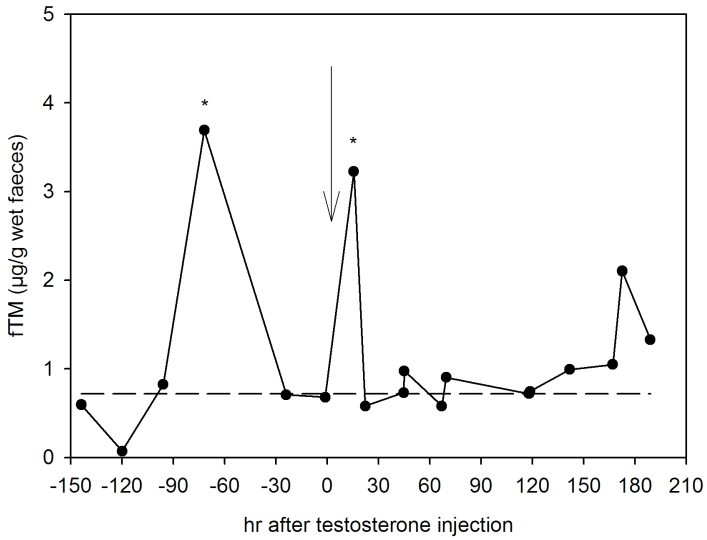

Figure 6.5. Changes in fTM concentrations in response to a testosterone challenge in a female spotted hyena. Faecal samples were collected from 6 days prior to injection until 8 days post-injection and were analysed with an epiandrosterone EIA following hydrolysis with β-glucuronidase from *Helix pomatia*. The arrow represents the time of testosterone injection; the dashed line indicates the baseline level. The * indicates peaks (values exceeding mean + 2SD).

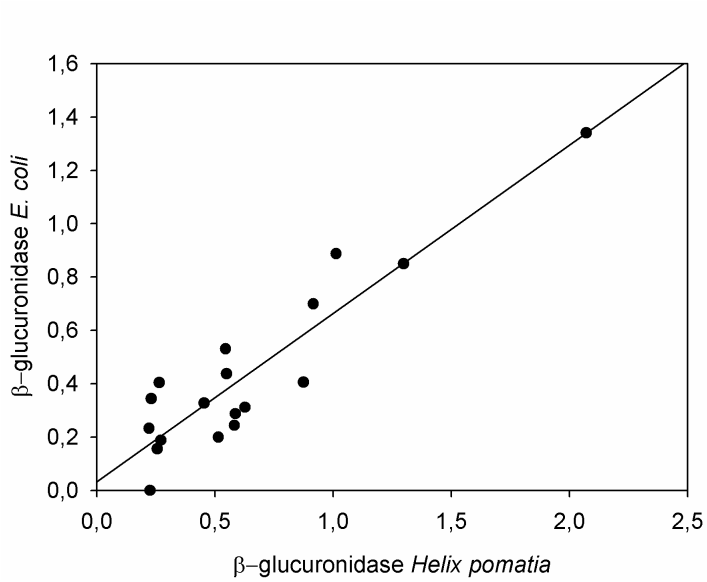

Figure 6.6. Comparison of testosterone immunoreactivity following hydrolysis with β-glucuronidase from *Helix pomatia* and from *Escherichia coli*. FTM concentrations were determined in faecal samples from the testosterone challenge in the epiandrosterone EIA following hydrolysis with β-glucuronidase from *Helix pomatia* and β-glucuronidase from *Escherichia coli*, respectively. The linear regression indicates that both hydrolysis methods are congruent, as the regression explains a large segment of the variance ($r^2 = 0.84$) and the intercept does not significantly differ from zero (see text for details).

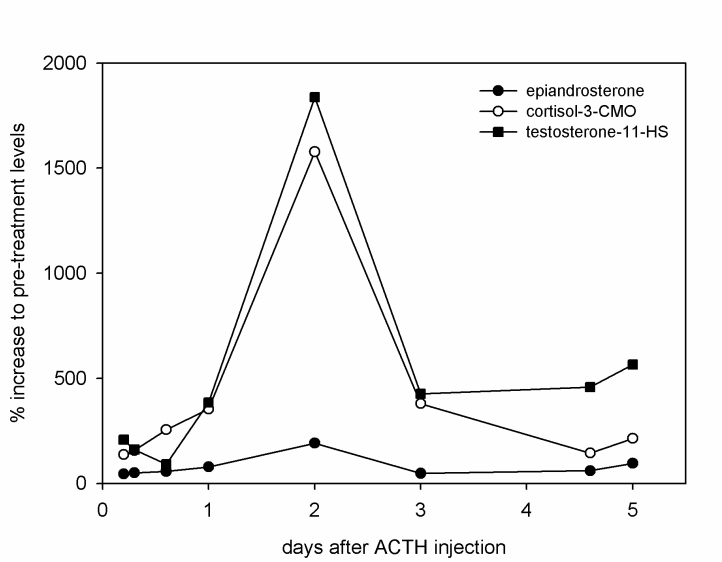

Figure 6.7. Comparison of faecal cortisol and testosterone immunoreactivity. Changes in fGM and fTM concentrations were determined in faecal samples from the ACTH challenge following hydrolysis with β-glucuronidase from *Helix pomatia* in the cortisol-3CMO, epiandrosterone and testosterone-11-HS EIAs, respectively. Levels of fGM and fTM are shown as percentage increase over pre-injection levels.

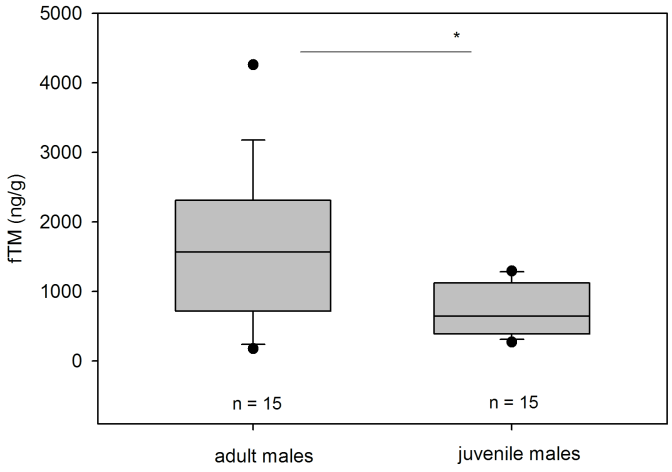

Figure 6.8. Hormonal data of juvenile and adult immigrant male hyenas. FTM concentrations (ng/g faeces) in juvenile male (n = 15) and adult male (n = 15) spotted hyenas determined in the epiandrosterone EIA following hydrolysis with β-glucuronidase from *Helix pomatia*. The * indicates a significant difference between both categories.

7. Article 2

Validation of an enzyme-immunoassay for the non-invasive monitoring of faecal testosterone metabolites in male cheetahs (*Acinonyx jubatus*)

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Contribution of each author to the manuscript: The experiments of the study were conceived, designed and performed by SP, BW, LC, AW and MD. The endocrine laboratory analyses were performed by SP. The data were analysed by SP, BW and MD. SP drafted the manuscript; all co-authors revised the manuscript.

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Abstract

In mammals, the sex hormone testosterone is the major endocrine variable to objectify testicular activity and thus reproductive function in males. Testosterone is involved in the development and function of male reproductive physiology and sex-related behaviour. The development of a reliable androgen enzyme-immunoassay (EIA) to monitor faecal testosterone metabolites (fTM) is a powerful tool to non-invasively assess the gonadal status of males. We validated an epiandrosterone EIA for male cheetahs by performing a testosterone radiometabolism study followed by high-performance liquid chromatography (HPLC) analyses and excluding possible cross-reactivities with androgenic metabolites not derived from testosterone metabolism. The physiological and biological relevance of the epiandrosterone EIA was validated by demonstrating (1) a significant increase in fTM concentrations within one day in response to a testosterone

injection, (2) a significant increase in fTM concentrations within one day in response to a gonadotropin-releasing hormone (GnRH) injection, which failed following a placebo injection, and (3) significant differences in fTM concentrations between adult male and adult female cheetahs and between adult and juvenile male cheetahs of a free-ranging population. Finally, we demonstrated stability of fTM concentrations measured in faecal samples exposed to ambient temperatures up to 72 h. Our results clearly demonstrate that the epiandrosterone EIA is a reliable non-invasive method to monitor testicular activity in male cheetahs.

7.1. Introduction

Cheetahs (*Acinonyx jubatus*) have undergone a substantial decline in population size due to habitat loss, degradation, fragmentation, and conflicts with humans attempting to decrease the threat to their livestock and game species (Marker et al., 2007; Purchase et al., 2007). Today, Namibia is considered to host the largest free-ranging cheetah population, with approximately 3100 to 5800 individuals (Hanssen and Stander, 2004). However, this population lives primarily outside protected areas on land used for livestock and game species (Marker et al., 2007). As the cheetah population is declining, captive breeding becomes important for species management and conservation. However, contrary to free-ranging cheetahs, which exhibit high reproductive performance, low cub mortality and low disease susceptibility (Thalwitzer et al., 2010; Wachter et al., 2011), captive individuals have low reproductive performance, high cub mortality and high disease predisposition (Lindburg et al., 1993; Marker-Kraus and Grisham, 1993). Marked differences in breeding success among institutions suggested that husbandry conditions and animal management may be crucial, because captive but not free-ranging cheetahs suffered from chronic stress (Terio et al., 2004). Chronic stress, characterised by long-term hyperadrenal activity, may have adverse effects on reproductive performance and testicular activity (Caro and Laurenson, 1994; Wildt et al., 1993; Wildt et al., 1988). For male cheetahs in captivity, permanent visual or olfactory contact with females may be detrimental for promoting natural interest in females and courtship behaviour (Bertschinger et al., 2008; Brown et al., 1996b).

Comparative analyses of adrenocortical and testicular steroid hormone excretion would improve our understanding of reproductive biology in male cheetahs and thus provide essential information for husbandry management to facilitate successful breeding in captivity. However, taking blood samples to objectify gonadal activity is often limited by restricted accessibility to study animals, particularly in wildlife species (Goymann, 2005;

Palme et al., 2005). In contrast, monitoring faecal testosterone metabolites (fTM) using enzyme immunoassays (EIAs) provides a valuable method to evaluate the hormonal status of an animal (Jewgenow et al., 2006; Pribbenow et al., 2015). Compared to snap-shots in blood plasma based on single samples, hormone metabolite concentrations in faeces represent averaged values of excreted hormones over a species-specific time period, and thus are less affected by the pulsatory hormone secretion pattern than blood hormone levels (Goymann, 2005; Palme et al., 2005). Furthermore, due to the non-invasive character of this method fTM concentrations are less affected by handling procedures such as capture, restraint and anaesthesia, which may alter circulating testosterone concentrations (Möstl and Palme, 2002; Palme et al., 2005; Sapolsky, 1982). The selection of an appropriate assay plays an important role in fTM analysis including physiological and biological validations to confirm that changes in testicular activity are reflected in fTM concentrations measured by the respective assay. The most widely used experiments to stimulate testicular activity and thus to physiologically validate an assay are challenge tests to provoke an increase in peripheral testosterone levels and to detect an elevation in faecal androgen metabolites following the injection of 1) exogenous testosterone (Pribbenow et al., 2015) or 2) a gonadotropin-releasing hormone (GnRH) analogue (Dloniak et al., 2004; Hogan et al., 2010; Wildt et al., 1984). For the development of a reliable androgen EIA it is also necessary to exclude possible cross-reactivities with structurally related androgenic hormone metabolites derived from adrenal glucocorticoid metabolism (Ganswindt et al., 2003; Palme, 2005; Young et al., 2004; Pribbenow et al., 2015).

The overall goal of our study was to develop and validate a reliable non-invasive method using an EIA to monitor testicular activity in male cheetahs. The first aim was to provide basic information on testosterone metabolism and excretion using a ^{3}H-testosterone radiometabolism study and to characterise fTM by HPLC analyses. The second aim was to physiologically validate the suitability of an epiandrosterone (epi-A) EIA to measure fTM by injecting exogenous testosterone and conducting a GnRH challenge. The third aim was to exclude cross-reactivities with androgenic glucocorticoid metabolites; samples from an adrenocorticotropic hormone (ACTH) challenge test (Ludwig et al., 2013) were used to investigate whether faecal glucocorticoid metabolites (fGM) cross-react with the epi-A antibody. The fourth aim was to biologically validate the epi-A EIA by measuring fTM concentrations of free-ranging adult male, adult female and juvenile male cheetahs on farmland in Namibia, with the expectation that the fTM concentration in adult males is higher than in adult females and juvenile males. Finally, we investigated the effect of time on the stability of fTM concentrations when faeces are exposed to natural environmental conditions at ambient temperatures prior to their collection.

7.2. Material and Methods

7.2.1. Study Animals and Sampling Procedures

Cheetahs kept in German zoos. Two adult males (M1 from Zoo Wuppertal, M2 from Zoo Muenster) and one juvenile male (M3 from Zoo Muenster) were involved in the experimental part of this study between January 2010 and July 2012. The injections of radiolabelled ^{3}H-testosterone, placebo (NaCl), GnRH analogue and testosterone were conducted with M1 with time periods of 11, 1 and 17 months between injections, respectively. The adult animals were individually housed in one enclosure each. The juvenile male was kept with two females in one enclosure. Faecal samples were collected twice per day, at approximately 08:45 am and 03:45 pm and stored at −20 °C until transported to the Leibniz Institute for Zoo and Wildlife Research (IZW). All procedures were performed in accordance with the requirements of the Ethics Committee on Animal Welfare of the IZW (permission numbers: 2009-10-02).

Captive cheetahs in Namibia. Faecal samples from two adult males (M4, M5) individually housed in large enclosures (1 ha) on a private farm in Namibia were used to test whether concentrations of fTM changed with exposure time to natural environmental conditions. Fresh faecal samples estimated to be not older than two hours were collected in the morning at approximately 06:00 am.

Free-ranging cheetahs in Namibia. A total of 76 faecal samples from 46 adult males, 18 adult females and 12 juvenile males were collected in the area of east-central Namibia (−21° 45' S to −22° 45' S and 16° 30' E to 18° 30' E). The animals were captured in box traps, immobilised and handled as described in Thalwitzer et al. (2010). Age was assessed following the age class categories described in Caro and Laurenson (1994). Faeces were taken manually from the rectum of the animals using a medical glove and either kept in a cool box with ice blocks until they were frozen in liquid nitrogen at the research station or frozen directly in liquid nitrogen when a dissection was being conducted at the research station (mean ± sd: 5.6 ± 6.6 hours later, range: 0.0 to 26.75 hours).

After transport to the IZW, all faecal samples were thawed, mixed well and subsamples of 1 g were divided into two aliquots of 0.5 g.

7.2.2. Experimental Studies

Radiometabolism study. To characterise excreted fTM, a radiometabolism study was conducted with M1. The cheetah was immobilised with a mixture of 10% ketamine (CP-Pharma, Burgdorf, Germany, 4.5 mg/kg) and medetomidine (Janssen Animal Health, Neuss, Germany, 0.04 mg/kg). A radiolabelled solution containing 0.25 ml of ~250 µCi

1,2,6,7-^{3}H testosterone (TRK407, Amersham Bioscience, UK) in ethanol was mixed with 2.25 ml sterile 0.9% NaCl solution and injected into the cephalic vein. Faecal samples were collected from 3 days before until 4 days after injection and stored at −20 °C until analyses. Aliquots of each sample (n = 12) were extracted for fTM determination and radioactivity counting. The sample collected 21 hours after injection contained the highest percentage of radioactivity (82%) and therefore was used for HPLC analyses (see 7.2.3). Radioactive counting was conducted in a MicroBeta TriLux 1450 liquid scintillation counter (Perkin Elmer, Waltham, Massachusetts, USA).

Because androgens may be converted into polar water soluble conjugates prior to excretion via faeces (Jewgenow et al., 2006; Möhle et al., 2002; Pribbenow et al., 2015; Velloso et al., 1998), the faecal extract was subjected to enzyme hydrolysis (see 7.2.4) before HPLC separation.

Testosterone challenge test. The testosterone challenge was conducted with M1 by injecting 20 mg of long-acting testosterone intramuscularly (for immobilisation protocol see 7.2.2). Faecal samples were collected 7 days before and 10 days after injection. The enclosure was checked for faeces once per day. FTM concentrations were examined in non-hydrolysed and hydrolysed extracts, respectively, which produced similar results (see 7.3.2). Therefore, all further analyses were performed using non-hydrolysed faecal extracts. As a control, faecal samples of M2 and M3 were collected twice per day over a period of 14 days and stored at −20 °C until analysis.

GnRH challenge test and placebo administration. The GnRH challenge test was conducted with M1 by injecting 1 ml of GnRH analogue (Receptal (Buserelin 0.04 mg) Intervet, Unterschleissheim, Germany) intramuscularly with a blow pipe to stimulate testosterone secretion. As a control, M1 received a placebo (saline) one month prior to the GnRH challenge consisting of 3 ml of 0.9% NaCl injected intramuscularly with a blow pipe. Faecal samples were collected 5 days before and 6 days following injection, and 4 days before and 5 days following injection, respectively. In both experiments the enclosure was checked for faeces twice per day and samples were stored at −20 °C until analysis.

7.2.3. HPLC Analyses of fTM

Aliquots of 150 µl of the faecal extract with the highest percentage of radioactivity from M1 and one fresh sample each from M4 and M5 (see 7.2.1) containing high concentrations of fTM were selected for reversed phase HPLC analyses, with and without enzymatic hydrolysis, respectively. Sample preparation on C18 columns and chromatographic separation using an Ultra Sep ES-RP 18/6 µm HPLC column (250x4 mm, Sepserv, Berlin, Germany) were conducted as described previously (Pribbenow et al., 2014). The elution positions

of native cortisol, corticosterone, testosterone, epi-A and dihydrotestosterone (DHT) on this column had been determined previously in separate HPLC runs.

7.2.4. Processing of Faecal Samples

Extraction of wet faeces. Masses of 0.5 g of wet faeces were extracted for 30 min with 4.5 ml of 90% methanol and gently shaken on a horizontal shaker (SM-30, Edmund Buhler, Hechingen, Germany) to allow extensive mixing. After centrifugation (15 min, 1000 g) the supernatant was transferred to a new tube. Aliquots of these extracts were used for HPLC analyses. For EIA analyses aliquots were diluted 1:2 with water and 20 µl were subjected to the epi-A EIA.

Hydrolysis with β-glucuronidase from *Helix pomatia*. Enzymatic hydrolysis was performed as previously described (Pribbenow et al., 2015). Briefly, 0.1 ml of faecal extracts in 90% methanol were hydrolysed in 900 µl of 0.05 M acetate buffer (pH 4.8) containing 4 µl β-glucuronidase and arylsulfatase from *Helix pomatia* (Roche Diagnostics GmbH). After triple extraction of faecal steroids with 2.5 ml of tert methylbutylether/petroleum ether (30:70, v/v), phase separation was achieved by freezing for 15 min at −80 °C. The extracts were combined, evaporated under nitrogen in a sample concentrator (Dri Block DB3, Techne, Staffordshire, UK) at 55 °C, dissolved in 1 ml of 40% methanol and stored at −20 °C until HPLC analyses.

7.2.5. Enzyme Immunoassay

Aliquots (20 µl) from methanolic faecal extracts were analysed with an "in-house" epi-A EIA as previously described for spotted hyenas (Pribbenow et al., 2015). The antibody was polyclonal and raised in rabbits against the 3-hemisuccinat (HS)-steroid coupled with BSA. The corresponding epiandrosterone-3-HS-peroxidase was used as label. Cross-reactivities of the antibody were determined as follows: 100% epiandrosterone (5α-androstan-3β-ol-17-one), 88% epiandrosterone glucuronide (5α-androstan-3β-ol-17-one glucosiduronate), 68% epiandrosterone sulphat, 60% androsterone (5α-androstan-3α-ol-17-one), 60% androstenedione (4-androsten-3, 17-dione), 24% androsterone sulphate (5α-androstan-3α-ol-17-one sulphate), 19% dehydroandrosterone (5-androsten-3α-ol-17-one), 17% dehydroepiandrosterone (5-androsten-3β-ol-17-one), and 0% for testosterone, cortisol and corticosterone. The assay was validated by demonstrating parallelism of faecal extracts to the epiandrosterone calibration curve (supplement, Fig. 12.2). The intra-assay and inter-assay coefficients of variation were determined using faecal extracts containing known concentrations of epi-A. The inter-assay coefficients were 6.3% (n = 6) and 11.2%

(n = 6) for extracts containing low and high concentrations of endogenous epi-A, respectively. The corresponding intra-assay coefficients were 8.3% (n = 6) and 11.6% (n = 6), respectively.

7.2.6. Cross-Reactivity of Epi-A EIA with Faecal Glucocorticoid Metabolites

To ensure that measurements of fTM based on the epi-A EIA were not biased by fGM that may cross-react with the epi-A antibody, samples from an ACTH challenge conducted previously with M1 and one adult female (F1) (Ludwig et al., 2013) were analysed with the epi-A EIA expecting no ACTH dependant increase in fTM. Detailed description of treatment and sample collection procedure has been described in Ludwig et al. (2013).

7.2.7. Data Analysis

Calculation of baseline values was performed using an iterative process excluding all values greater than the mean + 2SD (Brown et al., 1996a). Baseline values of testosterone concentrations are quoted as mean ± SD. Significant increases were defined as peaks with values exceeding the baseline +2SD. Statistical analyses were performed with Systat 13 (Systat Software, Inc., San Jose, Carlifornia, USA). The level of significance was set at 5% and all tests were two-tailed. A Wilcoxon signed rank test was performed to examine whether fTM concentrations of non-hydrolysed and hydrolysed extracts differed. To ensure both the biological validity of the epi-A EIA we compared fTM concentrations between free-ranging adult males, adult females and juvenile males by applying a Kruskal-Wallis test post hoc Dwass-Steel-Chritchlow-Fligner test. FTM concentrations are quoted as mean ± SD.

7.3. Results

7.3.1. Analytical Validation

HPLC analysis of radiolabelled testosterone metabolites. To characterise the radiolabelled fTM in male M1, a non-hydrolysed and hydrolysed faecal extract was analysed by reversed phase HPLC (Fig. 7.1 A). Thereby, fTM were separated according to their polarity with the more polar metabolites being eluted first. Arrows depict the elution positions of steroid standards of cortisol, corticosterone, testosterone, epi-A and DHT when applying the corresponding steroid hormone specific assays. In the non-hydrolysed faecal extract, only one major polar radiolabelled peak in fractions 1 – 5 was detected, indicating a cluster of conjugated metabolites. Enzymatic hydrolysis of the faecal extract distinctly

changed the elution patterns of radiolabelled fTM. Polar radiolabelled conjugates disappeared and were substituted by a major radiolabelled peak in fractions 39 – 42, co-eluting with the epi-A standard. Minor radioactive peaks were detected in fractions 34 – 36 and 36 – 39. No radiolabelled fTM were detected at the elution position of native testosterone, indicating that the circulating hormone itself is not present in faeces. Additionally, no radiolabelled fTM were detected at elution positions of native cortisol, corticosterone and DHT.

HPLC analyses of immunoreactive testosterone metabolites. Our epi-A EIA evaluation of a faecal sample from male M1 revealed HPLC immunograms (Fig. 7.1 B) which coincided with the profiles of radiolabelled fTM. Prior to enzymatic hydrolysis the epi-A EIA detected one polar immunoreactive peak, eluting in fractions 1 – 5. Similar to radiolabelled fTM, hydrolysis of the faecal extract affected the elution pattern of immunoreactive fTM. Deconjugation of steroid conjugates led to the appearance of one distinct immunoreactive peak in fractions 39 – 42, corresponding to the elution position of native epi-A and radiolabelled fTM. HPLC immunograms of faecal samples from two non-stimulated adult males (M4 and M5) yielded similar profiles (supplement, Fig. 12.3). Again, enzymatic hydrolysis led to the appearance of one major immunoreactive peak, corresponding with the elution position of native epi-A. In addition, similar to the HPLC profile of radiolabelled fTM, no immunoreactivities were detectable at the elution positions of any of the native steroid standards.

7.3.2. Physiological Validation

Testosterone challenge test. Faecal samples from the testosterone challenge of male M1 were analysed with the epi-A EIA, each with and without enzymatic hydrolysis (Fig. 7.2). In the non-hydrolysed extracts a significant increase in fTM was detected one day after the injection of testosterone, with a peak concentration of 9.2 µg/g, exceeding baseline levels (2.1 ± 1.0 µg/g) by approximately 4-fold (Fig. 7.2). FTM concentrations returned to baseline within 4 days after injection. Hydrolysis of faecal samples revealed a similar fTM profile with a peak concentration of 4.2 µg/g, also approximately 4-fold above baseline concentrations of 0.8 ± 0.4 µg/g (Fig. 7.2), indicating that course and amount of increase of fTM concentrations do not differ between both extraction procedures ($Z = -5.086$, $P < 0.0001$, Wilcoxon signed rank test). Therefore, all further analyses in this study were conducted without hydrolysis of faecal samples. As a control, fTM concentrations of non-hydrolysed faecal samples of two non-stimulated adult (M2) and juvenile males (M3) were measured, resulting in 0.6 ± 0.4 µg/g for M2 and 0.7 ± 0.4 µg/g for M3. In both

males, concentrations of fTM remained stable over the entire period of sample collection (Fig. 7.2).

GnRH challenge test and placebo administration. Administration of GnRH analogue resulted in a significant increase in fTM concentrations in male M1 (Fig. 7.3). Peak excretion of 17.6 μg/g occurred in the sample collected 21.5 hours following injection, which is 16-fold higher compared to baseline concentrations of 1.1 ± 0.5 μg/g. FTM concentrations returned to pre-treatment levels within 50 hours following injection. Both the GnRH and testosterone challenges revealed similar results regarding latency until peak fTM concentrations were reached, however, in the case of GnRH, the peak levels were approximately twice as high (compare Fig. 7.2 and Fig. 7.3). In contrast, administration of the placebo NaCl induced no increase in fTM concentrations, thus values remained constant over the entire period of sample collection (Fig. 7.3).

Exclusion of the epi-A EIA from cross-reactivities with faecal glucocorticoid metabolites. As validated by Ludwig et al. (2013), fGM in cheetahs can be measured non-invasively using an antibody directed against corticosterone-3-CMO detecting significant increases in fGM concentrations following ACTH injection. To exclude the possibility that the epi-A EIA detects androgenic steroids derived from cortisol metabolism, we analysed faecal samples from the above mentioned ACTH challenges in one captive adult male (Fig. 7.4 A) and adult female (Fig. 7.4 B) cheetah. In contrast to the fGM, fTM concentrations measured with the epi-A EIA did not show any ACTH induced changes (Fig. 7.4 A, B). These results indicate that our epi-A exclusively detects faecal testosterone metabolites but does not recognize androgenic glucocorticoid metabolites that might falsify fTM measurements in cheetahs.

7.3.3. Biological Validation — Comparison of fTM Concentrations Between Free-Ranging Cheetahs

There were significant differences in fTM concentrations between free-ranging adult male, adult female and juvenile male cheetahs (Fig. 7.5, $KW = 18.586$, $df = 2$, $P < 0.0001$). In adult males, fTM concentrations were 2.0 ± 1.5 μg/g, ranging from 0.32 to 6.90 μg/g, in adult females 0.7 ± 0.6 μg/g, ranging from 0.16 to 2.09 μg/g and in juvenile males 0.9 ± 0.5 μg/g, ranging from 0.14 to 1.79 μg/g, indicating high inter-individual variation. Post hoc pairwise comparisons revealed that adult males had higher fTM concentrations than adult females ($P < 0.0001$) or juvenile males ($P < 0.0001$), whereas juvenile males had significantly higher fTM concentrations than adult females ($P = 0.01$).

7.3.4. Stability of fTM Concentrations Under Field Conditions

FTM concentrations in samples from the captive males M1, M4 and M5 did not significantly change within 48 hours and 72 hours at ambient temperature, respectively, neither in a German zoo in winter conditions nor in Namibian farmland conditions in summer (Fig. 7.6, German zoo: $R^2 = 0.05$, $F = 2.613$, $df = 1,9$, $n = 31$, $P = 0.12$, regression equation: $y = 80.7 - 0.33\ x$); Namibian farmland: $R^2 = 0.002$, $F = 1.016$, $df = 1, 8$, $n = 10$, $P = 0.34$, regression equation: $y = 113.95 - 0.22\ x$). Noticeablye, in both time series fTM fluctuations occurred during the first 24 hours, but remained more stable thereafter.

7.4. Discussion

In this study we demonstrated that variations in fTM concentrations in male cheetahs can be reliably monitored non-invasively using the epi-A EIA because conjugated epi-A represents the major faecal testosterone metabolite in cheetahs. Our results showed the predicted peaks in faecal hormone excretion following testosterone injection and GnRH stimulation, indicating that faecal epi-A measured by this assay reflects biologically meaningful testicular activity.

In felids, previous studies demonstrated that gonadal steroids are mainly excreted via faeces as non-hydrolysable polar conjugates, steroid sulphates or glucuronides (Brown et al., 1996a; Brown et al., 1994; Jewgenow et al., 2006). In the male cheetah, our radiometabolism study confirmed that radiolabelled fTM are mainly excreted as polar conjugates. However, these conjugates were hydrolysable with enzymes from *Helix pomatia* liberating epi-A from its conjugates. The HPLC immunograms of a male from Zoo Muenster and two males from Namibia showed that the epi-A antibody detects both polar epi-A conjugates and free epi-A following hydrolysis. Moreover, the almost identical HPLC profiles of radiolabelled and immunoreactive metabolites in a male cheetah demonstrate that our epi-A EIA detects faecal metabolites derived from testosterone metabolism. We assume that epi-A-glucuronide is the major conjugate as its cross-reactivity towards the epi-A antibody (directed against the 3-HS-steroid coupled with BSA) was 88%. Thus, pre-treatment enzymatic hydrolysis seems non-essential given that analyses with and without hydrolysis resulted in similar biological response profiles following testosterone injection.

Based on structural similarities of androgen and glucocorticoid metabolites (Ganswindt et al., 2003; Pribbenow et al., 2015), antibodies directed against androgen metabolites might cross-react with glucocorticoid metabolites of androgenic structure. Thus, we examined whether the results of our epi-A EIA were biased by such cross-reactivities using faecal extracts from an ACTH challenge experiment (Ludwig et al., 2013). We demonstrated

in both sexes that an increase in fGM did not influence the measurements of fTM. This is consistent with our HPLC immunograms of fTM demonstrating that the epi-A EIA only detects metabolites derived from testosterone degradation, mainly consisting of epi-A and/or epi-A-conjugates. Epiandrosterone (5α-androstane-3β-ol-17-one), one of the major excreted 17-oxoandrogen metabolites, is derived from testosterone by oxidoreductive reactions at the A- and D-rings (Schänzer, 1996). To specify, the initial step in testosterone metabolism is the reduction of the C-4,5 double bond by 5α-reductase, followed by a hydroxy-reduction of the C 3-keto group by 3β-hydroxysteroid dehydrogenase (HSD). The final step is the enzymatic oxidation by 17β-HSD to form the 17-keto steroid (reviewed by Schänzer, 1996). Therefore, the conjugation of epi-A takes place at the position C-3, as this is the only hydroxy group. Epiandrosterone has also been reported as a major testosterone metabolite in faeces of macaques (Girard-Buttoz et al., 2009; Möhle et al., 2002) and spotted hyenas (Pribbenow et al., 2015). EIAs using antibodies directed against epiandrosterone have also been validated for gibbons (Barelli and Heistermann, 2012), spiny mice (de Bruin et al., 2014) and elephants (Ganswindt et al., 2002; Ghosal et al., 2013).

To evaluate the ability of our epi-A EIA to monitor testicular activity in the cheetah, a physiological validation had to be conducted. Therefore, we increased fTM concentrations with an injection of native testosterone and stimulated gonadal testosterone secretion by applying a GnRH analogue. FTM concentrations increased significantly within 1 day following both testosterone and GnRH injection, corresponding to the time course of excreted radiolabelled ^{3}H-testosterone metabolites. Our results are in line with previously reported excretion patterns of faecal steroid metabolites in felids (Brown et al., 1996a; Ludwig et al., 2013). In contrast, a placebo injection of NaCl did not result in elevated fTM concentrations, confirming that our epi-A EIA is an appropriate indicator of testicular activity.

The biological validity of the epi-A EIA was further demonstrated by the ability to distinguish between free-ranging adult male, adult female and juvenile male cheetahs. FTM concentrations were higher in free-ranging adult males than in adult females and juvenile males as expected. We did not find a difference between the adult (M2) and juvenile (M3) male from the zoo in Muenster. This may have been due to the small sample size or potentially to chronic stress in captive cheetahs. Terio et al. (2004) demonstrated significantly enlarged cortices, a measurement for chronic stress, in captive cheetahs housed in North American zoos, and higher fGM but lower fTM concentrations compared to free-ranging individuals from Namibia. This is consistent with our study as we found that free-ranging adult males had higher fTM concentrations (2.0 ± 1.5 μg/g) than the captive adult male M2 from Zoo Muenster (0.6 ± 0.4 μg/g).

In the last two decades, a number of EIAs have been developed and applied in a variety of species to evaluate endocrine function non-invasively. Various studies demonstrated that faecal hormone metabolites may differ considerably between individuals and sexes due to variations in steroid metabolism, excretion and composition of gut bacteria (Palme et al., 1996; Palme et al., 2005). However, faecal steroid metabolite concentrations may also be affected by faecal sampling regime and storage under field conditions. Because it is often difficult to collect fresh faecal samples from free-ranging animals, samples are exposed to various environmental conditions before being frozen. This might promote bacterial degradation and thus may alter faecal metabolite composition and affect measurements (Abáigar et al., 2010; Hodges and Heistermann, 2011; Terio et al., 2002; Wasser et al., 1988). In a given species, stability may differ between metabolites of different steroids (Terio et al., 2002) and may also affect different assays in different ways (Morrow et al., 2002). Thus, the effect of storage mimicking field conditions should be performed in each species for the steroid metabolite of interest. Similarly to fGM concentrations in cheetahs (Ludwig et al., 2013), we obtained no evidence for changes in fTM concentrations over time, suggesting that fTM concentrations in male cheetahs were unaffected by environmental conditions. Despite exposure to low (samples from Zoo Wuppertal) or high ambient temperatures and direct sunlight (samples from Namibia), fTM concentrations remained stable up to 3 days.

In conclusion, we demonstrated that the epi-A EIA provides a beneficial and practicable tool to evaluate testicular activity in male cheetahs. This non-invasive method allows the study of the impact of life history parameters, husbandry conditions, nutrition and environment in this species. Thus, comparative analyses of fTM and fGM can contribute to the improvement of male reproductive physiology and mating success in cheetahs.

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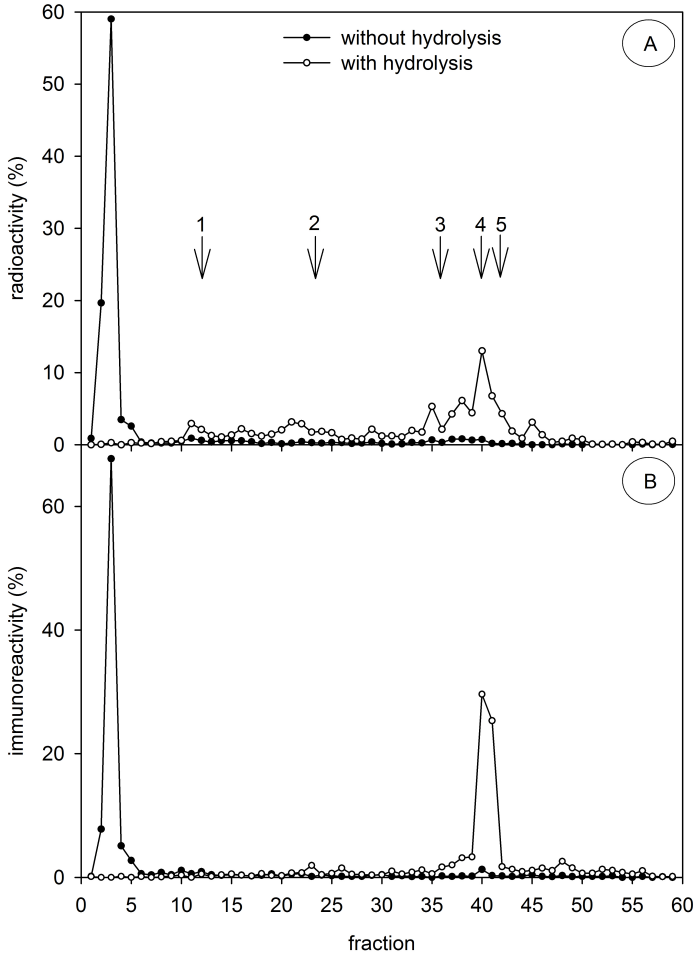

Figure 7.1. HPLC profile of (A) radiolabelled and (B) immunoreactive faecal testosterone metabolites (fTM). FTM were analysed with the epi-A EIA in a non-hydrolysed (black circles) and hydrolysed (white circles) faecal extract of a male (M1) that received an injection of radiolabelled ^{3}H-testosterone. Radiolabelled testosterone and immunoreactive metabolites are presented as percentage of overall eluted activity. Arrows indicate the elution positions of steroid standards: (1) cortisol, (2) corticosterone, (3) testosterone, (4) epi-A, and (5) DHT.

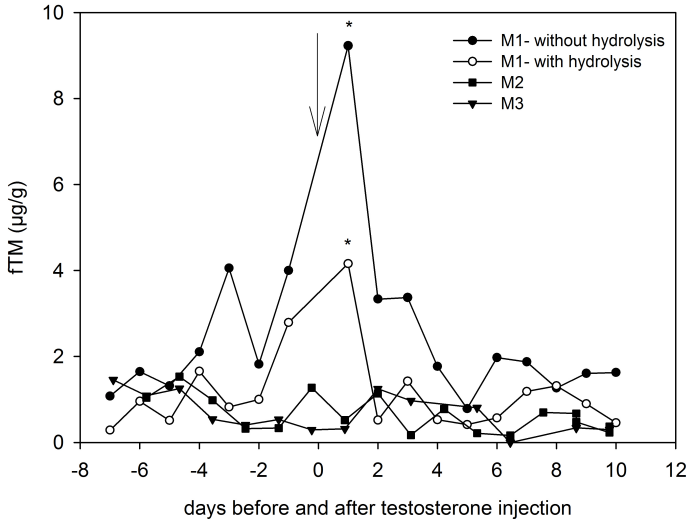

Figure 7.2. Changes in fTM concentrations in response to a testosterone challenge in an adult male (M1) in comparison to fTM concentrations in a non-stimulated adult male (M2) and juvenile male (M3). Concentrations of fTM were measured with the epi-A EIA after a testosterone injection, as indicated by the arrow, in non-hydrolysed (black circles) and hydrolysed (white circles) faecal extracts of M1 and in non-hydrolysed faecal extracts of M2 (black squares) and M3 (black triangles). Faecal samples were collected 7 days prior to the injection until 10 days after injection. The asterisks indicate fTM elevations exceeding baseline concentrations + 2SD.

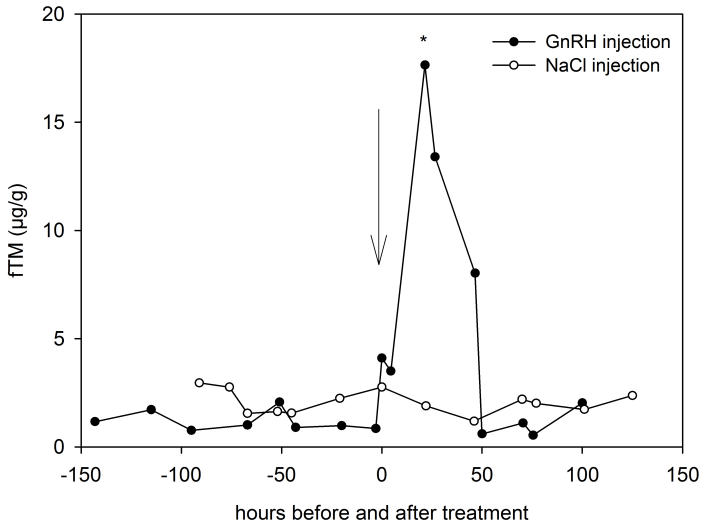

Figure 7.3. Changes in fTM concentrations in response to a GnRH challenge and placebo injection (NaCl) in the adult male M1. The arrow indicates the time of GnRH and NaCl administration, respectively. The asterisk indicates fTM elevations exceeding baseline concentrations + 2SD.

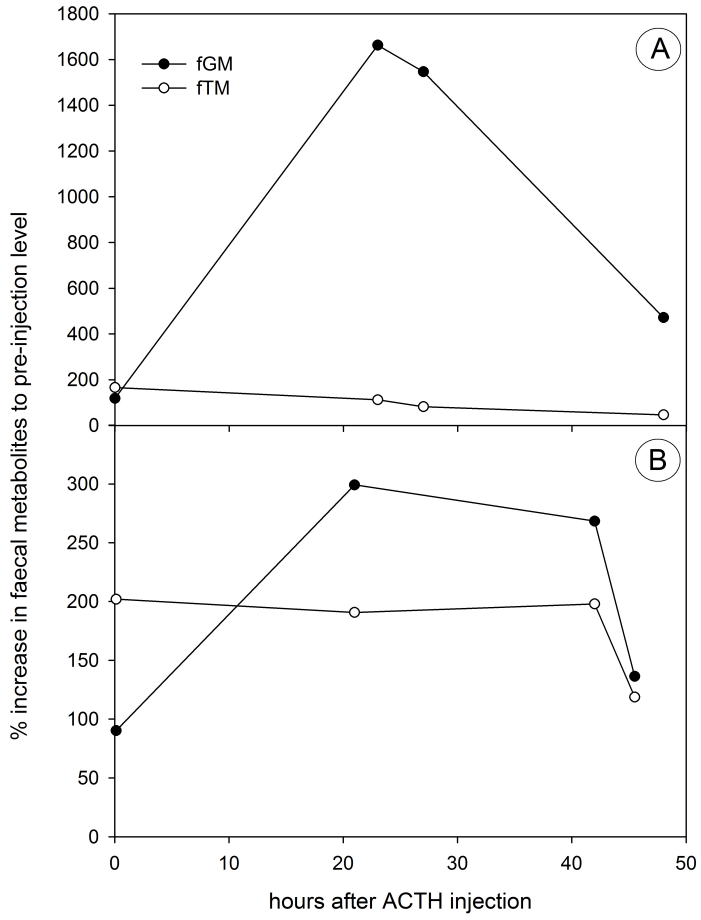

Figure 7.4. Percent increase in fTM and fGM concentrations compared to pre-injection levels in response to administration of synthetic ACTH in the (A) captive male M1 and (B) captive female cheetah F1. Faecal samples collected from 76 h before until 45 h after injection were analysed with the epi-A EIA and compared to fGM concentrations previously determined with the corticosterone-3-CMO EIA (Ludwig et al., 2013).

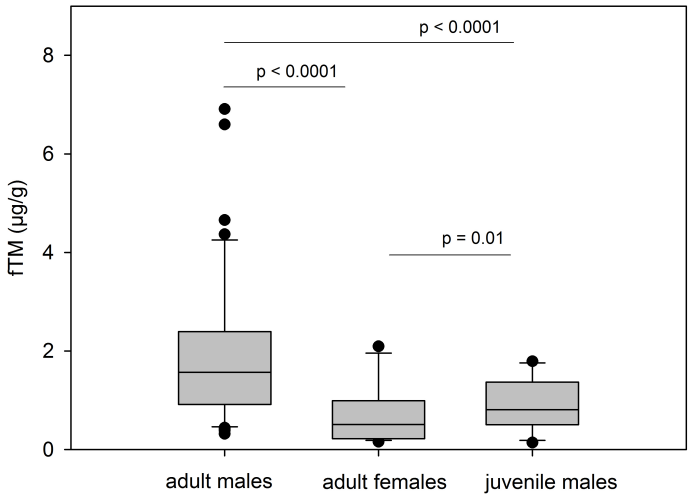

Figure 7.5. FTM concentrations of 46 adult male, 18 adult female and 12 juvenile male cheetahs determined with the epi-A EIA.

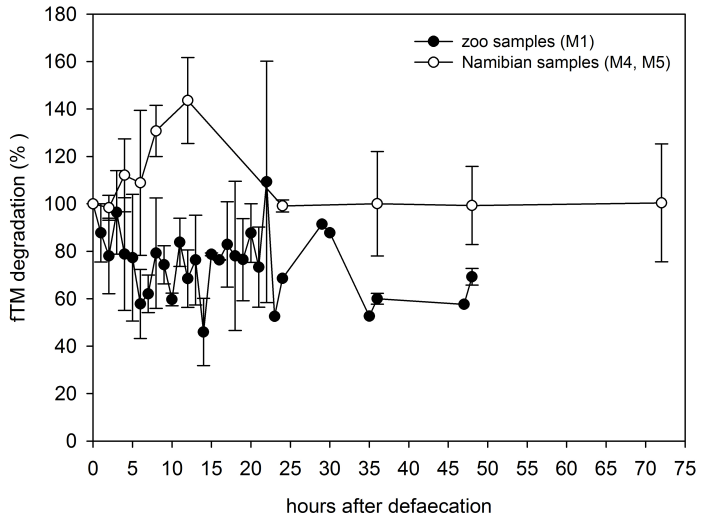

Figure 7.6. Relative degradation of fTM (%) in three samples of M1 over a period of 48 h and one faecal sample each from M4 and M5 over a period of 72 h, given as mean values ± SEM, respectively. Relative fTM concentrations were calculated in relation to the reference concentration of the sample frozen immediately after defaecation, representing 100%.

8. Article 3

Measuring fecal testosterone metabolites in spotted hyenas: choosing the wrong assay may lead to erroneous results

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Abstract

Enzyme-immunoassays (EIA) that detect fecal testosterone metabolites (fTM) are powerful tools to monitor gonadal activity non-invasively. However, a challenge with testosterone EIAs might be their potential for cross-reactivities with structurally similar glucocorticoid metabolites. Therefore, we aimed to verify the capability of four different testosterone EIAs to monitor fTM without reflecting changes in adrenocortical activity in spotted hyenas by analyzing fecal samples following testosterone and ACTH challenge tests. We demonstrated that none of the testosterone EIAs is appropriate to measure fTM as all of them showed substantial cross-reactivities to unknown metabolites. Our study underlines the importance of validating androgen EIAs.

Part III.

General Discussion

Testosterone is essential for male sexual development and maturation, development and maintenance of male reproductive organs and spermatogenesis as well as for the expression of sexual behaviour (Mooradian et al., 1987; Wingfield et al., 1990; Heinlein and Chang, 2002). The effective reproduction management of wildlife in both free-ranging and captive settings is assisted by knowledge of the basic reproductive physiology of a given species and the ability to assess the reproductive status of individuals (Schwarzenberger et al., 1996). In the past, analyses of plasma testosterone have been routinely conducted to assess an animal's gonadal status. However, drawing blood is often limited by restricted accessibility to study animals, particularly in wildlife species, and may negatively impact circulating testosterone concentrations owing to its invasive character (Möstl and Palme, 2002; Goymann, 2005; Palme et al., 2005). Thus, alternative strategies to accurately assess an animal's gonadal status are needed (Schwarzenberger et al., 1996; Schwarzenberger, 2007).

9. Monitoring Gonadal Activity Using an Epiandrosterone EIA

During the last three decades the non-invasive monitoring of faecal testosterone metabolites has become a widely accepted and important tool for the reproduction management and conservation of wildlife species, as it allows an integrated measure of reproductive activity even over long study periods without disturbing the animal (Goymann, 2005; Palme et al., 2005). Non-invasive hormone analyses using EIAs rely on the ability of the utilised antibody to detect the predominant species-specific faecal metabolites (Palme et al., 1996; Kersey and Dehnhard, 2014). Since steroids are extensively metabolised prior to excretion, cross-reactivities of an androgen antibody with "non-target" metabolites such as metabolites not derived from testosterone degradation represent a major challenge in the development of a reliable androgen EIA (Ganswindt et al., 2003; Palme et al., 2005). Thus, EIAs should be carefully validated for each specific hormone and species (Palme et al., 1996; Palme et al., 2005; Touma and Palme, 2005).

The aim of the presented dissertation was to establish and validate non-invasive methods to monitor gonadal activity in spotted hyenas and male cheetahs based on faecal metabolite analyses, presented in ***Article 1*** and ***Article 2***, respectively. In both species, our studies demonstrated the efficacy of an epiandrosterone EIA by performing all necessary validation procedures, including: (1) radiometabolism studies followed by HPLC analyses; (2) demonstration of a lack of cross-reactivities with "non-target" hormone metabolites; (3) physiological validation by artificially induced increases in circulating testosterone concentrations, and (4) by comparing fTM levels in free-ranging individuals (Pribbenow et al., 2015; Pribbenow et al., 2016). The third study, presented in ***Article 3***, highlights the challenge of undesired cross-reactivities of testosterone EIAs with metabolites not derived from testosterone metabolism. As the metabolism of androgens and glucocorticoids may result in metabolites with similar chemical structures, fTM results can be impacted by co-measurement of fGM (Ganswindt et al., 2003; Palme et al., 2005). By comparing the ability of different testosterone EIAs to monitor fTM in spotted hyenas we demonstrated that a careful methodological and biological validation is requested to ensure that fTM concentrations quantitatively reflect gonadal activity. We highly recommend to confirm that

stress-related increases in fGM are ignored by assays used to quantify fTM concentrations (Article 3, Pribbenow et al., *under review*).

As far as we know, our epiandrosterone EIA represents the first validated EIA for monitoring fTM in both spotted hyenas and male cheetahs which specifically detects metabolites derived from testosterone degradation (Article 1, Pribbenow et al., 2015; Article 2, Pribbenow et al., 2016). Furthermore, in both species we confirmed that testosterone is metabolised via the epiandrosterone pathway, resulting in the excretion of hydrolysable epiandrosterone conjugates (Article 1, Pribbenow et al., 2015; Article 2, Pribbenow et al., 2016). Epiandrosterone has also been identified as the major testosterone metabolite in male long-tailed macaques, *Macaca fascicularis*, (Möhle et al., 2002) and white-faced capuchins, *Cebus capucinus*, (Weltring et al., 2012). Epiandrosterone (5α-androstane-3β-ol-17-one) is one of the major excreted natural 17-oxoandrogen metabolites derived from testosterone by oxidoreductive reactions at the A- and D-rings of the molecule at positions C-3, C-5 and C-17, shown in Figure 9.1 (Schänzer, 1996). The initial step is the reduction of the C-4,5 double bond by 5α-reductase, followed by a hydroxy-reduction of the C-3-keto group by 3β-HSD. The final step is the enzymatic oxidation by 17β-HSD to form the 17-keto steroid (reviewed by Schänzer, 1996).

EIAs utilising antibodies directed against epiandrosterone have also been successfully applied to reliably reflect testicular endocrine activity in a variety of male mammals [primates: gibbon, *Hylobates lar* (Barelli and Heistermann, 2012), orang-utans, *Pongo spp.*(Weingrill et al., 2011), rhesus macaques, *Macaca mulatta* (Higham et al., 2013), Assamese macaques, *Macaca assamensis* (Ostner et al., 2011); ungulates: giraffes, *Giraffa camelopardalis* (Seeber et al., 2013), chamois, *Rupicapra rupicapra rupicapra* (Hoby et al., 2006), red deer, *Cervus elaphus* (Pavitt et al., 2015); rodents: spiny mice, *Acomys spinosissimus* (de Bruin et al., 2014); and African elephants, *Loxodonta africana* (Ganswindt et al., 2002)]. Although the above mentioned studies did not confirm the presence of faecal epiandrosterone, the broad applicability of epiandrosterone-based EIAs suggests a conserved metabolism of testosterone to epiandrosterone or epiandrosterone-like metabolites across mammalian taxa.

Androgens are also of major significance for female reproduction, as they are precursors of oestrogens and thus are essential for follicle maturation, especially in the early stages of folliculogenesis and granulosa cell activity (reviewed by Staub and De Beer, 1997; Gleicher et al., 2011). Elevations in plasma testosterone have been shown to be positively correlated with the pre-ovulatory LH surge in female domestic pigs, *Sus scrofa*, domestic dogs, *Canis lupus familiaris* and rhesus macaques (Enne et al., 1981; Wallen et al., 1984; Gudermuth et al., 1998). In Assamese macaques, fTM levels increased during premating season, potentially indicating the onset of ovarian cyclicity, and during early gestation (Fürtbauer

Figure 9.1. Testosterone metabolism to epiandrosterone. Epiandrosterone is derived from testosterone via oxidoreductive reactions at the A- and D-ring. 3α-HSD: 3α-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase. According to Schänzer (1996).

et al., 2012, 2013). However, in previous studies androgen EIAs have also been shown to cross-react with non-androgen-related faecal metabolites such as faecal oestrogen and progesterone metabolites and thus to reliably monitor the follicular phases in female sun bears, *Helarctos malayanus*, Indian rhinoceros, *Rhinoceros unicornis*, and domestic dogs (Möstl and Brunner, 1997; Schwarzenberger et al., 2000; Schwarzenberger et al., 2004). Thus, when applying an androgen EIA to assess the androgen output in females, a potential co-measurement of metabolites derived from oestrogen degradation rather than testosterone should be taken into careful consideration. Interpretation of fTM results in females requires careful consideration of reproductive status and behavioural parameters in order to prevent erroneous conclusions.

10. Impact of Sampling and Storage on Faecal Steroid Metabolites

Variables associated with sampling and storage methods may impact the quality of faecal steroid metabolite measurements and thus complicate the interpretation of results (Millspaugh and Washburn, 2004; Keay et al., 2006). I will now discuss the effects of sample collection and post-defaecation metabolism.

10.1. Sample Collection

Sample collection itself may introduce confounding issues for the analyses of faecal steroid metabolites. Ideally, faecal samples for hormone analyses are collected freshly after defaecation from individuals of known sex and age. In captivity, where animals are kept together in pairs or groups in an enclosure, colourants can be used to tag food provided to individual animals. These substances are excreted with faeces and guarantee the individual identification of the appropriate faeces (Keay et al., 2006). In free-ranging populations, collection of individually known faecal samples necessitates the careful observation of known individuals and the immediate collection upon defaecation (Kretzschmar et al., 2004; Schwarzenberger, 2007). Depending on the species and their defaecation rate, faecal steroid metabolite measurements may also reflect diurnal variations of plasma hormone levels (Keay et al., 2006; Schwarzenberger, 2007).

In animals with high defaecation rates such as small primates and rodents, distinct diurnal rhythms of steroid metabolite levels have been described (reviewed by Touma and Palme, 2005; Hodges and Heistermann, 2011). However, in species which defaecate less frequently, as this is the case for our study species, short-term fluctuations of plasma hormones should be dampened in faeces due to the relative long gut-time passage (Palme et al., 2005; Touma and Palme, 2005). Nevertheless, as they may occur, Schwarzenberger (2007) suggests to collect faecal samples defaecated at the same time of the day.

Steroid metabolites may be unevenly distributed within the faecal material. Comparative analyses of subsections of faecal samples from felid species and African elephants demonstrated a high variability of P_4/E_2 metabolite distribution across each sample (Brown et al., 1994; Wasser et al., 1996). Intra-sample variations have also been repor-

ted for fGM concentrations in white-tailed deer, *Odocoileus virginianus*, domestic pigs, domestic horses, *Equus ferus caballus*, and tigers, *Panthera tigris* (Palme et al., 1996; Millspaugh and Washburn, 2003; Parnell et al., 2015). However, the distribution of faecal steroid metabolites within a sample seems to be dependent on the type of metabolites and species, as fGM have been found evenly distributed in cattle, *Bos taurus*, and sheep, *Ovis aries* (Palme et al., 1996; Morrow et al., 2002). As faecal steroid metabolite analyses are typically obtained from small aliquots of the total samples, faeces should therefore be well-mixed before taking subsamples (Palme et al., 1996; Parnell et al., 2015). Contamination of faecal samples with urine or faeces of other animals should be avoided, as additional potentially structurally different metabolites may bias the measurements of steroid metabolite concentrations (Wasser et al., 1988).

10.2. Post-Defaecation Metabolism and Storage Conditions

Besides sampling, subsequent post-defaecation metabolism and storage conditions may artificially distort faecal steroid metabolites and thus affect metabolite measurements (Keay et al., 2006). As gastrointestinal bacteria are abundant in faeces, ageing of faecal samples implies bacterial degradation and the potential conversion of excreted metabolites to non-measurable entities. Winter and Bokkenheuser (1987) showed that C-19 steroids may be metabolised in several ways by the faecal flora: *Clostridia* species are known to synthesise reductases responsible for A-ring reductions, whereas *Bacteroides* species are active on 17-keto steroids (D-ring reduction). Furthermore, desmolase, an enzyme produced by various anaerobic bacteria, may catalyse an oxidative side-chain cleavage at C-17-C-20, resulting in the formation of androstane structures (Winter et al., 1984). Thus, naturally occurring bacteria and their enzymes may decompose steroid metabolites within hours after defaecation and potentially alter faecal steroid metabolite concentrations in unpreserved samples (Whitten et al., 1998; Möstl et al., 1999). Studies on domestic livestock demonstrated that the interval between defaecation and preservation of samples was critical: fGM concentrations in samples of domestic cattle, domestic horses and domestic pigs not only significantly increased within 1 h, 4 h and 24 h, respectively, after defaecation, the composition of excreted metabolites also changed when stored unpreserved at room temperature (Möstl et al., 1999). Age-dependent changes in faecal steroid metabolite concentrations have also been shown for wildlife species: In unpreserved samples of baboons, *Papio cynocephalus*, faecal progesterone (P_4) metabolite concentrations increased whereas faecal oestradiol (E_2) metabolite concentrations decreased within 6 h (Wasser et al., 1988). In cheetahs, fGM concentrations remained stable

over a time period of 22 h when stored at 0 – 4 °C and increased significantly afterwards (Ludwig et al., 2013). In contrast, we did not detect significant changes in fTM concentrations of samples exposed to ambient temperatures in a German Zoo or on a Namibian farmland within 2 days and 3 days, respectively, after defaecation (Article 2, Pribbenow et al., 2016). Similarly, Abáigar et al. (2010) demonstrated that fGM concentrations of Iberian lynxes remained stable for 1 week under field conditions. These contrasting findings are possibly a result of the differing composition and nature of animal faeces, sex-specific and species-specific patterns of metabolism as well as antibody-specific cross-reactivities with metabolites converted by faecal bacteria (Parnell et al., 2015). Lexen et al. (2008) showed that after storing faecal samples from domestic sheep at room temperature for 24 hours, fGM concentrations either increased or decreased as a function of the assay system used. It should be noted that post-defaecation metabolism can be intensified if faecal samples have been exposed to excessive temperatures and/or precipitation (Millspaugh and Washburn, 2004). Following artificial rainfall, Washburn and Millspaugh (2002) observed increasing fGM concentrations in white-tailed deer, suggesting that these created an optimal environment for the growth of faecal bacteria. FGM concentrations in tigers changed significantly as a result of natural weathering after 48 h (Parnell et al., 2015). To minimise metabolism by faecal bacteria or bacterial enzymes, the collection of fresh material is recommended (Millspaugh and Washburn, 2004; Hodges and Heistermann, 2011). Thus, the interval between defaecation, sample collection and preservation should be as short as possible (Millspaugh and Washburn, 2004; Hodges and Heistermann, 2011). The most effective way of preserving faeces for prolonged periods of time is undoubtedly freezing at −20 °C (Hodges and Heistermann, 2011; Palme, 2012).

In our studies, post-defaecation metabolism should have no or rare effects on the measured fTM concentrations, as faecal samples in the field were either collected immediately (within minutes) after defaecation or taken manually from the rectum and afterwards either stored in a cold box for less than 3 hours or frozen at −20 °C or in liquid nitrogen at the field station (Article 1, Pribbenow et al., 2015; Article 2, Pribbenow et al., 2016). We also made sure that faecal samples were well-mixed before taking subsamples for fTM analyses.

11. Impact of Biological Factors on Faecal Steroid Metabolites

Throughout their lives organisms are exposed to variations in environmental conditions. They have to cope with these challenges in order to ensure their own survival and success in reproduction. Hormones regulate many aspects of morphological, physiological and behavioural transitions in relation to predictable changes in the environment (Wingfield, 2008). Conversely, hormonal secretion may also be modulated by an organism's environment and behaviour (Hirschenhauser and Oliveira, 2006). Thus, biological factors may hamper the interpretation of fTM measurements. Here, I consider the link between testosterone, aggression and social status, the impact of stress on reproductive physiology, and potential dietary effects on fTM results.

11.1. Testosterone, Aggression and Social Status

Testosterone does not only regulate male reproductive physiology, it may also be correlated with many behavioural traits related to reproduction. Traditionally, testosterone is considered to be a determinant of aggressive behaviour in animals and men, where high testosterone is a prerequisite to aggressive behaviour or social dominance (Wingfield et al., 1990; Christiansen, 1998). Conversely, testosterone concentrations have also been shown to be the result of the outcome of agonistic interactions and dominance behaviour (Christiansen, 1998; Wingfield et al., 2006; Oliveira, 2009). The impact of testosterone on social status and agonistic behaviour has been demonstrated in a variety of rodent, primate and bird species (reviewed by Wingfield et al., 1987; Wingfield et al., 1990; Giammanco et al., 2005). It has been shown that dominant males have higher testosterone levels than subordinate individuals and that social challenges increase circulating testosterone in winners while it decreases in losers (Wingfield et al., 1990; Giammanco et al., 2005). Thus, high endogenous testosterone can be associated with dominance as either a prerequisite or as a consequence of social success, as variation in social status may influence testosterone secretion and subsequently the expression of aggressive behaviour (Giammanco et al., 2005; Wingfield et al., 2006).

The relationship between male social status, aggressive behaviour and testosterone concentration varies among species and even among social conditions within a given species (Wingfield et al., 1990; Wingfield and Hahn, 1994; Whitten et al., 1998; Sannen et al., 2004). To explain the dynamic relationship between testosterone and social behaviour, Wingfield et al. (1990) proposed the "challenge hypothesis". Within this framework they presented a three-level model of circulating testosterone:

> The first level represents the non-breeding baseline of circulating testosterone concentrations. In the second level testosterone rises to a breeding-season baseline stimulated by environmental factors such as photoperiod. This level is sufficient for spermatogenesis and the expression of sexual behaviour and rather not associated with pronounced increases in male aggression rates. However, in response to specific social challenges such as male-male competition over access to mates, territory defence or periods of social instability testosterone may increase to a physiological maximum and in turn, may elevate the intensity and frequency of territorial and mate-guarding aggression.
>
> *Wingfield et al., 1990*

In addition to the proposed framework, testosterone may also vary as a function of the mating system and parental care of a given species: Elevated testosterone secretion in socially monogamous species in which males provide parental care is less pronounced and shorter than in polygamous species with males providing little or no parental care (Wingfield et al., 1990; Wingfield et al., 2001). Inhibitory effects of testosterone on parental care may result in a decrease in the survival of offspring, and thus such effects have consequences in terms of Darwinian fitness, as demonstrated in avian and mammalian species (reviewed by Wingfield et al., 1990; Ziegler, 2000; Wingfield et al., 2001).

Although originally proposed for birds, the challenge hypothesis has also been successfully applied to mammals. In a variety of species, pronounced elevations in male testosterone concentrations have been demonstrated during breeding seasons characterised by increased mate-guarding, male-male interactions and presence of fertile or cycling females [golden lion tamarins, *Leontopithecus rosalia* (Bales et al., 2006); long-tailed macaques, (Girard-Buttoz et al., 2009); Assamese macaques (Ostner et al., 2011); red-fronted lemurs, *Eulemur fulvus rufus* (Ostner et al., 2002) and African wild dogs, *Lycaon pictus* (Creel et al., 1997)]. However, according to the challenge hypothesis an association between social dominance, high testosterone and aggression was only shown in unstable social settings when males experienced acute social challenges such as rank or group instability or risk of infanticide (Sapolsky, 1991; Creel et al., 1997; Cavigelli and Pereira,

2000; Wingfield et al., 2001; Ostner et al., 2002; Beehner et al., 2006). In stable dominance hierarchies social rank, rates of aggression and high testosterone levels are not related (reviewed by Sapolsky, 1993). In contrast, in long-tailed macaques and mandrills, *Mandrillus sphinx*, testosterone levels and dominance rank were positively correlated independently of social instability and mating season, suggesting that these males face permanent contests to maintain their rank to gain access to potential mates (Setchell et al., 2008; Girard-Buttoz et al., 2015). Thus, if male reproductive success is primarily determined by agonistic interactions (e.g. reproductive competition), variation among testosterone levels in males may be predicted by rank or rates of aggression (reviewed by Anestis, 2010), although it remain far from clear for any specific study whether testosterone levels are the cause or the consequence of agonistic interactions and of social dominance. In conclusion, testosterone may regulate aggressive behaviour and social dominance when it is beneficial in a reproductive context but disentangling cause and effect can be challenging (Wingfield et al., 1990).

11.1.1. Testosterone in Spotted Hyenas

Female spotted hyenas remain in their natal clan to breed, whereas males usually disperse and join other clans when they become reproductively active (Frank, 1986). Immigrant males enter the male dominance hierarchy at the lowest rank and improve their status by queuing instead of using agonistic contests (Frank, 1986; East and Hofer, 2001). While queuing, males establish and cultivate relationships with the more dominant females (East and Hofer, 1991, 2001). Females exercise strong mate-choice because the occurrence and position of their peniform clitoris ensures that copulation cannot occur without the complete cooperation of the female (Matthews, 1939; Kruuk, 1972; East et al., 1993; East et al., 2003; Browne et al., 2006; Höner et al., 2007). Thus, close relationships to females are essential for males to gain access to mates and successfully reproduce (East and Hofer, 1991; East et al., 1993; East and Hofer, 2001). Males foster close relationships with females through affiliative interactions; they also follow them ("shadowing") and defend them against other males (East and Hofer, 1991, 2001). In the context of both shadowing and defending, higher-ranking males actively follow and monopolise access to one or more females by preventing more subordinate males from approaching the female, thereby restricting the access of other males to females (East and Hofer, 2001; Goymann et al., 2003). Thus, there is a conflict between males to access females, resulting in male-male competition (Goymann et al., 2003).

In general, mammalian males are thought to be more aggressive than females (Giammanco et al., 2005). However, male spotted hyenas show only low rates and usually only a

low intensity of agonistic interactions towards other males and females (Frank, 1986; East et al., 1993; East and Hofer, 2001). The challenge hypothesis predicts (1) no relationship between testosterone levels and social status when agonistic interactions over access for mates are low and dominance hierarchies are stable, but (2) high testosterone levels in males during mate-guarding of females (Wingfield et al., 1990; Wingfield et al., 2001). Consistent with these predictions, Goymann et al. (2003) demonstrated that in male hyenas neither testosterone, DHT nor androstenedione levels were related to male social status, whereas males defending a female had significantly higher testosterone levels than males not defending a female.

Female dominance is a rare phenomenon amongst mammals and any possible endocrine mechanism associated with female dominance is still unclear. Staub and De Beer (1997) hypothesised that an increased responsiveness to androgens may result in the expression of typical male morphological and behavioural traits in females ("masculinisation" or "virilisation" of females). In spotted hyenas, however, Goymann et al. (2001) demonstrated that circulating androgen levels (e.g. testosterone, DHT and androstenedione) were lower in adult females than in postdispersal males which are known to be sexually mature and reproductively active. Furthermore, *in-utero* administration of antiandrogen drugs did not prevent the development of the peniform clitoris in female spotted hyena cubs (Drea et al., 1998). These results indicate that quantitative sex differences in androgen levels are not the main proximate mechanism underlying female dominance and the development of masculinised genitalia (Drea et al., 1998; Goymann et al., 2001). Similar results were presented for ring-tailed lemurs (von Engelhardt et al., 2000): Adult females did not have higher faecal androgen metabolite (fAM) levels than adult males. Although females showed an increase in rates of agonistic behaviour rates during the mating season, this was not accompanied by any elevation in androgen measurements. Thus, fAM levels, social status, and aggressive behavior were not associated with each other in female ring-tailed lemurs (von Engelhardt et al., 2000). Also, in a group of captive bonobos, *Pan paniscus*, which exhibit a "non-exclusive" female dominance hierarchy (e.g. females maintain the first three high ranks) urinary testosterone metabolite levels were not associated with social dominance, independently of social stability (Sannen et al., 2004). Similarly to spotted hyenas, dominance hierarchies within this study group appeared to be stable and aggression between males or females is rarely seen (Sannen et al., 2004).

In conclusion, neither testosterone-dependent behaviour nor body size appear to be proximately responsible for female dominance, it rather seems to be a feature of behavioural and social conditions (von Engelhardt et al., 2000; Goymann et al., 2001). As proposed by Goymann et al. (2001), female dominance in spotted hyenas appears to be a function

of matrilineal associations, coalitions between females, inheritance of maternal rank and lack of aggressiveness in males, rather than a trait driven by testosterone.

11.2. Stress

The survival of an organism relies on mediators that regulate homeostasis, a dynamic equilibrium at molecular, cellular, physiological and behavioural levels (O'Conner et al., 2000; Charmandari et al., 2005; Chrousos, 2009). Although there is no generally accepted definition of stress, it can be considered as a state of threatened homeostasis that compromises not only immediate survival but other key life history traits such as growth, immune function and reproduction (Hofer and East, 1998; Moberg, 2000; Moore and Jessop, 2003). Intrinsic or extrinsic challenges, including environmental, social and physical stimuli that lead to a departure from homeostasis, are called stressors (Dehnhard et al., 2001; Charmandari et al., 2005). The perception of stressful stimuli results in activation of a suite of physiological cascades and can generate a complex repertoire of physiological, immunological and behavioural responses (Charmandari et al., 2005; Palme, 2012). These responses are normally adaptive and limited in time, allow an individual to cope with challenging situations and ensure survival (Tilbrook et al., 2000; Charmandari et al., 2005; Palme, 2012). Thus, an appropriate responsiveness to stressors represents a crucial prerequisite for well-being and adequate performance (Charmandari et al., 2005). However, a variety of factors are known to modify the response system, thus stress responses may vary considerably between individuals (Dobson and Smith, 2000; Charmandari et al., 2005). For example, an individual's genetic background, sex, body condition, reproductive and social status, as well as exposure to adverse stimuli during pre- and postnatal life determines whether it copes well with a particular stressor (reviewed by Hofer and East, 1998; Dobson and Smith, 2000; Charmandari et al., 2005).

11.2.1. Physiological Response to Stress

The most commonly activated immediate physiological stress response comprises two pathways: the acute "fight or flight" reaction mediated by the sympatho-adrenomedullary (SAM) system, and the activation of the hypothalamus-pituitary-adrenal (HPA) axis (von Holst, 1998; Dehnhard et al., 2001; Chrousos, 2009). The SAM system is activated within seconds of a stressor impacting the organism and results in the release of catecholamines (e.g. epinephrine and norepinephrine) from the adrenal medulla which allows the rapid regulation of vital functions (Chrousos, 2009; Kyrou and Tsigos, 2009). This in turn stimulates accelerated cardiac and respiratory output and an increase in blood flow to

skeletal muscles and brain to maximise an individual's survival (Chrousos, 2009; Kyrou and Tsigos, 2009). Activation of the HPA axis takes place within minutes and results in the release of corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) from paraventricular nuclei of the hypothalamus into the hypophyseal portal system (Viau, 2002; Charmandari et al., 2005; Chrousos, 2009). CRH and AVP induce the synthesis and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH, in turn, stimulates the synthesis and release of adrenocortical glucocorticoids, e.g. cortisol and corticosterone.

Glucocorticoids are the final effector of the HPA axis and exert their effects through ubiquitously distributed intracellular glucocorticoid receptors (GR) that bind to specific glucocorticoid-response elements (GREs) located in the promoter region of target genes upon activation and regulate the expression of glucocorticoid-responsive genes (Charmandari et al., 2005). Similarly to ARs, GRs can also modulate gene expression independently of GRE-binding, by physiologically interacting with other transcription factors (Charmandari et al., 2005). Glucocorticoids stimulate a broad spectrum of physiological responses, primarily catabolic effects such as recruiting available energy resources by increasing gluconeogenesis, lipolysis and protein turnover to defend homeostasis against the imposed stressor (Charmandari et al., 2005; Kyrou and Tsigos, 2009). Glucocorticoids are also crucial for the appropriate termination of the stress response by providing negative feedback at the hypothalamic and pituitary level, as well as at higher brain centres (Charmandari et al., 2005; Kyrou and Tsigos, 2009).

11.2.2. Effects of Stress on Reproduction

Maladaptation to stressors results in a chronically activated HPA axis associated with prolonged glucocorticoid secretion. This may decrease the secretion of gonadal steroids, which in turn may lead to a reduction of sexual activity and behaviour as well as the suppression of reproductive functions (Rivier and Rivest, 1991; Tilbrook et al., 2000).

As reviewed by Tilbrook et al. (2000), stress may impact reproduction at all three levels of the HPG axis: Stress may inhibit the synthesis or secretion of GnRH by modifying the activity of hypothalamic GnRH neurons, alter the responsiveness of pituitary gonadotropins to GnRH, and modify the stimulatory effect of gonadotropins on gonadal hormones. Furthermore, stress may have an impact on the feedback mechanisms and actions of gonadal hormones on the hypothalamus or pituitary (Tilbrook et al., 2000). Studies in dairy cattle demonstrated that stressful clinical conditions and social stressors resulted in reduced fertility by interfering with mechanisms that regulate the precise timing of events within the follicular phase that led to ovulation (Dobson and Smith, 2000). Increased

adrenocortical activity has also been linked to low reproductive success in pygmy rabbits, *Brachylagus idahoensis*, and tigers (Scarlata et al., 2011; Bhattacharjee et al., 2015). In captive female Southern white rhinoceros, *Ceratotherium simum* reproductive acyclicity was associated with high fGM levels (Carlstead and Brown, 2005).

However, the stress-reproduction relationship seems to be context-specific, as elevations of glucocorticoids are not exclusively associated with suppression of reproductive behaviour and physiology, but rather may facilitate specific behavioural and physiological aspects of reproduction (Tilbrook et al., 2000; Moore and Jessop, 2003). In Iberian lynxes, we showed that introduction to a reproduction partner and copulation resulted in elevated fGM concentrations (Pribbenow et al., 2014). Reproductive behaviour has also been demonstrated to trigger adrenocortical activity in a variety of species (Szechtman et al., 1974; Wang et al., 1986; Terio et al., 1999; Kotwica et al., 2002; Howell-Stephens et al., 2012). It is thought that short-term elevations in plasma glucocorticoid concentrations may promote the production of LH and testosterone as the sensitivity of the pituitary to GnRH might be enhanced (Liptrap and Raeside, 1983; Dobson and Smith, 2000; Tilbrook et al., 2000). Thus, the effects of stress and thereby increased adrenocortical activity on reproduction depend on type, duration, and frequency of the given stressor (Dobson and Smith, 2000; Tilbrook et al., 2000).

11.2.3. Stress-Reproduction Relationship in Cheetahs

In captive animals, inappropriate social conditions or difficulties in coping with stressors related to captivity (e.g. public presence, noise, close presence of predators such as lions or spotted hyenas, lack of space and natural substrate) may cause chronic stress and persistent glucocorticoid secretion, which in turn may result in compromised reproductive function (Charmandari et al., 2005; Chrousos, 2009; Romano et al., 2010). Especially in felid species, it has been shown that reproductive success seems to be related to husbandry conditions (Mellen, 1991; Carlstead et al., 1993; Wielebnowski et al., 2002a).

Although cheetahs reproduce rather poor in *ex-situ* conditions, some institutions have bred captive cheetahs successfully, indicating that reproductive success seems to depend on optimal housing conditions and breeding management (Wildt et al., 1993; Bertschinger et al., 2008). Thus, captivity-related stressors are thought to be major factors contributing to the poor health and low reproductive performance of captive cheetahs (Caro, 1993; Terio et al., 2004). Terio et al. (2004) demonstrated evidence for chronic stress indicated by high fGM levels and cortical hyperplasia in captive cheetahs but not in their free-ranging counterparts. Additionally, Wells et al. (2004) showed that cheetahs held in “on-exhibit” enclosures had significantly higher fGM levels than cheetahs in “off-exhibit” enclosures.

In the free-ranging populations, male cheetahs often live in small stable groups or coalitions of two or three individuals, whereas female cheetahs live solitary or with dependent offspring (Caro, 1994). In contrast, captive cheetahs are often housed in pairs or groups that may be detrimental to promote natural courtship behaviour in both sexes (Brown et al., 1996b; Wielebnowski et al., 2002b). The impact of suboptimal housing conditions on reproduction, however, have only been analysed for female cheetahs. Cohabitation of females may result in reproductive suppression, as indicated by ovarian acyclicity (Brown et al., 1996b; Wielebnowski et al., 2002b). In addition, Jurke et al. (1997) demonstrated that acyclic females have higher fGM concentrations than cycling individuals. In male cheetahs, Koester et al. (2015) showed that sperm parameters are affected by housing conditions (e.g. on-exhibit vs. off-exhibit, number of keepers), but this influence was not reflected in faecal androgen output. For their analyses, Koester et al. (2015) used a widespread available EIA relying on an anti-testosterone-antibody that mainly cross-reacts with testosterone and DHT. However, we demonstrated that testosterone in male cheetahs is mainly metabolised via the epiandrosterone pathway (Article 2, Pribbenow et al., 2016). Accordingly, it might be questionable whether husbandry and management conditions really do not affect fTM levels, or if the findings presented by Koester et al. (2015) are driven by missing or minimal cross-reactivities of the used antibody with excreted fTM. In contrast, we confirmed that the epiandrosterone EIA exclusively detects faecal metabolites derived from testosterone degradation by performing a radiometabolism study (Article 2, Pribbenow et al., 2016). Thus, our recently validated epiandrosterone EIA allows a non-invasive, long-term evaluation of testicular activity in male cheetahs.

11.3. Diet

Plasma steroid patterns are reflected in faeces after a species-specific lag time or latency (Palme, 2005; Touma and Palme, 2005). This latency can be affected by digestibility of food, influencing the passage rate and time of digesta, thereby altering reabsorption rates of steroid hormones, metabolism processes, faecal water content, bacterial activity and faecal mass (Adlercreutz et al., 1987; Schwarzenberger et al., 1996; Rabiee et al., 2002). In humans it has been shown that vegetarian high fibre and high protein diet partially interrupted enterohepatic circulation of steroids, resulting in lower faecal oestrogen and testosterone concentrations, respectively, whereas high fat intake resulted in elevated faecal oestrogen concentrations, possibly by increased reabsorption of oestrogens from the intestine (reviewed in Adlercreutz et al., 1987). As the diet of free-ranging animals is rarely uniform, faecal steroid metabolites may be influenced by seasonal changes in food

availability, composition and volume (Schwarzenberger et al., 1996; Dantzer et al., 2011). However, the potential impact of diet on faecal steroid metabolite levels has mainly been analysed in herbivore and omnivore species. In red deer, Millspaugh et al. (2001) and Huber et al. (2003) demonstrated a relationship between seasonal food availability and variations in fGM levels. It is thought that lower food availability associated with decreased caloric intake results in elevated fGM concentrations, thereby reflecting a shift to catabolic metabolism as a physiological adaptation in the absence of adequate food resources, whereas high food availability might result in increased faecal mass, thereby potentially diluting fGM concentrations (Huber et al., 2003). A shift to high energy diet and enriched dietary fibre consumption associated with decreased and increased excreted faecal mass, respectively, has been demonstrated to significantly affect the concentration of excreted glucocorticoid and testosterone metabolites in red squirrels, *Sciurus vulgaris* (Dantzer et al., 2011), mice (Kalliokoski et al., 2015) and dairy cattle (Morrow et al., 2002). However, the impact of diet on faecal steroid metabolites has rarely been analysed in carnivores. Studies in felid species showed that dietary manipulations affect faecal characteristics such as pH, microbial composition and activity (Kerr et al., 2013; Pradhan et al., 2015), which in turn may alter the pattern and concentration of excreted steroid metabolites. Dloniak et al. (2004) determined whether dietary variations might affect fAM in captive spotted hyenas and showed that the presence or absence of bone in the diet did not affect mean fAM concentrations.

In order to minimise any potential dietary effect on fTM concentrations in free-ranging spotted hyenas we freeze-dried faecal samples prior to extraction procedures (Article 1, Pribbenow et al., 2015). Wasser et al. (1993) also suggested drying faeces before analyses to eliminate the influence of water. In both spotted hyena and cheetah there was no difference in the biological information when comparing fTM concentrations of wet and dried faecal samples. Thus, faecal water content did not seem to affect the measurement of fTM levels in either species (Pribbenow et al., unpublished data). This observation could be a result of the low defaecation rates (once or at most twice a day) in both species, so that the "integration" of fTM over a period of 15 h to 22 h may dampen any potential dietary effect. Nevertheless, diet may represent a variable that alters faecal steroid metabolite concentrations unrelated to the endocrine status of an animal, and thus should be carefully considered when conducting non-invasive hormone analyses.

In conclusion, potential post-defaecation metabolism, together with environmental conditions and intra-sample variability, as well as biological factors (e.g. reproductive behaviour and stress responses) should be taken into close consideration if an animal's endocrine status is supposed to accurately and reliably assessed in a non-invasive manner based on faecal samples.

12. Conclusion and Future Perspectives

Animals live in a changing environment in which biotic and abiotic conditions fluctuate over short-term and long-term periods. In this context, hormones are of great interest, as they regulate major biological functions such as development, growth, and reproduction as well as behavioural responses to adapt behaviour to environmental and social stimuli (Wielebnowski and Watters, 2007; Hau and Goymann, 2015). Understanding physiological responses and their regulation in relation to environmental and social conditions are of great importance especially in studies on behavioural ecology and conservation (Whitten et al., 1998; Schwarzenberger, 2007; Hau and Goymann, 2015). Thus, the evaluation of variation in hormonal levels in combination with behavioural, ecological and environmental variables among and within individuals provides insights into the nature of quantitative relationships between hormones and the traits they regulate (Hau and Goymann, 2015).

Non-invasive hormone monitoring provides a powerful alternative to the use of blood samples to assess an animal's endocrine status. Within the framework of the current dissertation we developed and validated methods for the non-invasive monitoring of gonadal activity in both spotted hyenas and male cheetahs using the epiandrosterone EIA. In future, these non-invasive techniques will contribute to the understanding of the basic reproductive physiology of both species and identify typical testosterone patterns of captive and free-ranging individuals, thereby providing insights into selective pressures that shaped the evolution of reproductive and species-specific mate choice strategies. fTM measurements will permit the determination of the impact of hormone-behaviour interrelationships on the reproductive success of individuals, and therefore the viability of populations or species. Since in both species adrenocortical activity can also be monitored non-invasively (Benhaiem et al., 2012; Ludwig et al., 2013), comparative analyses of fGM and fTM measurements will contribute to investigations of reproductive constraints imposed by environmental and social conditions associated with stressful situations. For male cheetahs, fTM measurements will provide essential information on whether and to what extent husbandry and management conditions impact the reproductive performance of captive individuals. Furthermore, monitoring fTM levels in spotted hyenas will allow to determine to what extent individual differences in fTM are related to the context and outcomes of behavioural interactions, reproductive status, social status, age or season, or possibly other behavioural or environmental factors.

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Appendix

Supplementary Article 1

Data on „Measuring faecal epi-androsterone as an indicator of gonadal activity in spotted hyenas (*Crocuta crocuta*)" S Pribbenow, ML East, A Ganswindt, ASW Tordiffe, H Hofer, M Dehnhard.

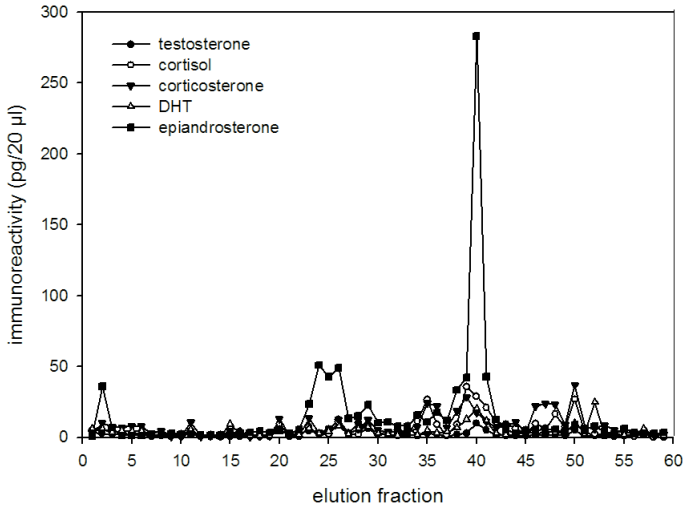

Figure 12.1. Comparison of epi-A immunoreactivity to immunoreactivities of control steroids. HPLC elution fractions from the captive female were analysed in the cortisol-21, corticosterone-21, testosterone and DHT EIAs in comparison to the epi-A EIA.

Table 12.1. HPLC analyses of steroid standards. Standards are measured in the respective EIA. Concentrations presented in pg/20 µl. DHT = dihydrotestosterone.

elution fraction	testosterone	cortisol	corticosterone	dihydrotestosterone	epi-A
1	0,6	0,9	1,6	1,2	0
2	1,1	2,8	7,3	3,7	1,9
3	0,5	1,6	1,5	2,1	1,1
4	0,2	0,6	0,9	1,6	0,6
5	0	0,6	0,9	2,1	0,5
6	0,4	0,6	1	1,5	0,7
7	0	0,8	0,8	2,6	1,5
8	0	0,4	0	0	0,8
9	0	0,4	0,2	0,5	0

Table 12.1. *Continuation.*

elution fraction	testosterone	cortisol	corticosterone	dihydrotestosterone	epi-A
10	0	0,4	0,8	1	1,5
11	0	2,8	0,7	0,5	1
12	0	91,3	0,6	0,9	0
13	0,4	78	0,5	1	0
14	0	5	0,3	0,9	0,9
15	0	1,1	0,3	1,5	1,9
16	0	0,4	0	0,8	1
17	0	0,4	0	0	0
18	0	0,4	1	0,5	1,7
19	0	2,2	1,1	0	1,4
20	0	0,4	0,8	0,8	0
21	0	0,4	1,8	0,8	0
22	0	0,5	34,7	1	0,6
23	0	0,4	1215,6	1,2	1,6
24	0	0,4	280,1	0,7	1
25	0	0	12,3	0	0,6
26	0,6	0,4	8,3	0	1,8
27	0	0,4	2,9	0	1,7
28	0	0,4	1,6	0,7	0
29	0	0,4	1,1	0,4	0
30	0	0,4	1,1	0	0,4
31	0	0,4	2,6	1,1	1,8
32	0	0,3	1,2	0	0,5
33	0	0,4	1,3	1	0
34	0	0,4	1,5	0,9	1,8
35	100	3,9	0,9	1,4	1,2
36	236,3	3,4	0,9	4,5	0
37	14,4	0,6	0	0,9	0
38	2,7	0,4	0	0,9	0
39	0,7	0,4	0	1,3	23,9
40	0,3	0,4	0,9	186,9	78,1
41	0,3	0,4	3,6	635,4	7
42	0	0,4	1	244	2,8
43	1,5	0,4	0,4	24,6	1,5
44	0,8	0,4	0,5	6	0,6
45	0	0,4	0	2	0,5
46	0	0,4	6,6	1,4	1,4
47	0	0,4	0	1,2	1,7
48	0	0,4	0,4	1,2	1
49	0	0,4	0,2	0,9	0,9
50	0	0,4	0,3	1	2,2
51	0	0,4	0	1	1
52	0	0,4	0	1,5	0,7
53	0	0,4	0	3	0,8
54	0	0,4	0	1,2	0,8
55	0	0,6	0	0,9	2,1
56	0	0,4	0,5	1,1	1,2
57	0	0,4	0,5	0,8	0,6
58	0	0,4	0,3	1,7	2,1
59	0,3	0,4	0	0,8	1,1

Table 12.2. Radiometabolism study female A after C18 purification. Radioactivity presented as cpm/100 µl.

Lab-ID	hr after injection	radioactivity
38	-144,0	0
40	-120,0	0
42	-96,0	0
44	-72,0	0
46	-24,0	0
48	-1,3	0
49	15,5	855
50	22,3	629
51	44,7	214
52	45,0	370
54	67,0	112
56	69,5	39
61	118,0	13
62	118,5	7
64	141,8	6
66	167,0	0
67	172,5	0
70	189,0	0

Table 12.3. Radiometabolism study male A after C18 purification. Radioactivity presented as cpm/100 µl.

Lab-ID	hr after injection	radioactivity
5	-18,2	37
9	-1,5	29
12	6,9	37
32	47,3	1041
36	71,3	263

Table 12.4. HPLC analyses of radiolabelled fTM of female A and male A after C18 purification without hydrolysis. Radioactivity presented in cpm/100 µl.

elution fraction	female A	male A
1	1	94
2	962	74
3	478	133
4	164	13
5	36	11
6	24	8
7	7	4
8	8	10

Table 12.4. *Continuation.*

elution fraction	female A	male A
9	10	7
10	5	9
11	3	3
12	8	15
13	6	14
14	16	18
15	14	10
16	14	4
17	11	5
18	3	0
19	10	9
20	5	8
21	2	5
22	9	9
23	10	11
24	6	9
25	9	13
26	45	7
27	10	8
28	12	4
29	23	3
30	23	6
31	13	8
32	12	6
33	10	8
34	10	3
35	16	15
36	9	13
37	5	19
38	3	8
39	11	8
40	26	30
41	5	19
42	13	19
43	10	18
44	7	16
45	5	19
46	11	20
47	11	9
48	8	12
49	3	5
50	5	1
51	5	6
52	9	14
53	6	8
54	0	7
55	0	6
56	0	14
57	7	5
58	7	13

Table 12.4. *Continuation.*

elution fraction	female A	male A
59	0	9

Table 12.5. HPLC analyses of radiolabelled fTM of female A and male A after C18 purification after hydrolysis using β-glucuronidase from *Hp.* Radioactivity presented in cpm/100 µl.

elution fraction	female A	male A
1	6	14
2	6	7
3	8	7
4	6	17
5	18	6
6	0	4
7	11	16
8	2	9
9	7	4
10	7	9
11	3	10
12	12	9
13	0	14
14	4	8
15	5	7
16	4	13
17	14	16
18	16	10
19	4	8
20	13	16
21	2	12
22	7	8
23	11	3
24	10	10
25	2	25
26	16	11
27	18	9
28	11	6
29	8	13
30	10	6
31	17	25
32	4	15
33	8	7
34	6	23
35	1	13
36	4	18
37	0	18
38	6	22
39	27	13
40	54	74

Table 12.5. *Continuation.*

elution fraction	female A	male A
41	26	21
42	4	23
43	14	7
44	13	16
45	12	40
46	0	17
47	10	18
48	15	17
49	10	13
50	8	11
51	13	9
52	6	10
53	4	7
54	4	17
55	4	17
56	12	3
57	5	1
58	5	5
59	5	12

Table 12.6. Comparison of epi-A immunoreactivity in faecal samples of female A to immunoreactivities of control steroids (cortisol, corticosterone, testosterone and DHT). Immunoreactivities were measured in the respective EIAs after hydrolysis using β-glucuronidase from *Hp*. Concentrations presented in pg/20 µl. DHT = dihydrotestosterone.

elution fraction	radioactivity	epi-A	cortisol	corticosterone	testosterone	DHT
1	6	0,9	4,9	3,4	3,9	5,9
2	6	36	5,3	10,1	2,7	5,9
3	8	6,6	4,4	7,5	2	3,4
4	6	1,2	3,3	7	2,3	4,5
5	18	1,1	1,8	8	1,4	4,2
6	0	1,1	3,8	7,7	1,7	4,6
7	11	1,8	0,6	2,7	0,9	1,4
8	2	1,6	1,3	4,5	1,6	2,3
9	7	2,8	0,5	0,7	0,7	1,4
10	7	2,4	0,5	0,8	0,8	1,5
11	3	2,3	4,5	10,6	2,9	5,2
12	12	1,7	0,3	0,7	0,5	0,9
13	0	1,8	0,4	1,2	0,7	1,4
14	4	1,5	0,2	1,2	0,8	1,2
15	5	4	0,6	1,4	0,9	9
16	4	2,6	0,7	1,3	3,9	3,3
17	14	3,4	0,4	0,3	2	1,3
18	16	4,5	0,5	0,8	1	1,1
19	4	3,4	0,7	1	0,8	1,2
20	13	4,4	9,8	13,1	5,3	5,4

Table 12.6. *Continuation.*

elution fraction	radioactivity	epi-A	cortisol	corticosterone	testosterone	DHT
21	2	2,7	0,4	1,4	1,7	1,7
22	7	5,3	0,3	1,4	2,3	1,6
23	11	23,5	7,2	13,4	4,6	6,3
24	10	51	2,2	3,2	2,4	2,5
25	2	42,6	2,5	5,9	5,9	4
26	16	49	10,6	12,2	12,4	7,6
27	18	13,3	2	2,5	2,8	2,4
28	11	15	1,9	5,9	10,1	6
29	8	23	7,3	12,3	6,1	7
30	10	10,3	3,3	4,8	2,3	2,5
31	17	10,6	1,7	3,7	1,7	2
32	4	8	1,6	3,8	0,9	1,3
33	8	7,9	2,9	3,2	1,5	7,3
34	6	15,7	11,4	7,2	1,2	1,6
35	1	10,9	26,6	24,2	3	4,9
36	4	17,5	9,1	22,1	2	2,9
37	0	12	1,6	6,9	1	3,7
38	6	33,4	9,1	18,5	1,9	6,5
39	27	42,2	35,4	28,5	2,9	12,7
40	54	282,8	28,9	17,3	9,8	20
41	26	42,7	21,1	11,4	5,1	11,5
42	4	12,4	10,5	6,8	1,4	2,7
43	14	5,4	9,3	8,2	1,3	1,6
44	13	2,8	6,2	10,7	0,9	1,5
45	12	5,2	2,3	2,8	0,9	4,7
46	0	4,8	9,7	21,9	1,8	2,8
47	10	6	6,1	23,7	1,6	3,7
48	15	5,6	16,4	23,3	1,5	3,5
49	10	3,8	7,1	8,9	1,2	2,9
50	8	9	26,8	37	4,9	9,6
51	13	6,1	2,9	7	1,5	3,8
52	6	7,8	2,1	6,2	0,9	24,6
53	4	4,1	1,7	8,5	0,8	2,4
54	4	2,4	1,2	5,2	0,5	2,6
55	4	5,9	0,8	3,3	1,1	1,6
56	12	3,5	0,8	3,1	1,2	1,8
57	5	2,4	2,9	2,8	1,9	5,9
58	5	2,7	0	1,7	0,9	1,5
59	5	3,4	0	2	0	1,3

Table 12.7. HPLC analyses of faecal samples from captive female A, captive male A, free-ranging female B and free-ranging male B without hydrolysis. Immunoreactivities were determined with the epiandrosterone EIA, presented as pg/20 µl.

elution fraction	female A	male A	female B	male B
1	1,3	1,4	2,4	3,9

Table 12.7. *Continuation.*

elution fraction	female A	male A	female B	male B
2	185,1	3	4,8	4,8
3	805	3,8	8,9	5,6
4	922,3	4,1	3,9	4,1
5	102,3	2,8	2,5	12,5
6	34,4	2,8	2,6	2,6
7	17,8	3,8	3,4	2,7
8	24,3	3,8	4,3	2,9
9	20,6	2,3	10,1	2,5
10	16,6	3,1	2,4	4,4
11	16,9	4,2	1,6	2
12	12,1	3,2	4,6	4,8
13	6,7	2,9	2,1	4,9
14	8,8	4,2	2,8	4,1
15	9,5	5,5	3,4	5,7
16	6,7	7,5	3,1	4,2
17	7,2	3,6	4,5	4,4
18	5,8	3,5	4,3	5,3
19	3,8	4,9	6,1	4,9
20	3,8	6,6	9,3	6,9
21	3	22,4	2,5	4,5
22	8,8	26,3	6,5	6,5
23	6,1	13,1	6,5	7,6
24	6,6	14,9	8,6	9,3
25	21,3	15,1	20,4	30,7
26	44,9	5,2	26,2	31,6
27	15	7,5	11,2	8,8
28	17,7	16,3	7,6	10,3
29	35,4	24,5	8,2	16,7
30	14,1	22,5	2,8	9
31	16	30,1	5,2	8,7
32	8	20,2	4,9	6,2
33	6,4	14	11,7	9,1
34	13,7	21,9	9,2	8,4
35	11	16,7	11,1	7,7
36	18,9	22,3	10,3	10,4
37	9,7	20,1	14,6	10,6
38	43,3	8,4	62,1	47,1
39	48,8	19	36,8	35,9
40	194,3	7,5	176,2	200
41	40,5	5,1	23,8	69,5
42	16,2	14,8	9,6	13,1
43	13,2	6,9	6,3	10,6
44	11,8	7,1	3,8	7,5
45	10,3	3,9	45,2	6,9
46	9,4	2,9	17,5	7,5
47	5,9	7,1	13,8	6,8
48	7,2	1,8	20,9	6,9
49	4,3	2	13,4	8,4
50	7,1	3	20,2	7,1
51	9	2	14,5	6,5

Table 12.7. *Continuation.*

elution fraction	female A	male A	female B	male B
52	8,5	2,5	5,9	6,4
53	3,6	2,8	4,2	4,1
54	5	2,5	3,1	3,4
55	7,1	1,7	5,4	9
56	8,4	0,9	6	4,3
57	6	0	3,7	7,2
58	4,2	1,1	5,8	7
59	5,8	4,3	3,1	4,9

Table 12.8. HPLC analyses of faecal samples from captive female A, captive male A, free-ranging female B and free-ranging male B after hydrolysis using β-glucuronidase from *Hp*. Immunoreactivities were determined with the epiandrosterone EIA, presented as pg/20 µl.

elution fraction	female A	male A	female B	male B
1	0,9	0	1,6	0
2	36	2,2	2	0
3	6,6	2	1	1,4
4	1,2	3,8	1,5	0
5	1,1	1,8	2,5	0
6	1,1	1,5	1,2	0
7	1,8	1,3	2,2	0
8	1,6	2,2	3	1,1
9	2,8	1,8	2,8	0
10	2,4	1,9	3,1	0
11	2,3	1,8	3,9	3,2
12	1,7	5,9	3,5	0,6
13	1,8	1,5	6,2	0
14	1,5	1,7	3,2	0
15	4	2,4	4,2	0
16	2,6	5,5	2,9	3,9
17	3,4	3,9	2,1	0
18	4,5	2,7	3,6	0
19	3,4	5,4	3,8	2
20	4,4	10,8	4,8	2,1
21	2,7	5,5	4,7	0
22	5,3	24,8	4,6	0
23	23,5	6,9	4,4	1,7
24	51	5,8	4,1	5,1
25	42,6	16,2	17,4	2,9
26	49	20,1	18,2	0,7
27	13,3	16,1	5,7	3,2
28	15	10,6	5,2	0,6
29	23	5,2	5,9	0
30	10,3	13,3	4,4	0
31	10,6	9,9	4,1	2
32	8	7,9	2,5	2,6

Table 12.8. *Continuation.*

elution fraction	female A	male A	female B	male B
33	7,9	17,6	5,9	0
34	15,7	18,7	5,5	0
35	10,9	15	5,4	0
36	17,5	19,7	4,8	0
37	12	15,6	9,3	2,6
38	33,4	26,3	37,4	3,1
39	42,2	25,9	24,5	5,7
40	282,8	92,5	89,9	35,4
41	42,7	19,8	17,3	5,1
42	12,4	8,7	6,1	3,5
43	5,4	4,5	5	12,6
44	2,8	3	4,8	1
45	5,2	7,3	3,6	0,4
46	4,8	9,4	15,5	0
47	6	4,6	9,8	0
48	5,6	20,7	12,7	2,2
49	3,8	1,3	8,6	0
50	9	7,3	18,8	1,9
51	6,1	11,8	7	0,9
52	7,8	4	4,4	1,9
53	4,1	2,2	2,8	3,6
54	2,4	2,3	4	0
55	5,9	3	4,3	1,6
56	3,5	10	5,8	2,9
57	2,4	2,1	6,1	0
58	2,7	1,7	5,8	0,6
59	3,4	2	5	2,6

Table 12.9. Testosterone challenge. Analyses of faecal samples from female A after testosterone injection. Immunoreactivity determined with the epiandrosterone EIA, presented as µg/g.

Lab-ID	hr after injection	epi-A
38	-144	0,6
40	-120	0,1
42	-96	0,8
44	-72	3,7
46	-24	0,7
48	-1,25	0,7
49	15,5	3,2
50	22,25	0,6
51	44,66	0,7
52	45	1
54	67	0,6
56	69,5	0,9
61	118	0,7
62	118,5	0,7

Table 12.9. *Continuation.*

Lab-ID	hr after injection	epi-A
64	141,75	1
66	167	1
67	172,5	2,1
70	189	1,3

Table 12.10. Comparison of immunoreactivities in faecal samples from the testosterone challenge in female A after hydrolysis with *Hp*-enzymes and *Ec*-enzymes. Immunoreactivity determined with the epiandrosterone EIA, presented as µg/g.

hr after injection	Hp	Ec
-144	0,6	0,2
-120	0,9	0,7
-96	1	0,9
-72	2,1	1,3
-24	0,9	0,4
-1,3	0,5	0,2
15,5	1,3	0,8
22,3	0,6	0,3
44,7	0,2	0,3
45	0,5	0,5
67	0,5	0,3
69,5	0,2	0,2
118	0,2	0
118,5	0,3	0,2
141,8	0,3	0,2
167	0,5	0,4
172,5	0,3	0,4
189	0,6	0,3

Table 12.11. Comparison of fTM concentrations of free-ranging juvenile male and adult male spotted hyenas. Immunoreactivities determined with the epiandrosterone EIA, presented as ng/g.

Sex	epi-A
juvenile1	391,9
juvenile2	800,5
juvenile 3	975,2
juvenile 4	555,9
juvenile 5	750,9
juvenile 6	1126,6
juvenile 7	340,5
juvenile 8	1277,8
juvenile 9	423,3

Table 12.11. *Continuation.*

Sex	epi-A
juvenile 10	272,7
juvenile 11	1295,1
juvenile 12	1232,7
juvenile 13	647,4
juvenile 14	513,2
juvenile 15	384,1
male1	1184,8
male2	2173,3
male3	2451,9
male4	2434,9
male5	1877,9
male6	1255,1
male7	4259,8
male8	804,2
male9	355,1
male10	179,3
male11	2310,5
male12	1767,3
male13	716,2
male14	283,9
male15	1566

Table 12.12. Mean ± SD fTM concentrations of juvenile male and adult male spotted hyenas. Immunoreactivities determined with the epiandrosterone EIA, presented as ng/g.

	juvenile males	adult males
mean fTM	732,5	1574,7
SD	365,2	1083,4

Supplementary Article 2

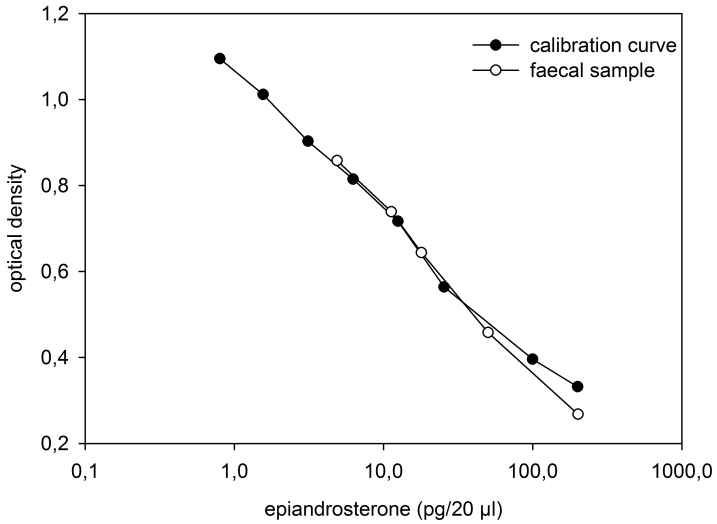

Figure 12.2. Semi-logarithmic description of a parallelism test for epiandrosterone (epi-A). Serial dilutions from a faecal extract of M1 were subjected to the epi-A EIA together with standards for epi-A ranging from 0.4 to 200 pg/20 µl. Similarity of the curves indicates a high degree of parallelism, excluding matrix effects.

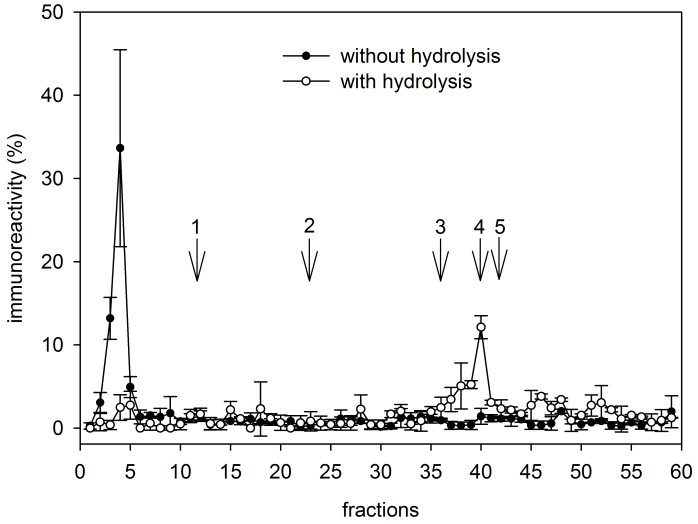

Figure 12.3. HPLC profiles of immunoreactive fTM in two captive Namibian male cheetahs (M4, M5). Immunoreactive fTM were determined with an epi-A EIA in non-hydrolysed (black circles) and hydrolysed (white circles) faecal extracts, respectively, and presented as percentage of overall eluted activity (mean ± SD). Arrows indicate the elution positions of steroid standards (see Fig. 7.1).