EXCRETION OF POTENTIALLY BIOLOGICAL ACTIVE COMPOUNDS OF GLUCOCORTICOID METABOLITES (C_{19}O_{3}) VIA ANIMAL MANURE

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By:


Omer Elfaroug Ibrahim Sid Ahmed Ismail

Vienna, October 2013
Supervisor:
A.Univ.Prof. Dr.med.vet. Erich Möstl
Department of Biomedical Sciences
Institute of Medical Biochemistry
University of Veterinary Medicine Vienna

Reviewer:
A.Univ.Prof. Dr.med.vet. Rupert Palme
Department of Biomedical Sciences
Institute of Medical Biochemistry
University of Veterinary Medicine Vienna
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Dedication

To my father’s soul,
To my mother, brother, sisters, nieces and nephews.
To my wife Shiraz with everlasting love.
Thank you all for your great support during hard days.

Omer,

Vienna, October 2013
Abbreviations

11,17-DOA  11,17-Dioxoandrostane
11-KT  11-Ketotestosterone
11-OE  11-Oxoetiocholanolone
11ß-OH-A  11ß-hydroxyandrostenedione
ACTH  Adrenocorticotrophic Hormone
AKR1D  Aldo-Keto Reductase Family 1 Dehydrogenase
AR  Androgen Receptor
BSA  Bovine Serum Albumin
CAFOs  Concentrated Animal Feeding Operations
DDW  Double Distilled Water
DMSO  Dimethyl sulfoxide
EDCs  Endocrine Disruptor Compounds
EPA  Environmental Protection Agency
FCM  Faecal Cortisol Metabolites
GC  Glucocorticoid
GR  Glucocorticoid Receptor
HPLC-ESI-MS  High Performance Liquid Chromatography-Electrospray ionization-Mass Spectrometry
HPLC-MS  HPLC-Mass Spectrometry
P4  Progesterone
RP-HPLC  Reverse Phase-HPLC
SCC  Side-chain Cleavage
SRD5A  Steroid 5α Reductase Gene
WK  week
WWTPs  Waste Water Treatment Plants
List of Figures

Figure 1 Numbering of carbon atoms around the glucocorticoid nucleus (A, B, C, and D-ring). ....11
Figure 2 Schematic illustration of side-chain cleavage of GC. ..............................................................11
Figure 3 The chemical structure of 5α-11-oxo-17β-hydroxyandrostan and 5α-11,17-dioxoandrostan configurations. ........................................................................................................................17
Figure 4 Production of 11β-hydroxyandrostedione. ..................................................................................38
Figure 5 Excretory profile (ng/g faeces) of 5α- and 5β-reduced 11,17-DOA metabolites in cattle before and after ACTH injection. ....................................................................................................48
Figure 6 RP-HPLC immunogram of C19O3 immunoreactive substances in ACTH injected cattle........50
List of Tables

Table 1 Different buffers and solutions used in the enzyme immunoassays........................................ 43
Table 2 Specificity of the 5α-androstan-11,17-dione antibody for different steroids having structural similarities.................................................................................................................... 46
Table of Contents

Acknowledgement .......................................................................................................................... 3
Dedication .................................................................................................................................... 4
Abbreviations ............................................................................................................................... 5
List of Figures ............................................................................................................................... 6
List of Tables .................................................................................................................................. 7
1. General Introduction .................................................................................................................. 10
   1.1. Glucocorticoid hormones: Biosynthesis, biomedical importance, and metabolism .......... 10
   1.2. Reduction of the ring-A of glucocorticoids to 5α/5β forms and their biological activity .... 11
   1.3. Cortisol metabolism in cattle and sheep ............................................................................ 12
   1.4. C₁⁹O₃ cortisol metabolites and animal welfare ............................................................... 13
   1.5. Steroid hormones and aquatic organisms ........................................................................ 13
   1.6. Microbial side-chain cleavage (SCC) in animal manure ................................................. 14
   1.7. Glucocorticoid metabolites as pheromones in fish ......................................................... 15
   1.8. C₁⁹O₃ metabolites derived from GCs in the environment ............................................... 16
   1.9. Aim of the investigations ................................................................................................. 16
2. Publication: Increased immunoreactive 11-ketotestosterone concentrations in sheep feces after 
   ACTH challenge ....................................................................................................................... 18
3. Formation of 5α-reduced C₁⁹O₃ immuno-reactive metabolites with an 17-oxo group in cattle 
   faeces following ACTH injection ............................................................................................ 37
   3.1. Introduction ....................................................................................................................... 37
   3.2. Materials and Methods ..................................................................................................... 41
      3.2.1. Animals and ACTH injection ..................................................................................... 41
      3.2.2. Extraction of faecal samples ...................................................................................... 41
      3.2.3. EIA procedure ........................................................................................................... 42
      3.2.4. Assay validation ......................................................................................................... 44
         3.2.4.1. Cross-reactivity, sensitivity and precision ............................................................... 44
         3.2.4.2. Physiological validation ....................................................................................... 44
         3.2.4.3. HPLC separation of immuno-reactive metabolites ............................................... 44
   3.3. Results .................................................................................................................................. 46
      3.3.1. Assay validation ........................................................................................................... 46
      3.3.2. Faecal excretory profile of 5α-reduced 11,17-DOA metabolites in cattle .................... 47
      3.3.3. HPLC separation of 5α- and 5β-reduced C₁⁹O₃ metabolites ..................................... 49
   3.4. Discussion ............................................................................................................................. 51
4. Conclusion and recommendation ................................................................. 55
5. Summary ........................................................................................................ 56
6. Zusammenfassung .......................................................................................... 58
7. References ..................................................................................................... 60
8. Appendix: ....................................................................................................... 66
1. General Introduction

1.1. Glucocorticoid hormones: Biosynthesis, biomedical importance, and metabolism

Glucocorticoids (GCs) including cortisol and corticosterone, are a group of steroid hormones synthesized in all vertebrates from the adrenal cortex (Zona fasciculata) via hypothalamic–pituitary–adrenal axis stimulation and have the property of binding to and activating the GC receptor (GR). GCs are essential for life because of their regulatory mechanisms of variety of important biological functions such as cardiovascular, metabolic, immunologic, and homeostatic. GCs containing a hydrogenated cyclopentano-perhydro-phenanthrene ring system are produced from cholesterol via 20,22-lyase activity through a set of enzymes. The general chemical structure of GCs and carbon atom numbering around the steroid nucleus is provided in figure 1. Glucocorticoids are frontline hormones to cope with stressful situations. After secretion into blood circulation, GCs are exposed to extensive metabolism in the body (hydroxylation, reduction of double bonds, conjugation with sulphates and glucuronides). A minor part is excreted without metabolism via the bile into the faeces or via the urine (TAYLOR 1971; PALME et al. 1996; TESKEY-GERSTL et al. 2000; SCHATZ and Palme 2001). In the gut, GCs are further converted by anaerobic intestinal bacteria (WINTER and BOKKENHEUSER 1979; BOKKENHEUSER et al. 1986) able to transform the ring-A and ring-D of the steroid or the 17-oxo-group or by C17-C20 side-chain cleavage (SCC) (MARTIN et al. 1975; GROH et al. 1993) forming C19 steroids (Figure 2).
11

Figure 1 Numbering of carbon atoms around the glucocorticoid nucleus (A, B, C, and D-ring).

![Figure 1](image1)

Figure 2 Schematic illustration of side-chain cleavage of C₂₁ GC at positions C-17 and C-20 via the activity of 17-hydroxylase/C-17,20-lyase to form C₁₉O₃ androgen metabolites.

![Figure 2](image2)

1.2. Reduction of the ring-A of glucocorticoids to 5α/5β forms and their biological activity

Reduction of GCs ring-A to 5α-reduced metabolites is achieved by hepatic enzymes that belong to a large family of genes encoded by *SRD5A1* and *SRD5A2* containing domains that catalyze the reduction of steroids ring-A to the 5α-configuration. Similarly, the 5β-reductase
enzymes are highly expressed in hepatic cells which are part of the aldo-keto reductase superfamily (AKR1D) (KIMURA et al. 1998). In mammals, it has been thought for long time that the reduction of endogenous GCs (cortisol and corticosterone) to their 5α- and 5β-reduced metabolites is the principal inactivation process in the liver to yield more polar metabolites that can be easily eliminated via kidneys. However, it is well demonstrated that 5α- and 5β-reduced metabolites possess biological activity and that they are even more potent than the parent steroid as for example the 5α-dihydrotestosterone (DHT) which is the reduced form of testosterone (binds tightly to the androgen receptor). Furthermore, the reduced forms of C21 GCs have the ability to bind and activate the glucocorticoid receptor (reviewed by NIXON et al. 2012).

1.3. Cortisol metabolism in cattle and sheep

In cattle urine, it has been recently reported that the 5β-reduced forms of tetrahydrocortisol are more abundant than the 5α-reduced metabolites (PAVLOVIC et al. 2013). Using high performance liquid chromatography-electro-spray-ion-mass-spectrometry (HPLC-ESI-MS) the authors suggested that the 5α-reduction is virtually absent in cattle and not like that of human where 5α-reduction is dominant. In sheep, LINDER (1972) reported that cortisol is excreted via the bile as tetrahydrocortisol and tetrahydrocortisone metabolites. Using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS), MÖSTL et al. (2002) and PALME et al. (1996) reported that the cortisol is metabolized yielding different C19O3 and C21 metabolites.
1.4. C$_{19}$O$_3$ cortisol metabolites and animal welfare

Over the last few decades, the high correlation between serum cortisol levels and its 5β-reduced C$_{19}$O$_3$ (faecal) metabolites, made the quantification of the later the method of choice for most researchers concerned with animal welfare issues in ruminants and some other species as there is no need to strain the animal for sampling which avoids sampling-induced stress. Therefore, with the advancement of enzyme immunoassay (EIA) techniques, several EIAs measuring 5β-reduced C$_{19}$ metabolites have been developed and validated in different animal species to monitor the adrenocortical activity and reflect the physiological status of the animals. From a chemical point of view, it has been shown that the increased formation of 5β-androstane-11,17-dione metabolites was obvious when bovine faecal samples were stored at room temperature for up to 24 h, suggesting a SCC of C$_{21}$ cortisol metabolites (i.e. 17α-hydroxylated corticoids) by faecal microorganisms (MÖSTL et al. 2002) as a matter of microbial 17,20 lyase activity. Additionally, a similar scenario in sheep faeces reported that the formation of 5β-reduced metabolites was increasing within 2 h following spontaneous defecation (LEXEN et al. 2008).

1.5. Steroid hormones and aquatic organisms

The contribution of livestock and humans to the environmental load of steroid hormones has become an international concern as some of the excreted biologically active gonadal metabolites are suspected to be androgenic/oestrogenic substances for aquatic organisms via soil runoff from concentrated animal feeding operations (CAFOs) where animal manure is spread. Several steroids with varying concentrations have been reported in different environmental matrices. For example, manure originating from cattle and poultry has been found as a source of
testosterone and oestrogens (LEE et al. 2003). The contribution of humans to the steroids introduced into the environment is mainly through treated and untreated effluents and sludge from waste water treatment plants (WWTPs) (LIU et al. 2012).

Over the last few decades several investigations have been done to detect anthropogenic and animal steroid hormones, or their metabolites released into the environment that may interfere with or disrupt hormonal homeostasis of aquatic organisms as endocrine disruptive compounds (EDCs). EDCs are defined by KAVLOCK et al. (1996) as “an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development and/or behavior”.

Soil runoff from agricultural fields where the animal dung is spread (mainly from CAFOs) constitutes the major source of biologically active steroid metabolites. The immediate survival of aquatic organisms is not linked directly to reproduction, but fecundity may be reduced due to frequent exposure to pheromones and/or androgens that produce behavioural responses and mediate complex and critical reproductive functions leading to decreased numbers in succeeding generations.

1.6. Microbial side-chain cleavage (SCC) in animal manure

In animal manure, microbial induced transformation of steroids to produce different chemical configurations varies between species with regard to pH optima, enzyme kinetics, and substrate specificity. For example, WINTER et al. (1984) reported that anaerobic colonic bacteria (Clostridium scindens) isolated from human faeces possess 17,20 lyase activity, which is capable to transform GCs to C19 steroids via the SCC at ring-D of the steroid. The authors stated that the 17,20 lyase enzyme has
specific substrate requirements such as an $\alpha$-hydroxy group at C-17 and preferably an $\alpha$-ketol group at C-20-C-21 position, while chemical configurations at position C-11 (hydroxyl group, ketone, or hydrogen) and the presence or absence of a conjugated double bond in ring-A had no effect on the enzymatic reaction. Furthermore, lyase activity of anaerobic bacteria from sewage and cat faeces has been shown to share some similarities (BOKKENHEUSER et al., 1986). These authors suppose species differences in terms of substrate specificity of these microbes. Since the 1950s, $5\beta$-reduced metabolites are known as important $C_{19}$ compounds of SCC of cortisol (BURSTEIN et al. 1953a; BURSTEIN et al. 1953b; DORFMAN 1954). However, knowledge about $5\alpha$-reduced metabolites and their biological activity in animal manure is still scarce.

1.7. **Glucocorticoid metabolites as pheromones in fish**

In fish, the olfactory epithelium constitutes the frontline of endocrine communication between males and females which governs a multitude of behavioral and reproductive responses when exposed to pheromones (STACEY et al. 2003). Interestingly, using electro-olfactography (EOG) technique, unconjugated 11-oxoetiocholanolone ($3\alpha$-hydroxy-$5\beta$-androstane-11,17-dione) which is a metabolite produced by reduction and side-chain cleavage of cortisol and its $C_{21}$ metabolites in animal manure, has been recently demonstrated to act as fish pheromone in round gobies (*Neogobius melanostomus*) by attracting non-reproductive mature females (TIERNEY et al. 2013). It has been shown that the fish olfactory epithelia can detect several steroids and prostaglandins with high sensitivity and specificity (reviewed by STACEY et al. 2003) related to different molecular configurations within the steroid nucleus which results in species specific attractants. Given the fact that different metabolic reduction reactions could occur within A-ring of $C_{21}$ steroids following
the SCC, several unconjugated chemical configurations can be formed (e.g., 3α/3β-hydroxy-5α/5β-11,17-dioxoandrostanes) that could be detected by fish species as reported in round gobies (TIERNEY et al. 2013). However, species specificity of the various C19O3 configurations in terms of the pheromonal substances has been scarcely investigated in fish.

1.8. C19O3 metabolites derived from GCs in the environment

Most research work thus far has focused on the quantification and estimation of excreted oestrogens, gonadal androgens, and gestagens in different farm animals (LANGE et al. 2002), municipal biosolids, and animal manure (LORENZEN et al. 2004), but the formation and excretion of C19O3-androgens either 5α- or 5β-reduced was neglected, even though available literature suggests that 5α- and 5β-reduced glucocorticoid metabolites possess potential biological activity. Interestingly, it has been recently reported that the C19O3 cortisol metabolite 5α-androstane-3,11,17-trione acts similarly as the major fish androgen, 11-ketotestosterone (11-KT) in medaka fish by causing the masculinization of female fish (GRILLITSCH et al. 2010). This finding should be considered as an emerging issue with regard to endocrine disruptive compounds originating from GCs transformation to produce possible active androgens mimicking the endogenously produced fish androgens. However, to the best of my knowledge no studies dealing with the formation of C19O3 metabolites derived from the adrenal or by the metabolism of GCs in animal manure have been performed up to now.

1.9. Aim of the investigations

As reviewed in the literature, excretion of potentially active GC metabolites (C19O3) in animal manure having a 5α-androstan-11-oxo-17β-hydroxy or 5α-androstane-11,17-dioxo configuration (Figure 3) has never
been investigated. Furthermore, available evidence has been recently reported that the cortisol metabolite 5α-androstane-3,11,17-trione acts as an active androgenic compound for medaka fish (GRILLITSCH et al. 2010). Therefore, the aims of this thesis were:

1. To investigate the formation of 5α-reduced C₁₉O₃ metabolites in sheep faeces by the development and biological validation of an 11-ketotestosterone EIA.
2. To investigate the formation of 5α-reduced C₁₉O₃ metabolites having an 11,17-dioxoandrostan (DOA) configuration in cattle faeces by the development and biological validation of an EIA capable to detect metabolites with this chemical configuration.

Figure 3 The chemical structure of 5α-11-oxo-17β-hydroxyandrostane (A) and 5α-11,17-dioxoandrostan (B) configurations.
Publication: Increased immunoreactive 11-ketotestosterone concentrations in sheep feces after ACTH challenge

OMER SID-AHMED, NINO ARIAS, RUPERT PALME, and ERICH MÖSTL*

Department of Biomedical Sciences, Institute of Medical Biochemistry, University of Veterinary Medicine, Vienna, Austria.

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* Address correspondence to erich.moestl@vetmeduni.ac.at.

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Abstract

11-Oxoetiocholanolone and related substances are important metabolites of cortisol and are excreted via feces in ruminants. To investigate whether 11-ketotestosterone (11-KT) or its immunoreactive metabolites are formed and excreted in ruminant feces, an enzyme immunoassay (EIA) was developed and validated. The antibody was raised in rabbits against 11-KT-3-CMO:bovine serum albumin with biotinylated 11-KT as a label. The assay showed a sensitivity of 0.3 pg/well. To validate the assay biologically, 6 rams were injected with a synthetic analogue of the adrenocorticotropic hormone (Synacthen, 2 mg/kg body wt). An aliquot was collected of each faecal portion spontaneously defecated 8 h before Synacthen injection to 24 h after injection and stored at -20 C until analysis. Samples (0.5 g) were extracted using 80% methanol and immunoreactive metabolites measured using the 11-KT EIA and an already established 11,17-dioxoandrostane (11,17-DOA) EIA. High-performance liquid chromatography separation revealed no peak in the same elution position as authentic 11-KT; therefore, reacting substances were referred to as 11-KT equivalents. In the case of 11-KT immunoreactive substances, the values increased from baseline (median, 136 ng/g feces) to a peak concentration (median, 424 ng/g) 10 to 14 h after Synacthen injection and declined afterwards. Concentrations of 11,17-DOA showed the same pattern, but the values were 2 to 4 times higher. From this data, the authors conclude that 11-KT-like substances, specifically C_{19}O_{3}-androgens with a 17β-hydroxy group, were present in the feces. These substances originate from the adrenals and are most likely cortisol metabolites.

Keywords: Manure; Endocrine disruptors; Environment Feedlot; Sewage
INTRODUCTION

In mammals, androgens (19-carbon steroids) are produced by the gonads and adrenal glands. These hormones can be grouped into C₁₉O₂-androgens (e.g., dehydroepiandrosterone, androstenedione, and testosterone) and 11-oxygenated androgens (C₁₉O₃-androgens, e.g., 11ß-hydroxyandrostenedione and 11-ketotestosterone). The 11-oxygenated androgens are important in teleost fish and produced mainly by the gonads, whereas 4-androstene-11ß-ol-3,17-dione is predominantly of adrenal origin and considered a weak androgen in humans [1]. However, the gonadal pathway to produce 11-ketotestosterone (11-KT) appears to be active in mammals; for example, 11-KT is produced in the gonads of immature mice [2]. Androgens are metabolized and excreted in the urine and feces [3]. Using the chicken-comb test, androgen activity was reported [4] while studying the feasibility of using bovine manure as a source of B vitamins for chickens. Furthermore, androgenic activity in the feces of cows but not bulls was described after 24 h incubation at 45 °C [5]. These findings were confirmed and evidenced by the excretion of androgenic metabolites in pregnant and progesterone-injected cows [6]. Later, the androgenic activity in cow feces was reported to be dependent on the presence of androgen precursors and microbial activity [7]. Cortisol is a potential precursor of faecal 11-oxygenated 19-carbon steroids because the glucocorticoid is converted by anaerobic intestinal bacteria [8], and the side chain of cortisol can be cleaved, resulting in the formation of C₁₉O₃-androstanes. These molecules are reabsorbed, metabolized again in the liver, and excreted into the gut via the entero-hepatic circulation [8-10]. In ruminants, appreciable amounts of steroid hormone metabolites are excreted in the feces [3]. Radiometabolism studies of ¹⁴C-cortisol in sheep have shown that authentic cortisol is not present in the feces, but a multitude of radioactive metabolites, including
C$_{19}$O$_3$-androgens, are excreted [11,12]. Consequently, an assay for measuring 11-oxoetiocholanolone was developed [11], and the concentrations of these immunoreactive substances are now widely used to measure adrenocortical activity in ruminants, reflecting the production of glucocorticoids in the adrenal glands [12,13]. Steady increases in C$_{19}$ metabolites, such as 5ß- C$_{19}$O$_3$-androstanes, have been reported for faecal samples stored at room temperature for up to 24 h, which can be explained as an indicator of side-chain cleavage by faecal microorganisms [12]. Some of the excreted gonadal metabolites are suspected to be androgenic substances; therefore, the contribution of livestock and humans to the environmental load of steroid hormones has become an international concern. Most research work thus far has focused on the quantification and estimation of excreted estrogens, gonadal androgens, and gestagens in different farm animals [14], municipal biosolids, and animal manure [15], but the presence of C$_{19}$O$_3$-androgens was neglected, even though they are the active androgens in fish. Data concerning the androgenic activity of C$_{19}$O$_3$-androstanes in mammals remain elusive.

In rats, 11ß-hydroxyandrostenedione has been found to have an osteotropic effect but little androgenic activity [16]. In contrast to reports in mammals, 11-KT is the most potent androgen in fish. Interestingly, the C$_{19}$O$_3$-cortisol metabolite 5α-androstane-3, 11, 17-trione was described recently to act similarly to 11-KT in medaka fish, causing the masculinization of females [17]. In addition, 5α-reduced C$_{19}$O$_2$-androstanes appear to act as androgens in some fish species. For example, the potency of 5α-dihydrotestosterone as an androgenic compound in the fathead minnow (Pimephales promelas) has been described [18]. Furthermore, an 11-oxygenated 5ß-androstane (i.e., 11-oxoetiocholanolone) has been shown to act as a putative pheromone in some fish species [19]. Until now, only 19-carbon 5ß-steroids with an oxo
group at position 17 of the molecule have been described in the feces of ruminants and used as noninvasive parametric values for animal welfare. The objective of the present study was to investigate whether ruminants should be considered as sources of 5α-androstanes with a 17β-hydroxy group configuration. Therefore, an enzyme immunoassay against 11-KT was developed and validated in faecal samples of sheep.
MATERIALS AND METHODS

Animal treatment and faecal sample collection

Feces from 6 male Austrian mountain sheep (age, 3–5 yr; weight, 74–107 kg) at the Teaching and Research Farm of the University of Veterinary Medicine Vienna were analyzed. Permission to conduct the animal experiments was obtained from the local authority (GZ 68.205/0108-II/10b/2009). The animals were apparently healthy and housed in metabolic cages (1.5 - 1.2 m). Hay and fresh water were provided ad libitum, and all animals had access to a mineral lick. All animals were intravenously injected with the synthetic Adrenocorticotropic hormone analogue (Synacthen, Novartis; 2 mg/kg body wt). Faecal samples were collected in plastic bags after each spontaneous defecation, homogenized, and stored immediately at -20 8C. The faecal samples containing 14C-cortisol metabolites were collected in an earlier experiment [3] in which 14C-cortisol was injected into rams to monitor excretion of the steroid hormone. Those samples were then stored at -20 8C. Feces extraction (0.5 g feces) was made on both Synacthen treated ram feces and on 14C-cortisol feces using 80% methanol method [11]. After centrifugation, an aliquot of the supernatant was transferred to a new vial, diluted (1:5 v/v) with assay buffer (Tris-hydroxyl-aminomethane [20 mmol/L], NaCl [0.3 mol/L], bovine serum albumin [1 g/L], Tween 80 [1 mL/L], pH 7.5), and analyzed by 11-KT and 11-oxoetiocholanolone enzyme immunoassays (EIAs).

Enzyme immunoassay

Both EIAs were performed as described previously [11], with the exception that protein-A was used to coat the microtiter plates instead of antirabbit immunoglobulin G (IgG). The microtiter plates (F96 MaxiSorp, No. 442404, Co. Nunc) were coated with 50 mg protein A (Sigma
Aldrich) dissolved in 25-mL coating buffer (0.25 mL/well) and left overnight at room temperature. The second coating used a buffer containing bovine serum albumin plus sodium azide, and the plates were stored at room temperature until use (up to 4wk). A new assay was developed to measure 11-KT immunoreactive substances. The antigen (11-ketotestosterone-3-CMO: bovine serum albumin) was kindly donated by A. Scott (Centre for Environment, Fisheries and Aquatic Science, Weymouth, UK) and sent to Pineda Antikörper Service (Berlin, Germany) to immunize rabbits. As a label, 11-ketotestosterone-3-CMO (10 mg) was linked to 10 mg EZ-Link Biotin PEO-amine-biotinyl-3,6-dioxaoctanediamine (10 mg; Pierce) using the mixed anhydride reaction. Label purification was performed as described previously (MÖSTL et al. 2002). Working dilutions of antibody (1:300000) and label (1:10^6) for the 11-KT assay were made by checkerboard titration with 11-KT as a standard. To monitor the stimulation of glucocorticoid production by the adrenal gland after Synacthen injection, a previously validated 11-oxoetiocholanolone EIA was used [11,13].

**Validation of the 11-KT assay**

The standard curve for 11-KT ranged from 0.3 pg to 80 pg per well. The 50% intercept was 2 pg/well. The sensitivity of the test was calculated as the least amount of 11-KT that was different from a concentration of zero at the 95% confidence limit (0.3 pg/well). The assay showed cross-reactions with 5α-androstan-17β-ol-3,11-dione (39%), 5β-androstane-17βol-3-one (3.6%), 5α-androstan-3α-ol-11,17-dione (0.3%), 4-androstene-3,11,17-trione (0.2%), 5α-androstan-3β-ol-11,17-dione (0.2%), 5α-androstan-17β-ol-3-one (0.1%), 4-androstene-17α-ol-3-one (0.1%), and 4-androstene-17β-ol-3-one (0.04%). To test the linearity of the assay in faecal sample extracts, a pooled sample from different animals was used, followed by serial dilution with assay buffer.
concentrations were measured using the 11-KT EIA. Different amounts of feces had no significant influence on the results expressed as nanograms per gram of feces. The intra-assay and interassay coefficients of variation for the low-level pool of the 11-KT assay were 3.9% and 8.7%, respectively.

**High-performance liquid chromatography separation of radiolabeled 14C-cortisol metabolites and immunoreactive substances**

To characterize the radiolabeled and immunoreactive metabolites in feces, a faecal sample containing the $^{14}$C-cortisol metabolites was used from a previous experiment [3]. A high performance liquid chromatography (HPLC) immunogram was produced as described previously [20]. Briefly, 1 mL of the methanol phase from the extract was diluted in 10-mL water and re-extracted using a Sep-Pak (Waters) C18 cartridge following the manufacturer’s instructions. The dried extract was dissolved in 50% methanol and separated using HPLC (Novapac C18, column dimension: 0.39 -15 cm; pumps: Waters 626; controller: Waters 6005; photodiode array detector: Waters 996) with water/methanol as the mobile phase (starting with 50% and up to 75% methanol within 35 min using a linear gradient) operated at room temperature. The flow rate was 1 mL/min.

Three fractions were collected per minute and the immunoreactivity measured in each fraction using the 11-KT and 11,17-DOA assay. The radioactivity was measured in all fractions using liquid scintillation counting. The elution positions of some standards were determined using an ultraviolet detector.

**Statistical analysis**

Statistical analyses were performed using SigmaStat 3.1 and SigmaPlot 11.0 was used for preparing the figures (SPSS). Due to normality test failure, results are expressed as median and the correlation between the
concentrations of 11-KT and 11,17-DOA was calculated as the Spearman correlation test.

RESULTS

The concentrations of immunoreactive 11-KT and 11,17-DOA in faecal samples of two rams are shown in Figure 1. High inter-individual variability was seen in the baseline and peak values of all animals. The baseline levels of 11-KT ranged from 76 ng/g to 240 ng/g feces (median, 136), and from 188 ng/g to 297 ng/g feces (median, 252) for 11,17-DOA. Following Synacthen injection, the faecal concentrations of both 11-KT and 11,17-DOA reached peak values between 10 and 12 h (Figure 1). Peak concentrations of 11-KT and 11,17-DOA ranged from 352 ng/g to 475 ng/g feces (median, 424) and 1211 ng/g to 1822 ng/g feces (median, 1570), respectively. After Synacthen injection, the increase (median) above baseline values of 11-KT and 11,17-DOA was 293% and 626%, respectively. In all individuals, the concentrations of both groups of metabolites highly correlated (n= 125 [total number of faecal samples from all 6 rams]; r= 0.863; p < 0.0001), but 11,17-DOA values were approximately 2 to 4 times higher. Although concentrations measured by the 2 EIAs started to decline gradually after the Synacthen peak, they were still significantly different from baseline levels (p < 0.001) at the end of the experiment. HPLC separation of glucocorticoid metabolites Reverse phase-HPLC analysis revealed that 14C-cortisol was extensively metabolized.
Figure 1 Concentrations of immunoreactive 11-ketotestosterone (11-KT) and 11,17-dioxoandrostan (11,17-DOA) before and after Synacthen injection in two rams. All immunoreactive 11-KT in all six rams is provided in the appendix.
All immunoreactive substances eluted between fractions 20 and 50, indicating that they are non-conjugated steroids. The 14C-cortisol metabolites eluted in 6 major peaks, 5 of which were in the same range as immunoreactive substances measured with the 11-KT and 11-oxoetiocholanolone EIA (Figure 2). Though the peaks were not positively identified, three major immunoreactive peaks of 11-KT (30–33, 37–41, and 46–49) coincided with the 14C-cortisol peaks. The RP-HPLC separation system did not detect authentic 11-KT at its respective elution position (fraction 22).
Figure 2 High-performance liquid chromatography elution pattern of radioactivity and immunoreactive substances. The black arrow indicates the elution position of 11-ketotestosterone (11-KT; fraction 22). The elution positions of cortisone, cortisol, corticosterone, $17\alpha,20\alpha$-dihydroxyprogesterone ($17\alpha20\alpha$ -P4), and $20\alpha$-hydroxyprogesterone ($20\alpha$-P4) are indicated by inverted triangles.
DISCUSSION

Endogenous steroid hormones of human or animal origin have been introduced to the environment for a long time [14]. However, the recent rise of intensive animal husbandry and urbanization has dramatically increased the amounts of steroid released into the environment. From a toxicological point of view, most research has focused on sex steroids and synthetic anabolic hormones in sewage treatment plants, which act as endocrine disruptor compounds (EDCs) to aquatic organisms. Glucocorticoid metabolites received less attention, with the exception of their wide use as a noninvasive measure of animal welfare. However, the possible role of glucocorticoid metabolites as EDCs remains unclear. To demonstrate that immunoreactive 11-KT metabolites are present in animal manure and from adrenal origin, an 11-KT EIA was developed and validated. The 11-KT-EIA exhibited very high sensitivity (0.3pg/well) compared to currently available commercial kits, providing a useful tool for noninvasive monitoring of androgen production in fish aquaria, where low amounts of steroids constitute a major drawback for the applicability of these assays. A second advantage of this EIA is that it does not need the use of radioactive tracers that constitute major environmental biohazards. The highest cross reactivity (39%) shown by 5α-androstan-17b-ol-3,11-dione (11-ketoandrostanolone), which is 5α-reduced 11-KT, would be quite useful and should not present a problem as far as the biological activity is concerned because 5α-reduced androgens (e.g., dihydrotestosterone) are more potent than 4-androstene (e.g., testosterone) [20]. Two points regarding the excretory profile of faecal 11-KT immunoreactive substances merit discussion. First, the 11-KT EIA detected immunoreactive substances, and their concentrations increased after stimulation by Synacthen, indicating that the substances or their precursors were produced by the adrenal glands. Second, peak values
were reached between 10 and 12 h (Figure 1), paralleling those of the 11,17-DOA EIA and showing the same excretory pattern (gut passage time) within a comparable time in sheep [13]. Reverse phase-HPLC separation revealed different immunoreactive metabolites with polarity similar to unconjugated steroids, but none of the peaks showed the same elution position as authentic 11-KT. Despite 11-KT EIA being raised against the 4-androstene structure, the assay showed cross reactions with $5\alpha$-reduced KT. As far as the linking position of the antigen at position C-3 in the new 11-KT EIA is concerned, it would make it difficult for the antibody to differentiate between $5\alpha$-reduced and delta-4 steroids as demonstrated by the cross reactions as described earlier [21].

Our new 11-KT EIA has to be considered as group specific for 11-oxo and 17β-hydroxy configuration, and the authentic 11-KT was absent in our samples. Though the HPLC peaks were not positively identified, some showed chromatographic behavior (2 fractions) with 11,17-DOA identified by Ganswindt et al. [22] using the same separation system for $^{14}$C-cortisol metabolites: 11β-hydroxyetiocholanolone at fractions 22 to 24 and 11-oxoetiocholanolone at fractions 36 to 40. Furthermore, using HPLC coupled with mass spectrometry, MÖSTL et al. [12] reported a mixture of C-21 and C-19 metabolites coincided in a single radioactive peak. Therefore, due to steroid metabolite complexity in faecal matter, the 11-oxoetiocholanolone EIA [11,13] picked a group of unconjugated metabolites (11,17-DOA) similar to the new 11-KT EIA. However, the major peak of 11-KT immunoreactive substances in fractions 36 to 40 coincided only with $^{14}$C-cortisol metabolites. Based on our results for cross reactivity and HPLC chromatographic separation, we can assume that rams excrete C$_{19}$O$_{3}$-androgen metabolites with a $5\alpha$-configuration plus a 17β-hydroxy group in the feces. The origin of these metabolites or their precursors is the adrenal gland, as the concentrations of these
substances were indicated after Synacthen injection and the 11-KT EIA coincided with some $^{14}$C-cortisol metabolites in the HPLC immunogram (Figure 2). These steroids may be a product of the sheep itself, or most by side-chain cleavage induced by micro-organisms in the gut using adrenal steroids as precursors. If adrenal androgens are the cause of the faecal androgen concentrations, one has to expect a certain amount of 17α-hydroxylated androgens and lower concentrations of 11-oxoetiocholanolone. It has been shown that 11-oxoetiocholanolone is rapidly converted by red blood cells of ruminants into 17α- and 17β-hydroxylated steroids [23]. A similar scenario of C-17 oxygenated metabolite transformation would be expected, as other steroids have been reported in ruminant feces [24]. Measurement of 11,17-DOA in feces is an established assessment of adrenocortical activity in response to stressful situations in various species, including ruminants [11-13], cats [25], monkeys [26], and nonhuman primates [27]. However, quantification of biologically active glucocorticoid metabolites in animal manure has received no attention, though available evidence confirmed that mammalian faecal glucocorticoid metabolite (5α-androstane-3,11,17-trione) masculinized the sex characteristics of the anal fin in female medaka in a dose dependent manner, and this effect was comparable to the effects of the major fish androgen, 11-KT [17]. In conclusion, the present study did not show the presence of 11-KT in animal manure after Synacthen injection, but crossreacting metabolites were detected. However, metabolites could constitute a major problem for aquatic organisms; therefore, the assessment of the androgenic potential of 11-oxoetiocholanolone and 17β-hydroxymetabolites originating from animal manure is important. It may also be of interest to measure the amount of C$_{19}$O$_{3}$-androgens in sewage water.
**Acknowledgment**

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REFERENCES


3. Formation of 5α-reduced C_{19}O_{3} immuno-reactive metabolites with an 17-oxo group in cattle faeces following ACTH injection

3.1. Introduction

It has been thought for a long time that both chemical configurations of A-ring reduced GC metabolites (5α and 5β) are biologically inactive. However, the biological activity of 5α-reduced metabolites of corticosterone (C_{21} steroid) attracted considerable clinical interests for its anti-inflammatory properties with apparent paucity of undesired metabolic effects (YANG et al. 2011). Beside the widely believed inactivation of GCs via ring-A reduction, the activity of 11β-hydroxysteroid dehydrogenase II (11β-HSDII) has also been implicated to convert cortisol to its inactive form, cortisone (PAVLOVIC et al. 2013).

Production of C_{19} androgens in mammals is achieved by the gonads and adrenal glands. These steroids can be chemically grouped into C_{19}O_{2}-androgens (e.g., dehydroepiandrosterone, androstenedione and testosterone) and 11-oxygenated androgens (C_{19}O_{3}-androgens, e.g., 11β-hydroxyandrostenedione (11β-OH-A)). 11β-OH-A can be produced directly from cortisol via the widely known side-chain cleavage (SCC) process in the liver or from androstenedione via the activity of 11β-hydroxylase in the adrenal zona reticularis (Figure 4).
Figure 4 Production of 11β-hydroxyandrostenedione (11β-OH-A; C_{19}O_{3} androgen) by:
(1) Side-chain cleavage of cortisol (C_{21} glucocorticoid) and (2) from androstenedione
(C_{19}O_{2}-adrenal androgen) via the activity of 11β-hydroxylase.

Direct production of C_{19}O_{3} androgens by the adrenal cortex (e.g., 11-oxo-
androstenedione (adrenosterone)) prior to 5α ring-A reduction has been
reported (SAVARD et al. 1953; BRADLOW et al. 1967). Furthermore,
biosynthesis of 11β-OH-A from cortisol by the side-chain cleavage of
human intestinal clostridium bacteria has also been reported
(BOKKENHEUSER et al., 1984). Moreover, it has been shown that the
metabolites of adrenosterone and 11β-OH-A are predominantly 5α-
reduced (SHACKLETON et al. 2008) in humans. Although 5α-reduced
11-oxygenated C_{19}O_{3} androgen metabolites constitute a significant
amount of the steroids produced and excreted by humans as indicated in
the previously mentioned pathways, to my knowledge, their formation and
presence in cattle manure has never been investigated.

The role of the major mammalian androgens, testosterone and its 5α-
reduced metabolite (DHT) that are both C_{19}O_{2} androgens has been
described and documented at several levels from androgen receptor
binding, anabolic effects, maintenance of skeletal homeostasis, gene
expression and gametogenesis. It is interesting that the C_{19} 11-oxygenated
androgens are important in teleost fish and are produced mainly by the
gonads, whereas 11β-OH-A is predominantly of mammalian adrenal origin and considered as a weak androgen in humans (GOWER 1975). It has been found to possess osteotropic effect but little androgenic activity (SUZUKI et al. 2000). However, the gonadal pathway to produce 11-ketotestosterone which is the C-11 oxidized form of testosterone (17β-hydroxyandrost-4-ene-3,11-dione) appears to be active in mammals. For example, 11-KT is produced in the gonads of immature mice (YAZAWA et al., 2008) after stimulation of the organs using luteinizing hormone/human chorionic gonadotropin, indicating conservation and evolution of the androgen metabolic pathway. Furthermore, we have recently reported that immuno-reactive substances measured with an 11-KT (C₁₉O₃ metabolites) are excreted in sheep faeces after an ACTH challenge test (SID-AHMED et al., 2013), suggesting that these substances originate from the adrenals. The physiological role of C₁₉O₃ metabolites remains elusive in mammals. Interestingly, it has been recently shown that the C₁₉O₃ cortisol metabolite (5α-androstane-3,11,17-trione) possesses androgenic activity in a dose-dependent manner similar to that caused by 11-KT for the masculinization of female meddaka fish (GRILLITSCH et al., 2010). It is well documented that the 5α-reduced androgens are more potent than the 4-ene as has been previously demonstrated by the actions of DHT. However, the role of chemical configurations at carbon positions C-11 and C-17 (keto or hydroxyl moiety) in biological activity of 4-androstene or 5α-reduced C₁₉O₃ metabolites remains elusive.

The experiment described here was primarily intended to investigate the formation of 5α-reduced C₁₉O₃ metabolites with a 17-oxo group in cattle faeces and to indicate the polarity of those metabolites using HPLC. This
work is a preliminary stage for forthcoming studies concerning the biological activity of animal-manure derived metabolites.
3.2. Materials and Methods

3.2.1. Animals and ACTH injection

All animals (three females and one male Austrian cattle) and the ACTH injection in this study were previously described by PALME et al. 1999.

3.2.2. Extraction of faecal samples

Faecal samples were extracted using 80% methanol according to the method described by PALME and MÖSTL (1997). Briefly, 0.5 g faeces was weighed and extracted with 5 mL (80% methanol) on a vortex for 30 min (Rapidvap Labconco, Vacuum systems, USA) followed by centrifugation at 2500 x g for 15 min (Beckman coulter, Allegra X-12R centrifuge, USA). After centrifugation a 0.1 mL aliquot of the supernatant was mixed with 0.4 mL of assay buffer (tris-hydroxyl-aminomethane [20 mmol/L], NaCl [0.3 mol/L], bovine serum albumin [1 g/L], Tween 80 [1 mL/L], pH 7.5), slightly vortexed and kept at -20°C until assayed with an 11-oxoisoandrosterone and an 11-oxoaetiocholanolone EIAs (PALME and MÖSTL 1997).

5α-androstan-3ß-ol-11,17-dione (11-oxoisoandrosterone)-hemisuccinate:BSA (bovine serum albumin) was synthesized in our lab and used as antigen and sent to Pineda Antikörper Service (Berlin, Germany) to immunize rabbits. 5α-androstan-3ß-ol-11,17-dione-3-glucuronide: biotin was used as enzyme label. In a step the glucuronide of 5α-androstan-3ß-ol-11,17-dione was produced as described by CASATI et al. (2009) for 17ß-boldenone. Afterwards, the glucuronide ester was linked to diaminodioxooctane–biotin using the mixed anhydrid reaction. Working dilutions of antibody (1:20000) and label (1:50000) were found to produce the best optical density and displacement as measured by checkerboard titration with 5α-androstane-3ß-ol-11,17-dione as standard.
3.2.3. EIA procedure

Protein A-coated microtiter plates were washed three times with 0.35 mL/well of washing solution (Table 1) using an automated microtiter plate washer (ELX 405, BioTek instruments, USA). All duplicates (0.05 mL; 1:5 diluted with assay buffer) of faecal extracts and standards prepared in assay buffer (Table 1) were pipetted into respective wells with the aid of a diluter dispenser (Hamilton Micorlab dispenser 1000, Netherland) followed by the addition of 0.1 mL biotin-labelled steroid and 0.1 mL of antibody solution into each well except the wells marked for non-specific binding (NSB) which received assay buffer (0.15 mL) and biotin-labelled steroid (0.1 mL). Microtiter plates were covered with parafilm and incubated overnight at 4°C under constant agitation. The plates were washed four times with the washing solution. 0.25 mL of streptavidin: peroxidase solution was dispensed into each well, covered with parafilm and incubated again at 4°C under constant agitation for 45 min. After incubation a second washing step was performed as previously mentioned. The colour reaction was performed by adding 0.25 mL of substrate solution (table 1) into each well and incubated at 4°C under constant agitation for 45 min. The reaction was stopped by the addition of 0.05 mL of stop solution. The optical density of formed yellow colour was measured at 450 nm in a microtiter plate reader (BioTek-EL808, USA) coupled with a PC and equipped with software for the measurement of steroid concentrations. Specifications of different solutions that were used in EIA are provided in Table 1.
Table 1 Different buffers and solutions used in the enzyme immunoassays.

<table>
<thead>
<tr>
<th>No</th>
<th>Reagent</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coating buffer</td>
<td>1.59 g Na₂CO₃; 2.93 g NaHCO₃, dissolved and filled up to 1 L with DDW; pH 9.6</td>
</tr>
<tr>
<td>2</td>
<td>HCl (1mol/L)</td>
<td>920 mL DDW + 80 mL (37% HCl)</td>
</tr>
<tr>
<td>3</td>
<td>Assay buffer</td>
<td>2.42 g trishydroxyaminomethane; 20 mmol/l, 17.9 g NaCl (0.3 mol/l); 1 g Bovine serum albumin 1 mL Tween 80, dissolved and filled up to 1 L (DDW); adjusted to pH 7.5 with 1 M HCl (920 mL H₂O+80 mL HCl (37%))</td>
</tr>
<tr>
<td>4</td>
<td>Second coating buffer</td>
<td>3.146 g trishydroxyaminomethane; 23.3 g NaCl; 13 g BSA; 1.3 g Sodium azide, dissolved and filled up to 1.3 L (DDW); pH 7.5; filtered through SEP-PAK C18 (see 1.3.1.)</td>
</tr>
<tr>
<td>5</td>
<td>Washing solution</td>
<td>0.5 ml Tween 20; add 2.5 L DDW</td>
</tr>
<tr>
<td>6</td>
<td>Substrate buffer for peroxidase</td>
<td>1.36 g sodium acetate (10 mmol/L), dissolved and filled up to 1L (DDW); adjusted to pH 5.0 with 5% acetic acid (10%)</td>
</tr>
<tr>
<td>7</td>
<td>Enzyme solution for Streptavidin-reaction</td>
<td>30 mL assay buffer, 0.001 mL Streptavidin-POD-conjugate (=0.5 U; , 500 U) mixed on a magnetic stirrer prior to use</td>
</tr>
<tr>
<td>8</td>
<td>Substrate solution for peroxidase</td>
<td>30 mL of substrate buffer + 0.5 mL 3,3',5,5'-Tetramethylbenzidine (0.4%= 0.4gm (Fluka) dissolved in 100gm DMSO (Fluka) + 0.1 mL H₂O₂ (0.6%; 0.3mL H₂O₂ (Merck) + 17.5 mL DDW)</td>
</tr>
<tr>
<td>9</td>
<td>Stop solution (2 mol/L H₂SO₄)</td>
<td>900 mL DDW + 100 mL H₂SO₄ (95-97%)</td>
</tr>
</tbody>
</table>
3.2.4. Assay validation

3.2.4.1. Cross-reactivity, sensitivity and precision

The cross-reactivity of steroids and steroid metabolites that are structurally related to 5α-11,17-dioxyandrostanes was assessed for the assay. Sensitivity was calculated as the least amount of metabolites that could be distinguished from zero concentration of standard as calculated from 95% confidence limits at the zero point of the standard curve. To measure assay precision the intra-assay coefficient of variation which is the measure of repeatability and the inter-assay coefficient of variation which is the measure of reproducibility were assessed. The intra and inter assay coefficient of variations were determined using pooled faecal extracts from both sexes.

3.2.4.2. Physiological validation

To evaluate if these metabolites are produced by adrenal precursor steroids, physiological validation was performed using faecal samples from ACTH injected cattle (three females and one male: PALME et al. 1999). Faecal samples were collected following each spontaneous defecation (before and after) ACTH injection. Faecal metabolites concentrations having 5α- and 5β-DOA configurations were measured.

3.2.4.3. HPLC separation of immuno-reactive metabolites

To confirm assay specificity and to indicate the polarity of immuno-reactive C19O3 metabolites produced in faecal samples, 1 mL of the methanolic phase from extracts of the high concentration pool (from both sexes) obtained after the ACTH injection were diluted in 10 mL water separately and subjected to reverse-phase-HPLC (RP-HPLC). An HPLC immunogram was produced as described earlier (MÖSTL et al. 2002;
SID-AHMED et al. 2013) with minor modification. Briefly, 1 mL of the methanol phase from the pooled extract was diluted in 10 mL water and further purified using a Sep-Pak® (Waters, Milford, Mass, USA) C18 cartridge following the manufacturer’s instructions. The dried extract was dissolved in 50% methanol and separated using HPLC (Novapac® C18, column dimension: 0.39×15 cm, Waters 626 pumps, Waters 6005 controllers, Waters photodiode array detector 996, Waters, Milford, Mass, USA) with water/methanol as the mobile phase (starting with 50%, up to 75%, methanol within 35 min using a linear gradient) run at room temperature. The flow rate was 1 mL/min. Three fractions were collected per min and each fraction was analysed in monoplicate by both enzyme immunoassays. The elution positions of standards (cortisol, cortisone, and 4-androstene-3,11,17-trione) were determined using an ultraviolet detector, while the two diastereoisomers (5α and 5β-reduced) of C19O3 metabolites were determined using respective EIAs.
3.3. Results

3.3.1. Assay validation

The cross-reactivity of different steroids with the antibody revealed a very high specificity for 5α-androstan-3β-ol-11,17-dione (11-oxoisoandrosterone). The other 5α- and 5β-reduced steroids which are structurally related to 5α-11,17-DOA are provided in Table 2.

Table 2 Specificity of the 5α-androstane-11,17-dione antibody for different steroids having structural similarities.

<table>
<thead>
<tr>
<th>Steroid/Steroid metabolites</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5α-Androstan-3α-ol-17-one</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5α-Androstan-3β-ol-17-one</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5α-Androstan-11β-ol-3,17-dione</td>
<td>7</td>
</tr>
<tr>
<td>5α-Androstane-3α,11β-diol-17-one</td>
<td>7</td>
</tr>
<tr>
<td>5β-Pregnan-3β-ol-11,20-dione</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5β-Pregnan-3α-ol-11,20-dione</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5β-Androstane-3,17-dione</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5β-Androstan-3α-ol-17-one</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5β-Androstan-3α-ol-11,17-dione</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5β-Androstane-3,11,17-trione</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

The least amount of metabolites that could be distinguished from zero concentration was 15 pg/well. The intra- and inter-assay coefficients of variations for low and high pools were less than 10%.
3.3.2. Faecal excretory profile of 5α-reduced 11,17-DOA metabolites in cattle

The patterns of faecal 5α-11,17-DOA are provided in Figure 5. Inter-individual variability was seen in baseline and peak values. Baseline levels of 5α-11,17-DOA ranged from 6.5 to 128.9 (median: 42.8 ng/g faeces) and from 53.5 to 128.4 (median: 42.2 ng/g faeces) for 11-OE. Following the ACTH injection, the concentrations of faecal 5α-11,17-DOA and 11-OE metabolites reached the peak values between 12 to 24 h. Peak concentrations of 5α-11,17-DOA were 6 to 298.6 (median: 94.6 ng/g faeces) and 27.5 to 535 (median: 135 ng/g faeces) for 11-OE. Peak concentrations of both assays were 91.4% to 232% (median: 221%) and 78% to 417% (median: 320%) above baseline levels for 5α-11,17-DOA and 11-OE, respectively.
Figure 5 Excretory profile (ng/g faeces) of 5α- and 5β-reduced 11,17-DOA metabolites in three females and one male before and after ACTH injection.
3.3.3. HPLC separation of 5α- and 5β-reduced C₁₉O₃ metabolites

RP-HPLC separation of methanolic extracts from female and male ACTH injected animals is provided in Figure 6. HPLC in both sexes yielded several polar peaks indicating a large number of different metabolites with varying degree of polarity. The 5α-11,17-DOA metabolites co-eluted with 11-OE immuno-reactive substances in almost all fractions. However, 11-OE concentrations were higher than those of 5α-11,17-DOA metabolites.
Figure 6 RP-HPLC immunogram of C₁₉O₃ immunoreactive substances in ACTH injected females and male cattle. Immunoreactivity in each fraction was determined with 11-OE and 5α-11,17 DOA EIAs. The retention positions of standards detected by UV absorbance (C: cortisol; CC: corticosterone; 4AT: 4-androsten-3,11,17-trione) or their immunoreactivity (1: 5α-androstan-3β-ol-11,17-dione; 2: 5β-androstane-3,11,17-trione; 3: 5α-androstan-3α-ol-11,17-dione ; 4: 5β-androstan-3α-ol-11,17dione) are indicated by inverted triangles.
3.4. Discussion

Aquatic organisms are exposed to a multitude of endocrine disruptive compounds (EDCs) originating from agricultural practices via soil runoff. Steroid hormones constitute a main source as EDCs due to their application for anabolic purposes and/or therapeutic indices in concentrated animal feeding operations that have been recently introduced to meet the demand of an increasing human population. In the environment, steroid hormones are exposed to several metabolic transformations by different microbial organisms in the animal manure or in the soil. Endogenous and synthetic sex steroids received great attention by environmental researchers (e.g., in sewage treatment plants) as EDCs for aquatic organisms, while glucocorticoid metabolites having different chemical configurations received less attention despite the fact that available evidence confirms the presence of immuno-reactive metabolites of the major fish androgen (11-ketotestosterone) in the faeces of the sheep after an ACTH injection (SID-AHMED et al. 2013). Furthermore, metabolites (11-OE ) that are formed in animal manure by the side-chain cleavage of cortisol metabolites (as for example MÖSTL et al. 2002; PALME and MÖSTL 1997) have just received the attention for their use as animal welfare markers and they are now widely used in both domestic and wildlife animals as non-invasive parameter for the assessment of adrenocortical activity. However, it has been recently reported (TIERNEY et al. 2013) that 11-OE acts as a fish pheromone by attracting the non-reproductive females of round gobies fish (Neogobius melanostomus).

It is well known that glucocorticoids are extensively metabolized to different chemical configurations. Thus, the chance of forming different hydroxyl and keto moieties around the steroid nucleus either by the endogenous enzyme systems of the animal or by the intestinal microbial
transformation is high. Although it has been reported that the 5β-reduced forms of tetrahydrocortisol are predominant metabolites in cattle (PAVLOVIC et al. 2013), the formation of potentially biological active C_{19}O_{3} metabolites having the 5α-androstane-11,17-DOA structure in cattle faeces cannot be excluded but has never been investigated. Interestingly, the 5α-reduced faecal glucocorticoid metabolite (5α-androstane-3,11,17-trione) has been recently reported (GRILLITSCH et al. 2010) to masculinize the sex characteristics of the anal fin in female medaka in a dose dependent manner similar to that caused by the major fish androgen (11-KT). However, if the formation of this androstane is the effect of intestinal transformation or an endogenous product of the animal itself remains elusive. Therefore, the aim of the current study was to investigate the formation of C_{19}O_{3} having a 5α-11,17-dioxoandrostane (5α-DOA) configuration in cattle faeces.

The EIA at first showed a low sensitivity, but when the enzyme label was formed via a glucuronide bridge it improved the sensitivity. This newly developed EIA is the first detecting the presence of 5α-reduced C_{19}O_{3} metabolites with a 11,17-dioxo configuration in animal manure. Cross-reactions with structurally similar steroids give a good evidence of a highly specific EIA as the maximum cross-reaction was less than 7% (Table 2). The excretory profile of the 5α-11,17-DOA immuno-reactive substances in bovine faeces confirmed that these metabolites might be directly derived from the adrenal gland as they followed the expected intestinal gut passage pattern after the ACTH injection. It is also possible that these metabolites might be glucocorticoids that have been exposed to microbial transformation in the animal gut.

Reverse-phase-HPLC separation has shown different immuno-reactive peaks in male and female. However, both sexes share three peaks with additional minor peaks detected only in the samples of the female. In both
sexes chromatographic polarity of the measured metabolites indicated unconjugated steroids. The male showed the higher concentration in both metabolites (11-OE and 5α-11,17-DOA) when compared with female. Several studies have reported sex differences in the amount of excreted metabolites. However, due to the limited number of animals used in this study it is difficult to attribute these differences to the sex.

Based on cross-reactions and the HPLC chromatographic separation, cattle could be assumed to excrete both 5α and 5β-reduced androstanes with a 11,17-dione configuration and both metabolites are produced from the adrenals as their concentrations were elevated after the ACTH injection. It is well known that the 5α-reduced metabolites are supposed to possess higher biological activity than the parent 4-ene steroids as for example dihydrotestosterone (DHT) (WILSON et al. 2002). The current findings open the door for new research perspectives considering glucocorticoid metabolites in the animal manure as potentially biological active compounds that may affect different sexual and reproductive fish behavior. However, a 5β-reduced metabolite (e.g. 11-OE) has been recently reported as fish pheromones in round gobies (TIERNEY et al. 2013). Pheromones in fish are highly specific due to different chemical structures that could be produced in A-ring (reviewed by STACEY et al. 2003). Alternative moieties in the A-ring at positions C-3 and C-5 will give the chance of forming several free 11-OE configurations via oxidation-reduction reactions. In animal manure, different microbial organisms although not yet isolated or identified are capable of achieving different transformation reactions either via reduction of the A-ring or via the SCC at the D-ring. Furthermore, the usefulness of measuring 11-OE is now widely applied in many wild and domestic animal species as non-invasive parameter for the assessment of the animal welfare status. However, 5α-reduced metabolites could constitute a major problem for
aquatic organisms via soil runoff. Therefore, the assessment of the biological activity of different 5α/5β-androstane-11,17-dione metabolites detected in the HPLC fractions either in fish model or cell-culture is important.
4. Conclusion and recommendation

The current studies showed for the first time that two different chemical configurations of \(\text{C}_{19}\text{O}_3\) metabolites (5α-11-oxo-androstanes having either a 17β-hydroxy or a 17-oxo group) are present and excreted in the faeces of two ruminant species (sheep and cattle), respectively. Their possible formation and origin either from the adrenal or via side-chain cleavage of glucocorticoid metabolites in the intestine is evident by their excretion pattern following the ACTH injection which was validated and reported in both species. The chromatographic separation (RP-HPLC) of both chemical configurations revealed the presence of metabolites of different polarity in both species. However, the molecular characterization and biological activity in fish or cell-culture models to assess their possible endocrine disruptive activity to the aquatic organisms need further investigations in order to develop certain withdrawal plan for the animal manure derived from these two animal species.
5. Summary

Over the last few decades, steroid hormones of anthropogenic and animal origins received great attention as potential hazard for the aquatic organisms. Although several studies were previously conducted to investigate the possible effects of endogenously produced and synthetic sex steroids as endocrine disruptive compounds (EDCs) on aquatic organisms, glucocorticoid (GC) metabolites having different chemical configurations received no attention in spite of their use (e.g., 11-oxoaetiocholanolone (11-OE)) as non-invasive measures of adrenocortical activity in wild and domestic animal species. However, it has been recently reported that 11-OE acts as fish pheromone by attracting the non-reproductive females of round gobies (*Neogobius melanostomus*).

Following secretion, GCs are extensively metabolized in the liver. In the gut, GCs are further metabolized by the intestinal bacteria and C$_{19}$O$_{3}$ steroid formed after side-chain cleavage. The principal focus of this thesis was to investigate the formation of 5α-C$_{19}$O$_{3}$ cortisol metabolites having a 17β-hydroxy group (as the major fish androgen 11-ketotestosterone) or a 17-oxo configuration by developing enzyme immunoassays (EIAs) against these chemical configurations and validating them in sheep and cattle faeces after adrenocorticotropic hormone (ACTH) injection. A group specific assay measuring 11-oxo-17β-OH-5α-androstanes was developed (antigen: ketotestosterone-3-CMO:BSA; label: ketotestosterone-3-CMO- biotinyl-3,6-dioxaoctanediamin and validated and is already published (SID-AHMED et al., 2013). The results of the validation experiment showed that such steroids are excreted via faeces in ruminants and the precursors originate from the adrenal glands. In view of the possible formation of 5α-reduced metabolites in bovine faeces, a group specific EIA was developed against 11-oxoisoandrosterone
(antigen: 11-oxoisoandrosterone-hemisuccinate:BSA, label: 11-oxoisoandrosterone-3-glucuronide- biotinyl-3,6-dioxaoctanediamin. The assay was validated in cattle (three females and one male) injected with ACTH. Reversed phase high performance liquid chromatography (RP-HPLC) revealed several polar peaks of immunoreactive 11-OE and 5α-11,17-DOA metabolites in both sexes. Based on this data, cattle are considered to excrete both 5α- and 5β-reduced C₁₉O₃ metabolites with a 11,17-dione configuration and both metabolites are derived from the adrenal as their concentrations were elevated after the ACTH injection. The current work is the first describing the presence of 5α-C₁₉O₃ metabolites with a 17β-hydroxy group similar to the major fish androgen 11-ketotestosterone and a 17-oxo group in animal faeces. Forthcoming research should characterize these metabolites and test their biological activity in fish models or using cell culture techniques.
6. Zusammenfassung

Chemische Verbindungen, die wie Hormone wirken und das Endokrinium von Menschen oder Tieren stören, werden als endokrine Disruptoren (endocrine disrupting chemicals, EDCs) bezeichnet. Untersuchungen über potentielle EDCs in menschlichen oder tierischen Ausscheidungsprodukten haben in den vergangenen Jahrzehnten eine gewisse Bedeutung erlangt.

Bisher lag der Fokus dieser Studien auf dem Nachweis von gonadalen und synthetischen Steroidhormonen, die Glukocorticoide (GC) und ihre Metaboliten (GCM) wurden jedoch im Hinblick auf ihr Potential als EDCs kaum erwähnt. Die bisherigen Untersuchungen der GCM konzentrierten sich vorwiegend auf ihre Anwendung als Parameter für Belastungen bei Tieren, da die Konzentration dieser Verbindungen in den Ausscheidungsprodukten mit der Cortisolproduktion positiv korreliert ist.

Für solche Messungen hat sich bei einigen Spezies die Quantifizierung von immunreaktivem 11-Oxoäthiocholanolon (11-OE) bewährt. Dabei handelt es sich um 5ß-reduzierte Androstane. Solche Verbindungen entstehen durch Seitenkettenabspaltung der GC, die entweder im Körper selbst oder durch die Mikroorganismen des Verdauungstrakts durchgeführt wird. Im Gegensatz zu den gonadalen Androgenen und ihren Metaboliten weisen die von GC stammenden Verbindungen drei Sauerstoffatome im Molekül auf (C19O$_3$), während die gonadalen Androgenmetaboliten nur zwei Sauerstoffatome haben (C$_{19}$O$_2$). Auch die C$_{19}$O$_3$-Verbindungen haben biologische Wirkungen. So ist bekannt, dass 11-OE bei Schwarzmund-Grundeln (Neogobius melanostomus) als Pheromon wirkt und dass ein 5α-reduzierter Cortisolmetabolit (5α-Androstan-3,11,17-trion) bei Fischen als Androgen wirkt. Dies ist insofern naheliegend, als 11-Ketotestosteron, eines der beiden dominierenden Androgene bei Fischen, strukturähnlich zu 5α-Androstan- 3,11,17-trion ist.


Die Ergebnisse zeigten, dass beide 5α-Androstane in den Faeces von Wiederkäuern vorkamen. Der Ausscheidungsverlauf war parallel zu dem der 5β-Androstane, was dafür spricht, dass die 5α-Androstane bzw. ihre Vorstufen von der Nebenniere gebildet werden. Die Ergebnisse der HPLC-Analysen ergaben, dass es sich bei den immunreaktiven Substanzen überwiegend um unkonjugierte Verbindungen handelte, die in mehreren Peaks aus der Säule eluierten.

Diese Ergebnisse zeigen, dass die potentiell androgenaktiven 5α-Androstane in den Faeces von Wiederkäuern vorkommen. Es sollte daher in der Folge untersucht werden, welche Wirkungen diese Verbindungen auf Fische bzw. Säugetiere haben.
7. References


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8. Appendix:

Data related to chapter two: Excretory profiles of 11-KT and 11-OE metabolites in all rams