From the Institute of Medical Biochemistry, Department for Biomedical Sciences, University of Veterinary Medicine Vienna, Austria

Increased androgen concentrations in cow faeces and urine during storage

K. HORAK and E. MÖSTL*

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Summary

There are many of reports concerning the occurrence of oestrogens and gonadal androgens ($C_{19}O_2$ compounds) in animal manure. To date, $C_{19}O_3$ androgens, another group of chemicals that interfere with the endocrine system, have not been included in the investigations as they are considered to be weak androgens in mammals. However, 11-ketotestosterone is a potent natural androgen in many species of fish and 11-oxoaetiocholanolone is a potent odorant/pheromone in at least one species of goby. In the present study we investigated the source and stability of two $C_{19}O_2$ androgens (testosterone and androstenedione) and $C_{19}O_3$ androgens (11-oxoaetiocholanolone and 11β-hydroxyaetiocholanolone) in the manure of cows during storage. Fresh faeces and spontaneously voided urine samples collected from twelve cows were incubated for up to two months. Additionally, bile fluid was analysed to see whether $C_{19}O_3$ steroids are excreted as such via the bile. Urine samples were extracted using diethyl ether, whereas for faecal samples methanol was used for extraction and the concentrations of immunoreactive steroids were determined by enzyme immunoassays. To characterize of the polarity of immunoreactive metabolites, high performance liquid chromatography was used. In urine, there was a significant increase in the

Zusammenfassung

Die Konzentrationen von immunreaktiven Androgenen in Urin und Fäzes von Rindern steigen bei Lagerung an

Einleitung

Während bei Säugetieren Testosteron und Androstendion die dominierenden Androgene sind, kommt bei Fischen zusätzlich 11-Ketotestosteron vor und ist bei diesen Spezies wichtig für die Sexualfunktion. Bei einigen Fischspezies spielen auch Pheromone bei der Fortpflanzung eine Rolle. So wird vermutet, dass 11-Oxoätiocholanolon (ein Steroid mit 19 C-Atomen und drei Sauerstoffatomen, ein $C_{19}O_3$-Steroid) Pheromonwirkung aufweist.

$C_{19}O_3$-Verbindungen kommen auch in den Ausscheidungen von Säugetieren vor. Sie entstehen im Rahmen der Metabolisierung von Glukokortikoiden ($C_{21}O_3$-Verbindungen), wobei speziell Mikroorganismen im Darm eine Rolle spielen. Sie spalten von Glukokortikoiden eine Seitenkette ab, wodurch $C_{19}O_3$ Verbindungen entstehen. Gelangen diese Verbindungen in die Gewässer (z.B. durch die Ausbringung von Gülle auf landwirtschaftliche Flächen), kann potentiell die Fortpflanzung der darin lebenden Fische beeinflusst werden. Wir untersuchten deshalb, ob es nach dem Kot- bzw. Harnabsatz zum weiteren Konzentrationsanstieg von Androgenen kommt, was auf eine Seitenkettenabspaltung durch Mikroorganismen zurückzuführen wäre.

Material und Methode

Kot- und Harnproben wurden bis zu 61 Tage (zwei Monate) gelagert, dann extrahiert (Kotproben mit Methanol, Harnproben mit Diethyläther) und der Gehalt an immunreaktivem Androstendion, Testosteron, 11-Oxoätiocholanolon und 11β-Hydroxyätiocholanolon mittels verschiedener Enzymimmunoassays (EIAs) gemessen. Zusätzlich wurde Gallenflüssigkeit untersucht, um herauszufinden, ob die Verbindungen bereits in der Galle vorhanden sind, oder erst im Darm entstehen.

Ergebnisse

In der Galle waren sowohl immunreaktives 11-Oxoätiocholanolon als auch 11β-Hydroxyätiocholanolon nachweisbar. Im Urin stieg die Konzentration aller gemessenen Steroide bei der Lagerung rasch an, was auf eine Spaltung der im Harn vorkommenden konjugierten Verbindungen zurückzuführen sein kann. In den Fäzes kam es während der Lagerung zu einem Absinken der Konzentration von immunreaktivem 11β-Hydroxyätiocholanolon, die Konzentration der anderen drei Steroide stieg hingegen in der Anfangsphase der Inkubation an. Alle Steroide waren jedoch auch nach 61 Tagen nachweisbar.

concentrations of all four ether-extractible steroids within the first day of incubation. In the bile sample, immunoreactive 11-oxoaetiocholanolone and 11ß-hydroxyaetiocholanolone were present and eluted predominately as unconjugated steroids. In faecal samples, the values of immunoreactive steroids were much higher than in urine samples, reaching median values of up to 210 ng/g faeces in the case of androstenedione. The median immunoreactive 11ß-hydroxyaetiocholanolone concentration was also higher than 200 ng/g and declined during the storage period. The concentration of other steroids significantly ($p<0.1$) increased within the first few days of the incubation period and there was only a slow decline thereafter.

From our data we conclude that these biologically active molecules may be associated with a certain environmental burden for aquatic organisms caused if they are leached into the water.

Diskussion

Da in den Fäzes von Wiederkäuern maximal Spuren von konjugierten Steroidhormonmetaboliten vorkommen, ist der Grund des Konzentrationsanstieges vermutlich die Seitenkettenabspaltung von Vorläufermolekülen. Dadurch kommt es nach der Ausscheidung der Fäzes zum weiteren Anstieg von potentiell androgen-wirksamen Substanzen. Die Umwandlung von Glukokortikoiden zu Androgen-ähnlichen Verbindungen sollte speziell wegen der Umweltrelevanz genauer untersucht werden. In Gebieten mit hohen Tiermassierungen kann es zum Eintrag von biologisch aktiven Steroidmetaboliten in Oberflächengewässer kommen, wodurch sich Auswirkungen auf die Reproduktion von Fischen ergeben könnten. Außerdem sollte ermittelt werden, ob Abwässer von Kläranlagen solche Verbindungen enthalten.

Abbreviations: DHT = Dehydrotestosteron; EDC = endocrine disrupting chemical; EIA = enzyme immunoassay; HPLC = high performance liquid chromatography; 11,17-DOA = 11,17-dioxoandrostenone; 11 ß -OH,17-oxo-A = 11 ß hydroxy,17-oxo-androgen

Introduction

There is some concern about endocrine disrupting chemicals (EDCs) in the environment (GROVER et al., 2011), such as the endogenous and synthetic hormones (for example ethinylestradiol) that are present in sewage effluents and adjacent surface waters. At the moment there is no consensus about the hazard of these substances for human health (TOURAUD et al., 2011), but effects are seen in fish. One of the observations is a relationship between feminization of male fish (known as intersex) and the discharge of steroidal oestrogens downstream of sewage treatment plants (for example see HUO and HICKEY 2007). The increasing density of livestock has also caused apprehension about the environmental releases of various steroid hormones produced by animals in concentrated animal feeding operations (LANGE et al., 2002; JOHNSON et al., 2006; SUZUKI et al., 2009; CHEN et al., 2010; ZHAO et al., 2010; BARTELT-HUNT et al., 2012).

To date, oestrogenic substances have received most attention, but androgens should not be neglected. The anabolic steroid trenbolone, for example, is used for beef production in the USA and is known to have androgenic (ANKLEY et al., 2003) and genotoxic (BOETTCHER et al., 2011) effects.

Androgens have a variety of chemical structures and are not defined via their chemical formula but by whether they have an androgenic action. The natural androgens produced by vertebrates are steroid hormones with 19 carbon atoms and are formed by the gonads and adrenal glands of vertebrates from precursor molecules with 21 carbon atoms. They are metabolized in the liver and are excreted via urine and faeces and reach the environment via sewage effluents or animal manure. Especially in areas with urban agglomerations or concentrated livestock feedlots, these substances have the potential to disrupt the endocrine system of other vertebrates living in the vicinity. The environmental load of natural androgens produced by farm animals has been calculated (LANGE et al., 2002). The authors estimated that calves excreted about 120 mg androgens (mainly calculated as androstenedione plus testosterone) per animal per year, and bulls excreted approximately 390 mg.

The presence of androgenic activity in the faeces of ruminants has been known for a long time. Using the ‚chicken-comb-test', the presence of androgens was reported by HAMMOND (1942) while he was studying the use of bovine manure as a source of B-vitamins for chickens. Sex differences have also been described as RILEY and HAMMOND (1942) detected androgenic activity in the faeces of cows but not in those of bulls after 24 h of incubation of faeces at 45 °C. LEUKER et al. (1960) concluded that the androgenic activity of the cows' faeces was due to the effect of microbial activity on other steroids that were provided by the cows.

SOTO et al. (2004) investigated the androgenic and oestrogenic activity of feedlot effluents and concluded that they contained sufficient concentrations of hormonally active substances to show possible effects on aquatic ecosystems. MANSELL et al. (2011) postulated a potential risk for aquatic organisms caused by oestrogens and androgens from feedlots. The authors collected soil samples and added manure and simulated rainfall. In fresh manure, 17α-oestradiol, testosterone and progesterone were detected, whereas after two weeks 17ß-oestradiol, oestrone and androstenedione were present in the soil.

In some countries certain steroids are allowed for use in finishing in the beef industry and there are data concerning the longevity of these substances

in the environment (SELLIN et al., 2009; KHAN et al., 2008).

Androgens can be degraded by microorganisms and some efforts have been made to understand degradation processes to diminish the load of the runoff water (FAN et al., 2011; LIU et al., 2012). YANG et al. (2011) described aerobic degradation pathways of testosterone in swine manure by manure-borne bacteria.

Androgens produced by vertebrates can be classified according to the number of oxygen molecules in the steroid molecule into $C_{19}O_2$ and $C_{19}O_3$ androgens. In mammals, $C_{19}O_2$ androgens such as testosterone or androstenedione are mainly produced by the gonads, whereas $C_{19}O_3$ androgens (for example 11β-hydroxyandrostenedione), are mainly produced by the adrenals. Some of these substances also are also derived from metabolism of cortisol (PALME and MÖSTL, 1997). The basic principle of the formation of $C_{19}O_2$ and $C_{19}O_3$ androgens from precursor molecules is shown in Figure 1.

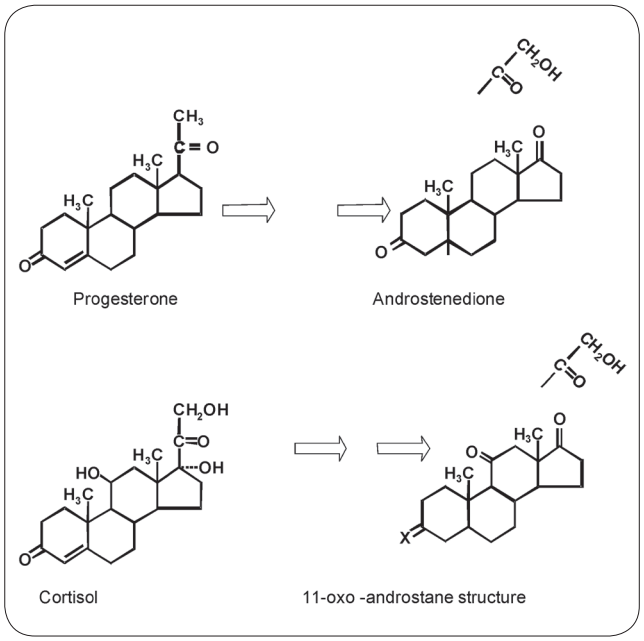

Fig. 1: Formation of $C_{19}O_2$ and $C_{19}O_3$ androgens from precursor molecules

A key enzyme for androgen synthesis is 17-hydroxylase/C17,20-lyase (CYP17). This adds an oxygen group to the 17 position of progesterone and then cleaves off the two-carbon side chain to form androstenedione. The enzyme is controlled by the luteinizing hormone (MURAYAMA et al., 2012). SHACKLETON et al. (2008) described investigations in humans lacking CYP17 as a result of a genetic mutation. One would expect that an individual with such an enzyme defect to be unable to produce $C_{19}O_3$ androgens from exogenous cortisol, but the results showed that the formation of 11-oxo-C19 steroids was normal. The authors concluded that there is some enzyme other than CYP17 that is able to perform side-chain cleavage (perhaps directly on cortisol) in humans.

Some strains of anaerobic bacteria present in the gut of humans and cats are also able to cleave the side chain of cortisol (BOKKENHEUSER et al., 1986).

According to BELANGER et al. (1993), the androgenic activity of $C_{19}O_3$ androgens such as 11β-hydroxyandrostenedione is low using the guinea pig as a model organism. SUZUKI et al. (2000) used rats as experimental animals and also described a weak androgenic activity of this adrenal androgen although they detected an osteotropic effect.

Despite being weak androgens in mammals, the $C_{19}O_3$ androgen 11-ketotestosterone is a strong androgen in teleosts. The testes of some teleosts also naturally form 5α- and 5β-reduced androgens in their gonads (ARBUCKLE et al., 2005), and the 5β-reduced $C_{19}O_3$ steroids are biologically active (GRILLITSCH et al., 2010). The 5ß-reduced metabolite 11-oxoaetiocholanolone (also a $C_{19}O_3$ androgen) has been shown to be a potent odorant (LAFRAMBOISE et al., 2011) and a putative pheromone (CORKUM et al., 2008) in the round goby.

In sheep, PALME et al. (1996) described that about 30% of radioactivity is excreted via faeces after infusing radioactive cortisol. However, MÖSTL et al. (2002) showed that cortisol was completely metabolized in the faeces of ruminants. PALME and MÖSTL (1997) showed that the measurement of androgens (especially 11,17-dioxoandrostanes, 11,17-DOA) in the faeces of sheep could be used to monitor glucocorticoid production. This was subsequently confirmed in other ruminant species (MÖSTL et al., 2002; KLEINSASSER et al., 2010; ROUHA-MÜLLEDER et al., 2010). Measuring glucocorticoid metabolites in faeces as a parameter for glucocorticoid production is now widely used for monitoring disturbances, but requires freezing of samples soon after collection as for example LEXEN et al. (2008) described an increasing concentration of cortisol metabolites if samples were allowed to stand at room temperature after defecation. The authors concluded that formation of 11,17-DOA in the faeces originates from the side chain cleavage of cortisol metabolites by microorganisms.

This neo-formation of C19 steroids by microorganisms has so far been neglected as a potential source of endocrine disrupting substances until now. The aim of our investigation was to obtain information on potential environmental pollution caused by cattle manure formed in the excreta of cows.

Material and Methods

To mimic conditions of the manure of cows in the environment, various incubation conditions (different temperatures, aerobic/anaerobic) were used. Fresh faeces and spontaneously voided urine samples were collected from 12 cows (non-pregnant or at the beginning of pregnancy) at the research farm of the

University of Veterinary Medicine Vienna. A fraction of each sample was placed in glass test tubes (16x105 mm) and frozen immediately at -20 °C to avoid further metabolism (PALME et al., 2005) and stored until analysis. The other part of the samples was brought to the lab for the incubation experiment. Glass tubes were filled with 5 ml urine (non-sterile) or 2 g (aerobic storage) or 10 g (non aerobic storage) faeces and closed with plastic plugs. For aerobic storage, two small holes with a diameter of approximately 2 mm were pierced into the plugs of the tubes to allow air circulation.

Samples from six cows were incubated at 20 °C, the excreta of the other six cows were incubated at 38 °C for 1, 2, 3, 4, 5, 6, or 7 days, two weeks, three weeks, four weeks and two months. After incubation, the samples were stored in a freezer at -20 °C until analysis to inhibit further metabolism. Additionally, bile fluid was collected from one cow at necropsy.

Sample extraction and analysis

Before analysis, sample weight losses caused by evaporation of water during the incubation period were compensated by adding distilled water up to the original weight. Afterwards the samples were vortexed for 15 minutes.

Urine samples (300 µl) were extracted for 30 min in glass tubes using 5 ml diethyl ether. After freezing the water phase at -20 °C overnight, the organic phase was transferred to a new vial and evaporated under a stream of nitrogen. The extract was redissolved in 300 µl assay buffer. Faecal samples were extracted as described by PALME and MÖSTL (1997) and the methanolic supernatants were used for analysis after dilution with assay buffer. As representatives for gonadal androgens the concentrations of the two androgens testosterone and androstenedione were measured using enzyme immunoassays (PALME and MÖSTL, 1994). In case of $C_{19}O_3$ androgens, two groups of 5ß-reduced substances were measured, 11,17-DOA and 11ß-hydroxy, 17-oxo-androgens (11ß-OH,17-oxo-A). These enzyme immunoassays (EIAs) have been described by PALME and MÖSTL (1997) and FRIGERIO et al. (2004). 11-oxoaetiocholanolone and 11ß-hydroxyaetiocholanolone were used as standards.

The intra- and inter-assay coefficients of variation of the assays were 7.9 and 12.9 for testosterone, 10.2 and 15.8 for androstenedione, 12.2 and 14 for 11,17-DOA and 9.1 and 13.1 for the 11ß-OH,17-oxo-A. A high performance liquid chromatography (HPLC) separation of the immunoreactive 11,17-DOA into a high- and a low-level pool sample was performed to characterize the immunoreactive metabolites before and after two months of incubation. One ml of each pool (methanolic faecal extracts) was transferred into a new vial and diluted with distilled water (9 ml). The samples were extracted using Sep-Pak® C18 cartridges (solid phase extraction). After rinsing the column with 4 ml water and eluting the cartridge with methanol (4 ml), the organic solvent was evaporated using a stream of nitrogen. The bile sample (1 ml) was diluted with water and extracted for HPLC as described for the cleanup procedure for HPLC analysis of faeces samples. The extracts for chromatography were re-dissolved in 400 µl of 40% methanol. 100 µl were used for HPLC (Novapac® C18 column, diameter 0.39 x 15 cm, Fa. Waters, Milford, Mass.; solvent: water/methanol; start 40% methanol; linear gradient from 40% methanol to 80%; flow 1 ml per min; 3 fractions per minute were collected). 60 fractions were tested for immunoreactive substances reacting with the 11-oxoaetiocholanolone assay. The elution of 11-oxoaetiocholanolone (standard) was also determined.

Statistical analysis

The concentrations of all four incubation conditions were combined and grouped according to the duration of incubation as the sum of the various conditions may reflect potential conditions in the environment. We calculated whether there were statistically significant differences between the results of the four steroid assays on day 0 (no incubation) compared to those at the other time points. Data were analysed using the software package SPSS (IBM SPSS Statistics, Friedman test) and by hand according to SIEGEL and CASTELLAN (1988). The graphs were prepared using SigmaPlot 9.0.

Results

In ether extracts of the urine samples frozen immediately after collection, the concentrations of all four immunoreactive steroids were close to the detection limit of the EIAs. Comparing the concentrations measured in the samples of spontaneously voided samples and samples after one day of incubation there was a significant ($P<0.001$) increase under all incubation conditions, and the median values reached maximum values within the first week (Fig. 2).

The concentrations remained elevated during the entire period investigated. The variation of the steroid concentrations increased with increasing duration of the incubation and the increase was more pronounced at 38 °C than at room temperature. Highest median concentrations of all four substances were measured by the androstenedione assay (median value on day 6 10.1 ng/ml), followed by the 11-oxoaetiocholanolone assay (median 4.7 on day 5). The concentrations of 11ß-OH,17-oxo-A reached a peak of 2.5 ng/ml on day 7 of the incubation (Tab. 1). The median of ether-extractable immunoreactive testosterone concentration in urine was the lowest of all C19-steroids investigated. The maximum of the median concentrations was seen after two weeks (1.1 ng/ml).

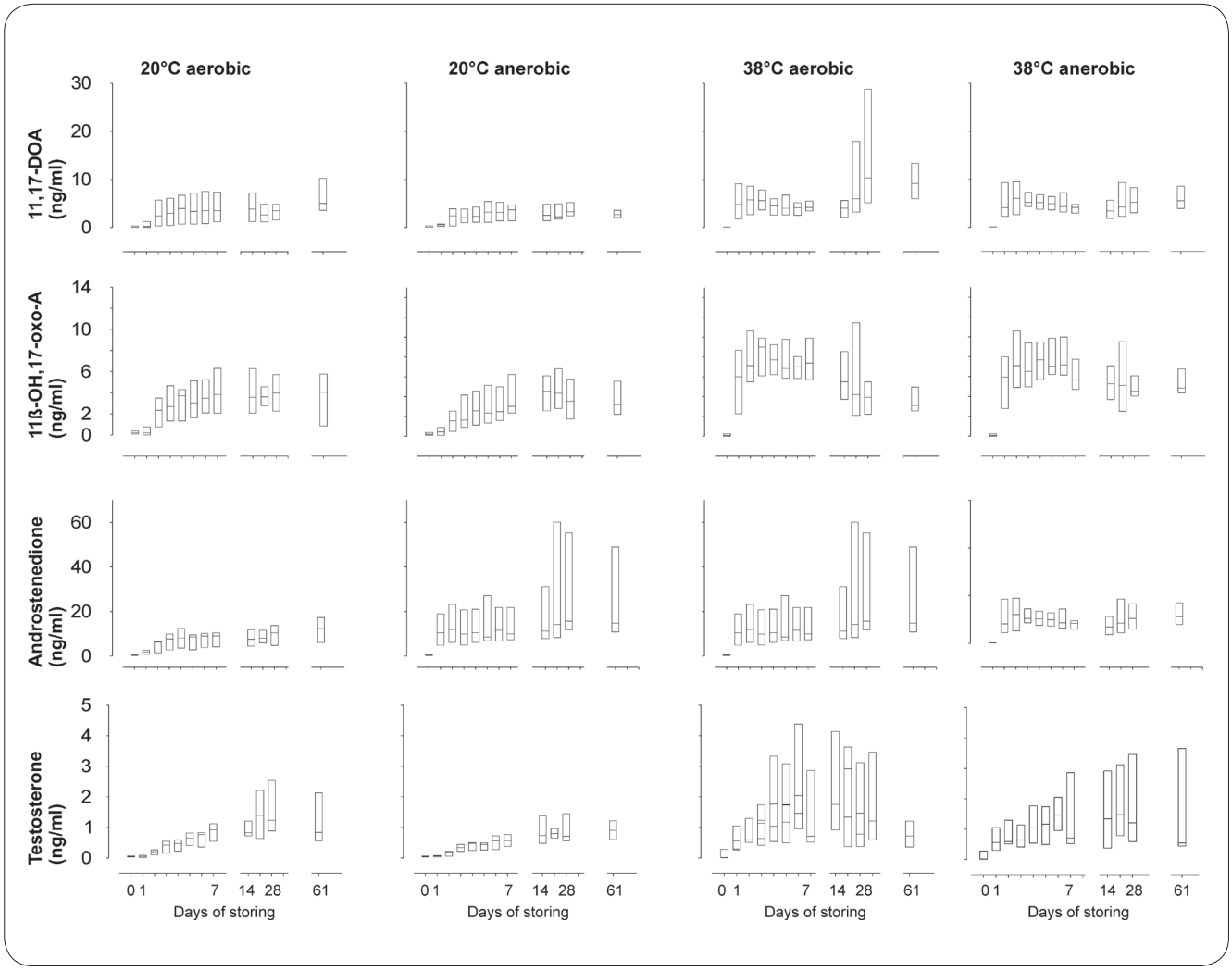

Fig. 2: Box-plots of immunoreactice androgens in the ether extracts of incubated urine samples

In faeces, the concentrations of immunoreactive steroid metabolites were much higher than in urine (Tab. 1). Highest concentrations were measured in the assay for 11ß-OH,17-oxo-androgens in samples frozen immediately after collection (median 203 ng/g). The concentrations of these immunoreactive steroids declined during incubation and the values measured were significantly ($P \leq 0.05$) lower at the end of the incubation period compared with non-incubated samples. In the other three groups of metabolites, peak concentrations were reached after one day of incubation (Tab. 1 and Fig. 3), the values were significantly higher on day one compared to samples frozen immediately after defecation.

In faeces, as in urine, androstenedione showed higher concentrations than the other metabolites measured, followed by 11ß-OH and 17-oxo-A. In faeces, the values of the 11,17-DOA, androstenedione and testosterone increased significantly during the first day of incubation.

The analysis of the bile fluid showed that immunoreactive 11-oxoaetiocholanolone and 11ß-hydroxyaetiocholanolone were present and eluted predominantely as unconjugated steroids (Fig. 4). As shown by the results of the analysis of the HPLC fractions of faecal samples (Fig. 5), storing for two months had no significant influence on the immunoreactive substances measured using the 11,17 DOA assay.

Three immunoreactive peaks were detectable in fresh samples using this assay, with the peak at fraction 32 eluting as authentic 11-oxoaetiocholanolone. Analysing incubated samples, two additional smaller peaks were detected, which eluted earlier from the column.

Discussion

Urine

The concentration of immunoreactive C19-steroids was much higher in faeces than in urine but it should be noted that only ether-extractable androgens were monitored in urine. PALME et al. (1996) showed that in sheep, most of the infused radioactive testosterone or cortisol was excreted via urine and most of these metabolites were not extractable using diethyl ether

Tab. 1. Immunoreactive androgens (median values) in faeces and urine after storing. The values represent equivalents of the standard used (n.d. = non detectable).

	Faeces				Urine			
Day	11ß,17-oxo-A	11,17-DOA	ADN	Testosterone	11ß,17-oxo-A	11,17-DOA	ADN	Testosterone
0	203	18	45	17	0.1	0.1	0.4	n.d.
1	140	59	210	41	0.4	1.5	2.8	0.1
2	118	24	178	36	0.9	3.0	7.7	0.4
3	140	23	161	29	1.5	4.7	8.2	0.5
4	119	25	133	30	1.8	4.3	8.3	0.6
5	121	20	118	24	1.8	4.0	9.0	0.7
6	111	16	95	23	2.1	3.4	10.1	0.9
7	107	13	95	18	2.5	4.0	9.9	0.8
14	108	9	81	14	2.3	3.1	8.4	1.1
21	75	23	102	11	2.5	4.0	11.4	0.9
18	142	24	111	13	1.8	4.8	12.6	1.0
61	44	17	126	13	1.8	5.2	11.9	0.8

(i.e the steroids are conjugated). In our experiment, the increase of all four steroids measured in urine can be attributed to either deconjugation of precursor molecules (conjugated androgens) by microorganisms or by neo-formation of androgens from C21-precursor molecules (as microorganisms were present in urine samples). It is pssible that both mechanisms may be involved. The microbial conversion of steroids in urine is already known and some researchers recommend the use of substances such as sodium azide for preservation (SAUDAN et al., 2006). TSIVOU et al. (2009) investigated the use of preserving substances in human urine sent to the laboratory for doping control and found that a mixture of antibiotics, antimycotic substances and protease inhibitors is able to prevent steroid degradation at 37 °C.

Bile

The presence of immunoreactive 11-oxoaetiocholanolone and 11ß-hydroxyaetiocholanolone in the bile fluid indicates that those metabolites are excreted by the liver of the cows. As steroids undergo an enterohepatc circulation the amount of $C_{19}O_3$ steroids produced by the cows and the amount formed by bacterial enzymes cannot be determined but $C_{19}O_3$ steroids are already present in the bile.

Faeces

To our knowledge, the presence of immunoreactive 11ß-OH,17-oxo-A in the faeces of cows had not previously been described. The decrease of the 11ß-OH,17-oxo-A values during storage can be explained by a conversion of the 11ß-hydroxy group into an 11-oxo group (caused by the enzyme 11ß-hydroxysteroid-dehydrogenase).

The increase of the 11,17-DOA values during the first day of storing sheep samples at room temperature was described by LEXEN et al. (2008). These authors proposed that microorganisms in the gut are able to cleave the side chain of an unknown cortisol metabolite. Another source of these metabolites could be the 11ß-OH,17-oxo-A, as a simple conversion of the oxo-group at position C 11 will form 11,17-DOA and this enzyme may be present in microorganisms of the gut. A deconjugation of conjugated precursor molecules into unconjugated and immunoreactive 11,17 DOAs is less plausible as PALME and MÖSTL (1997) showed that conjugated glucocorticoid metabolites are not

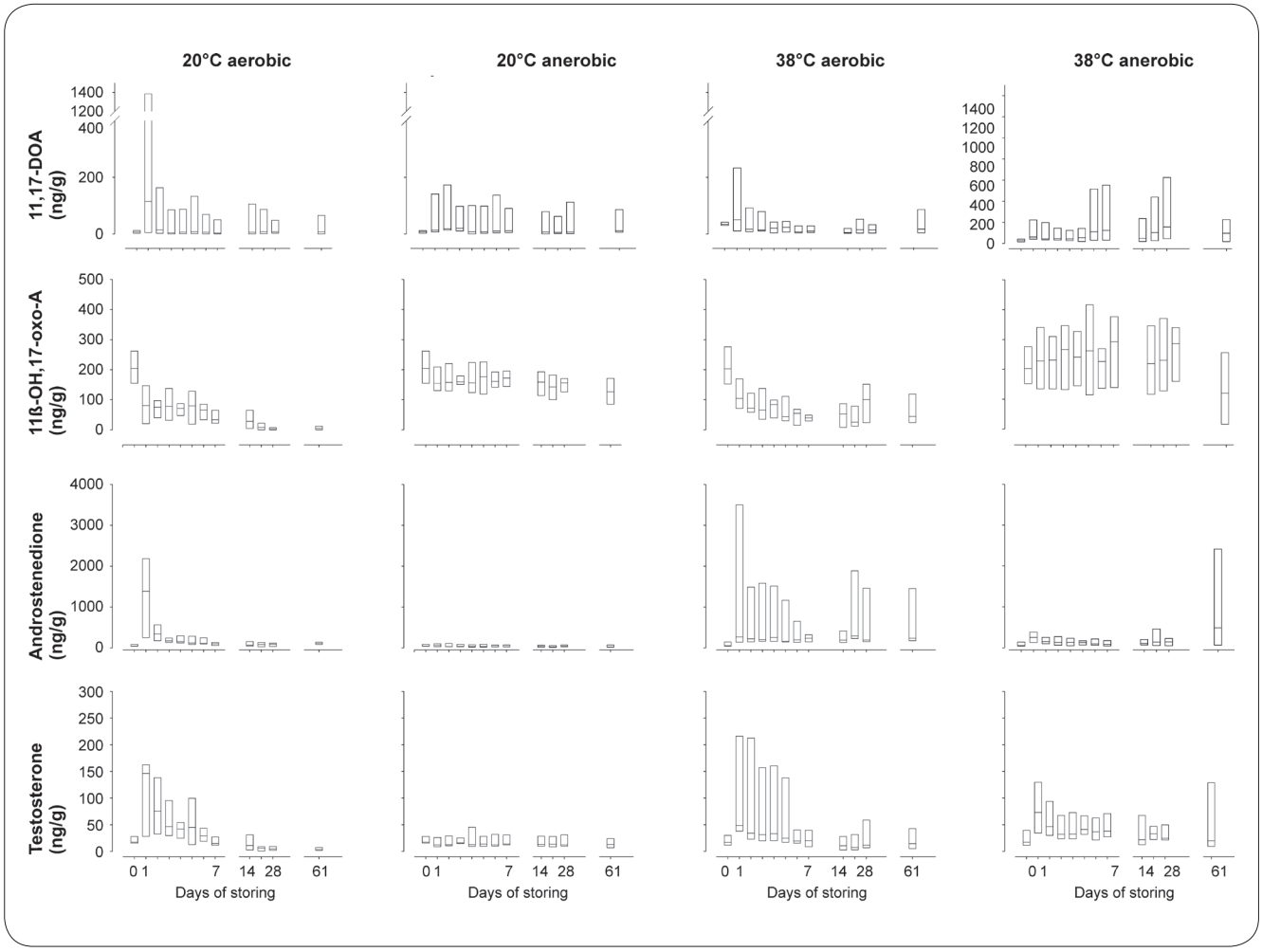

Fig. 3: Concentrations of the immunoreactive androgens (shown as box-plots) of incubated faecal samples stored under different conditions during the incubation period

present in faecal samples of cows.

The levels of the immunoreactive gonadal androgens, androstenedione and testosterone, also increased within the first 24 hours of incubation. This may be explained by a similar mechanism as postulated for 11,17-DOA (side chain cleavage of a precursor molecule).

Another explanation for the increasing androgen concentrations in the manure may be a conversion of 17α-hydroxyandrogens into the 17β-OH or 17-oxo-form. It is known that ruminants (especially cows) convert androstenedione into epitestosterone and this conversion is showed by red blood cells. So a great deal of androgens voided by ruminants may be excreted in the form of epitestosterone (VELLE, 1976) and related substances and those 17α-hydoxylated compounds could be reconverted into substances with a 17-oxo or 17β-hydroxy group such as androstenedione or testosterone.

As the results showed that all four steroids are quite stable in the faeces, some of these substances may potentially reach the surface water. Gonadal androgens are potential endocrine disruptors and these substances (including synthetic androgens such as trenbolone) have been shown to persist in two soil types (KHAN et al., 2008) and livestock feedlot effluent in surface waters (SOTO et al., 2004).

The higher concentrations of $C_{19}O_3$ androgens in faeces than in urine may be caused by the production of these substances in the gut by microorganisms. The increase of the 11,17-DOA concentration after defecation is a further indicator that microorganisms are the source of these metabolites in the manure of the cows.

HPLC analysis of faecal extracts using the 11-oxo-aetiocholanolone EIA showed three peaks of immunoreactive material in the pool samples. The peak eluting around fraction 31 had the same chromatographic mobility as authentic 11-oxoaetiocholanolone and we suggest that this peak represents authentic 11-oxoaetiocholanolone. That more than one immunoreactive substance reacts in the 11,17-DOA-assay has previously been described described by MÖSTL et al. (2002) using a straight phase system and results from the cross reactions of the assay used. This assay is described as ‚group specific', as it does not discriminate be- tween different substances in the position 3 of the steroid molecule, but all three peaks are most probably 5ß-androstanes with an 11,17-dioxo-group.

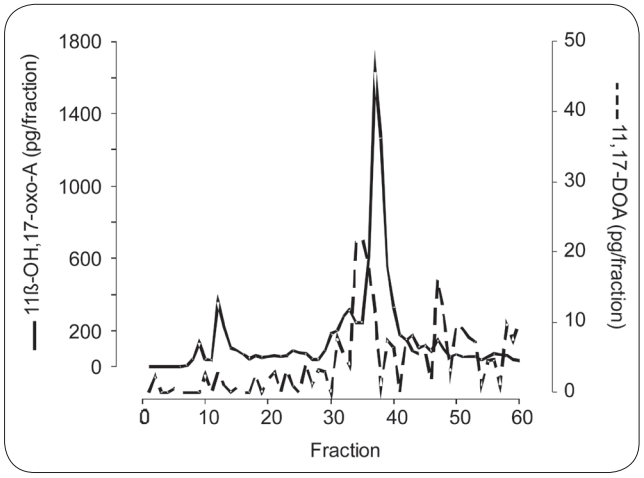

Fig. 4: Elution pattern of immunoreactive 11-oxoaetiocholanolone and 11β-hydroxyaetiocholanolone in the extract of a bile sample

Storing faecal samples for 61 days did not alter the pattern of immunoreactive metabolites although that the values were somewhat higher. From these data we conclude that 11-oxoaetiocholanolone remains in faeces for a long period.

The results of PALME and MÖSTL (1997) showed that cortisol metabolites in faeces are best dissolved in 80% methanol, whereas the solubility in water is poor and the metabolites remain in the particles of the faeces. This may be a hint that in the environment rainfall will only slowly dissolve these steroids. The experiment incubating urine and faeces may not reflect the situation in dung piles, where the manure is stored under anaerobic conditions and elevated temperatures.

In swine manure, DERBY et al. (2011) investigated the effect of composting under aerobic and anaerobic (static manure pile) conditions. The authors measured the oestrone and oestradiol concentrations and described a reduction by 74% in the static manure pile and 79% in the composted pile after three months.

In our experiments storing cow manure, the immunoreactive concentrations of androstenedione, testosterone and 11,17-DOA in faeces were higher in stored samples than in samples frozen immediately after collection.

It would be worth investigating whether the fate of $C_{19}O_3$ androgens in the environment is similar to that described by Mansell et al. (2011) for $C_{19}O_2$ androgens. To date, most investigators have focused on the fate of the ‚classical' androgens such as testosterone and androstenedione and the potential environmental impact of the metabolites has been neglected. It is well known that the testosterone metabolite dihydrotestosterone (DHT) is a stronger androgen than testosterone in mammals, and using fish (Fathead Minnow, *Pimephales promelas*) as an experimental model MARGIOTTA-CASALUCI and SUMPTNER et al. (2011) showed DHT to be a potent androgen. Similar results were obtained when the androgenic potency of a 5α-$C_{19}O_3$-androgen

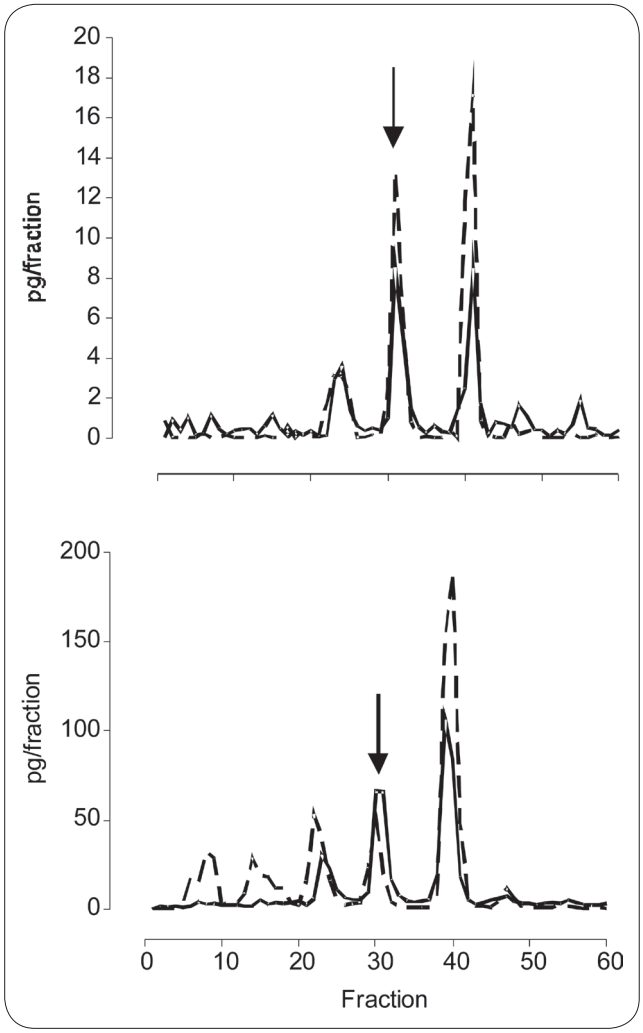

Fig. 5: Elution pattern of immunoreactive substances of two faecal pool samples without (solid lines) and after 61 days of incubation (dashed lines) using the 11-oxoaetiocholanolone assay. The arrows represent the elution position of authentic 11-oxoaetiocholanolone.

investigated by GRILLITSCH et al. (2010). As PALME et al. (1997) showed that after infusion of progesterone 5α- and 5β-reduced metabolites are excreted via faeces it is expected that 5α-$C_{19}O_3$-androgens are excreted as cortisol metabolites in ruminants.

From our results we conclude that substantial amounts of $C_{19}O_3$ and $C_{19}O_2$ steroids are present in the faeces of cows and persist for some time after defecation. As there is some evidence that these substances are hormonally active in fish, their presence in the environment might be of importance.

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***Corresponding author's address:**
Erich Moestl
University of Veterinary Medicine Vienna
Veterinärplatz 1, 1210 Vienna, Austria
email: erich.moestl@vetmeduni.ac.at