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Androgens and their metabolites in faeces of non-pregnant and pregnant cows and sows

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Summary

The gonads, the adrenals and in many mammal species also the placenta produce steroid hormones. The production and function of gestagens and oestrogens are well documented but less is known about androgens in females, where they are often considered as mere precursors of oestrogens.

We believe that substantial amounts of various androgens of placental origin may be present in the faeces. To test this notion, we evaluated and characterized faecal androgens/metabolites in sows and cows during oestrus and at different stages of pregnancy. We took faecal samples from eight sows and six cows on the day of oestrus and 16 days later as well as from eight pregnant sows on day 110 of gestation, from ten cows on day 150 of gestation and from eight cows on day 240 of gestation. We used enzyme immunoassays to measure the concentrations of aetiocholanolone (Et) and epiandrosterone (Ep) as representatives of the 5ß- and 5α-17-oxo-androstanes and of testosterone (T) and 5ß-dihydrotestosterone

Zusammenfassung

Androgene und ihre Metaboliten in den Faeces von nicht-trächtigen und trächtigen Kühen und Sauen

Einleitung und Fragestellung: Steroide mit 19 Kohlenstoffatomen (C19-Steroide, Androstene und Androstane) werden in den Gonaden, den Nebennieren und bei einigen Säugetierspezies auch in der Plazenta gebildet und in das Blut abgegeben. Die C19-Steroide werden in der Leber metabolisiert und dann über den Urin oder die Galle ausgeschieden. Bisher konzentrierten sich die Untersuchungen von physiologisch vorkommenden C19-Steroiden in den Faeces überwiegend auf Testosteron. Die Ausscheidung von Androstanen mit einer Oxo-Gruppe an der Position 17 des Moleküls wurde kaum untersucht, obwohl es sich teilweise um bioaktive Stoffe handelt. Unsere Hypothese war, dass über die Faeces vor allem Androgenmetaboliten ausgeschieden werden.

Material und Methoden: Kotproben wurden von acht klinisch gesunden Schweinen und sechs Kühen an den Tagen 0 und 16 des Zyklus, sowie von acht Schweinen am Tag 110 der Gravidität, von zehn Rindern am Tag 150 und von acht Rindern am Tag 240 der Gravidität, entnommen und bis zur Probenvorbereitung bei -20 °C eingefroren. Nach Extraktion wurden die Proben mittels Enzymimmunoassays zum Nachweis von zwei 17-Oxosteroiden (Ätiocholanolon und Epiandrosteron) sowie zwei 17ß-Hydroxysteroiden (5ß-DHT und Testosteron) analysiert. Um die gemessenen Steroide zu charakterisieren, wurden zusätzlich RP-HPLC-Analysen durchgeführt. Weiters wurde die Androgenaktivität in den Kotproben mittels eines Androgen-Rezeptor-Tests gemessen.

Ergebnisse: In allen Proben konnten mittels Immunoassays verschiedene Androgenmetaboliten gemessen werden. Die gemessenen Konzentrationen stiegen während der Gravidität an. Bei beiden Spezies waren die Konzentrationen gegen Ende der Trächtigkeit signifikant

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(5ß-DHT) as representatives of the 17ß-hydroxyandrostanes. We measured the concentration of receptor-active androgenic substances (expressed as testosterone equivalents) using an androgen receptor assay. We used reversed-phase high performance liquid chromatography (RP-HPLC) immunograms to characterize the immunoreactive substances.

Immunoreactive and receptor-active androgens and androgen metabolites were present in all samples, with higher amounts in cows than in sows. The immunograms showed that only unconjugated steroids were present in the faeces. All C19-steroid concentrations increased significantly towards term, indicating that the placenta produces these steroids or their precursors. During the end of pregnancy, the prevailing metabolites in cows cross-react with the Et assay; those in sows react with the Ep assay. Near the end of gestation, there was a distinct increase of the receptor-active androgens. In pregnant sows, the concentration of receptor-active androgens was higher than the results of the immunoassay but the increase close to the end of gestation was not as pronounced as in cows. In cows, the androgen receptor assay showed significantly higher concentrations than the testosterone immunoassay on days 0, 16 and 240 but not on day 150, whereas the concentrations in faecal samples of sows were significantly higher only on day 16. The difference in the concentrations measured by the two assays may be a hint that androgenic substances other than T and 5α-DHT are present in the faeces, especially in cows.

Our results show that high concentrations of C19-steroids are excreted in the faeces of pregnant cows and sows. We conclude that the placenta is the main source of these molecules, especially in the later periods of gestation.

Abbreviations: ARE = androgen-responsive element; BSA = bovine serum albumin; CL = *corpus luteum*; DHEA = dehydroepiandrosterone; DHT = dihydrotestosterone; EIA = enzyme immunoassay; Ep = epiandrosterone; Et = aetiocholanolone; hAR = human androgen receptor; RP-HPLC = reversed-phase high performance liquid chromatography; PCOS = polycystic ovarian syndrome; T = testosterone

höher als am Tag des Östrus. Auch die Untersuchung mittels des Androgen-Rezeptor-Assays ergab ähnliche Ergebnisse, wobei höhere Werte als mit dem Testosteronimmunoassay gemessen wurden. Die HPLC-Analysen zeigten, dass jeweils mehrere immunreaktive Metaboliten vorhanden waren. Aufgrund des Elutionsverhaltens handelte es sich dabei ausschließlich um unkonjugierte Verbindungen.

Interpretation und Diskussion der Ergebnisse: In den Faeces beider Spezies stiegen während der Gravidität die Konzentrationen aller untersuchten C19-Steroide an. Bei beiden Spezies dominierte die Ausscheidung von 17-Oxoandrostanen (Ätiocholanolon und Epiandrosteron) gegenüber den 17ß-Hydroxyandrogenen (Testosteron und 5ß-DHT). Das 5ß-reduzierte Androstan Ätiocholanolon war in den Faeces von Rindern in höherer Konzentration vorhanden als die entsprechende 5α-reduzierte Verbindung Epiandrosteron. In den Faeces von Sauen war während der Gravidität Epiandrosteron dominierend. Aufgrund der signifikant erhöhten Ausscheidungen von Steroiden in der Endphase der Gravidität ist anzunehmen, dass die Plazenta beider Spezies die Bildungsstätte dieser Steroide oder deren Vorläufer ist. Die Ergebnisse des Rezeptor-Assays sind eine Bestätigung dafür, dass im Kot beider Spezies androgenaktive Substanzen ausgeschieden werden. Dabei war auffällig, dass die mit dem Androgen-Rezeptor-Test im Rinderkot erhobenen Werte an den meisten untersuchten Tagen signifikant höher waren als die mittels Testosteron-Immunoassays ermittelten. Das kann dadurch erklärt werden, dass in den Faeces von Rindern noch andere Androgene außer Testosteron und 5α-DHT enthalten sind. Insgesamt zeigen die Ergebnisse, dass speziell in der Endphase der Gravidität erhebliche Mengen an Androgenen in der Plazenta gebildet werden. Möglicherweise gibt es bei Kühen und Sauen für die Androgene im Rahmen der endokrinen Regulation von Gravidität und Geburt Wirkmechanismen, die über die Rolle als Östrogenvorstufe hinausgehen.

Introduction

Natural androgens are steroid hormones and contain 19 carbon atoms in their molecule. By definition, they can bind to androgen receptors to trigger the development and maintenance of male characteristics but the term androgen is also commonly used for their metabolites and other C19-steroids such as androstenedione, dehydroepiandrosterone (DHEA) and sulfonated DHEA (DHEAS) that have low activity at the androgen receptor (Schuler et al. 2018). Androgens are best known as male sex hormones but also have essential functions in female reproduction.

Androgens, their precursors and synthesis in mammals

Steroids are widespread in eukaryotic organisms (Möstl 2021). The precursor of steroid hormones (except vitamin D) is pregnenolone, which is mainly produced in the adrenals, gonads, the brain and in some species in the placenta. Pregnenolone can be converted into progesterone, a hormone involved in the regulation of the oestrus cycle and pregnancy. As pregnenolone, it also functions as an intermediate in the synthesis of androgens, oestrogens and glucocorticoids. The main sources of progesterone in cows and sows are the *corpora lutea* (Meyer 1994) but progesterone is also produced in the ovarian follicles, the adrenals and

the placenta. In sows, the *corpora lutea* are required for the maintenance of pregnancy during the entire gestation period, whereas the placenta can maintain pregnancy in cows without luteal progesterone from day 180 until day 240 (Schuler et al. 2018).

Vertebrates produce androgens from pregnenolone or progesterone (C21 precursor molecules) by the enzymatic side-chain cleavage of a two-carbon group catalysed by the enzyme 17,20-lyase (for example, see Miller et al. 1997). There are three known pathways for the synthesis of androgens, depending on the precursor molecule from which the side chain is cleaved. The Δ-5 pathway starts from pregnenolone and proceeds via 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA), a C19-steroid, which is the pre-hormone of androstenedione (Lee & Kim 2022). The route in the Δ-4 pathway is via progesterone, 17α-hydroxyprogesterone to androstenedione. Both pathways result in the production of androstenes (DHEA, androstenedione and testosterone, T), which are converted to androstanes by two types of enzyme, 5α- and 5ß-reductases. In the case of 5α-reductases, the product (e.g. 5α-dihydrotestosterone, 5α-DHT) has a planar structure like that of the precursor molecules and is a strong androgen, whereas 5ß-reduction, resulting in e.g. aetiocholanolone (Et), causes a non-planar structure with a 90° bend between rings A and B of the steroid (Fig. 1).

In humans, a third pathway is described: the "backdoor pathway". 17α-hydroxyprogesterone is 5α-reduced and then converted to 5α-androstane-3,17-dione and finally to 5α-DHT (Miller & Auchus 2019; O'Shaughnessy et al. 2019). This pathway bypasses DHEA, androstenedione and T but also results in the formation of 5α-DHT, an even more potent androgen than T. Unlike the case in the Δ-5 or Δ-4 pathway, the C19-steroids in the backdoor pathway cannot function as oestrogen precursors because they lack the double bond in ring A. Fig. 2 shows the three pathways for the synthesis of androgens.

Androgens in sows and cows

During the oestrus cycle, sows and cows mainly produce androgens in the ovarian follicle and the adrenal glands. The follicular fluids of sows with a higher average follicular size have higher oestradiol-17ß concentrations than follicular fluids from sows with smaller follicles but the concentrations of other steroids, including 19-norandrostenedione and T, do not differ between the two groups (Costermans et al. 2020). There are significant differences in the concentrations of progesterone, 17α-hydroxyprogesterone, androgens and oestrogens in the fluid of bovine ovarian cysts with granulosa cell layers and those without granulosa cell layers. The latter are not capable of forming 17α-hydroxyprogesterone, androgens or oestrogens but have a high concentration of progesterone. The steroid metabolism of these cysts is functionally similar to that of the bovine *corpus luteum*, which has a restricted capacity to produce androgens and oestrogens (Choi et al. 1983).

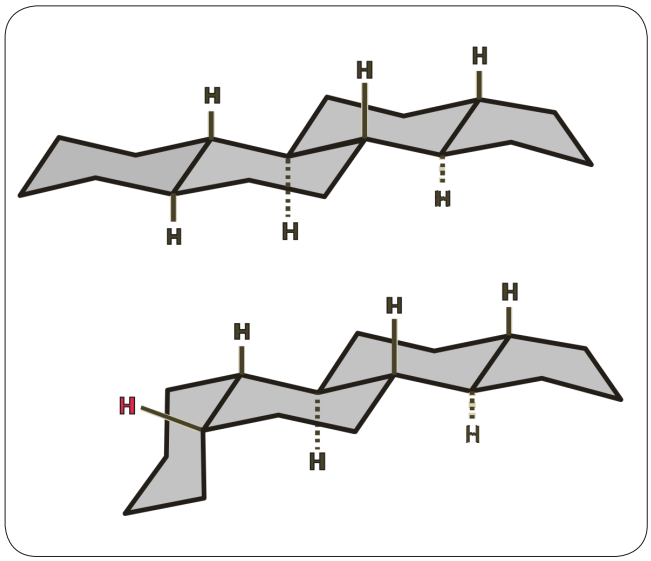

Fig. 1: Different configurations of a 5α-steroid (top; a planar molecule) and a 5ß-steroid (bottom, with a bend between the first two ring systems); modified from Hill et al. (1991) / Verschiedene Konfigurationen eines 5α-Steroids (oben planare Molekülstruktur) und eines 5ß-Steroids (unten, mit einem Knick zwischen den ersten zwei Ringsystemen); nach Hill et al. (1991)

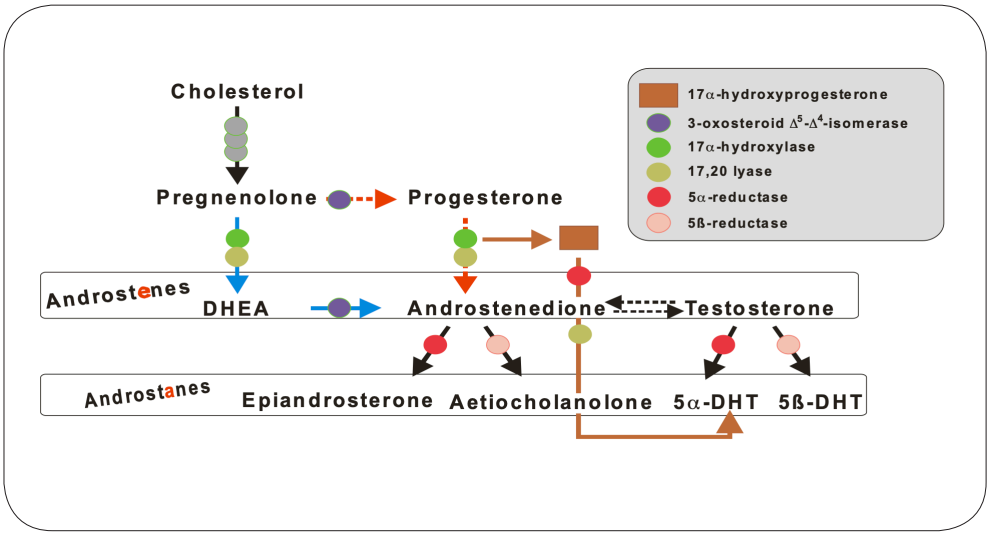

Fig. 2: Δ-5 pathway (blue), Δ-4 pathway (red) and the backdoor pathway (brown) for androgen synthesis. Enzymes are symbolized by coloured points. / Der Δ-5 Pfad (blau), Δ-4 Pfad (rot) und der "backdoor" Pfad (braun) für die Androgensynthese. Enzyme sind als farbige Punkte dargestellt.

The concentrations of the 5α-reduced metabolites are extremely low and fairly constant in bovine follicular fluids with high or low oestrogen concentrations (Prevost et al. 1989). By incubating pieces of the bovine follicular wall with radioactive pregnenolone or progesterone, it was shown that pregnenolone is by far the more effective precursor, so the bovine follicle predominantly produces androgens and oestrogens via the Δ-5 pathway (Fortune 1986).

In pregnant animals, the source of androgens for oestrogen synthesis depends on the species-specific capacity of the placenta to cleave the side chain of the androgen precursors (pregnenolone, progesterone). Human and equine placentae depend on the provision of androgens such as androstenedione and T from extraplacental sources (Raeside 2017), which can be converted into oestrogens, whereas placentae in species such as the cow (Schuler et al. 1994) can produce androgens themselves. The bovine placenta predominantly uses the Δ-5 pathway for oestrogen synthesis, as it has minimal 17-20-lyase activity that would be required for the Δ-4 pathway (Nguyen et al. 2012). The sheep placenta also preferentially uses the Δ-5 pathway for oestrogen biosynthesis (Reynolds et al. 2018).

At the end of gestation, the foetus of cows and sheep produces a high amount of cortisol, which causes an increase of 17α-hydroxyprogesterone, a direct androgen precursor (Ricketts et al. 1980). The cortisol increase is the signal for the start of parturition. It can be imitated by injecting synthetic glucocorticoids into the dam and both glucocorticoids cause an increase of androgens in the blood of cows (Möstl et al. 1985).

The androgen receptor

The androgen receptor is located in the cytoplasm and is activated by binding to any androgenic hormone such as T or 5α-DHT. After binding, the receptor-androgen-complex translocates into the nucleus where it functions as a DNA-binding transcription factor, regulating gene expression (for review see Davey & Grossmann 2016). In addition to binding to the classical intracellular androgen receptor, in various species androgens can also rapidly act via membrane receptors that activate adenylate cyclase (for review see Thomas 2019). 5ß-androstanes do not bind to the classical intracellular androgen receptor because they have a different shape than that of 5α-reduced steroids (Fig. 1). Their biological effects are mediated by other mechanisms (see e.g. Li et al. 2007).

Effects of 5ß-androstanes

5ß-androstanes are often considered as mere degradation products of androgens but some of them show biological effects. For example, under the influence of the 5ß-steroid Et human leukocytes produce a substance that causes fever (Kappas et al. 1957). Similarly, 5ß-androstanes stimulate the growth of early and late human erythroid progenitor cells in cell culture together with erythropoietin (Urabe et al. 1979) and human osteoblasts treated with various androstenes and androstanes, including Et, have improved proliferation and differentiation (Wu & Zahng 2018). The two steroids androsterone and Et (5α- and 5ß-androstanes) have anticonvulsant properties in rodents with epilepsy. Although of low potency, they are present in high concentrations and could represent endogenous modulators of seizure susceptibility (Kaminski et al. 2005).

In a human *in vitro* model, androstenes and androstanes have a relaxing influence on the contractility of isolated myometrium. All androgens tested caused a concentration-dependent inhibition of spontaneous contractility activity, with 5ß-dihydrotestosterone far more potent at inducing myometrial relaxation than the other androgens (Perusquia et al. 2005).

Androgen effects in female reproduction

- **Modulation of the *corpus luteum* (CL) during pregnancy**

The administration of flutamide (a selective androgen receptor antagonist) to pregnant sows influences androgen and oestrogen metabolism in the CL. The effects of injecting flutamide depend on the time period of gestation. During days 43–49, anti-androgen treatment increases the progesterone concentration in blood, whereas injection of flutamide later in gestation causes a decrease in the concentration of the hormone. This shows that androgens regulate the function of the *corpora lutea* in pregnant sows (Grzesiak et al. 2014).

- **Effects of androgens in the uterus and cervix**

Some complications during human pregnancies are thought to be caused by androgens. Some pregnancy disorders in humans (pre-eclampsia, gestational diabetes, intrauterine growth restriction and polycystic ovarian syndrome PCOS) are associated with abnormal androgen levels or androgen signalling (Parsons & Bouma 2021). Very little is known about the role of androgens in pregnancy disorders in ruminants and pigs (Hord et al. 2020), although an excess of T in pregnant sheep causes placental lipotoxicity and fibrosis (Kelley et al. 2019).

In a murine model, 5α-reduced androgens are essential for cervical ripening (Mahendroo et al. 1999). When the gene encoding the 5α-reductase (type1) is disrupted, the animals become pregnant but only 33 % of them deliver their litters on day 19 of pregnancy (control group: 100 %). Animals with a disrupted gene have a prolonged and distressed labour on day 21 or 22 with vaginal bleeding, dystocia and malaise. A quarter (25 %) of the females die and 46 % resorb or expel dead foetuses. The defect can be reversed by supplying the mice with 5α-androstanes, while administration of 5ß-androstanes has no effect.

- **Androgens and sexual differentiation in mammals**

During the embryonic state, sexual development is caused by the sex chromosome differences between males and females. The expression of the phenotype is controlled by the action of hormones, esp. 5α-DHT. Androgens are thus essential for the differentiation of male internal and external genitalia as well as of other sexual organs and general body composition (for review see Hiort 2013).

- **High or low androgen levels during pregnancy can cause reproductive diseases in the offspring**

Sheep have been used as a model to study human PCOS. Sheep foetuses exposed to increased androgen concentrations during early or mid-gestation exhibit ovarian dysfunction after puberty (Forsdike et al. 2007). The timing of follicle formation and steroidogenic activity may vary between the offspring of different breeds as well as in response to androgens. Even low concentrations of androstanes can have detrimental effects on female reproduction (Comim et al. 2015). Androgens also have a role in pregnancy in mice. Female mice with a deficit mutation in steroid 5α-reductase type 1 have smaller litters (Mahendroo et al. 1997). Plasma levels of androstenedione and T are 2- to 3-fold higher on gestation day 9, while oestradiol levels are elevated by 2- to 3-fold throughout early and mid-gestation. Inhibiting the effects of oestrogens by the administration of an oestrogen receptor antagonist or inhibitors of aromatase reverses the high rate of foetal death in the mutant mice. The results suggest that the 5α-reduction of androgens in female animals has a crucial role in protecting the foetus against oestrogen toxicity during pregnancy.

Excretion of androgens in farm animals

There has been little work on the excretion of androgens and their metabolites in faeces, although excreted steroids are potentially bioactive. Approximately 3.9 x 10^{12} kg faeces are produced worldwide per year, largely by farm animals (Berendes et al. 2018). In non-pregnant sheep 44 % of infused ^{14}C-labelled T is excreted in the faeces, compared to only 14 % in non-pregnant pigs (Palme et al. 1996). The major source of environment steroids from livestock animals seems to be cattle and chicken manure and the flow of hormonal steroids into the environment could be drastically reduced by established techniques, such as composting (Shore & Shemesh 2003). The level of steroid hormones in composted beef cattle manure is reduced by 79–87 % (Bartelt-Hunt et al. 2013).

The aim

It may be expected that most of the androgen metabolites are excreted in the 5ß-configuration, as the liver contains a high activity of the 5ß-reductase required for the formation of bile acids. We are unaware of any data on which metabolites dominate in the faeces of pregnant farm animals. We now report an examination of the excretion of androgen metabolites in the faeces of pregnant cows and sows and show which configuration of androstanes (5α or 5ß) predominates.

Material and Methods

The collection of faecal samples was approved by the institutional ethics and animal welfare committee of the University of Veterinary Medicine Vienna in accordance with GSP guidelines and with national legislation (ETK-001/01/2020).

At the research farm of the University of Veterinary Medicine, Vienna, fresh faecal samples were collected from clinically healthy cows (n=6) and sows (n=8) on the day of oestrus and 16 days after oestrus, from another eight sows on day 110 of gestation, from another ten cows on day 150 (when the CL is necessary to maintain pregnancy) and from another eight cows on day 240 of gestation (when pregnancy can be maintained without the CL, Schuler et al. 2018). Faeces were frozen within 10 minutes of collection and stored at -20 °C until analysis.

The samples were thawed at 60 °C and 0.5 g was placed in a glass vial. 5 ml of 80 % methanol were added (Palme et al. 2013) and the samples were extracted for 30 min using a multivortex (Labconco Rapid Vap Schüttler), then centrifuged for 10 min at 2500 *g* (Allegra X - 12R). The extracts were frozen at -20 °C until further analysis.

Measuring immunoreactive C19-steroids using enzyme immunoassays (EIAs)

There is a testosterone EIA available to measure androstenes and 5α-androgens with a 17ß-hydroxy-group (immunoreactive T including 5α-DHT, Auer et al. 2020). As representative for 5α-androstanes with an oxo-group at position 17, we used an epiandrosterone EIA (immunoreactive Ep, first described by Palme & Möstl 1994). We also elected to perform group-specific assays for 5ß-DHT (5ß-androstan-17ß-ol-3-one) and Et (5ß-androstane-3α-ol-17-one) to measure 5ß-17ß-androstanes and 5ß-17-oxoandrostanes. It was necessary to develop assays for these two groups of substances.

Antibodies against Et and 5ß-DHT were raised in rabbits. Et (STERALOIDS, Wilton, NH, USA) was converted into the hemisuccinate and 5ß-DHT (STERALOIDS, Wilton, NH, USA) into the carboxymethyloxime derivate as described (Kohen et al. 1975). To purify derivates, the reaction mixture was evaporated and re-dissolved in 5 % sodium bicarbonate. After extraction with diethylether, the aqueous solution was acidified and the precipitate dried after centrifugation. One portion

Tab. 1: Characteristics of the newly developed enzyme immunoassays (EIAs) for androgen metabolites / Charakteristika der kürzlich entwickelten Enzym-Immunoassays (EIAs) für Androgenmetaboliten

EIA	Aetiocholanolone	5ß-dihydrotestosterone
Standard	aetiocholanolone 3α-hydroxy-5ß-androstan-17-one	5ß-dihydrotestosterone 17ß-hydroxy-5ß-androstan-3-one
Immunogen Antibody titre	aetiocholanolone-3-HS:BSA 1:250,000	5ß-dihydrotestosterone-CMO:BSA 1:250,000
Label (titre)	aetiocholanolone-3-HS-biotin (1:250,000)	5ß-dihydrotestosterone-CMO-biotin (1:350,000)
Sensitivity	1.5 pg/well	0.8 pg/well
Intra/inter-CV %	8.3 %/10.3 %	3.7 %/9.7 %

of each steroid linked to bovine serum albumin (BSA) by a mixed anhydride reaction (Kohen et al. 1975). The two BSA-steroid conjugates were dialysed and sent to PINEDA (Berlin, Germany) for immunising rabbits (two animals per steroid). The remaining portion of each steroid was linked to biotin (THERMO SCIENTIFIC, EZ-Link™ Amine-PEG2-Biotin) and purified using high performance liquid chromatography (HPLC) as described (Möstl et al. 2002). Table 1 provides the details of the EIAs for Et and 5ß-DHT.

We observed the following cross-reactions of the **Et EIA:** 3α-hydroxy-5ß-androstan-17-one, 100 %; 5ß-androstane-3,17-dione, 209 %; 5ß-androstane-3α,11ß-diol-17-one, 26.1 %; 3α-hydroxy-5ß-androstane-11,17-dione, 10.5 %; 4-androsten-3,17-dione, 20.9 %; 17ß-hydroxy-5ß-androstan-3-one, 0.7 %; and testosterone, epiandrosterone and 17α-hydroxy-5ß-androstan-3-one, <0.1 %. **The 5ß-DHT EIA** showed the following cross-reactions: 17ß-hydroxy-5ß-androstan-3-one, 100 %; testosterone, 9.0 %; 5ß-androstane-3α,17ß-diol, 2.6 %; 5ß-androstane-3ß,17ß-diol, 2.6 %; 5α-dihydrotestosterone, 2.3 %; 17α-hydroxy-5ß-androstan-3-one, 0.4 %; 5α-androstane-3α,17ß-diol, 0.6 %; and 5-androstene-3ß,17ß-diol and 3α-hydroxy-5ß-androstan-17-one, <0.1 %.

HPLC immunograms

To test whether immunoreactive substances other than the standards were present in the samples, we performed HPLC immunograms on cow faeces from day 240 of gestation and on sow faeces from day 110 (for details of the HPLC see Touma et al. 2003).

After extraction with methanol (Palme et al. 2013), we diluted a portion of the extract (100 µl) with water (5 ml) and re-extracted it with a primed Sep-Pak C18 cartridge (Fa. Waters, Milford, MA, USA) according to the manufacturer's instructions. We used the methanolic extract for HPLC (Novapak C18 column, 0.39 x 15 cm, solvent: methanol/water, linear gradient 20 to 80 % methanol), collecting 90 fractions from the faecal extracts of both species and testing them for immunoreactive substances with the four immunoassays.

Measuring androgens using a receptor assay

We used a commercial *Saccharomyces cerevisiae* strain (Xenometrix) for the androgen receptor assay, following the supplier's instructions. This strain has been genetically modified to react to a variety of compounds that interact with the human androgen receptor (hAR). hAR is constitutively expressed from a chromosomal locus and drives the expression of a plasmid-encoded lacZ gene under the control of the androgen-responsive element (ARE). Upon binding to the AREs, the hAR induces the enzyme β-galactosidase, which converts the yellow substrate chlorophenol red-β-D-galactopyranoside (CPRG) into a red product that can be colorimetrically detected at 570 nm. We monitored potential cell lysis with an additional measurement at 690 nm.

Yeast cells were grown in YAS-Minimal medium (YAS-MM 2 % agar-agar) from stocks stored at -80 °C. After incubation at 30 °C, single colonies were picked and resuspended in fresh liquid YAS-MM containing 0.1 g/l CPRG to a final OD_{600nm} of 0.2. 200 µl of the suspension were added to each well of a flat-bottomed, clear, 96-well microtiter plate, followed by 10 µl of methanolic sample solution. One well on each plate was left blank and 10 µl of pure methanol added to correct for the cytotoxic effect of the methanolic solution. Each plate also included a reference series with known concentrations of T for calibration. Plates were sealed with oxygen-permissible foil (Breathe-Easy™) to avoid evaporation, incubated at 30 °C and measured hourly at 570 nm and 690 nm for up to 48 h in a plate reader (Biotec Synergy H1). The final timepoint for each plate was chosen based on the development of the calibration series. Each sample was measured at least in triplicate. T was used as standard, so the results represent T equivalents.

Statistics

The groups were defined according to the species, the day of sampling and the steroid measured. A t-test or Mann-Whitney Rank Sum Test (where appropriate)

was performed using SigmaStat 4.0 to calculate statistical differences between the groups (day 110/240 versus day 0). The results of the testosterone EIA and the receptor assay were compared directly within each group by a paired t-test (or Signed Rank Test).

Results

We found immunoreactive androgens and androstanes in all samples of non-pregnant and pregnant animals. The concentrations of all four C19-steroids increased towards term in cows and sows (Fig. 3). In both species, all EIAs gave significantly higher (see Fig. 3) concentrations in samples collected on days 110 and 240 of pregnancy than in samples collected on the day of oestrus.

In cows, the androgen receptor assay (expressed as T equivalents) gave significantly higher concentrations than the T immunoassay on days 0 ($p=0.002$), 16 ($p=0.002$) and 240 ($p=0.002$) but not on day 150, whereas the concentrations in faecal samples from sows were significantly higher only on day 16 ($p=0.007$).

The immunograms of the faecal extracts showed more than one peak in each EIA. The immunoreactive steroids eluted from the column like unconjugated androgen standards, so steroid concentrations should be considered as equivalents of the standards used in the assays (Fig. 4). The 5ß-steroids (Et and 5ß-DHT) dominated in cows and 5α-steroids in sows. Most of the C19-steroids investigated had an oxo-group at position 17 (17-oxoandrostanes, Et and Ep; Fig. 3).

Discussion

The presence of androgens in the faeces of cows has been known since 1942, when the use of cow faeces as a feed supplement and source of vitamins and minerals for chickens was found to cause a comb as in cocks (Riley & Hammond 1942). Interesting, the androgenic activity of cow faeces is not shared by bull faeces, after 24-h incubation at about 45 °C (Lueker et al. 1960). The finding was interpreted as an indication that androgen activity depends on an androgen precursor that is present only in cow faeces and on microbial activity that is present both in bull and in cow faeces.

Our results confirm that cow faeces contain androgens. We also found substantial amounts of other C19-steroids in faeces during the oestrus cycle. The source of the steroids may be the adrenal glands and/or the follicles. A certain amount of C19-steroids may be formed by microorganisms in the digestive tract, as some bacteria can cleave the side chain of C21-steroids (Pernigoni et al. 2021). The extent to which the C19-steroid metabolites in the faeces originate from DHEA, androstenedione or T is unknown.

Our results show that higher amounts of androgens and androstanes are excreted in the faeces of pregnant sows and cows towards the end of gestation. The concentration of DHEA is higher in the blood of pregnant cows than of non-pregnant cows and decreases after parturition (Marinelli et al. 2007). Together with our results, this finding indicates that the placenta is the source of elevated concentrations of steroids.

The concentrations of androgens and androstanes in the faeces of cows were higher than those in the faeces of sows. This may be due to the fact that the proportion of steroid hormones and their metabolites excreted in the faeces (compared to via the urine) is much lower in pigs than in ruminants (Palme et al. 1996).

The HPLC results show that all immunoassays reacted with unconjugated C19-steroids in the faeces of the two species. We assume that conjugated androstanes are deconjugated by the microorganisms of the gut, as has been described for the glucocorticoid metabolite 11-oxoaetiocholanolone: polar immunoreactive 11-oxoaetiocholanolone (most probably conjugated) can be detected in the bile but not in faecal samples (Horak & Möstl 2013). Our HPLC results also show more than one peak of immunoreactive substances in the individual assays. This is caused by the specificity of the assays, which show cross-reactions with C19-steroids that differ at position 3 (the immunogens for preparing the anti-steroid antibodies were linked to BSA at this position).

There have been reports of very low concentrations of 17-oxoandrogens in the serum or plasma of cows (e.g. Schuh et al. 2022). This may relate to the conversion of androstenedione and other 17-oxosteroids into 17α-hydroxysteroids by erythrocytes (Möstl et al. 1980). The half-life of androstenedione (a 17-oxoandrogen) in the blood of cows is about 6 min, so it is necessary to stop the breakdown during blood collection for meaningful results.

The dominance of 17-oxosteroids (Et and Ep) in faeces may be caused by metabolism, either in the liver or in the intestines. In the rat, the main portal vein metabolite of T is androstenedione, so oxidation of T by the intestinal mucosa and not metabolism in the liver could account for the failure of oral T as androgen replacement therapy of human hypogonadism (Farthing et al. 1982). Gut bacteria in farm animals may also have a role in the dominance of 17-oxidized androgen metabolites, as they can oxidize 17ß-hydroxy- into 17-oxosteroids.

In cows, the dominant metabolite in faeces is Et, a 5ß-reduced 17-oxosteroid, whereas Ep is the main C19 steroid in sow faeces. The bovine endometrium largely produces 5α-reduced steroid hormone metabolites, whereas the conceptus mainly forms 5ß-reduced metabolites (Eley et al. 1983). The ratio of 5α/5ß metabolites in faeces may not reflect the ratio in the steroid-producing organs, as androstenedione or T may be reduced in the liver of the dam by 5ß-reduc-

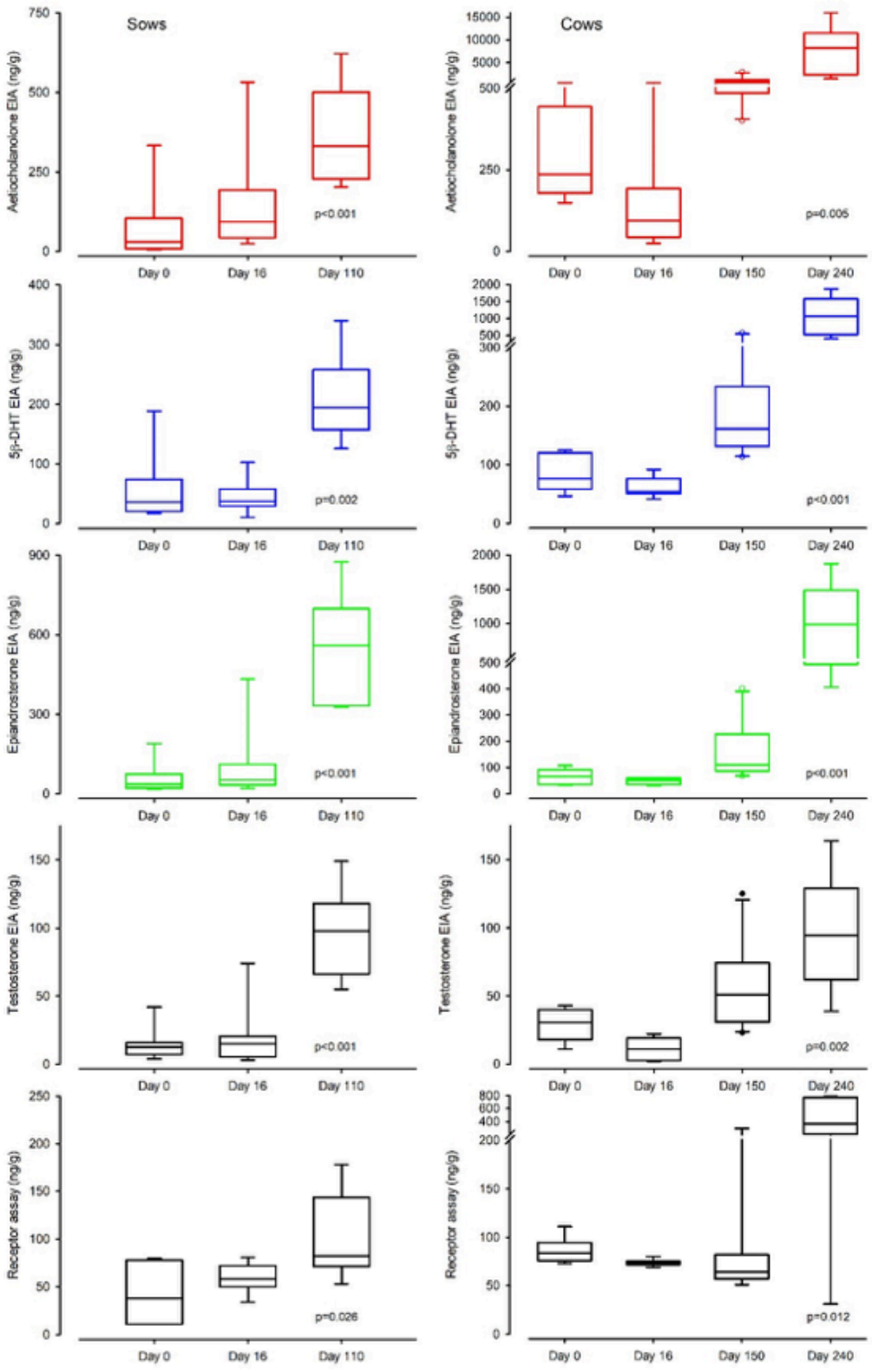

Fig. 3: Concentrations (boxplots) of C19-steroids measured with four immunoassays and the androgen receptor assay in sows and cows; Day 0 = day of oestrus, Day 16 = 16 days after oestrus, Day 110/240 = days of pregnancy. Please note the break in the y axis in some results in the faeces of cows. Concentrations of all steroids were significantly higher (p values are given below the boxplots) at the end of gestation (day 110/240) than on day 0. / Konzentrationen (Boxplots) von C19-Steroiden, die mit vier Immunoassays und einem Androgenrezeptorassay für Sauen und Kühe gemessen wurden; Day 0 = Tag des Östrus, Day 16 = 16 Tage nach dem Östrus, Day 110/240 = Tage der Gravidität. Bitte beachten Sie die Unterbrechung in der y-Achse in einigen Diagrammen. Die Konzentrationen aller Steroide waren am Ende der Gravidität (Tag 110 oder 240) statistisch signifikant höher (p-Werte sind unterhalb der Boxplots angegeben) als am Tag 0.

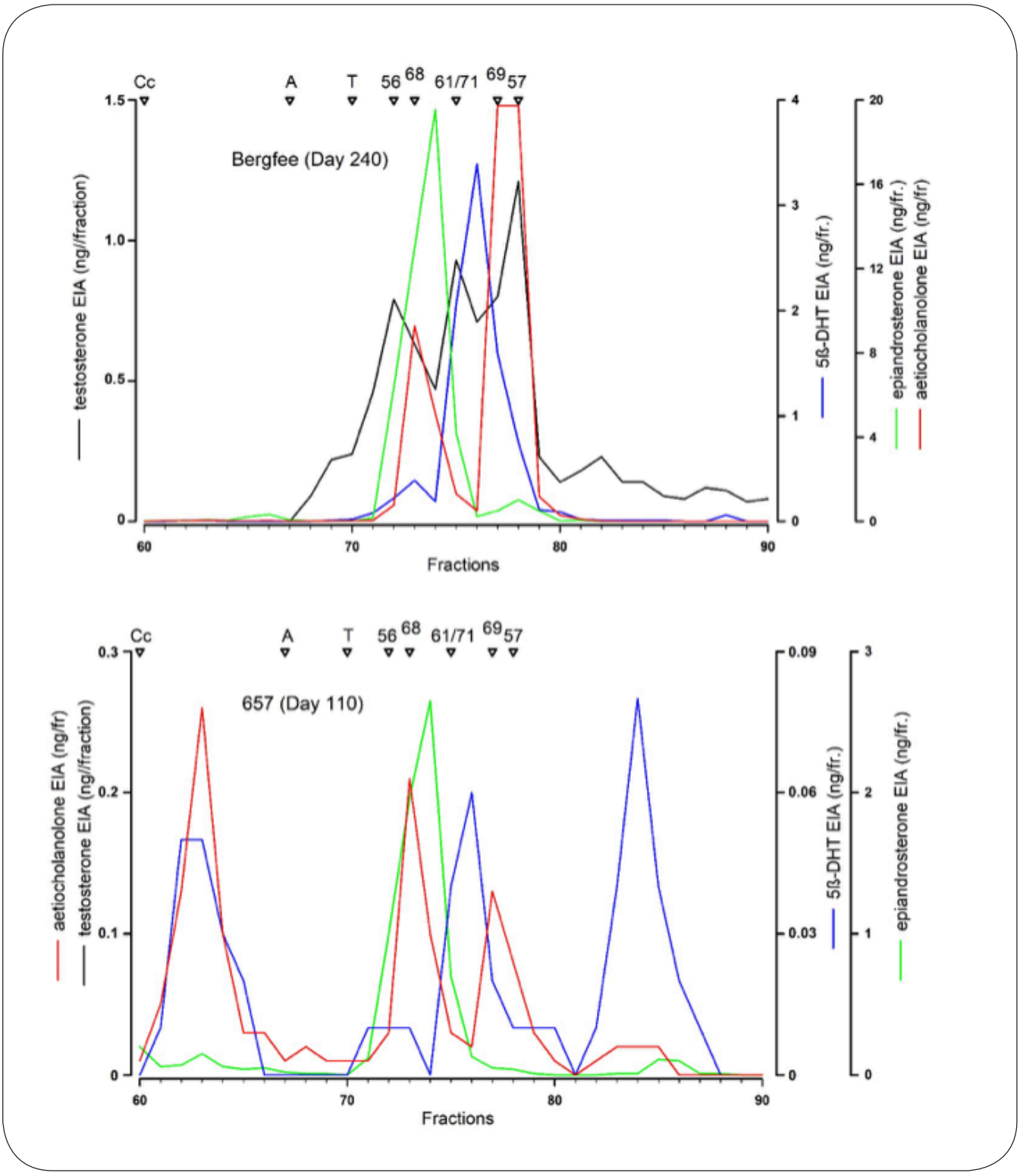

Fig. 4: Immunoreactive substances in the HPLC fractions measured with the various EIAs. The sample of the cow "Bergfee" was taken on day 240 of gestation, the sample of sow 657 on day 110 of gestation. The triangles mark the elution position of some steroid standards (Cc: Corticosterone, A: androstenedione, T: testosterone, 56: 5α-androstanedione, 68: 5ß-androstanedione, 61/71: 5α-DHT/5ß-DHT, 69: aetiocholanolone, 57: androsterone). / Immunreaktive Substanzen in den HPLC Fraktionen von einer mit EIAs untersuchten Probe. Die Probe der Kuh "Bergfee" wurde am Tag 240 der Gravidität, die der Sau 657 am Tag 110 der Gravidität genommen. Die Dreiecke markieren die Fraktionen einiger Steroidstandards (Cc: Corticosteron, A: Androstendion, T: Testosteron, 56: 5α-Androstandion, 68: 5ß-Androstandion, 61/71: 5α-DHT/5ß-DHT, 69: Ätiocholanolon, 57: Androsteron).

tase (AKR1D1), which can perform the 5ß-reduction of a broad spectrum of steroids, including androgens (Chen et al. 2011). Et also dominates in non-pregnant sows, although the 5α-reduced metabolite epiandrosterone dominates on day 110 of gestation. The shift between 5α/5ß-reduced metabolites may be caused by the production of C19-steroids in the porcine placenta.

The concentration of the receptor active androgens was higher than that of the immunoreactive T. The testosterone EIA cross-reacts with 5α-DHT but the receptor assay covers a broader spectrum of substances. The microorganisms of the bovine gut can produce boldenone (an anabolic androgen) and related androgens (Pompa et al. 2006). As substances such as 11-oxo-aetiocholanolone (a 5ß-C19-metabolite of cortisol) are present in ruminant faeces (Palme & Möstl 1997), it is possible that corresponding 5α equivalents (that can be expected to have androgen activity) are present in cow faeces. In humans, 11-oxygenated C19-steroids, which are cortisol metabolites, are active androgens (Pretorius et al. 2017).

The amount of aetiocholanolone, 5ß-DHT and epiandrosterone was close to the µg/g faeces range and such high concentrations may have receptor-independent effects similar to those described for 5ß-androstanes (Kaminski et al. 2005; Wu & Zahng 2018). The high concentrations of C19-steroids in the placenta of cows and sows raises questions about their role in the later stages of pregnancy and parturition. It seems plausible that androgens have an independent role in the endocrine control of the parturition in both species, for example for the maturation of the cervix (as described by Mahendroo et al.1997).

The environmental effects of excreted C19-steroids need to be considered and the work should include the 5α-17-oxoandrostanes, which can be converted into biologically highly active 17ß-hydroxy compounds such as 5α-DHT in a single enzymatic step. In view of the high amount of faeces excreted by farm animals (Berendes et al. 2018), the environmental aspects warrant closer investigation, as androgens are soluble in water and run-off water may contain concentrations of androgens that are sufficiently high to influence the development of aquatic organisms, esp. fish species (Grillitsch et al. 2010; Zhang et al. 2019; Huanyu et al. 2022).

As faecal samples are easy to collect and sampling does not disturb the animals, non-invasive monitoring can bypass the problem of measuring 17-oxosteroids in the blood of cows. Measuring steroid hormone metabolites in faecal samples is a standard procedure, especially in field endocrinology, and more than 1600 papers reported the use of this matrix for measuring glucocorticoid production in various vertebrate species (Palme, 2019). There are also many publications on measuring progesterone or its metabolites in faecal samples, although there are fewer reports on androgen excretion. The disparity may be caused by the fact that immunoassays for androstanes with a 5ß,17-oxo configuration (such as aetiocholanolone) are not easily available. Our newly developed Et and 5α-DHT assays may spur research on the role of androgens during the period around parturition and at puberty, on the hormonal situation in males during the reproductive season and on the environmental effects of excreted androgens.

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Fazit für die Praxis:
Trächtige Kühe und im geringeren Ausmaß trächtige Schweine scheiden große Mengen an Androgenen bzw. deren Metaboliten mit dem Kot aus. Unsere Ergebnisse deuten auf die Plazenta als Quelle hin. Die Funktionen dieser Steroidhormone während der Trächtigkeit und Geburt (z.B. auf die Reifung der Cervix) ebenso wie die Bedeutung der ausgeschiedenen Hormone und Hormonmetaboliten für die Umwelt bedürfen weiterer Untersuchungen.

Conflict of interest: The authors declare no conflict of interest.

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