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Intersexuality in pigs:Impact on veterinary public health and food safety

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ABSTRACT

Cornillie P, Mortier V, Verheyden K, Simoens P, De Brabander HF, Vanhaecke L, Intersexuality in pigs:Impact on veterinary public health and food safety, Online J Vet Res 13(1):63-75, 2009. During a routine inspection on a pig farm, nortestosterone was detected unexpectedly in faecal samples of sows. An intersexual pig, held responsible for this finding, was slaughtered and investigated. Macroscopic inspection revealed a mainly female reproductive tract consisting of a normally developed caudal part including a vagina, cervix and uterus, and on the left side a normal ovary and uterine tube. However, on the right side a hypoplastic testis and epididymis were found together with a well-developed plexus pampiniformis-like structure. Histologically, no spermatozoa were found in this testis, but Leydig cells were numerous. Chemical analysis showed the presence of 17β-nortestosterone in the urine, fat, kidneys and testicular tissue, as well as the precursor noradrenostenedione in the urine, kidneys and testis. As intersexuality is a frequent phenomenon in pigs, veterinarians who are responsible for veterinary public health and food safety must be aware of this phenomenon which may interfere with the control on the abuse of illegal growth promoters.

Key words**:** Nortestosterone, Endogenous source, Hermafroditism, Intersex, Sus scrofa

INTRODUCTION

The European Commission regulates the screening measure for the presence of residues and contaminants in slaughter animals of the European Union (Directive 96/23/EC). This regulation was already implemented in Belgium by the federal act of 15th of July 1985 concerning the use of drugs having a hormonal, anti-hormonal, β-adrenergic or production stimulating activity in animals. In contrast to most other EU countries, Belgium has a regular monitoring program of pig farms on which faeces samples are collected instead of urine. During a routine examination on a pig farm, 17β-nortestosteron (17β-NT) was identified in faeces samples of an apparently female animal. Since the presence of 17β-NT in pigs other than boars was not documented at

that time, a possible illegal use of 17β-NT as growth promoter was suspected. Further research however, showed the presence of an intersexual pig on the farm. The first results of this study were published in the Flemish Veterinary Journal (in Dutch) (Van Cruchten et al 2002) and are now completed with findings resulting from additional experiments.

Nortestosterone: NT is a steroid with two isoforms (17α-NT and 17βNT), which belongs to the group A-substances (banned substances) according to the Council Directive 96/23/EC. The 17β form of NT is also one of the most powerful androgenic anabolic steroids. In the past 17β-NT was often used for fattening purposes, because of its positive effects on improved weight gain and feed efficiency in animals. Its use has also been reported in human sports doping**.**

Until the early eighties, whenever residues of 17β-NT or its metabolites were identified in urine of oxes, race horses, or human athletes including body-builders, the exogenous administration was considered to be proven, since literature did not contain evidence of the endogenous character of this molecule in cattle, horses and men. The chemical structures of 17β-NT, its precursor 19-norandrostenedione (NAED) and its metabolites are illustrated in Image 1, below.

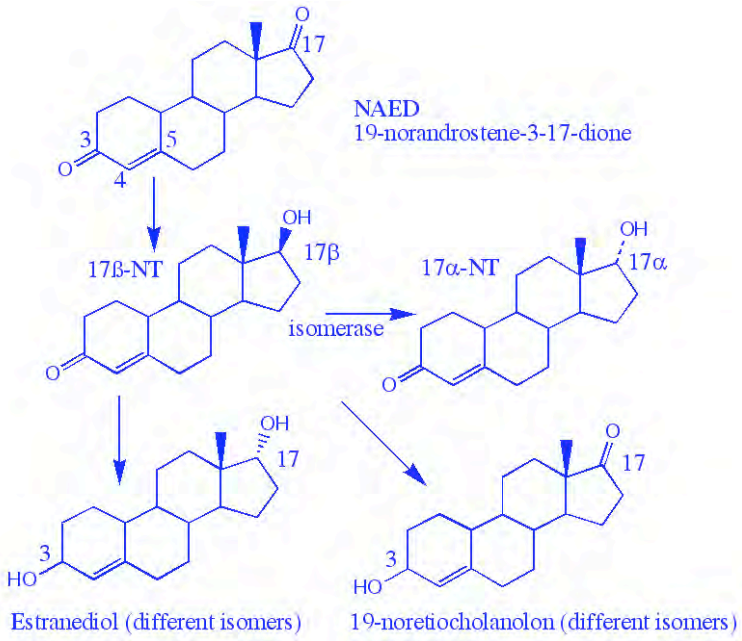

Image 1 - Chemical structures of 17β-NT, its precursor NAED and its metabolites

These products were previously only detected in animals which have been treated with 17β-NT or one of its derivates (e.g. esters of NT or nandrolone (Laurabolin® or Durabolin®)) (Delahaut et al 1988, Daeseleire et al 1993, McEvoy 1998a, McEvoy 1999a).

However, in 1984, Houghton demonstrated that 17β-NT was naturally present in the urine of stallions. A short time afterwards, Belgian and Dutch investigators found that 17β-NT is present in urine and edible tissues of boars. These results were first discussed in workgroups and were

published afterwards by Maghuin-Rogister (1988), van Ginkel et al (1989) and Debruyckere et al (1990). In this context, it is important to mention that an isomerase to convert 17β-NT to 17α-NT is absent in the pig. In cattle on the other hand, this isomerase is present and the 17α-form is the most abundant isoform found in bovine urine. In horses, both isomers are detected. In 1993 Rizzo and others reported the endogenous origin of 17β-NT by the analysis of boar testicles. Since then, the presence of 17β-NT in boar matrices is no longer considered as of exogenous origin.

The problematic nature of NT in bovines has also gained much attention. The first observations on the endogenous presence of 17β-NT in calves were based on an analytical artefact and confusion between 17β-testosterone and 17β-NT (De Ridder 1989). Rapp and Meyer described in 1989 the possibility of 17β-NT being excreted by non-treated calves fed with milk substitutes contaminated with 17β-NT. Vandenbroeck et al (1991) were the first to suggest the endogenous presence of 17β-NT (and not 17α-NT) in the urine of pregnant cows. Meyer et al (1992) reported the presence of relatively high concentrations of 17α-NT in the urine of a periparturient cow and in the newborn calf. 17α-NT has also been detected in pregnant animals of other species (Clouet et al 1997, McEvoy et al 1998b). The testicular production of nortestosterone has also been demonstrated in several animal species, including the boar and stallion, but not yet in the bull (McEvoy et al 1999b). Most of these findings could be confirmed and supplemented with additional data during a large-scale experiment involving several European residue laboratories (De Brabander et al 1994, 2004; Poelmans et al 2005).

During an antidoping control in sport in 1997, evidence was gained for the presence of endogenous 19-nortestosterone and its metabolites 19-norandrosterone and 19-noretiocholanolone in human urine (Le Bizec et al 1999). A new NT problem emerged in 1999 when two long distance swimmers were accused of abusing NT during a competition in Brazil. Both athletes claimed not to have abused drugs and ascribed the positive results of their urine sample to the consumption of “Sarapatel”, a typical Brazilian dish based on pork which may contain intact testicular tissue of boars. The attorneys of the swimmers called in advice from experts, who carried out some experiments including the consumption of meat and organs of a mature boar by three volunteers (Le Bizec et al 2000). During approximately one day the metabolites 17β-NT, 19-norandrosterone and 19-noretiocholanolone could be identified in the urine samples of the three test subjects in a concentration > 2 $\mu g.kg^{-1}$ (official International Olympic Committee cut-off level for norandrosterone). In the meat and organs of boars, including the consumed animal 17β-NT and its precursor 19-norandrostenedione **(**NAED) were identified (De Wasch et al 2001). NAED is a reduced testosterone precursor which does not carry a methyl group in the 19^{th} position. When the NAED molecule is processed by the liver, a reduction of the 17-keto group occurs leading to 17β-NT (17β-hydroxysteroid dehydrogenase). Recently, new data on NT in the horse were described (Houghton et al 2004). 19-Norandrost-4-ene-3,17-dione detected in urine from a fertile stallion following a solvolysis procedure may arise as an artefact of decarboxylation of a precursor 19-carboxylic acid (Image 2). A similar conclusion was drawn for the presence of 19-NT.

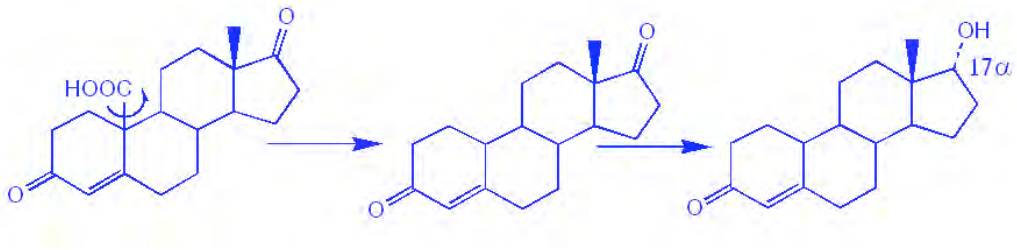

Image 2 - Decarboxylation of OxAED with formation of NAED and 17αNT

Intersexuality: An intersex is defined as an individual having both male and female sexual characteristics and organs. This phenomenon occurs in all domestic animal species, but is most frequently encountered in cattle, goats and pigs (Koch 1961). A distinction can be made between true hermaphrodites (*Hermaphroditismus verus*) and pseudo-hermaphrodites (*Pseudohermaphroditismus*). True hermaphrodites possess gonadal tissue of both genders, viz. one ovary and one testis (lateral hermaphroditism), or two ovotestes both containing testicular as well as ovarian tissue (bilateral hermafroditism), or a combination of one testis or ovary with an ovotestis (unilateral hermafroditism) (Halina et al 1984, Bansal et al 2005). Pseudohermaphrodites only contain gonads of one single gender, mostly opposite to the animal's external sexual characteristics. Depending on whether these gonads are testes or ovaries, such individuals are called *Pseudohermaphroditismus masculinus* or *Pseudohermaphroditismus femininus,* respectively (Cribiu and Chaffaux 1990). The term "*Freemartinism*" is also often used in literature. This form of intersexuality is almost exclusively found in cattle, but has also sporadically been reported in sheep, goats, deer, pigs, horses and camels (Padula 2005). Freemartins are virilized female animals and originate when a female foetus gets XY-leukocytes and male hormones from her twin brother by vascular anastomoses in the chorioallantois (Ladds 1993). When single freemartins are born without twin sibling, they are supposed to be the result of an early foetal loss of the male calf co-twin (Padula 2005).

Intersexuality is a frequent phenomenon in pigs and affects 0.5% of the European porcine population (Pinton et al 2002). Most intersexual pigs have a female phenotype (XX / SRY negative) whilst XX/XY chimeric pigs are rare (less than 5% of the intersexes) (Hunter and Greve 1996). Their morphological appearance is characterised by the presence of an enlarged clitoris, often enveloped by a prepuce, and a dorsally directed ventral commissure of the vulva (Image 3, next page).

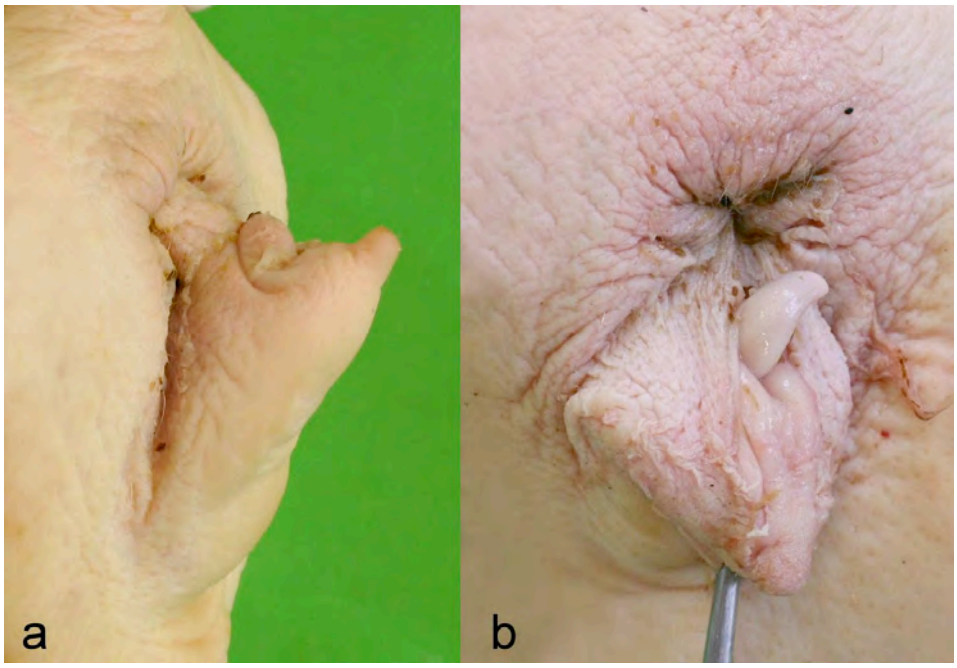

Image 3 - Typical appearance of the external genital organs of an intersex pig, a: left lateral view showing the enlarged ventral commissure of the vulva orientated in dorsal direction, b: The enlarged clitoris is clearly visible after pulling the ventral commissure of the vulva downwards (caudal view).

While the gonads may show all variations as described above, the morphology of the internal reproductive organs in intersexual pigs can assume all intermediate stages between females and males. As a result, the uterine horns and body can either be normal, hypoplastic or even aplastic, and they can co-exist with or be replaced by a pair of deferent ducts merging with the caudal part of the vagina. In the case of male pseudohermaphroditism, both testes can either be located in their original intra-abdominal position (cryptorchidism) or can have descended into an inguinal or scrotal position (personal observations and Halina et al 1984, Świtoński et al 2002).

Intersexuality in pigs is supposed to be a hereditary condition. In isolated herds of pigs in the northern islands of the Vanuatu archipel, where intersexual pigs were considered to be holy animals by the native Melanesians, the incidence of male pseudohermaphroditism reached up to 10 to 20 % of all boars due to selective breeding (Pacchioli 1997). An autosomal recessive gene is suggested to be the main cause of intersexuality in pigs (Parma 1999, Pailhoux et al 2001, Pinton et al 2002, Świtoński et al 2002). Pinton et al (2002) showed that intersexuality in pigs is correlated to an anomaly on chromosome 9. However, more research is still required to further elucidate this genetic component.

Most intersexual pigs are infertile. They often show aggressive behaviour, have a more rigid and hairy skin and can spread the typical boar odour from the age of puberty onwards (Hunter and Greve 1996). These qualities can form a major economic loss in the pig industry. Furthermore, these animals can show increased levels of steroidal hormones that can be mistaken for exogenously administered hormones, resulting in severe sanctions for the farmer.

MATERIAL AND METHODS

Morphological analysis: The intersexual pig was slaughtered at the local farm and its internal genital organs, together with samples of fat, muscle, liver, kidneys, urine and faeces, were collected for further morphological and chemical analysis respectively. The vulva and clitoris could not be investigated because these structures were resected early during slaughter. After macroscopic photography of the reproductive system, all genital organs were sampled for histological analysis. The tissue samples were fixated in a phosphate-buffered 3.5 % formaldehyde solution during 24 hours. After fixation the samples were embedded in paraffin by an automated system (Shandon Citadel Tissue Processor, Cheshire, UK) and subsequently cut in 8 µm thick sections and stained with haematoxylin and eosin (H.E.), PAS and Van Gieson. The sections were analysed with an Olympus BX61 light microscope provided with an Olympus DP50 digital camera (Olympus, Tokyo, Japan).

Chemical analysis: Different methods, depending on the matrix, were developed for the extraction and purification steps prior to chemical analysis. Five grams of meat and testicle matrices were digested with subtilisine, extracted with n-hexane and purified onto an aminopropyl solid phase extraction (SPE) cartridge. Twenty-five grams of liver and kidney were directly extracted by solvent and purified onto an aminopropyl SPE cartridge. Twenty-five millilitres of urine were hydroysed with glucuronidase-sulfatase before performing a liquid-liquid extraction (LLE). Purification was performed onto an aminopropyl SPE column and finally fractionated onto a HPLC system. Derivatization was achieved with twenty µl N-trimethyl-silyltrifluoroacteamide (MSTFA)/trimethyliodosilane (TMIS)/ dithioerythriol (DTE) (1000:5:5,v/v/w).

The chemical analysis of nortestosterone and noradrenostenedione in fat, urine and faeces was carried out by gas chromatography followed by multiple mass spectrometry (GC-MS^n). The chromatographic and spectrometric analyses were performed using a Trace GC 2000 Gas Chromatograph fitted with a Polaris ion trap mass spectrometer (Thermo Finnigan, Austin, TX, USA) with a Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA). Helium (99.99 % purity, Air Liquide, France) was used as carrier gas at a flow rate of 1 $ml.min^{-1}$. FC43 (Perfluorotributylamine) (Ultra Scientific, North Kingstown, USA) was used as calibration gas. A volume of 1 µl was injected (spit flow 60 ml/min, splitless time 1 min). Separation of the target analytes was performed on a BPX-5 (SGE Inc., Austin, TX, USA) (25 m x 0.22 mm I.D.) fused silica capillary column with 5 % phenyl-polysilphenylene-siloxane liquid phase (film thickness 0.25 µm). Injector, ion source and transfer line temperature were respectively 250 °C, 200 °C and 275 °C. Temperature program: initial 100 °C; ramp at 17 $°C.min^{-1}$ to 250 °C; ramp at 2 $°C.min^{-1}$ to 300 °C (hold 1.30 min). The spectra were obtained in Electron Impact (EI) mode at 70 eV.

Analysis of liver, kidneys, muscles and testes for these substances was performed by liquid chromatography again coupled to a multiple mass spectrometric analysis (LC-MS^n). Chromatographic separation was achieved using a Symmetry C_{18} column (Waters, 5 µm, 150 mm x 2.1 mm). A 1100 series quaternary gradient pump (Agilent, Palo Alto, CA, USA) was used. The mobile phase (methanol: A/0.02 M formic acid in water: B; 55:45) was maintained for 20 min and increased to A/B; 100:0 (v;v) in 4 min. The flow rate was 0.3 ml/min. The MS detector was a Finnigan LCQ deca ion trap (ThermoFinnigan, San Jose, CA, USA) equipped with an APCI interface in the positive ion mode MS/MS full scan.

Anatomical and histological characteristics: Macroscopical examination of the female reproductive system (Image 4, below) showed a well developed vestibulum vaginae and vagina as well as a normal uterus comprising the uterine cervix, body and two horns. On the left side, a well developed ovary with a normal uterine tube were identified, but on the right side a testis, epididymis and a deferent duct with a large plexus pampiniformis-like structure could be observed. A thin, tubular structure was found between the testis and epididymis, but could not be further identified macroscopically.

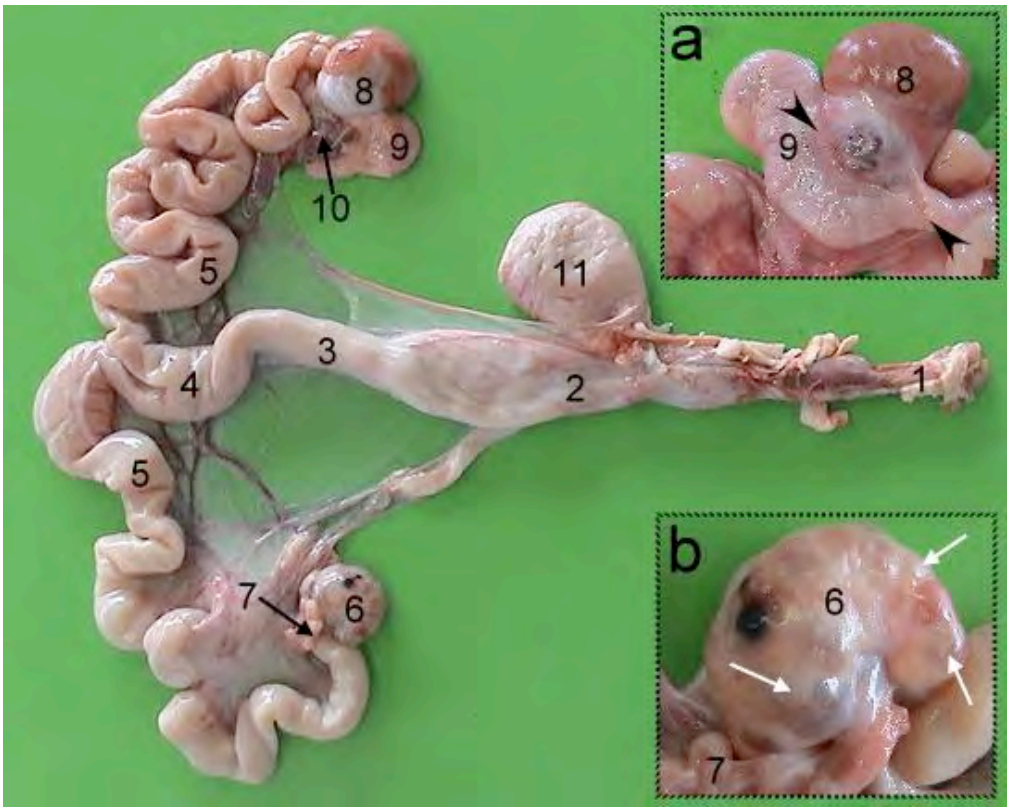

Image 4 - Dorsal view of the internal genital organs of the intersex pig. The female components consist of the vestibulum vaginae (1), vagina (2), cervix uteri (3), corpus uteri (4), both cornua uteri (5), left ovary (6) and left uterine tube (7). The male components are a hypoplastic right testis (8) and epididymis (9), provided with a plexus pampiniform-like structure (10). The urinary bladder (11) is also present. Insert a: detail of the hypoplastic testis and epididymis. The arrowheads points to a thin, undefined tubular structure in between both organs. Insert b: detail of the ovary. Some vesicular follicles are indicated by white arrows.

The vagina, uterus and the single uterine tube all had a normal histological constitution. The left-sided ovary consisted on histological examination of a peripheral cortex with many atretic

and some normal vesicular follicles, and a central medulla with a normal histological appearance (Image 5, below).

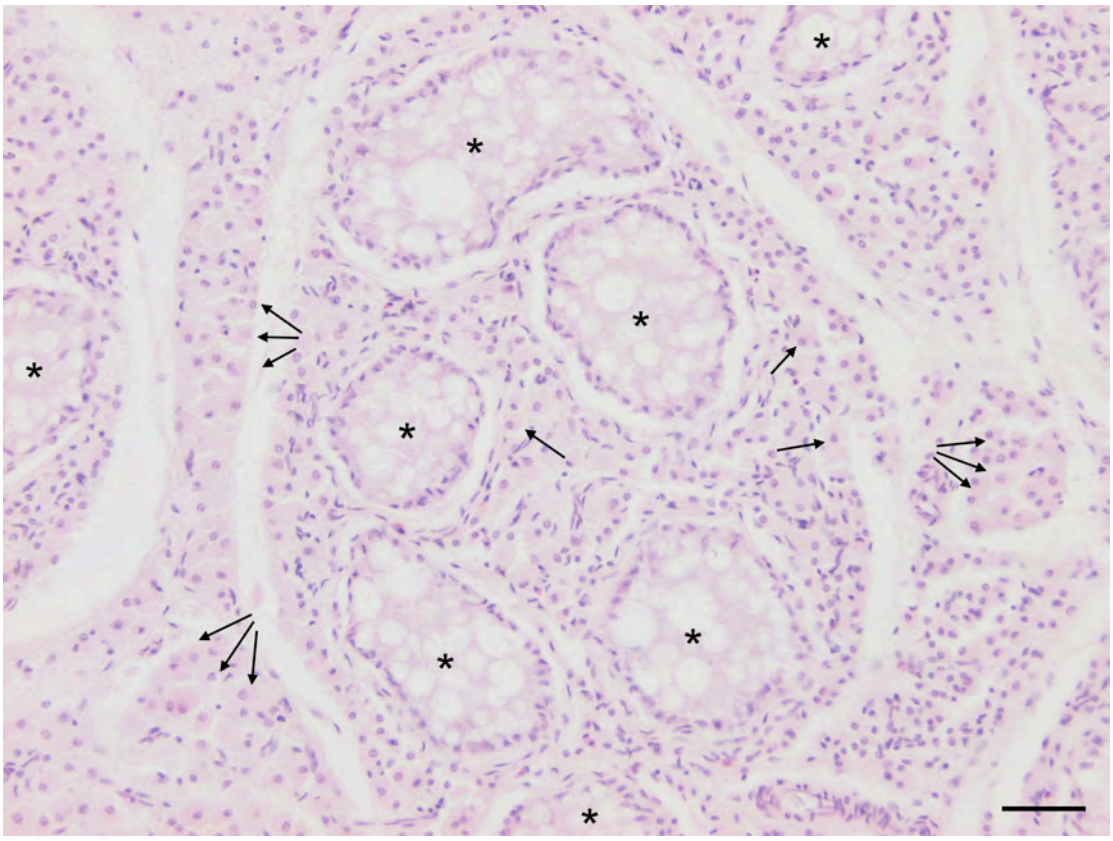

Image 5: Histological appearance of the ovarian cortex of the intersex pig showing several atretic follicles (*). Haematoxylin & eosin staining, scale bar = 100 µm.

On the histological sections of the right-sided testicle, some seminiferous tubules could clearly be identified, but they were less densely distributed within the testicular tissue compared with sections of normal adult boar testicles (Image 6). The walls of the tubules consisted only of a single layer of cells, in which sustentacular Sertoli cells could not be distinguished from spermatogonia. The seminiferous tubules did not contain any spermatozoa. Interstitial Leydig cells were clearly identifiable in the stroma between the seminiferous tubules and were often clustered (Image 6).

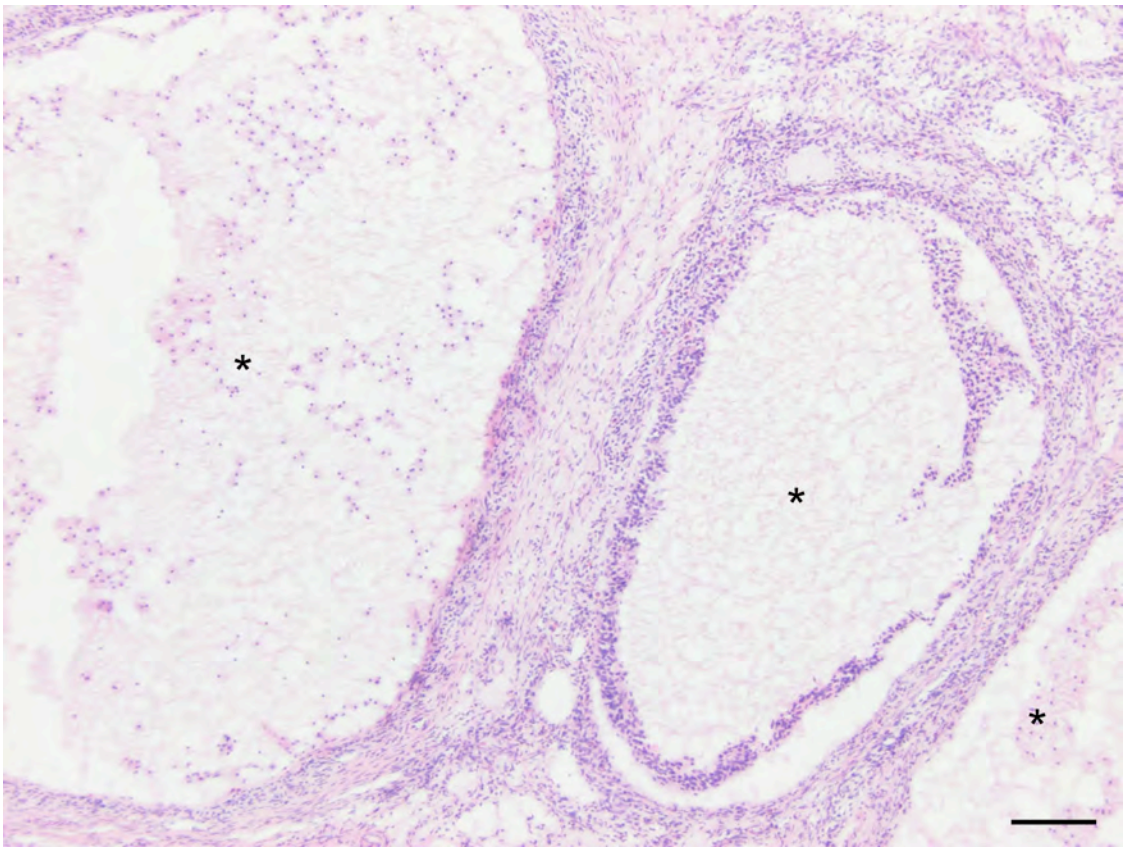

Image 6: Histological appearance of the hypoplastic testis of the intersex pig. The tubuli seminiferi (*) consist of a single layered epithelium in which spermatogonia cannot be distinguished from Sertoli cells. Interstitial Leydig cells (arrows) are numerous. Haematoxylin & eosin staining, scale bar = 50 µm.

Histology of the ductus epididymidis showed a normal pseudostratified columnar epithelium, but no spermatozoa could be detected in the central lumen. The thin undefined tubular structure between the testis and epididymis consisted of an inner squamous epithelium, a thick layer of loose subepithelial connective tissue, with many twisting PAS-negative cell strings which sometimes were surrounding a lumen, and an external smooth muscle layer.

Chemical analysis:17β-nortestosterone was detected in the fat, urine, kidneys and testes (Table I, next page). The concentration (27 $\mu g.l^{-1}$) was highest in the urine and also relatively elevated in the testes (ca. 5 $\mu g.kg^{-1}$). The concentrations in fat and kidneys were lower and near to the limit of detection. No nortestosterone was detected in faeces, liver and muscles (Table II). The precursor of nortestosterone, norandrostenedione, was present in low concentrations in the urine, kidneys and testes. The fat surrounding the kidneys as well as the faeces and muscles contained no detectable concentrations of norandrostenedione.

Table I: Results of the chemical detection of 17-beta-19-nortestosteron (µg/kg) in porcine matrices

Sample	Intersex	Sow	Old boar
Fat	ca 0.3	/	/
Urine	ca 27	1.3 - 1.6	51 - 344
Faeces	-	/	/
Liver	-	-	1 - 63
Kidney	ca 1.6	0.2 - 1.5	2.5 - 232
Meat	-	0.5	0.7 - 13.4
Testes	ca 5.3	/	24 - 144

- = below the limit of detection, / = no material available

Table II: Results of the chemical detection of 19-norandrostenedione (µg/kg) in porcine matrices

Sample	Intersex	Sow	Old boar
Fat	-	/	/
Urine	ca 0,5	0.9 - 18	5 - 109
Faeces	-	/	/
Liver	trace	3.1 - 8.3	0.1 - 24
Kidney	ca 1.1	2.7 - 18	2.3 - 535
Meat	-	0.04	0.1 - 5.5
Testes	ca 0.9	/	6.2 - 110

- = below the limit of detection, / = no material available

DISCUSSION

The gross anatomical and histological appearances of the internal reproductive organs of the investigated intersex concord with that of a real hermaphrodite. In this intersexual pig, detectable levels of NT and NAED were demonstrated in different matrices by chemical analysis. The highest concentration nortestosterone was found in urine (ca. 27 $\mu g.l^{-1}$), but the concentration was also high in the testes (ca. 5 $\mu g.kg^{-1}$). The precursor of NT, NAED, was also found in the urine, kidneys and testes, but the concentrations were lower than those of NT. The kidney fat, the faeces and the muscle tissue didn't contain any traceable levels of both products, probably because the concentrations were lower than the detection limit of the analytical method.

In an international study performed by 3 European laboratories in cooperation with the USDA, a well-documented overview of the status of the presence of 17β-NT, NAED and 17β-boldenone in swine (i.e. intersex, sow and boar) was made. Clear differences between the investigated tissues could be observed in all cases (De Wasch et al 2001, Poelmans et al 2005). The highest concentrations were always measured in the urine, kidneys and testes. Therefore it is recommended to use those matrices for the detection of 17β-NT and NAED. Concentrations are much lower and even below the limit of detection in fat, faeces, liver and muscle tissue. The data illustrate that boars produce higher concentrations of 17β-NT and NAED in comparison with intersexes and sows. NAED concentrations were higher in all investigated sow matrices than in the matrices from intersexual pigs. On the other hand, samples from intersexual pigs contain a higher concentration of 17β-NT than the 17β-NT concentration range of sows.

Whenever the presence of one or more intersexual pigs within a female pig population is not discovered during sampling for the analysis of illegal growth promoters, the pig breeder can

wrongly be suspected of illegal use of these hormones in his lifestock. Therefore, a proper and early identification of intersexual pigs is essential for the correct interpretation of the results drawn from the chemical and serological screening.

One of the initial indications of an intersex pig is the typical anatomy of the vulva. The ventral commissure of the vulva is enlarged and in 80% of the cases it is pointing in dorsal direction, making these animals urinate skywards (Hunter and Greve 1996). By spreading the lips of the vulva, an enlarged clitoris can be observed. Other obvious anatomical characteristics are the more hairy and rigid skin. A wide range of variations can be observed in the behaviour of intersexes. Some animals have a standing oestrus like sows, while other intersexes exhibit a masculine behaviour, act more aggressively, produce high amounts of saliva in the presence of a male, and show copulation behaviour in the presence of a female. As hypertrophy of the clitoris and other male characteristics in presumptive female pigs also can be the result of exogenous administration of androgens such as testosterone, methyltestosterone and trenbolone to normal sows, chemical analysis results must be interpreted cautiously in case of high prevalence of the described phenotype in a pig population.

The detection of an elevated level of nortestosterone in presumptive female pigs can only be suggestive for the (pseudo)hermaphroditic nature of the animal, but it cannot entirely rule out the exogenous administration of growth promoters. In such cases, only a morphologic examination of the internal genital organs obtained after slaughter can conclusively distinguish an intersexual pig from a sow exogenously supplied with androgens.
It can be concluded that public health inspectors responsible for the screening of illegally used growth promoters on pig farms, must be aware of the possible presence of intersexes and must have knowledge of the anatomical characteristics and behaviour pattern of these animals, as to be able to recognise them. Every suspected sample should be correlated to gender and age of the animal in question. If intersexuality is suspected from laboratory analysis, it should be confirmed by further morphological analysis at slaughter. A high prevalence of intersexual pigs in a farm can be caused by one of the breeding boars. In that case, zoötechnical measurements should be taken as to prevent great economic losses.

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