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Presence and metabolism of endogenous androgenic–anabolic steroid hormones in meat-producing animals: a review

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The presence and metabolism of endogenous steroid hormones in meat-producing animals has been the subject of much research over the past 40 years. While significant data are available, no comprehensive review has yet been performed. Species considered in this review are bovine, porcine, ovine, equine, caprine and cervine, while steroid hormones include the androgenic–anabolic steroids testosterone, nandrolone and boldenone, as well as their precursors and metabolites. Information on endogenous steroid hormone concentrations is primarily useful in two ways: (1) in relation to pathological versus 'normal' physiology and (2) in relation to the detection of the illegal abuse of these hormones in residue surveillance programmes. Since the major focus of this review is on the detection of steroids abuse in animal production, the information gathered to date is used to guide future research. A major deficiency in much of the existing published literature is the lack of standardization and formal validation of experimental approach. Key articles are cited that highlight the huge variation in reported steroid concentrations that can result when samples are analysed by different laboratories under different conditions. These deficiencies are in most cases so fundamental that it is difficult to make reliable comparisons between data sets and hence it is currently impossible to recommend definitive detection strategies. Standardization of the experimental approach would need to involve common experimental protocols and collaboratively validated analytical methods. In particular, standardization would need to cover everything from the demographic of the animal population studied, the method of sample collection and storage (especially the need to sample live versus slaughter sampling since the two methods of surveillance have very different requirements, particularly temporally), sample preparation technique (including mode of extraction, hydrolysis and derivatization), the end-point analytical detection technique, validation protocols, and the statistical methods applied to the resulting data. Although efforts are already underway (at HFL and LABERCA) to produce more definitive data and promote communication among the scientific community on this issue, the convening of a formal European Union working party is recommended.

Keywords: steroids; food; metabolism; endogenous; natural; meat; bovine; porcine; ovine

Introduction

The European Union Council Directive 96/22/EC (European Union 1996a) states that 'substances having a hormonal action' are prohibited for use in animals intended for meat production. As well as purely novel steroids not existing in nature, the Directive therefore also covers synthetically produced versions of steroids that are known to occur naturally in certain species under particular circumstances. However, in some countries, including the USA, Canada and Australia, some (combinations of) steroids and a related synthetic compound zeranol are officially registered for use as hormonal growth-promoting compounds. Due to their anabolic and/or partitioning effect, they increase the profit per unit head for the farmer. European Union Council Directive 96/23/EC (European Union 1996b) (European Union 2002) lays down the requirements for residue testing in order to ensure compliance with the European Union prohibition. For a succinct overview of the context of the different European Union directives and the resulting methods that have been applied in recent years, see Stolker and Brinkman (2005).

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The steroid hormones considered in this review are the androgenic–anabolic steroids that potentially derive from precursors within the body such as cholesterol and pregnenolone (Figure 1). These include testosterone, androstenedione, nandrolone, boldenone and DHEA, as well as their numerous catabolic products and any precursor compounds that might potentially lead to conversion to these steroids within the body. The task of detecting the abuse of synthetically produced hormones that are also known to be endogenous under certain conditions, dubbed 'pseudo-endogenous' or 'grey zone substances' due to their dual synthetic/endogenous nature (Van Thuyne 2006), is problematic for many reasons. The most significant challenge arises because when they are shown to occur naturally within a particular type of animal, a simple qualitative demonstration of their presence does not necessarily prove abuse. Most, but not all, steroid preparations are ester versions of these potentially endogenous steroids. However, a simple demonstration of the presence of the steroid ester as proof of abuse is not always possible (with the exception of hair and injection/implant sites in some cases) due to the ester being cleaved by the time it reaches the test matrix, i.e. plasma or urine. Some type of quantitative uni- or multivariate threshold approach is therefore usually required in order to confirm abuse. Where particular steroids are believed not to be endogenous in an animal at a particular limit of detection (LOD), who is to say that as analytical limits decrease, they will not be discovered as endogenous at a lower concentration? These and some further analytical and physiological considerations are taken up again later in this review.

Analytical methods of various kinds have in the past been employed to identify and quantify endogenous steroids, their metabolites and precursors, but their effectiveness and the harmonization of their application in different countries and situations is questionable. For example, Van Ginkel et al. (1993) highlight the wide range of different analytical methods and thresholds that have been applied in different European Union countries in the past. Since the authors are aware of no comprehensive published review on the concentrations and metabolism of such steroids in food-producing animals, the overall aim of the work reported herein was therefore to carry out a survey of the existing literature, which will then guide further

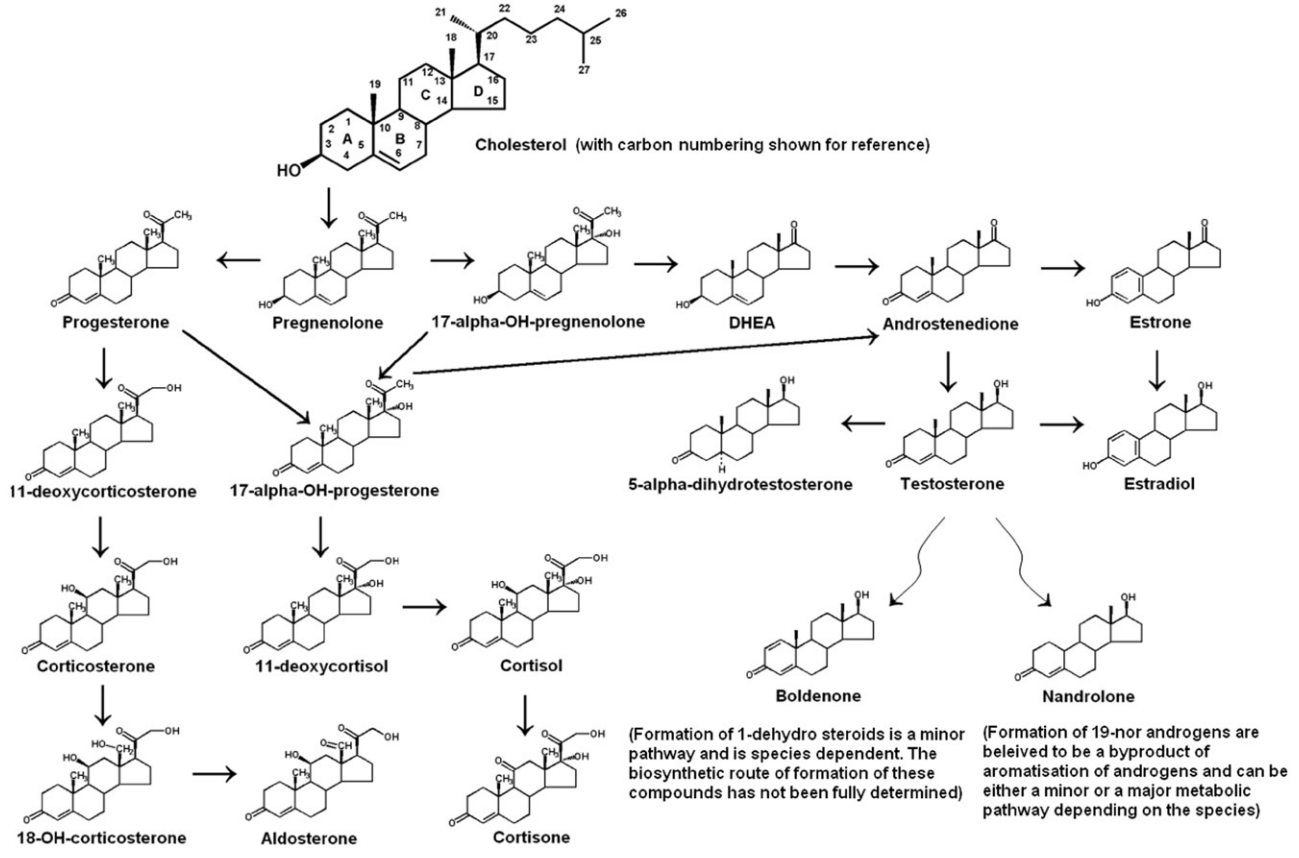

Figure 1. Schematic of the biosynthetic pathways for endogenous steroids in mammalian species. Many of the reactions that involve oxidation or reduction of hydroxyl and ketone groups, respectively, are reversible. Wavy arrows indicate a putative pathway only.

practical work in order to increase knowledge or to develop more effective testing methods.

Literature survey methods

The overall aim was to collect as much published and unpublished data as possible in order to provide for the most comprehensive evaluation of the field. Literature searches were conducted using the Pubmed facility of the US National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), Scopus (http://www.scopus.com), the Web of Science (http://www.scientific.thomson.com/products/wos/) and various 'grey' literature sources (referring to literature sources not easily found using common search facilities), such as government websites, reports and libraries. Many researchers internationally were also contacted so that as much data as possible from individual animals and particularly for values below the reported LODs, detection capability (CCα) or decision limit (CCβ) could be obtained. This had the advantage that some appreciation of concentrations could be obtained when analyte concentrations were currently too challenging for fully rigorous quantitative analysis.

In the following sections, the occurrence of precursors and metabolites of testosterone, nandrolone and boldenone, in bovine, porcine and ovine matrices is reviewed in narrative fashion, as well as precursors and metabolites of nandrolone and boldenone in equine, cervine and caprine matrices. Ideally, a statistical analysis of results using meta-analysis (defined here as 'the statistical analysis of a large collection of analysis results for the purpose of integrating the findings'; Glass 1976). would be a desirable outcome, but due to a lack of sufficient data this approach could not be used. Where differences between results in this review are stated to be 'statistically significant', this refers to comparisons of controlled populations within a single study and not between results of different studies. Unless otherwise stated, the results reviewed derive from controlled studies where the use of banned steroids can be ruled out.

As many relevant matrices as possible have been considered in this review. However, due to the magnitude of the literature and the overall scope of this review being predominantly targeted at control of abuse rather than the safety implications of steroids in food, there is an inevitable bias in the output toward plasma, urine, bile, faeces and hair over tissues such as muscle and fat, etc. In addition, longitudinal studies of animals using solid tissues are not often possible because this usually means slaughter of the animal, hence ending the longitudinal study. In addition to the individual studies reviewed herein, several reviews dealing with the concentrations of steroid hormones in different food products are also available (Henricks 1976; Velle 1976; Hartmann et al. 1998; Fritsche and Steinhart 1999; Arnold 2000; Daxenberger et al. 2001; Stephany et al. 2004; Mouw et al. 2006).

Physiological and analytical considerations regarding comparisons of steroid concentrations within and between different species

A basic understanding of the analytical and physiological context of natural steroids is assumed in this review. Nevertheless, some information of specific relevance is given below and the biosynthetic and catabolic pathways of some representative natural steroids are summarized in Figures 1 and 2 and Table 4. Further background information on general analytical aspects can be found in Makin et al. (1995) and Stolker and Brinkman (2005) while further physiological information can be found in Mason (2002) and Hadley and Levine (2006).

Although the background given here is separated into analytical and physiological factors, there are areas of overlap between the two. A critical theme that will become apparent is that even though it may sometimes be desirable to take into account a particular variable for analysis, the lack of reporting of this information (at least in a standard format) often means that rigorous quantitative comparisons are often not possible. It was also necessary to limit the number of parameters chosen for study. The remaining analytical parameters subject to full analysis were chosen by consideration of a combination of their impact on any results as well as the frequency and reliability of their reporting.

Analytical factors

Although most published methods rely on direct identification and/or quantification of analytes, indirect biomarker approaches have also been investigated. Groot (1992) for example describes 'Histological Screening for illegal administration of growth-promoting agents in veal calves'. This technique showed some potential for using the effects of androgens and oestrogens on male prostate or female clitoris/Bartholin gland as a biomarker of abuse, but was not widely applied. For the purposes of the current review, however, studies were limited to direct detection/quantification using such techniques as immunoassay (IA), high-performance liquid chromatography with ultraviolet detection (HPLC-UV), liquid or gas chromatography coupled to mass spectrometry (LC- and GC-MS, respectively) and combustion isotope ratio mass spectrometry (GC-C-IRMS).

When comparing data between studies, it becomes apparent that while 'true' differences between data

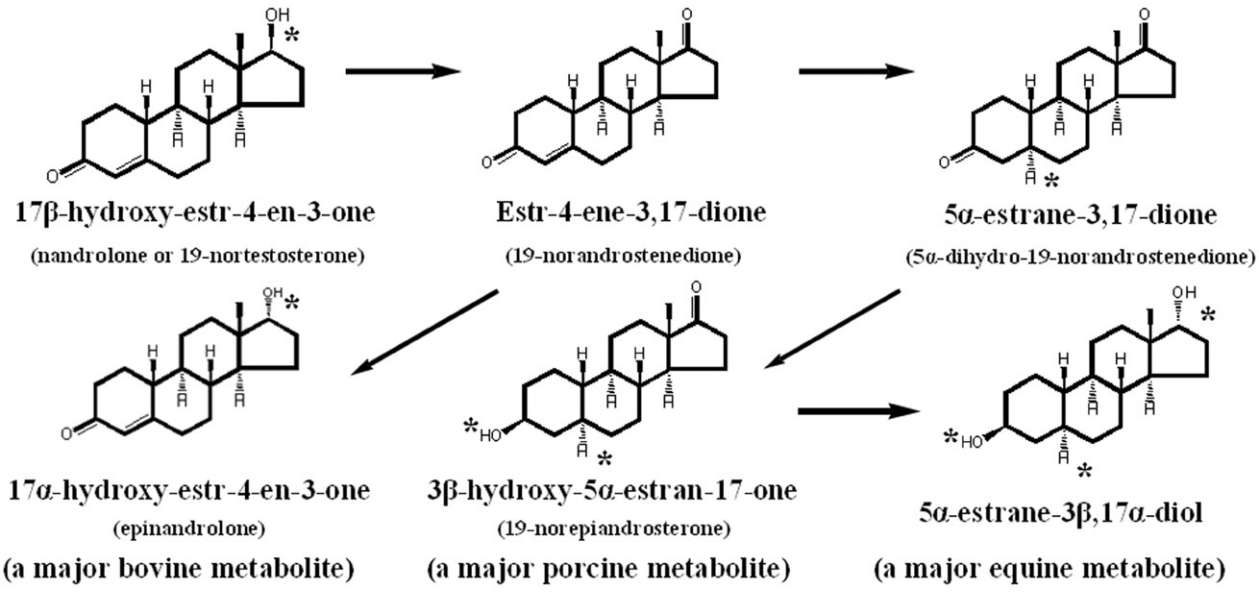

Figure 2. Schematic representation of the phase 1 metabolism of nandrolone in the three species where most information on this steroid's metabolism is available (bovine, porcine and equine).
*Sites of possible metabolic epimerization. For space purposes and due to the number of potential isomers, it is only possible to depict the major metabolites of nandrolone. For the same reasons it is also not possible to give schematic metabolic pathway representations for all the individual steroids covered in this review, but it is worth noting that the same functional groups liable to metabolism in nandrolone are also liable to metabolism in other androgenic–anabolic steroids.

points and populations do exist, that variation can also be caused by biases in sampling designs or the type of analysis used. In many cases, comparison of data is further complicated by the reporting of different types of information, i.e. LOD or CCα/β, are often not reported. Some examples of analytical aspects that can lead to variation within the data are given below:

- *Qualitative, semi-quantitative and fully quantitative data.* While the ultimate aim of this review was to consider concentrations of natural steroids in a fully quantitative fashion, it was also recognized that a number of useful studies only reported data in a qualitative or semi-quantitative fashion. While the results of these analyses were not subject to any statistical analysis, they were considered useful in answering certain qualitatively focussed questions, e.g. does boldenone occur naturally at any concentration in species X? Where qualitative or semi-quantitative data are analysed, this will be highlighted and any assumptions stated. Even within fully quantitative data, a number of factors (as exemplified in the rest of this section) can lead to a lack of precision and accuracy in the data set, so it is important to understand that even this data set has a degree of uncertainty attached. In most studies, insufficient validation data were available to assess fully the degree of certainty of the results.
- *Type of calibration line used for quantification.* When dealing with endogenous substances, quantification can sometimes be complicated by the difficulty of finding a true blank matrix. In cases where a blank matrix of the same type as the study samples is not available then one can either use standard addition, where known amounts of steroids are added 'on top' of the existing concentrations present, or alternatively a surrogate matrix can be used. If using a surrogate matrix devoid of endogenous steroid, then appropriate measures need to be taken to ensure the chosen matrix behaves in a similar way to the actual sample matrix so that it can control for any variation in the analytical procedure. Neither of the two aforementioned measures are ideal and the result can be that concentrations of steroid quantified using different calibration line approaches can lead to different reported values for the same data set due to differential matrix effects or recovery of analyte. In many published reports, the actual calibration range applied was not explicitly given. This made it difficult to evaluate whether individual results fell within a linear range. Due to the need to limit the number of factors that were being taken into account in this review, adjustment of analytical data for recovery and matrix effects was not attempted. In any case,

many of the aforementioned parameters were not always reported by authors.

- *Limit of detection (LOD) or CCα/β*. For some steroids in certain physiological situations, a large number of reported concentrations are 'not detectable' (ND). This causes problems for two main reasons, one statistical and the other regulatory. From a statistical angle, the existence of large numbers of concentrations below the LOD are problematic because these values have to be effectively treated as zero, making it more difficult to define the population distribution and hence set threshold levels. If the LOD or CCα/β is not reported at all then this further complicates meta-comparisons between data sets. From a regulatory point of view, because LODs generally decrease as technologies improve, this sometimes means that steroids once thought to be purely synthetic appear to exist naturally at very low levels. However, it is difficult to assess whether new clusters of positive findings at such low levels are due to an increased abuse of steroids or natural occurrence. The issue in this context is therefore not the LOD per se, but the LOD in the physiological, analytical and regulatory context. Where possible, authors of published works were contacted and information on LODs or CCα/βs requested. Also requested (where relevant) were any results that were quantified, but below the LOD or CCα/β. In these cases, an estimation of the reliability of the additional trace concentration data was also requested.
- *Sample collection and subsequent preparation technique*. Before analysis, most techniques require some degree of sample preparation, usually involving extraction of the analytes of interest from unwanted or interfering matrix components. The treatment of the sample once taken from the animal can influence the analytical results in several ways, all of which highlight the need to stabilize samples appropriately and to take into account any artefactual processes occurring before analysis. For example, it is known that a number of meat producing species, e.g. bovine, ovine and equine, but not porcine, have the capability to convert 17β-hydroxy or ketone functions into 17α-hydroxy compounds (Gaiani et al. 1984). Bovine plasma in particular is known to be capable of catalysing this reaction (Gaiani et al. 1984), but the addition of methanol to the matrix has been shown to inhibit the activity.

  It has also been shown that the new-born of the ovine, caprine and bovine display very high rates of 20α-hydroxysteroid dehydrogenase activity (acting on progestagens and corticosteroids) and that this activity diminishes rapidly (although not completely) with age, possibly due to the replacement of foetal with adult erythrocytes (Nancarrow 1983). Due to a general dearth of knowledge on the metabolism of steroids in caprine, and ovine species, the significance of this finding for surveillance of steroid abuse is currently difficult to assess. However, for all the species concerned, it does suggest that the choices of age of any animals that are used as reference populations in the settings of thresholds are chosen very carefully so that they reflect those likely to be encountered in routine surveillance programmes.

  Bovine faeces is known to be capable of producing boldenone and other 1-dehydro steroids as metabolites from some steroidal precursors *ex-vivo* (Pompa et al. 2006). It is therefore recommended that sampling of bovine urine be devoid of faecal contamination in order to avoid boldenone false positives (De Brabander et al. 2004).

  It has also been shown that nandrolone related compounds can be formed from testosterone derivatives in human urine. The authors of this work showed that this reaction can be partially stabilized by adding EDTA to the samples (Grosse et al. 2005).

  Many steroids can also be conjugated with polar moieties such as sulphuric and/or glucuronic acid. Samples are often hydrolysed before analysis in order to produce the 'free' steroid. Hydrolysis can be performed before or after preliminary extraction or group separation and even then can be performed by a variety of methods. *Helix pomatia* digestive juice is the most often applied enzymatic form of deconjugation and this method affords hydrolysis of glucuronic acid conjugates and aryl sulphates at optimum pH, but is known to contain hydroxylase and oxidoreductase enzyme activity that can artefactually oxidize or reduce some steroids (Houghton et al. 1992). Another preparation that is frequently used is the β-glucuronidase enzyme from *E. coli*, which as its name suggests cleaves glucuronic acid conjugates but not sulphate conjugates (Houghton et al. 1992). In a two-fraction extraction, glucuronic acid conjugates may be cleaved by the *E. coli* enzyme, while sulphate conjugates can be cleaved by acid solvolysis (James Scarth, personal observation). Another alternative is to cleave both types of conjugates simultaneously using methanolysis (methanol and acetyl chloride), but this can lead to more complex mixture of

components retained within extracts (James Scarth, personal observation). The use of a number of different hydrolysis (or no hydrolysis at all) steps in the literature, all with varying capacities to deconjugate steroids, is another factor that potentially leads to variation in the reported concentrations.

A mixture of purification/concentration approaches were identified in the literature including solid-phase extraction, liquid–liquid extraction, protein precipitation, immunoaffinity column chromatography, supercritical fluid extraction, accelerated solvent extraction (ASE) and some very elaborate, but often effective, multi-step HPLC fractionation processes. Results using these methods are generally not compared in this review, unless there was specific relevance to a result:

- *Type of analytical method used*. A major factor leading to variation between reported values lies in the type of end-point detection method used. These included (in approximate descending order of reported use), immunoassay (IA), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), high-performance liquid chromatography-ultraviolet detection (HPLC-UV) and thin-layer chromatography-ultraviolet or -fluorescence detection (TLC-UV/FL). Immunoassay and mass spectrometry techniques generally afford higher sensitivity over HPLC-UV or and TLC-UV/FL and are also generally more selective. Mass spectrometry is considered to offer more selectivity than IA, predominantly due to variable extents of cross-reactivity of steroids against the IA antibody, although the impact of any cross-reactivity can be reduced by performing HPLC separation of sample extracts before analysis. Although more selective, mass spectrometry is still subject to matrix effects such as ion suppression or enhancement (LC-MS generally more so than GC-MS), but these can usually be overcome through the use of matrix matched standards (when available). As a general rule, it has been observed that IA tends to overestimate oestrogen levels at low concentrations while underestimating them at high concentrations (Stephany et al. 2004).
- *Statistical analyses used within the studies reviewed*. Depending on a number of factors, including the steroid, species, matrix and analytical LOD, a Gaussian distribution of steroid concentration population data may or may not be determined. In this respect, a large number of parametric and non-parametric approaches were reported by authors, reflecting the different findings under varying conditions. With such major differences in statistical reporting, such as mean versus median or standard deviation versus interquartile range, it is very difficult to make quantitative comparisons between data sets. It is also important to highlight a major difference between a statistical method being able to discriminate a control from a steroid treated population (i.e., a *t*-test result) and a statistical method that allows a workable threshold to be calculated (i.e., allowing a degree of certainty that at a particular threshold a false positive will not occur). There can be a significant amount of overlap in individual steroid concentrations from control and treated steroid populations that can be discerned using a *t*-test, but this does not necessarily mean they are significant enough differences to allow a realistic threshold to be fixed.

It is also important to add that the uncertainty of measurement was very rarely reported in the studies reviewed and was not easily calculated from the data available, further adding to the difficulty in making quantitative comparisons between data sets.

Physiological factors

Some physiological considerations regarding steroid concentrations are given in the analytical third section. In addition to inter-individual differences, there are many further factors that lead to variation in the observed concentrations. Challenger (2004) has reviewed the peak ovarian cycle plasma/serum oestradiol and progesterone concentrations in mammalian species. It was found that oestradiol concentrations spanned around four orders of magnitude while those for progesterone spanned three orders of magnitude. Oestradiol concentrations were on average two orders of magnitude lower than progesterone concentrations and there were significant differences between different animal orders. Maximum oestradiol concentrations were more variable in artiodactyls and primates than in carnivores. Absolute oestradiol concentrations were not correlated with dietary niche, but the progesterone to oestradiol ratio was lower in artiodactyls and primates compared with carnivores. Although relating to oestrogens and progestagens rather than androgens (a comparable study for androgens could not be found by the authors), this study highlights the significant differences in steroid concentrations between species and identifies the need to obtain endogenous population data for hormones in each species before detection strategies for regulatory surveillance are devised. As many references in this review will demonstrate, intra- and inter-species genetic variation may be responsible

for a large proportion of the observed variation between animals:

- *4- versus 5-ene pathways*. As well as the absolute differences in oestradiol and progesterone described above, species are also known to vary in their utilization of the 4- and 5-ene pathways for the production of steroids, which can be traced back to differences in the substrate requirements of the CYP17 enzyme (Mason 2002). This means that some species produce more steroid precursors with a 4-ene group (e.g., 4-androstenedione) whereas others produce more with a 5-ene group (e.g., DHEA [dehydroepiandrosterone]). Of relevance to meat-producing animals, 5-ene precursors are relatively high in bovine, porcine, ovine and equine species, while the cervine is lower in 5-ene and higher in 4-ene steroids (Wichmann et al. 1984).
- *Pregnancy and pseudo-pregnancy*. It is well known that pregnancy can lead to extremely high concentrations of certain relevant steroids, so pregnant animals are usually excluded from threshold value calculations. However, a phenomenon termed 'pseudo-pregnancy' (also known as phantom pregnancy or pseudo-cyesis) also exists, which in some species can lead to the physiological appearance of a state of pregnancy (including raised steroid concentrations), but without an actual foetus being conceived (Johnson and Everitt 2000). The effect is certainly frequent in rodent and canine species, but some references to its occurrence in the porcine (Pusateri et al. 1996), caprine (Lopes Junior et al. 2004) and ovine–caprine hybrids (Maclaren et al. 1993) were also obtained. While the condition does seem to occur naturally at a high incidence in some caprine species, the porcine reports were of artificially induced pseudo pregnancy by administering oestradiol. No reports of pseudo-pregnancy in bovine species could be found in the published literature.
- *Oestrous synchronization*. The effects of oestrous synchronization devices are not covered in this survey, but the subject has received comprehensive review in Rathbone et al. (1998).
- *Route of excretion*. Endogenous and artificially administered steroids are predominantly excreted from the body via the urine and faeces. The excretion of steroids is species and compound dependent, with some species preferentially excreting in faeces and some in urine. Consideration of whether urine, bile or faeces are the most suitable choices for a particular steroid/species combination depends on a number of factors (taken up later in this review), but their relative excretion in the form of recovered radioactivity in urine versus faeces is one consideration. Although an important factor, a predominance of radioactivity in one or other matrices does not always imply greater suitability for that matrix, as a smaller proportion of radioactivity present as one analyte may be more useful than a larger proportion of radioactivity present as many metabolites. Differences in the total volume of excreted

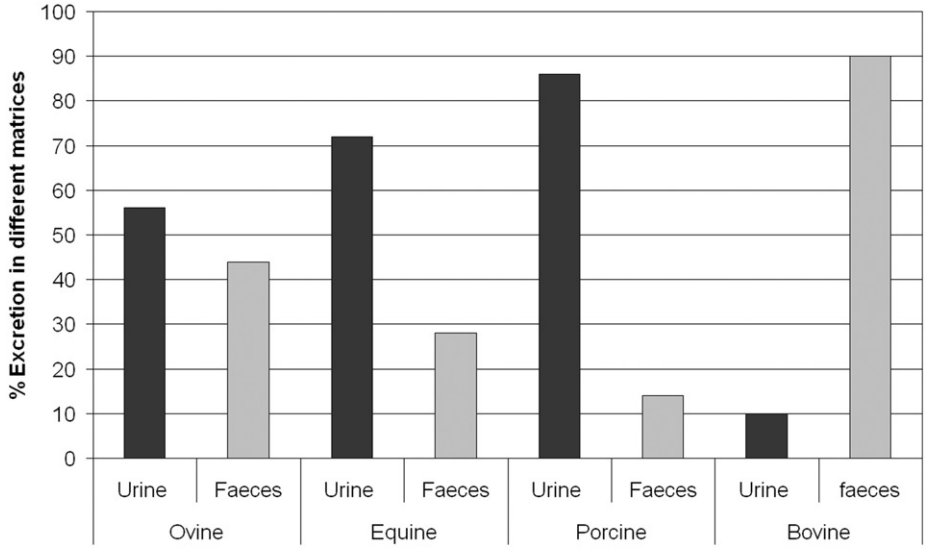

Figure 3. Percentage excretion of radioactivity in different waste products after intravenous infusion of testosterone into different species (adapted from Martin 1966; Calvert and Smith 1975; Velle 1976; Palme et al. 1996).

material can influence resulting concentrations, while urine generally suffers less analytical matrix effects and residual *ex-vivo* metabolism than faeces on the whole. Figure 3 exemplifies the range of different excretion patterns that have been observed for some steroids.

- *Hydration status*. The concentrations of steroids in some matrices, especially urine, can be affected by the hydration status of the animal (Wolfgang Korth, personal observation). One could predict that this might be a particularly important factor in countries that have experienced frequent droughts in recent years, for example Australia. The adjustment of urinary steroid concentrations for the hydration status of the animal (often measured as the specific gravity or the creatinine concentration of the urine) therefore has potential to reduce the variation in steroid values among the population. Like many physiological variables, it is also possible that dehydration may be a stressor that affects minor metabolic pathways such as the rate of biosynthesis/catabolism of steroids, but the authors are not aware of any studies that have assessed this particular variable.
- *Other variables*. Many other physiological variables can affect the concentrations of steroids in different animals. Previously proposed regulatory thresholds for natural steroids in meat producing species (i.e., Arts et al. 1991; Scippo et al. 1993) have taken into account at least the age and sex of the animal when constructing thresholds. In the current review, some of the factors that were analysed include the steroid in question, matrix, age, sex, herd demographics, gestation and castration status, geographical factors, housing conditions, season and time of day, disease, stress, medication, housing conditions, diet and breed.

Natural androgenic–anabolic steroid concentrations in the bovine

General trends in the data

Figures 4 and 5 summarize the different analyte/matrix and analytical technique/analyte combinations found for the bovine studies reviewed. This analysis was not repeated for other species because the bovine was atypical in the large numbers of studies performed and it was apparent that a similar analysis of other species would not provide sufficient data for a meaningful comparison.

As Figure 4 shows, testosterone has most often been analysed using IA, whilst nandrolone and boldenone by GC or LC-MS. The predominant use of IA (most often radioimmunoassay [RIA], followed by enzyme immunoassay [EIA]) is mainly due to its ease of application, its cost effectiveness, the fact that many studies are carried out in research laboratories that do not have mass spectrometry facilities or only use them for confirmatory analysis, and because of its high sensitivity in determining analytes present at low concentrations. Nandrolone and boldenone on the other hand are most often analysed by GC-MS or LC-MS. This can be explained partially due to the fact that proportionally more research on these analytes is carried out by residue screening laboratories that are more likely to use mass spectrometry However, it may also be because of the ambiguous status of these analytes and their metabolites, i.e. are they endogenous or not?

As Figure 5 shows, testosterone has most often been analysed in plasma or serum, whereas nandrolone and boldenone have most often been analysed from urine/bile or urine/faeces, respectively. As in the case of the explanation for the use of different analytical techniques for different analytes, these differences can

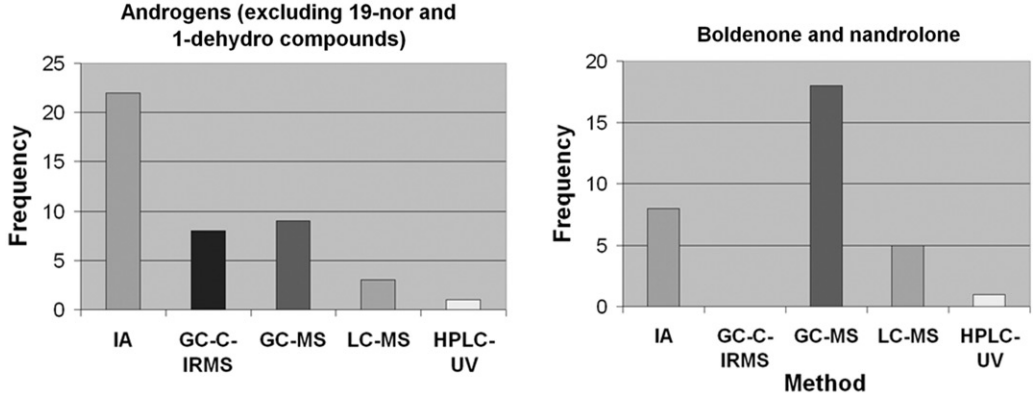

Figure 4. Summary of the use of different analytical techniques used in the bovine studies reviewed in this review.

be in part explained by quantitative biases in the type of research being carried out: either (1) physiology research looking at matrices indicating relevant circulating concentrations (i.e., plasma) for testosterone or (2) research for residue control in matrices more relevant to the detection of abuse (i.e., concentrated amounts in urine, bile or faeces for nandrolone and boldenone).

Data on the endogenous presence of androgenic–anabolic steroids in the bovine are summarized in Table 3 while details of the major phase 1 metabolic products following exogenous administration are given in Table 4.

Testosterone and related androgens in the bovine

Endogenous occurrence

As a general rule for all steroids, circulating plasma and tissues from non-excretory organs contain relatively high concentrations of unchanged steroid while excretory products such as urine, bile or faeces contain relatively higher concentrations of metabolites. As well as a relative difference in the proportion of each steroid/metabolite present, excretory products generally contained higher absolute concentrations of total analyte/metabolite due to a concentrating effect.

Testosterone and related steroids such as epitestosterone, 4-androstenedione and DHEA are ubiquitous among male and female animals of all mammalian species, so differences among various groups and times are purely quantitative. When surveying the ranges of mean, minimum and maximum values among the published studies (over 1000 papers for all species concerned), an approximate overall rank order of absolute concentrations can be constructed. It must be stressed that some positions within this rank may be caused by biases in the amount of information reported for each steroid in different matrices. An approximate rank order for testosterone concentrations in the bovine is hair > urine ~ fat ~ faeces ~ kidney > plasma > liver ~ muscle. An approximate rank order for the significant testosterone metabolite epitestosterone is urine > faeces > plasma > muscle > hair. There were no data available for epitestosterone in fat, liver or kidney. In terms of absolute values, testosterone and epitestosterone were present at similar concentrations in muscle and plasma, testosterone was at least a factor of 10 higher in hair, while epitestosterone was around a factor of 10 higher in urine and faeces. There were no data for comparison of fat, liver or kidney. There was more variation among epitestosterone values relative to those for testosterone. As mentioned elsewhere in this review, the majority of plasma results that contributed to the aforementioned results do not use sample hydrolysis. However, Scippo et al. (1993) showed that while the maximum testosterone concentration found in bull plasma were 5752 and 965 pg ml^{-1} for unconjugated and conjugated, respectively, the reverse was seen

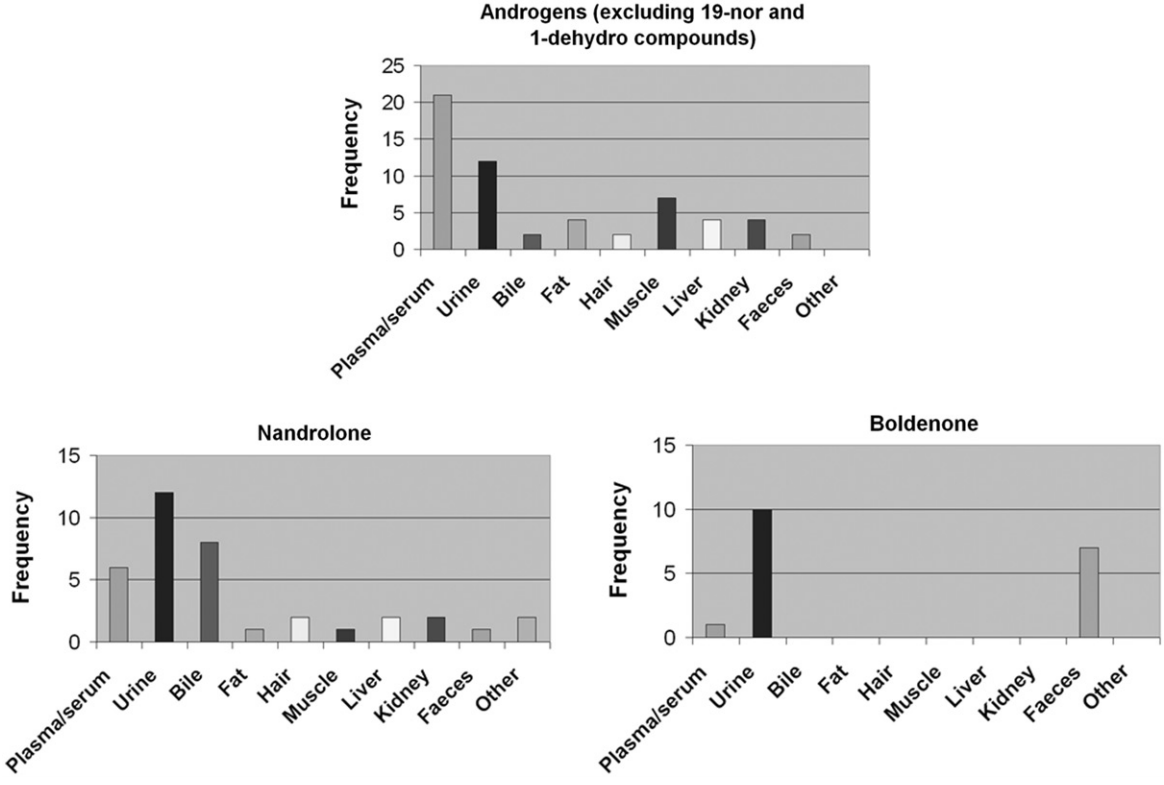

Figure 5. Summary of the matrices used in the bovine studies reviewed in this review (all methods of analysis included).

Table 1. European Economic Community (EEC) decision limits for testosterone in plasma (as proposed by Heitzmann 1994).

Age/sex of animal	Decision limit in plasma ($ng\,ml^{-1}$)
Female (non-pregnant)	0.5
Male (<6 months)	10
Male (>6 months)	30

for epitestosterone with values of 974 and 1750 $pg\,ml^{-1}$ for unconjugated and conjugated, respectively. This could lead to artificially low reported concentrations of epitestosterone in plasma relative to testosterone.

Several other precursors including DHEA, androstenediol isomers and 4-androstenedione were also occasionally quantified in plasma/tissue, but not in excretory products and there may be value in monitoring perturbations of endogenous steroid feedback loops after exogenous steroid administration.

Existing European Union guidelines for positive decision limits (as proposed by Heitzman 1994) in the bovine already rely on separation of sex, age and gestation status as summarized in Table 1.

From the current review, ranges of mean plasma/serum concentrations of testosterone and epitestosterone were found to be approximately ten-fold higher in intact mature males relative to females. No plasma/serum testosterone data were available for steers. One significant finding was of a study that stated that plasma testosterone was exceptionally high for a very brief time during the late luteal phase of the normal female oestrous cycle exceeding 1.8 $ng\,ml^{-1}$ (Dobson et al. 1977). All other ranges of testosterone reported by this author were in line with those of other studies, so if real, this phenomenon could have a serious, negative impact on the validity of the existing European Union decision limit for females.

From the current review, ranges of mean urinary concentrations of testosterone and epitestosterone were found to be approximately three-fold higher in mature males relative to females. No urinary testosterone data were available for steers. Ranges of mean muscle concentrations of testosterone were found to be approximately ten-fold higher in mature males relative to females or steers, although epitestosterone was similar between steers and bulls. There were no data for females. Ranges of mean liver and kidney concentrations of testosterone were found to be approximately ten-fold higher in mature males relative to females. There were no data for androgens in steers or epitestosterone in any sex. Ranges of mean hair concentrations of testosterone were found to be approximately three-fold higher in mature males relative to females and steers. There were no data for epitestosterone. There were insufficient data to compare testosterone concentrations by sex in faeces, fat or bile.

Several studies have assessed the effect of age on the plasma/serum concentrations of testosterone in males, although the different ages, matrices and conditions under which the animals were studied and a lack of standardization in reporting the uncertainty of measurement makes meaningful comparisons difficult. The results from three of the most informative studies are summarized as follows:

- Bagu et al. (2006) showed that mean male serum testosterone concentrations at 4 weeks of age were around 0.1 $ng\,ml^{-1}$. Concentrations then rose to 1.0 $ng\,ml^{-1}$ at 20 weeks, then dropped back to 0.4 $ng\,ml^{-1}$ at 28 weeks and rose again to 1.1 $ng\,ml^{-1}$ at 32 weeks. The authors of this study also referenced other studies that have shown a trough in testosterone concentrations between 20 and 32 weeks of age.
- Looking at older animals, Moura and Erickson (2001) reported that mean male serum testosterone concentrations were 1.8 $ng\,ml^{-1}$ at 26 weeks of age, rose to 8 $ng\,ml^{-1}$ at 43 weeks and then dropped to 6.5 $ng\,ml^{-1}$ at 52 weeks. In the same study, 4-androstenedione was 0.45 $ng\,ml^{-1}$ at 17 weeks and dropped to 0.25 $ng\,ml^{-1}$ at 52 weeks.
- The most informative single study on the effect of age was published by Arts et al. (1990). Median male plasma testosterone concentrations at 15 weeks of age were 0.8 $ng\,ml^{-1}$ and then rose to 1.3 $ng\,ml^{-1}$ at 28 weeks. Concentrations of epitestosterone, however, dropped from 7.1 $ng\,ml^{-1}$ at 15 weeks to 0.8 $ng\,ml^{-1}$ at 28 weeks. In the same study, median male urinary testosterone concentrations were 1.0 $ng\,ml^{-1}$ at 15 weeks and rose to 3.7 $ng\,ml^{-1}$ at 28 weeks. Epitestosterone concentrations did not change with age and values 15 and 28 weeks were 40 and 41 $ng\,ml^{-1}$, respectively. As a result of the aforementioned testosterone and epitestosterone concentration changes with age, the epitestosterone:testosterone ratio fell significantly from 15 to 28 weeks of age. On this note, the testosterone: epitestosterone ratio has been found to be a good indicator of testosterone abuse in humans and horses (due to selective elevation of testosterone after testosterone doping), but Angeletti et al. (2006) showed it to be of less use in the bovine, probably due to the relatively high 17α-hydroxylase enzyme activity.

Relatively fewer studies have analysed the effect of age on female testosterone concentrations. Nakada et al. (2000) reported that mean female plasma testosterone concentrations immediately after birth was $0.075\,ng\,ml^{-1}$ but then fell, ranging between means of 0.015 and $0.021\,ng\,ml^{-1}$ between birth and puberty. Mean (SEM) age to puberty was 43.3 (1.3) weeks, with a range of 38–55 weeks. The same study by Arts et al. (1990) that reported male testosterone data by age also reported female data. Median female plasma testosterone concentration was less than the LOD at both 15 and 28 weeks, while median plasma epitestosterone was less than the LOD at 15 weeks and then rose to $0.2\,ng\,ml^{-1}$ at 28 weeks. Median female urinary testosterone concentration at 15 weeks was less than the LOD and then rose to $1.1\,ng\,ml^{-1}$ at 28 weeks. Median female urinary epitestosterone at 15 and 28 weeks were 6 and $17\,ng\,ml^{-1}$, respectively. There were insufficient data to compare the effect of age on testosterone or related metabolites/precursors in faeces, liver, kidney, bile, muscle, hair or fat.

Data on the concentration of testosterone in any matrix from pregnant females was not available, but it would be expected to be elevated relative to non-pregnant females in line with other steroids (see later sections). However, mean plasma concentrations of DHEA and androst-5-ene-3β,17β-diol were found to be approximately three-fold higher in pregnant females (Gabai et al. 2004).

Plusquellec and Bouissou (2001) showed that lactating cows of the Herens breed (artificially selected for fighting ability) had significantly higher ($p < 0.05$) median plasma testosterone concentrations compared with Brune des Alpes animals, with values of 0.21 and $0.11\,ng\,ml^{-1}$, respectively. This conforms to the observation that aggression correlates with increasing testosterone levels. The biochemical observations were also borne out by secondary sexual characteristics, which were more prominent in the Herens breed.

Moura and Erickson (2001) showed that bulls suffering spermatic arrest had only slightly lower serum testosterone concentrations than healthy controls. However, serum 4-androstenedione in one diseased animal was $>0.8\,ng\,ml^{-1}$ at 12 months, relative to a mean of $0.25\,ng\,ml^{-1}$ in healthy controls. No reports on the effect of other factors known to increase the androgen output in other species were found, i.e. stress or congenital adrenal hyperplasia.

No studies were found that directly compared concentrations of testosterone or related precursors/metabolites in similar breeds under different housing conditions or in different countries, nor of diet, time of day or season on testosterone or related precursor/metabolite concentrations.

In most species long-term treatment with gonadotrophin-releasing hormone (GnRH) agonists such as deslorelin decrease luteinzing hormone (LH) output (and therefore testosterone secretion) due to desensitization of the pituitary gland. However, Aspden et al. (1997) reported that testosterone concentrations in mature bulls are increased following deslorelin administration, although another effect of this drug is that LH pulsatility is lost, leading to a flat LH secretion profile. On the other hand, Renaville et al. (1996) showed that administration of GnRH to immature bulls between 70 and 150 days of age delayed puberty relative to controls with mean pubarche ages of 180 and 120 days, respectively. No reports of the effects of other non-steroidal medications on androgen concentrations were found, but several types of medication in other species are known to affect increase or decrease in concentrations, e.g. cytochrome P450 enzyme-inducing inhibiting drugs.

Metabolism following administration

Many studies have reported the changes in natural steroid profiles after exogenous steroid administration and some general trends as well as the results of one informative study, whose results are in line with other reports, are summarized below. Testosterone itself was most often reported as being given in esterified form as an implant in the ear or as an intramuscular (IM) injection, either alone or in combination with oestradiol esters. A general trend in the bovine is for animals of both sexes to epimerize 17β-hydroxyl groups to the corresponding 17α isomer. While this epimerization results in significant amounts of 17α-hydroxy steroids in urine, the enzyme(s) responsible for the activity are also present in plasma/serum, highlighting the need to consider steroid stability studies in all matrices in order to ensure that appropriate action to maintain stability is taken.

Biddle et al. (2003) reported the effect of component-EH administration (a testosterone plus oestradiol preparation) to heifers and steers on the concentrations of urinary, serum and bilary androgens and compared the results to 'natural' populations (sampled from the field so not considered a fully 'controlled' population with respect to ruling out steroid abuse). For heifers and steer serum, basal androgen levels were very low and no significant changes were noted after administration of component-EH, In heifer and steer urine, epietiocholanolone, 5β-androstane-3α,17β-diol and 5β-androstane-3β,17α-diol were present at similar levels and were around ten times the concentration of testosterone. No data for epitestosterone were reported. After component-EH administration, the most significantly increased urinary metabolite relative to baseline values in both male and females was 5β-androstane-3β,17α-diol. Testosterone was not detectable in the majority of steer and heifer bile samples collected at slaughter. Epietiocholanolone and 5β-androstane-3α,17β-diol were detectable somewhat

more frequently, with 5β-androstane-3β,17α-diol being present at higher concentrations and therefore detectable in the majority of samples. However, no significant differences were observed in any of these analytes in animals administered component-EH. Overall, concentrations of testosterone metabolites were present in the following rank order of concentration: bile > urine > serum. It is important to add that the conclusions reached in the above study may be partially dependent on the analytical detection capabilities, i.e. if significantly reduced LOQs were applied then it could theoretically be possible to discern steroid abuse from the natural population.

Nandrolone and related 19-nor androgens in the bovine

Endogenous occurrence

Nandrolone was once thought to be a solely synthetic steroid, but in the 1980s it was isolated as a natural hormone in the stallion (Houghton et al. 1984) and boar (Maghuin-Rogister et al. 1988). Since then, nandrolone related compounds have also been detected in matrices originating in the bovine (Vandenbroecke et al. 1991), ovine (Clouet et al. 1997), caprine (Sterk et al. 1998), human (Dehennin et al. 1984) and cervine (Van Hende 1995). Non-phenolic C18 steroids (19-nor androgens) such as nandrolone are likely to be produced predominantly as minor pathways of the normal aromatization process of androgens that gives rise to the phenolic oestrogens. C18 androgens are therefore most often encountered in situations of high oestrogen output (Van Eenoo et al. 2001) such as pregnancy, although other contributory causes such as consumption of contaminated dietary products (Le Bizec et al. 2000), increased physiological stress (De Geus et al. 2004) and *in*-situ formation in stored urine samples (Grosse et al. 2005) (including while stored in the bladder before sampling and hence not subject to experimental control), have also been implicated. As the later section on the equine describes, the possibility that 19-nor androgens may arise as artefactual products of 19-carboxy compounds also has to be considered because most methods to date have not taken this possibility into account (Houghton et al. 2007). It is also possible, however, that the production of these 19-carboxy compounds are limited to certain species such as the equine due to unique genetic sequences in these species. A representative selection of studies reporting endogenous bovine nandrolone are summarized below:

Daeseleire et al. (1993) reported that traces of 5α-estrane-3β,17α-diol could be detected in urine from a pregnant control cow and 19-nor-etiocholanolone was detected in two control steers by GC-MS (the breed was not reported). Nandrolone and epinandrolone could not be detected in the cow or steer urine (method not quantitative so no LOD reported). No C18 androgens were present in calf urine.

De Brabander et al. (1994) reported the results of a multi-laboratory study on the natural occurrence of C18 androgens in the bovine. Although no nandrolone could be detected at an LOD of 0.5 ng ml^{-1}, all four laboratories involved found that urine from pregnant cows (the breed was not reported) contained epinandrolone for up to 4 months pre-partum and 2 days post-partum when analysed by GC-MS. Although most laboratories agreed when epinandrolone was or wasn't present in a sample (with some exceptions probably due to concentrations close to LODs), there was significant variation in the quantified concentrations when the compound was found (i.e., between 0.7 and 4.3 ng ml^{-1} for one sample). This study is seminal in that it highlights the huge uncertainty of measurement that can surround the determination of steroid concentrations. It highlights the need to standardize as many experimental factors as possible and then cross-validate methods before the results of such studies can be compared with confidence. It also serves as caution when trying to make too detailed a comparisons between data already in the published literature.

McEvoy et al. (1998) studied the natural occurrence of nandrolone and epinandrolone in the bile of pregnant Friesian cows using GC-MS. Nandrolone itself was not detectable in all samples from all stages of pregnancy (LOD not reported), but epinandrolone was detected from 120 days pre-partum onwards. Concentrations at 120 days pre-partum were around 1 ng ml^{-1}, rising to 37 ng ml^{-1} at parturition and then dropping to not detectable within 1 week post-partum. Cows carrying male foetuses had higher ($p < 0.001$) epinandrolone concentrations than those carrying female foetuses. Cows had lower ($p < 0.001$) epinandrolone concentrations during their second relative to first pregnancy. McEvoy (1999) subsequently showed that the bile of steers and bulls derived from an untreated population did not contain nandrolone or epinandrolone above a GC-MS LOD of 0.4 ng ml^{-1}. However, some bile samples from steers (but not from bulls) suspected of nandrolone abuse did contain epinandrolone.

Metabolism following administration

Van Ginkel et al. (1989) reported that in calves administered nandrolone (animal and administration details not available), that concentrations of epinandrolone were always greater than nandrolone, while concentrations of each were greater in bile than urine.

Samuels et al. (1998) used GC-MS to study plasma, urine and bile steroid levels after IM administration of a 1:1 mixture of nandrolone and a deuterated

labelled analogue to cull cows, steers and heifers, with only urinary results being reported in this paper. Between zero and 12 h after administration, urinary epinandrolone and 5β-estran-3α,17β-diol peaked at around 200 ng ml^{-1} while 5β-estran-3α,17α-diol and 5α-estran-3β,17α-diol were lower at around 160 and 80 ng ml^{-1}, respectively. However, between 12 and 24 h 5β-estran-3α,17α-diol and 5α-estran-3β,17α-diol were predominant with concentrations of around 120 and 90 ng ml^{-1}, respectively, followed by epinandrolone and 5β-estran-3α,17β-diol at concentrations of 30 and 40 ng ml^{-1}, respectively. The aforementioned compounds were detected for both the deuterium labelled and non-labelled mass transitions, suggesting that they were direct metabolites of the administered nandrolone laurate rather than through latent natural metabolic pathways being activated.

Biddle et al. (2003) reported the effect of Laurabolin (nandrolone ester) to heifers and steers on the concentrations of urinary, serum and bilary C18 androgens and compared the results to 'natural' populations (sampled from the field so not considered a fully 'controlled' population with respect to ruling out steroid abuse). The majority of steer and heifer plasma samples contained natural concentrations of nandrolone, epinandrolone, 5α-estrane-3β,17α-diol and 5β-estrane-3α,17α-diol that were below the LOQ and there was no significant increase in C18 androgens after administration relative to the 'natural' population. The majority of steer and heifer urine samples contained concentrations of nandrolone, epinandrolone, 5α-estrane-3β,17α-diol, 5β-estrane-3α,17α-diol, 5β-estrane-3α,17β-diol and 19-noretiocholanolone that were below the LOQ. Laurabolin administered to females produced significant increases in epinandrolone, 5α-estrane-3β,17α-diol, 5β-estrane-3α,17α-diol and 19-noretiocholanolone concentration, 5β-estrane-3α,17β-diol was also elevated but to a much less marked extent. However, administration of Laurabolin, to males only produced a slight rise in epinandrolone and 5α-estrane-3β,17α-diol concentrations. In bile, the majority of steer, heifer and bull samples contained concentrations of nandrolone, epinandrolone, 5β-estrane-3α,17β-diol, 5α-estrane-3β,17α-diol and 5β-estrane-3α,17α-diol that were below the LOQ. In post-administration samples taken at slaughter from both heifers and steers (no samples for bulls), concentrations of 5β-estrane-3α,17α-diol were the most obviously increased of the steroids relative to samples from the 'natural' population. Nandrolone itself was not detectable in post-administration heifer bile samples while interpretation of nandrolone data in steers and epinandrolone data in heifers and steers was complicated by the co-elution of a large interfering peak in the relevant mass transition windows, so results could not be reported.

Boldenone and related 1-dehydro androgens in the bovine

Endogenous occurrence

As in the case of nandrolone, boldenone and other 1-dehydro steroids were once thought not to be endogenous. Since the 1990s however, boldenone related compounds have been detected in different matrices from several species including microbes (Mahoto and Garai 1997), maggots (Verheyden et al. 2007), crustaceans (Verslycke et al. 2002), rats (Song et al. 2000), pigs (Poelmans et al. 2005), horses (Ho et al. 2004), and cattle (veal calves) (Arts et al. 1996). The most likely origin of 1-dehydro compounds in the bovine is through faecal conversion of precursors such as phytosterols or other steroids by gut microbes (Pompa et al. 2006). Extra-enteral production of 1-dehydro steroids within the body (i.e., testes) has not been demonstrated in the bovine (De Brabander et al. 2004), but boldenone has been identified in porcine testes (Poelmans et al. 2005). A very comprehensive review of the presence and metabolism of boldenone in various animal species was published by De Brabander et al. (2004). The endogenous occurrence of 1-dehydro steroids in the bovine has been the subject of some debate. 1-dehydro compounds appear to be present in some animal populations but not others. The extent to which this is due to real physiological variation versus experimental design is hard to establish, but the general consensus (James Scarth, various personal observations and communications) is that it is likely a mix of both. The following summaries of two studies highlight many of the issues:

Draisci et al. (2003) analysed urine samples for boldenone, epiboldenone and androsta-1,4-diene-3,17-dione by LC-MS from 25 untreated animals. Boldenone (LOQ = 0.2 ng ml^{-1}), epiboldenone (LOQ = 0.5 ng ml^{-1}) and androsta-1,4-diene-3,17-dione (LOQ = 0.2 ng ml^{-1}) were not detected above the LOQ in any of the urine samples from the untreated animals.

Pompa et al. (2006) studied the concentrations of boldenone, epiboldenone, androsta-1,4-diene-3,17-dione, testosterone and epitestosterone in the urine, skin swabs and faeces of Friesian calves and also assessed the effect of drying the faeces on the resulting faecal steroid concentrations. In urine, LODs for all steroids were 0.1 ng ml^{-1} and in faeces LODs for all steroids were 0.5 ng g^{-1} (based on $S{:}N > 3{:}1$). Boldenone, epiboldenone and androsta-1,4-diene-3,17-dione in urine were not detected in any of the samples from ten calves. Boldenone was detected in faeces sampled directly from the rectum (rectal faeces) in all the calves at concentrations ranging from 28 to 89 ng g^{-1}. Epiboldenone in rectal faeces was not detectable in six calves and between 2.6 and 5.9 ng g^{-1}

in the other four animals. Androsta-1,4-diene-3,17-one was not detected in the rectal faeces from nine calves while one calf had 21 ng g^{-1}. Results from faeces scraped from the skin, faeces taken from the stall floor and faeces stored for up to 13 days at room temperature in a cowshed showed that the concentrations of all steroids increased significantly (but variably) over time. This is especially true of epiboldenone and androsta-1,4-diene-3,17-dione, which by day 13 of storage are present in high concentrations, while boldenone was reduced to not detectable by day 13. This study exemplifies the need for avoiding faecal contamination of urine during sampling and to ensure swift storage and analysis of any samples taken.

Metabolism following administration

Van Puymbroeck et al. (1998) studied the metabolism of boldenone in the bovine using semi-quantitative GC-MS analysis of urine and faecal samples. In urine from animals given various boldenone ester administrations, epiboldenone was the major metabolite in urine, followed by significant amounts of two 5-reduced metabolites with unidentified stereochemistry at positions 3, 5 and 17 with molecular masses 2 units higher than boldenone. Other urinary metabolites identified were, 6*z*-hydroxyboldenone, androsta-1,4-diene-3,17-dione and 5β-androst-1-ene-3,17-dione (where a '*z*' indicates that the isomer configuration has not been established). In faeces, boldenone, androsta-1,4-diene-3,17-dione and 6*z*-hydroxyboldenone were absent, while only small amounts of epiboldenone, 5β-androst-1-ene-3,17-dione and some unidentified reduced metabolites were found.

Sterk et al. (2004) used GC-MS to study the effect on urinary and faecal metabolites after IM boldenone ester administration to veal calves. Not all experimental details or results were reported, but the authors stated that 'both 17α and 17β-boldenone were almost 100% present in urine as glucuronic acid conjugates. The 6*z*-hydroxy-boldenone metabolite was present as a sulphate conjugate. In faeces boldenone was present in the non-conjugated form'.

Le Bizec et al. (2006) used GC-MS to analyse the urinary metabolite profile after various boldenone administrations in cattle. Treated animals received either a single oral boldenone plus androsta-1,4-diene-3,17-dione dose, a daily oral boldenone ester dose for 3 days, a daily oral androsta-1,4-diene-3,17-dione dose for 6 days, a daily oral boldenone ester dose for 5 days or a single IM injection of boldenone esters. In all treated animals' hydrolysed urine, epiboldenone was by far the most predominant metabolite, while 17α-hydroxy-5β-androst-1-en-3-one, 17β-hydroxy-5β-androst-1-en-3-one and 3α-hydroxy-5β-androst-1-en-17-one were the only other metabolites always present. Analysis with and without hydrolysis suggested that the majority of metabolites were glucuronic acid conjugated, with boldenone having a variable degree of sulphate conjugation. Analysis using LC-MS was also carried out and it was found that urine from treated animals contained boldenone sulphate conjugate whereas urine from untreated animals did not. The authors therefore proposed looking at boldenone sulphate on a larger scale to see if it can be used as an indicator of boldenone administration.

Biddle et al. (2005) reported a quantitative metabolism study of boldenone in the bovine. Two steers were sequentially treated with (A) bolus 400 mg boldenone IM injection, (B) followed 14 days after the bolus injection by an oral 500 mg androsta-1,4-diene-3,17-dione administration, and (C) followed 10 days after the oral administration by a 700 mg boldenone undecylenate IM injection. Samples of plasma and urine were analysed as 'free' and 'glucuronic acid conjugated' fractions using differential extraction and *E. coli* hydrolysis. Analysis was by GC-MS and was qualitative/semi-quantitative only. **Note:** Where an analyte is preceded by a question mark in the remainder of this section, it signifies that the structure is putative (although with strong evidence to suggest its structure) as no reference standard was available. After oral androsta-1,4-diene-3,17-dione administration, the free plasma metabolites were of the order of epiboldenone > ?6β-hydroxyepiboldenone > boldenone ~ 6β-hydroxyboldenone. The plasma glucuronic acid conjugate fraction metabolites were also of the order of epiboldenone > ?6β-hydroxyepiboldenone > boldenone ~ 6β-hydroxyboldenone, but absolute concentrations were approximately ten times those of the free fraction. Of these, boldenone and 6β-hydroxyboldenone were only transiently visible at low concentrations. The free urinary metabolites were of the order of epiboldenone > ?5β-androst-1-ene-17α-ol-3-one > 6β-hydroxyboldenone ~ 5β-androst-1-ene-17β-ol-3-one. The urinary glucuronic acid conjugate fraction metabolites were of the order of epiboldenone > ?5β-androst-1-ene-17α-ol-3-one > 5β-androst-1-ene-17β-ol-3-one > ?6β-hydroxyepiboldenone > 6β-hydroxyboldenone. After IM boldenone undecylenate administration, the only free plasma analyte present was boldenone, which was detected for the whole 56 days after administration. The glucuronic acid conjugate plasma metabolites were of the order of epiboldenone > ?6β-hydroxyepiboldenone > boldenone. Of the glucuronic acid conjugated metabolites, ?6β-hydroxyepiboldenone had the longest detection time after administration. Boldenone itself was present in greater quantities in the free than glucuronic acid conjugate fraction in plasma. The free urinary metabolites were of the order of epiboldenone > boldenone. The glucuronic

acid conjugated urinary metabolites were of the order of ?6β-hydroxyepiboldenone ~ ?5β-androst-1-ene-17α-ol-3-one ~ 5*z*-androst-1-ene-3*z*-ol-17-one ~ epiboldenone > 5β-androst-1-ene-17β-ol-3-one. Boldenone and epiboldenone were present at higher concentrations as glucuronic acid conjugates than free steroids.

European Union recommendations regarding boldenone testing

The outcome of an experts meeting on the control of boldenone in veal calves in September 2003 (European Commission, Health and Consumer Protection Directorate-General 2003) recommended the following:

On the basis of the scientific information available, the experts of the Member States agreed that the presence of boldenone conjugates at any levels in urine from veal calves is proof of illegal treatment. In order for positive results for boldenone to be used as evidence of illegal treatment, the following must be fulfilled:

- That sampling of urine must be done without faecal contamination of the samples. The samples should be frozen as soon as possible after collection in order to avoid hydrolysis of the conjugates.
- Analytical results related to boldenone residues (boldenone or epiboldenone) must always be specified as free or conjugated forms, with the explicit identification of the animal species, including breed, gender and age of the animal.

There is sufficient scientific knowledge to conclude that the presence of epiboldenone in urine and faeces of bovine animals can come from other sources than illegal treatment. A number of explanations are currently being investigated by the scientific community. If only epiboldenone is found and if the levels are above $2\,ng\,g^{-1}$ in urine of veal calves, additional investigations would need to be carried out before concluding on illegal use of boldenone.

An MRPL for the analytical methods for the detection of boldenone and epiboldenone in urine of veal calves should be set at $1\,ng\,g^{-1}$. Further studies of appropriate marker metabolites of boldenone are encouraged. The member states should transmit existing and future data to the CRL in Bilthoven. This position could be amended in the light of additional data from ongoing and future research.

Testosterone and related androgens in the ovine

Data on the endogenous presence of androgenic–anabolic steroids in the ovine are summarized in Table 3, while details of the major phase 1 metabolic products following exogenous administration are given in Table 4.

Endogenous occurrence

In this review, ranges of mean plasma/serum testosterone concentrations were found to be between three- and 100-fold higher in rams relative to ewes (depending on the season), while concentrations in wethers were similar to those of ewes. Concentrations of 4-androstenedione in the only report of concentrations in wethers were similar to the lowest mean concentrations reported in rams, but approximately 60-fold lower than the highest mean concentrations reported in rams.

Plasma/serum concentration levels of testosterone in late pregnant ewes were similar to those of non-pregnant animals, while pregnant ewe DHEA concentrations were similar to concentrations in rams. 4-Androstenedione concentrations in pregnant ewes were similar to the maximum mean concentrations reported in rams and approximately 60-fold higher than the only report of concentrations in wethers.

Several studies have assessed the effect of age on plasma/serum testosterone concentrations in rams. Fahmy (1997) studied the serum concentrations of testosterone from the ages of 10–34 weeks in rams of the Romanov breed as well as the Booroola Merina × DLS breed. Testosterone concentrations increased in a linear fashion with age in both breeds ($p < 0.01$). Mean concentrations in the Romanov breed at 10 weeks were $3.8\,ng\,ml^{-1}$, rising to $13\,ng\,ml^{-1}$ at 34 weeks. Mean concentrations in the Booroola Merina × DLS breed were $0.8\,ng\,ml^{-1}$ at 10 weeks, rising to $8.0\,ng\,ml^{-1}$ at 34 weeks. Concentrations were significantly higher ($p < 0.05$) in the Romanov breed at all ages other than 14 weeks, which corresponded to a temporary reduction in concentrations in the Romanov breed.

Langford et al. (1998) reported the serum concentrations of testosterone in each of the Canadian Arcott, Outaouais Arcott, Rideau Arcott and Finnish Landrace breeds at 6, 8 and 12 months then at 3 years. Mean concentrations in the Canadian Arcott, Outaouais Arcott, Rideau Arcott and Finnish Landrace at 6 months were 3.3, 4.0, 3.5 and $3.9\,ng\,ml^{-1}$, respectively, at 8 months were 3.1, 5.0, 5.5 and $8.2\,ng\,ml^{-1}$, respectively, at 12 months were 3.5, 5.3, 5.4 and $7.8\,ng\,ml^{-1}$, respectively. Mean concentrations at 3 years were 8.0, 7.0, 5.7 and $7.0\,ng\,ml^{-1}$, respectively. Concentrations were significantly higher ($p < 0.05$) in Finnish relative to Canadian Arcott male lambs at 8 and 12 months, but none of the other differences was significant ($p < 0.05$).

There were insufficient data to compare concentrations of testosterone or related precursors/metabolites

between sexes, ages, gestation and castration statuses in any other matrix.

No reports on the effect of time of day or geographical factors were found, but two reports on the effect of diet and several reports on the effect of season and housing conditions were retrieved. Schanbacher and Ford (1979) reported the effect of transferring animals from a 12-h light, 12-h dark photoperiod to either short day photoperiod (8-h light, 16-h dark) or long day photoperiod (16-h light, 8-h dark) on serum testosterone concentrations in Suffolk–Hampshire rams. The animals exposed to short days had increased testosterone concentrations, heavier testes, larger seminiferous tubules and produced more sperm relative to the long-day rams. This effect of photoperiod has obvious implication for resulting testosterone concentrations, dependent upon the type of artificial light conditions and the hemisphere inhabited. Borque and Vazquez (1999) studied the effect of season on the concentration of plasma testosterone in Manchego rams living in the northern hemisphere (Spain). Concentrations varied with the season, such that mean peak values were in the second week of September at $7.2\,ng\,ml^{-1}$, while trough concentrations were $0.40\,ng\,ml^{-1}$ in the second week of February. The authors also commented that increasing testosterone concentrations were correlated with a decreasing photoperiod.

Rosa et al. (2000) studied the effect of housing males with females on plasma testosterone concentrations in Texel and Suffolk rams. Testosterone concentrations were increased on introduction to ewes ($p < 0.05$), but the effect did not depend on whether ewes were in oestrous or not. Mean basal concentrations in different groups varied from 5 to $10\,ng\,ml^{-1}$, while after introduction to ewes, concentrations increased by between 3 and $5\,ng\,ml^{-1}$ to between 8 and $15\,ng\,ml^{-1}$.

Parkinson et al. (2001) studied the effect of intersex relative to concentrations in 'normal' rams and ewes during the breeding season. Freemartin ewes (XX/XY chimeras) were classified as either 'male (MF)' type or 'undifferentiated (UF)' depending upon the masculinization of their genitalia. In one of the experiments (1b) mean basal testosterone concentrations in MF, UF, ewes and rams were 0.79, 0.29, 0.14 and $2.4\,ng\,ml^{-1}$, respectively. The testosterone concentrations in Freemartins were significantly higher ($p < 0.05$) than in ewes, but lower than in rams.

Stellflug et al. (2004) studied the effect of administering the opioid antagonist naloxone on plasma testosterone concentrations in rams with different sexual orientations. Mean basal testosterone concentrations in the first experiment were $2\,ng\,ml^{-1}$ for all animals. After naloxone administration, mean testosterone concentrations rose significantly ($p < 0.01$) after 60 min in both sexually active and male oriented rams to 8.0 and $8.2\,ng\,ml^{-1}$, respectively, but a rise in testosterone concentrations in sexually inactive rams to $3.5\,ng\,ml^{-1}$ at 60 min was not reported as significant. Although this example on it's own may have little relevance to the regulation of steroid abuse, it highlights the fact that concomitant medication can sometimes have effects on natural steroids and may therefore need to be considered.

Metabolism following administration

Only one study analysing androgen profiles after testosterone administration to sheep was found. Yamamoto et al. (1978) reported the effect of infusing a ram with radiolabelled testosterone and 4-androstenedione and a second ram with radiolabelled epitestosterone and testosterone on resulting androgen profiles in bile and urine. Between 80 and 90% of the doses were excreted as either glucuronic acid or sulphate conjugates. Urine contained mainly glucuronic acid conjugates, while bile mainly sulphate conjugates. Epitestosterone, along with some unidentified polar compounds, was the major metabolite in urine and bile, with androsterone, etiocholanolone, 5β-androstane-3α,17β-diol and two other androstanediols also identified. The study was not quantitative and metabolites were identified on the basis of their co-crystallization with standards using thin-layer chromatography (TLC).

19-Nor and 1-dehydro steroids in the ovine

Very few reports on the occurrence of 19-nor-androgens in the ovine were found, and none of these concerned steroid administrations, just endogenous concentrations. Vandenbroecke et al. (1991) reported that the urine of eleven rams/lambs and ten pregnant/non-pregnant ewes were positive for nandrolone around the concentrations of $2.5\,ng\,ml^{-1}$ when analysed by RIA, but that the samples could not be confirmed positive by GC-MS (the LOD was not reported).

Van Hende (1995) analysed the urine of four ewes at different stages of pregnancy and the amniotic fluid of one ewe for the presence of epinandrolone. Animals were sampled between 43 and zero days before parturition, some at multiple times and others only at one time point. Using semi-quantitative GC-MS analysis, the amniotic fluid did not contain epinandrolone above the LOD of $1\,ng\,ml^{-1}$. The urine of the four pregnant animals was found to contain epinandrolone at concentrations ranging from below the LOD to above $2\,ng\,ml^{-1}$. There was no clear correlation between the stage of gestation and the concentration of epinandrolone determined. The ages, parity and foetal number of the animals were not reported.

Clouet et al. (1997) reported the analysis of urine from 30 pregnant and non-pregnant French Vendenne 4-year-old ewes for the presence of nandrolone and epinandrolone using GC-MS with an LOD of $0.2\,ng\,ml^{-1}$. Nandrolone was not detected in the pregnant ewe's urine and neither analyte was detected in the non-pregnant ewe's urine. However, epinandrolone was detected at somewhere between the LOD and $0.5\,ng\,ml^{-1}$ (insufficient experimental details available to allow the quantitative significance of this statement to be determined) in pregnant animals at 120 to 39 days before parturition, with concentrations then increasing to $3.4\,ng\,ml^{-1}$ at 7 days before parturition. There was no correlation between the sex or number of foetuses with the epinandrolone concentrations found.

The above results contrast with those of Sterk et al. (1998) who could not detect nandrolone or epinandrolone in the urine of five Flevolander ewes during early or late stages of pregnancy using a GC-MS method with an LOD of $0.5\,ng\,ml^{-1}$. The ages, parity and foetal number of the animals were not reported.

Casson et al. (2006) reported on the use of LC-MS to assess whether nandrolone and epinandrolone were natural in a population of 130 male and female sheep in the UK. Although the population could not be guaranteed 'clean', the authors report that no evidence of steroids abuse was found on any of the farms tested. The background to this research was an observation that the incidence of nandrolone positives rose once an LC-MS screening method with an LOD of $0.5\,ng\,ml^{-1}$ replaced an ELISA method with an LOD of $2\,ng\,ml^{-1}$ in 2004. The authors commented the following:

> Nortestosterone seems endemic in British sheep; primarily as the 17α-isomer, but also with some 17β-present. There does not seem to be much correlation with age or sex of the animal, although the majority of the population tested was 6–12 months and some of the other categories contained very few samples (e.g. all 5 males of over 12-months contained the 17α-isomer at $0.4\,ng\,ml^{-1}$ or greater). In light of this, it seems unwise to extrapolate the '17α- in male animals indicates abuse' rules from cattle to sheep. An exercise to test the urine of a controlled population will validate these conclusions, and demonstrate any link to other physiological factors such as breed or feedings regime.

Testosterone and related androgens in the porcine

Data on the endogenous presence of androgenic–anabolic steroids in the porcine are summarized in Table 3 while details of the major phase 1 metabolic products following exogenous administration are given in Table 4.

Endogenous occurrence

From the current review, ranges of mean plasma/serum concentrations of testosterone were found to be between seven- and 500-fold greater than those observed in barrows (castrated males), while those in gilts/sows were around five-fold lower than those in barrows. There were insufficient data to compare the concentrations of any other steroids by sex as there were few reports of steroid concentrations in barrows and gilts/sows.

The most extensive study of steroid concentrations with age in boar plasma (Yorkshire breed) was carried out by Schwarzenberger et al. (1993). Before detailing the results, it is important to point out that the concentrations reported seem to be much higher than those reported by other authors. The authors commented that steroids were almost exclusively present in plasma as sulphate conjugates, so unless indicated, the results below are for sulphated steroids. 4-Androstenedione and DHEA sulphate were measured directly, whereas other steroids were measured after extraction and solvolysis of separated fractions using acidified ethyl acetate (a standard approach used to cleave predominantly the sulphate conjugates; James Scarth, personal observation), thus inferring, but not proving, the status of most conjugates. For testosterone, concentrations after birth were around $1\,ng\,ml^{-1}$ for both free and sulphate conjugates, rising to around $3\,ng\,ml^{-1}$ for each at 1 month. Concentrations of each then dropped back to around $1\,ng\,ml^{-1}$ at 5 months where they then increased again, in the case of free testosterone to around $3\,ng\,ml^{-1}$ at 6 months and in the case of testosterone sulphate to around $6\,ng\,ml^{-1}$ at 7 months. Concentrations of free and sulphated testosterone seemed to dip slightly after 7 months. For free 4-androstenedione, the pattern was similar to testosterone such that concentrations after birth were around $6\,ng\,ml^{-1}$, increasing to around $24\,ng\,ml^{-1}$ at 1 month, then dropping to around $6\,ng\,ml^{-1}$ until 5 months. Concentrations then increased again to around $18\,ng\,ml^{-1}$ at 6 months and then dipped to around $9\,ng\,ml^{-1}$ at 8 months. For DHEA sulphate (the authors report sulphate as $<10\%$ was present as free), the pattern was again similar to testosterone such that concentrations after birth were around $3\,ng\,ml^{-1}$, increasing to around $80\,ng\,ml^{-1}$ at 1 month, then dropping to around $15\,ng\,ml^{-1}$ at 5 months. Concentrations then increased to around $80\,ng\,ml^{-1}$ at 6 months and then dropped to around $60\,ng\,ml^{-1}$ at 8 months. For 5α-androstane-3β,17β-diol sulphate (the authors report sulphate as $<10\%$ was present as free), the pattern was again similar to testosterone such that concentrations after birth were around $3\,ng\,ml^{-1}$, increasing to around $12\,ng\,ml^{-1}$ at 1 month, then dropping to around $3–6\,ng\,ml^{-1}$ at 5 months. Concentrations then increased to around $35\,ng\,ml^{-1}$ at 7 months. There was then only a very slight drop between 7 and 8 months. Therefore, overall, sulphate conjugates were (or were inferred to be) the most predominant

steroids measured in plasma (with the exception of 4-androstenedione, which was measured as free only). Concentrations of most steroids peaked first at 1 month after birth and then dropped until around 5 months, before increasing again at 6–8 months (with some dropping slightly after 7 months). The rank order of absolute determined concentrations in plasma was DHEA sulphate > 5α-androstane-3β,17β-diol sulphate > 4-androstenedione > testosterone sulphate.

McCoard et al. (2003) studied the concentrations of plasma testosterone in Meishan and White Composite foetal and neonatal boars. Foetal testosterone concentrations were not significantly different ($p < 0.05$) in Meishan and White Composite boars, with values ranging from around 1 to 2 ng ml^{-1}. However, testosterone concentrations in both breeds increased neonatally, peaking at 14 days post-partum with concentrations of around 5.5 and 7 ng ml^{-1} in Meishan and White Composites, respectively (significantly higher in White Composites at $p < 0.05$). Concentrations then dropped to around 4 ng ml^{-1} in both breeds by 25 days post-partum. Park and Yi (2002) reported serum testosterone concentrations in Duroc versus Yorkshire boars living in the northern hemisphere country South Korea during spring, summer, autumn and winter. Mean testosterone concentrations in Duroc boars during spring, summer, autumn and winter were 3.1, 0.73, 1.3 and 1.4 ng ml^{-1}, respectively, while in Yorkshire boars were 5.1, 2.6, 2.5 and 2.6 ng ml^{-1}, respectively. The higher testosterone concentrations in Spring occur at a time when photoperiod is increasing in this country, but peak photoperiod (during the summer) does not correlate with peak testosterone concentrations. The testosterone concentrations in Yorkshire boars were significantly higher ($p < 0.05$) than for Duroc boars at all stages of the year. Testosterone concentrations were higher in all breeds in spring compared with the rest of year ($p < 0.05$). Walker et al. (2004) measured plasma testosterone concentrations in Duroc boars that had or had not been subject to selection for high testosterone concentrations over ten generations. Mean testosterone concentrations in boars selected over ten generations were 49 ng ml^{-1}, which were significantly higher ($p < 0.01$) than controls with a mean testosterone concentration of 28 ng ml^{-1}.

No reports of the effect of pregnancy, diet, housing conditions or geographical factors on testosterone concentrations were found.

Metabolism following administration

No studies assessing the metabolism of testosterone following administration to porcine animals were found.

19-Nor and 1-dehydro steroids in the porcine

Nandrolone

Poelmans et al. (2005) reported the results of the most comprehensive analysis of nandrolone and boldenone related compounds in the porcine to date. Samples of muscle, liver, kidney, testicles and urine from boars, cryptorchids, barrows, gilts and sows derived from France, the Netherlands, Belgium and the USA were analysed for boldenone, nandrolone and

Table 2. Concentrations of nandrolone, 19-nor-4-androstenedione and boldenone quantified by Poelmans et al. (2005b) in different matrices from different sex porcine animals.

	Gilt	Sow	Boar	Barrow	Cryptorchid
Nandrolone (ng g^{-1})					
Meat	< (11)	0.4–0.5 (11)	0.7–13.4 (11)	0.7–11.8 (11)	0.1–2.4 (11)
Liver	0.1–0.9 (11)	< (11)	1–63 (11)	< (11)	0.2–12.3 (14)
Kidney	0.2–0.5 (11)	0.2–1.5 (11)	2.5–232 (11)	0.1 (10)	1.3–78 (14)
Urine (ng ml^{-1})	1.3–2.8 (11)	1.3–1.9 (9)	51–344 (11)	0.5–16.3 (11)	8.6–343 (14)
Testes	–	–	24–144 (5)	–	2.2–101 (11)
19-Nor-4-androstenedione (ng g^{-1})					
Meat	< (11)	0.04–0.07 (11)	0.1–5.5 (11)	0.05–0.8 (11)	0.04–0.4 (11)
Liver	0.3–1.3 (11)	3.1–8.3 (11)	0.1–24 (11)	1.9–16 (11)	0.4–3.5 (14)
Kidney	8.3–25 (11)	2.7–18 (11)	2.3–535 (11)	0.1–15 (10)	0.2–159 (14)
Urine (ng ml^{-1})	1.8–17 (11)	0.9–18 (8)	5–109 (11)	1.1–16 (11)	9.9–103 (14)
Testes	–	–	6.2–110 (5)	–	1.3–25 (11)
Boldenone (ng g^{-1})					
Meat	<(11)	<(11)	0.5–2.5 (11)	<(11)	0.7 (11)
Liver	<(11)	<(11)	1.3–4.9 (11)	<(11)	0.5–2.3 (11)
Kidney	<(11)	<(11)	0.8–9.2 (11)	<(10)	0.3–8.1 (14)
Urine (ng ml^{-1})	0.5–0.6 (11)	<(11)	5.1–120.5 (11)	1.1 (11)	0.9–57.6 (14)
Testes	–	–	2.1–16 (5)	–	0.6–15.1 (11)

Notes: The number of animals is given in parentheses. <, Concentrations below the limit of detection (LOD).

19-nor-4-androstenedione using GC-MS and LC-MS (with $n =$ between 5 and 14 for the different sexes). The results from an inter-sex animal were also reported, but the 19-norandrogen results for this animal appear to be the same as those given in Van Cruchten et al. (2002), so will not be reproduced again here. The results of this study are summarized in Table 2. Limits of detection were not reported. Since barrows are castrated males, the major source of steroid production in this animal is likely to be the adrenal gland. While it is theoretically possible that androgens produced by the adrenal gland in the barrow may be converted into 19-nor-androgens and/or their precursors in the systemic circulation, i.e. in adipose tissue, a study by Miyashita et al. (1990) suggests that these compounds may be directly secreted from the adrenal gland.

Van Cruchten et al. (2002) reported the presence of 19-nor-androgens in what initially appeared to be a female pig. However, close inspection determined that the animal was in fact as a hermaphrodite (inter-sex animal) due to the presence of both a left ovary and a right undescended testicle (with functioning Leydig cells, but no spermatozoa). Samples of urine, fat, faeces, liver, kidney, muscle and testes were analysed for nandrolone and 19-nor-4-androstenedione using GC-MS for fat, urine and faeces and LC-MS for the remaining matrices. Concentrations of nandrolone in fat, urine, faeces, liver, kidney, muscle and testes were 0.3 ng ml^{-1}, 27 ng ml^{-1}, not detectable, not detectable, 1.6 ng ml^{-1}, not detectable and 5.3 ng ml^{-1}, respectively, while concentrations of 19-nor-4-androstenedione in these matrices were not detectable, 0.5 ng ml^{-1}, not detectable, not detectable, 1.1 ng ml^{-1}, not detectable and 0.9 ng ml^{-1}, respectively. The authors proposed visual inspection of pig external sexual organs in female pigs suspected of nandrolone abuse at slaughter in order to discern false positives due to inter-sex.

Debruyckere and Van Peteghem (1991) presented the results of a metabolism study of nandrolone in Gottinger × Vietnamese mini-pigs before and after injection of nandrolone laurate. One boar, one barrow and one sow (all 2 years old) were used and GC-MS was employed in a qualitative fashion to identify the compound present in urine. Nandrolone was detected in boar urine, but not barrow or sow urine, before nandrolone administration and in all animals after administration. No 17-keto, A-ring reduced compounds were detected in any of the animals before nandrolone administration, but after administration 19-noretiocholanolone and 19-norepiandrosterone were detected in all animals. 19-Norandrosterone was detected in barrows after nandrolone administration, but not in the boar or sow. 19-Norepietiocholanolone was not detected after nandrolone administration in any animal. No oestranediols were detected before nandrolone administration in any animal. Neither 5α-estrane-3α,17β-diol or 5α-estrane-3β,17α-diol were detected after nandrolone administration, but two other oestranediols, proposed to be 5β-estrane-3α,17β-diol and 5α-estrane-3β,17β-diol, were found in some of the animals.

More recently, Roig et al. (2007) reported the results of a quantitative metabolism study following IM nandrolone laurate injection to a 1-week-old boar. Only post-administration urine results were reported. Following the administration, the boar urine contained nandrolone in predominantly the sulphate fraction (the most abundant analyte in this fraction) with peak concentrations of around 80 ng ml^{-1} at day 1 dropping to around 10 ng ml^{-1} by day 4. Peak concentrations of different analytes in the glucuronic acid conjugate fraction were generally around 20 ng ml^{-1} or lower while in the free fraction, norepiandrosterone, noretiocholanolone and 5β-estrane-3α,17β-diol were most abundant with peak concentrations of around 40, 60 and 100 ng ml^{-1}, respectively.

A major consideration regarding 19-nor-androgens in the porcine is whether or not these compounds are excreted as 19-nor compounds or whether they are, in fact, artefacts of sample preparation produced from an initial 19-carboxy metabolite, analogous to what is experienced in the male horse (Houghton et al. 2007). Several lines of evidence support this hypothesis, including the above detailed observation that no A-ring reduced metabolites are found in untreated boar urine (Debruyckere and Van Peteghem 1991). These A-ring reduced metabolites would be expected to occur as metabolic products if free nandrolone and/or 19-nor-4-androstenedione were present *in-vivo*. Their absence suggests that nandrolone and/or 19-nor-4-androstenedione are naturally present as carboxylic acid precursors because these would be unlikely to undergo A-ring metabolism, but could be cleaved to form nandrolone and 19-nor-4-androstenedione during sample preparation procedures that involve a derivatization step or even a minor downward pH change. The 19-carboxylic metabolite of 4-androstenedione has also previously been identified in porcine granulosa cells (Garrett et al. 1991). Lastly, an unusually high ratio of 19-nor-androgens in boar urine, relative to faeces, has been observed (H. De Brabander, unpublished observation). This adds further weight to the theory of 19-nor-androgens in the porcine actually being the 19-carboxylic acid, as these acidic compounds could be substrates for organic anion transport proteins in the liver/gut that actively transport compounds from one area to another and maintain large concentration gradients (something that is less likely to occur for neutral steroids). If the compounds in porcine tissues that are currently thought to be 19-nor-androgens are proven to be predominantly

19-carboxylic acids, similar to the situation in the equine, then this could have major implications for nandrolone residue screening methods in the porcine. For example, Roig et al. (2007) showed that the major metabolites of nandrolone after exogenous administration to the boar are excreted in the sulphate and glucuronic acid conjugate fraction, while Debruyckere and Van Peteghem (1991) showed that A-ring reduced metabolites are only detectable after nandrolone administration. It may therefore be feasible to use a threshold of nandrolone sulphate or an A-ring reduced metabolite as an indicator of nandrolone abuse in this species.

Boldenone

Only one published study relating to the occurrence of boldenone in the porcine was found. In a study on boars, barrows, gilts and sows, Poelmans et al. (2005) reported the concentrations of endogenous boldenone and 19-nor-androgens in muscle, liver, kidney, testicles and urine using GC-MS and LC-MS methods. Calibration ranges and LODs were not reported, but the resulting concentration ranges in the different tissues were reported. The results from this study are presented in Table 2 along with the nandrolone data discussed above. In addition to the data in Table 2, the authors looked at levels of boldenone in an intersex animal, with muscle, liver, kidney, urine and testicles found to contain no boldenone above the LOD. More studies to determine the endogenous occurrence of boldenone in the porcine are clearly warranted.

Natural androgenic–anabolic steroid concentrations in the equine

Data on the endogenous presence of androgenic–anabolic steroids in the equine are summarized in Table 3, while details of the major phase 1 metabolic products following exogenous administration are given in Table 4.

Testosterone

Concentrations of testosterone in equine matrices were not reviewed as part of the current survey, but testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations. A large amount of data is available on the detection of steroid abuse in horseracing, the results of which are relevant to residue testing in meat production because the strategies for detection apply equally. The approach to controlling testosterone abuse in horseracing varies depending upon the sex and gestation status of the animal (James Scarth, personal experience with the application of these assays). There is no threshold in the intact male due to the high and variable concentrations produced in this animal. In the gelding and filly/mare, population studies have allowed urinary threshold concentrations for testosterone confirmation to be set at 20 and 55 ng ml^{-1}, respectively, in order to control the abuse of this steroid. A maximum urinary ratio threshold of testosterone:epitestosterone of 12:1 was in the past adopted in the filly/mare (analogous to the 4:1 ratio for either sex in human urine), but the absolute threshold of 55 ng ml^{-1} is now considered more robust (personal experience of authors J. Scarth and P. Teale). The major phase 1 urinary metabolites of testosterone after exogenous administration have been reported as 5α-androstane-3β,17β-diol, 5α-androstane-3β,17α-diol and 3β-hydroxy-5α-androstan-17-one (Dumasia and Houghton 1981).

Nandrolone

Early nandrolone administration studies on castrated males (geldings) in the 1970s and early 1980s suggested that 19-nor-androgens did not occur naturally in the horse (Houghton 1977; Houghton et al. 1978; Houghton and Dumasia 1980; Houghton and Teale 1981; Dumasia and Houghton 1984). Indeed, since 19-nor-androgens have not been shown to be endogenous in the gelding or filly/mare, their presence at any concentration is considered evidence of abuse in horseracing (at least with current detection capabilities). Roig et al. (2007) have recently confirmed the findings of some of the earlier studies on nandrolone metabolism after exogenous administration to geldings. While pre-administration samples contained no 19-nor-androgens, the major metabolites, post-administration, were 5α-estrane-3β,17α-diol in the glucuronic acid conjugate fraction followed by nandrolone in the sulphate fraction. However, Houghton et al. (1984) showed that the urine of untreated intact male horses appeared to contain 19-nor androgens. A threshold approach utilizing a ratio of estranediol:estrenediol of greater than 1:1 as indicative of abuse was introduced for intact male horses (Houghton et al. 1984) and is still in use as a confirmatory method in horseracing today (although work is underway to replace this with a threshold for the nandrolone metabolite 5α-estrane-3β,17α-diol; E. Houghton, personal communication). More recently, Sterk et al. (1998) have shown that pregnant mares may excrete epinandrolone naturally in urine at concentrations ranging from less than their LOD of 1 ng ml^{-1} up to 26 ng ml^{-1}, highlighting the need to consider the gestation status in mares. Nandrolone was not detected at any concentration above the LOD of 1 ng ml^{-1}.

Although intact male horse urine has for many years been considered to contain 19-nor-androgen sulphates, Houghton et al. (2007) have recently

Table 3. Summary of the endogenous occurrence of androgenic–anabolic steroids in mammalian meat-producing animal species.

Steroid	Details of endogenous presence in different species					
	Bovine	Ovine	Porcine	Equine	Cervine	Caprine
Testosterone	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations
Nandrolone and related 19-nor androgens	Epinandrolone detectable during pregnancy. Most other studies find no nandrolone or related metabolites in males, but some find trace amounts in male and female urine, i.e. following casualty (G. Kennedy, personal communication)	Epinandrolone detectable during pregnancy. One report of nandrolone and epinandrolone in both male and female urine in the UK, but population not controlled	Nandrolone and 19-nordrostenedione detected in urine and some other matrices of animals of all sexes (including inter-sex animals) at different concentrations	Detected in urine of pregnant mares and at high concentrations in stallions (probably as a by-product of the high concentration of aromatization in the testes) but not in geldings or fillies	Urinary epinandrolone detected in a pregnant red deer, but no other animals studied. One of 35 urines from an Australian NMP contained epinandrolone but not nandrolone	Epinandrolone, but not nandrolone, detected in urine during pregnancy, while neither analyte detected in non-pregnant females. Studies on endogenous concentrations in males is lacking
Boldenone and related 1-dehydro androgens	Boldenone and related compounds have been detected in urine and faeces, possibly secondary to their formation by gut bacteria. Some phase 1 plus 2 metabolites only detected after boldenone administration	Insufficient data to draw any conclusions, although two of 961 urines from an Australian NMP contained low concentrations of epiboldenone	Boldenone detected in urine and some other matrices of boars, cryptorchids, gilts and barrows at different concentrations, but not above the LOD in sows or an inter-sex animal	Boldenone detected at low concentrations in the urine of stallions, but not geldings or fillies	Insufficient data to draw any conclusions, although zero of 35 urines from an Australian NMP contained boldenone or epiboldenone	Insufficient data to draw any conclusions

Note: NMP, National Monitoring Programme, therefore not controlled with respect to steroid abuse.

Table 4. Summary of the major catabolic pathways of endogenous androgenic–anabolic steroids in mammalian meat-producing animal species.

	Details of major phase 1 urinary metabolic products after administration to different species					
Steroid	Bovine	Ovine	Porcine	Equine	Cervine	Caprine
Testosterone	Epitestosterone, epietiocholanolone, 5β-androstane-3α,17β-diol plus 5β-androstane-3β,17α-diol	Epitestosterone, androsterone, etiocholanolone plus 5β-androstane-3α,17β-diol*	Insufficient data to draw any conclusions	5α-Androstane-3β,17β-diol, 5α-androstane-3β,17α-diol plus 3β-hydroxy-5α-androstan-17-one	Insufficient data to draw any conclusions	Insufficient data to draw any conclusions
Nandrolone	Epinandrolone, 5β-estrane-3α,17β-diol, 5β-estrane-3α,17α-diol, 5α-estrane-3β,17α-diol	Insufficient data to draw any conclusions	19-Norepiandrosterone, 19-noretiocholanolone plus 5β-estrane-3α,17β-diol	5α-Estrane-3β,17α-diol	Insufficient data to draw any conclusions	Insufficient data to draw any conclusions
Boldenone	Epiboldenone, 17α-hydroxy-5β-androst-1-en-3-one, 6β-hydroxyepiboldenone, 17β-hydroxy-5β-androst-1-en-3-one plus 3α-hydroxy-5β-androst-1-en-17-one	Insufficient data to draw any conclusions	Insufficient data to draw any conclusions	Epiboldenone	Insufficient data to draw any conclusions	Insufficient data to draw any conclusions

Note: *More definitive quantitative studies required in order to confirm results.

shown that these compounds are predominantly artefactual in origin. The authors showed that it is 19-carboxy-nandrolone and 19-carboxy-4-androstenedione that occur naturally in intact male horse urine and that their decarboxylation under acidic sample preparation procedures leads, respectively, to the artefactual production of nandrolone and 19-nor4-androstenedione. The finding that nandrolone and 19-norandostenedione are in fact de-carboxylation artefacts of sample preparation is significant as nandrolone administration studies in horses have confirmed that, exogenously, nandrolone *is* excreted as a sulphate (Teale et al. 2000). Houghton et al. (2007) suggested that a threshold for nandrolone sulphate in the horse may be an effective way of discerning nandrolone abuse from the endogenous situation and a paper reporting a study of its levels in normal animals is currently in preparation (Ed Houghton, personal communication). This study also raises the possibility that 19-nor androgens found in other species, i.e. the porcine, may also be artefactual in origin.

Boldenone

Boldenone has recently been shown to be naturally occurring as a sulphate conjugate in the urine of intact males (Ho et al. 2004), but has to date not been reported to be endogenous in geldings or fillies/mares. The major urinary metabolite of boldenone after exogenous administration is epiboldenone (Houghton and Dumasia 1979).

Natural androgenic–anabolic steroid concentrations in the cervine

Data on the endogenous presence of androgenic–anabolic steroids in the cervine are summarized in Table 3. Data on the major phase 1 metabolic products following exogenous administration were not available.

Concentrations of testosterone in cervine matrices were not reviewed as part of the current survey, but testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations (Hamasaki et al. 2000). Only one report on the natural occurrence of 19-nor-androgens in the cervine was found. Van Hende (1995) analysed the urine of pregnant red deer for the presence of epinandrolone. Using semi-quantitative GC-MS analysis, epinandrolone was not detected at an LOD of $1\,ng\,ml^{-1}$ in two of the animals and was somewhere between 1 and $2\,ng\,ml^{-1}$ in the third animal. The ages, parity, foetal number and gestation status of the animals was unknown. No reports on the endogenous or post-administration levels of boldenone or related compounds in the cervine were found, but one of 35 cervine urine samples analysed for nandrolone and epinandrolone in an Australian national monitoring programme (not therefore a controlled population with respect to ruling out steroid abuse) was above the $1\,ng\,ml^{-1}$ LOD for epinandrolone with a concentration of $4.5\,ng\,ml^{-1}$. No samples were above the $1\,ng\,ml^{-1}$ LOD for nandrolone. Clearly, more work is required to establish the endogenous versus exogenous nature of 19-nor and 1-dehydro-androgens in deer of different ages, sexes, gestation/castration status and breeds, etc.

Natural androgenic–anabolic steroid concentrations in the caprine

Data on the endogenous presence of androgenic–anabolic steroids in the caprine are summarized in Table 3. Data on the major phase 1 metabolic products following exogenous administration were not available.

Concentrations of testosterone in caprine matrices were not reviewed as part of the current survey, but testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations (Flint and Burrow 1979; Ahmad et al. 1996). Only two reports on the occurrence of 19-nor-androgens in the caprine were found and both relate to female urine. Van Hende (1995) analysed the urine from two pregnant animals and a pool of urine from a number of pregnant animals for the presence of epinandrolone. Using semi-quantitative GC-MS analysis, the two individual samples were found to contain epinandrolone at a concentration above $2\,ng\,ml^{-1}$, while the pooled urine contained concentrations somewhere between 1 and $2\,ng\,ml^{-1}$. The ages, parity, foetal number and gestation status of the animals was unknown. Sterk et al. (1998) analysed the urine from two pregnant animals throughout gestation and four non-pregnant animals for the presence of both nandrolone and epinandrolone using GC-MS (LOD of $1\,g\,ml^{-1}$ for each). Nandrolone was not detected in any of the samples while epinandrolone was only detected in one sample from one animal at 16 weeks into pregnancy with a concentration of $2\,ng\,ml^{-1}$. The ages and parity of the animals was not reported. No reports on the endogenous or post-administration concentrations of boldenone or related compounds in the caprine were found. Clearly, more work is required in order to establish the endogenous versus exogenous nature of 19-nor and 1-dehydro-androgens in goats of different ages, sexes, gestation/castration status and breeds, etc.

Summary tables of the occurrence and metabolism of endogenous steroids in meat-producing animals

Data on the endogenous presence of androgenic–anabolic steroids in the species currently reviewed are summarized in Table 3 while details of the major phase

1 metabolic products following the exogenous administration of testosterone, nandrolone and boldenone are given in Table 4.

Multivariate analysis of steroid profiles

Up to this point, the discussions within this review have concentrated primarily on univariate measurements of steroid concentrations and their resulting implications for control of abuse. However, another technique that may be of use in differentiating natural from abusive situations relies on multivariate analysis of steroid profiles. This involves generating data using multiresidue methods and then analysing the data by such techniques as principal component analysis (PCA) or neural networking. Indeed, a small number of multivariate approaches have already been published, both with respect to steroid concentrations (Norli et al. 1995) and the carbon isotope composition of a particular steroid (Aguilera et al. 1996) (the topic of which is taken up further in the next section). Whether or not multivariate approaches are suitable as a confirmatory technique or if instead they are more suitable as screening tools to detect steroid abuse remains to be determined, but further studies are clearly warranted in order to investigate their potential.

Combustion isotope ratio mass spectrometry studies

Results of gas chromatography combustion isotope ratio mass spectrometry studies (GC-C-IRMS) are considered separately from those quantifying concentrations in bovine tissues because GC-C-IRMS measures the relative composition of ^{12}C and ^{13}C atoms of steroids rather than their absolute concentrations. However, since the aim of using GC-C-IRMS is to be able to distinguish natural from exogenous steroids, a consideration of the results of studies using this technique is relevant to the current review.

The majority of published studies relate to the bovine, but one study looked at porcine tissues. The following overview of the potential use of this technique was based on results of published studies as well as an executive summary from the European Union supported ISOSTER project GRD1-2001-40085 (which coordinated several of the published studies; ISOSTER 2006).

The technique of GC-C-IRMS relies on detecting differences in the relative ^{12}C and ^{13}C composition of steroids between the natural and synthetic states. Synthetic steroids are typically synthesized from a single C3 plant (often soy), while the natural diet of the bovine is usually a mixture of both C3 and C4 plants (Balizs et al. 2005). The terms C3 and C4 refer to the type of metabolic pathway used by the plant in synthesizing organic compounds during photosynthesis, utilizing either 3 or 4 carbon-chain metabolites, respectively. The significance of this lies in the fact that the two types of pathway display differing degrees of discrimination against ^{13}C and thus result in different ^{13}C to ^{12}C ratios. C4 plants have lower discrimination against ^{13}C than C3 plants, resulting in higher ^{13}C to ^{12}C ratios in C4 plant material (Balizs et al. 2005). Since steroids produced within the body will derive carbon from both C3 and C4 plant material of dietary sources, the resulting ^{13}C to ^{12}C ratio will be lower after exogenous steroid administration (mainly C3 plant material derived) relative to the endogenous state.

The method itself requires substantial sample preparation before analysis including hydrolysis, solid-phase extraction, liquid–liquid extraction and HPLC fractionation steps, as the influence of matrix interferences need to be minimized. Suitable derivatization of the extracts followed by separation using gas chromatography further purifies the extract before introduction into a furnace. The furnace then combusts the introduced sample, which is then analysed alongside a reference gas by mass spectrometry in order to determine the relative levels of ^{13}C and ^{12}C (Preä vost et al. 2004).

The resulting ^{13}C to ^{12}C ratio determined by GC-C-IRMS is usually expressed as a $\delta^{13}C$ value, calculated as follows:

$$\delta^{13}C = \left[\frac{(^{13}C :^{12}C)_{sample} - (^{13}C :^{12}C)_{reference}}{(^{13}C :^{12}C)_{reference}} \right] \times 1000‰$$

The range of $\delta^{13}C$ values for C3 plants (hence also abused steroids) is around -25 to $-35‰$, whereas for C4 plants is around -11.9 to $-15.2‰$ (Mason et al. 1998). With diets usually containing both C3 and C4 plants, the range of $\delta^{13}C$ values for endogenous steroids is usually somewhere between these two ranges. However, as will become apparent later in this section, the exact location on this continuum will depend on the type of diet fed to the animal (Buisson et al. 2005). The $\delta^{13}C$ values of the steroid or metabolite being measured is usually referenced to an endogenous reference compound (ERC) such as DHEA that is not affected by exogenous steroid administration (Ferchaud et al. 1998). This is to ensure that the endogenous make up of the animal has not been affected in some way (i.e., diet) so as to give low $\delta^{13}C$ values for all steroids. A high relative difference between the $\delta^{13}C$ values of the steroid or metabolite and the ERC can therefore be used as an indicator of abuse. The absolute sensitivity of the method depends to some extent on the analyte and matrix in question, but 10 ng is typically required, meaning that only some analyte matrix combinations are currently suitable (ISOSTER project [GRD1-2001-

40085]; for a publishable executive summary, see ISOSTER 2006; also, personal observation of Bruno Le Bizec).

As mentioned elsewhere in this review, diet is a major factor influencing the $\delta^{13}C$ value of endogenous steroids. In the UK for example, animals are typically fed a much higher base of C3 plants than in Europe, leading to a lower difference in the $\delta^{13}C$ values between administered steroids and the ERC (Mason et al. 1998). However, as studies by Buisson et al. (2005) and Hebestreit et al. (2006) have shown, the difference after steroid administration to animals with a C3 diet is still usually sufficient to discern testosterone abuse from the natural situation. Hebestreit et al. (2006) showed that after testosterone administration, the difference in $\delta^{13}C$ between the testosterone metabolite etiocholanolone and the ERC DHEA for C4 plant fed animals (maize) was typically around 10‰, whereas the difference for C3 fed animals (grasses) was typically around 4‰. In the endogenous state, the difference in $\delta^{13}C$ between the testosterone metabolite etiocholanolone and the ERC DHEA was a mean of 0.9‰ with a standard deviation of 0.7‰. Applying a confidence interval of 3 standard deviations to either side of the mean, endogenous $\delta^{13}C$ difference between etiocholanolone and the ERC DHEA allowed a 100% discrimination of samples as either positive or negative.

The ISOSTER project successfully validated the use of this technique for detecting testosterone abuse in urine via monitoring of etiocholanolone values in relation to DHEA as the ERC. The method was also applied to steroids in other tissues, but in most cases the steroid or ERC contents were too low to be of use in the technique. In addition to testosterone, Buisson et al. (2005) showed that detection of oestradiol abuse in the bovine was possible via monitoring the $\delta^{13}C$ value of the urinary oestradiol metabolite epioestradiol (17α-estradiol) relative to DHEA. GC-C-IRMS has also been used to analyse porcine nandrolone and 19-nor-4-androstenedione concentrations in testicles, liver and kidney, although post administration samples were not included (Prévost et al. 2004).

One question that still remains to be answered (several researchers, personal communications) is what the final statistical approach to determining the threshold for $\delta^{13}C$ value differences between target steroids and the ERC will be. If the use of an ERC is adopted as standard, then it is theoretically possible that some positive samples would be called negative due to a high proportion of C3 plant in their diet, but the converse situation of calling false positives is less likely than using the technique without an ERC.

The laborious nature of the sample preparation technique currently makes the technique unsuitable for use as a screening tool (various researchers, personal communications), but it has already found use as a confirmatory technique for confirming testosterone abuse in human sports (Saudan et al. 2006). The executive summary of the ISOSTER project GRD1-2001-40085 (ISOSTER 2006) showed that the GC-C-IRMS method for detecting testosterone abuse in bovine urine was successfully validated in several European laboratories. It is therefore recommended that the use of this technique be further explored in order to act as a confirmatory method following screening using a uni- or multivariate threshold of some kind.

Detection of intact steroid esters in hair

The majority of injectable steroid preparations contain steroids in esterified forms. The direct detection of steroid esters in matrices from an animal may therefore be indicative of steroid abuse. The only exception to this would be if the ester is also known to occur naturally. For example, oestradiol and testosterone have been shown to be present in enzymatically hydrolysed fractions, but not unhydrolysed fractions, of human high density lipoprotein and rat adipose tissue, respectively (Borg et al. 1995; Hockerstedt et al. 2006). While the exact nature of steroid esters were not unequivocally identified in either of these studies, the oestradiol related compound identified by Hockerstedt et al. (2006) co-crystallized with an oestradiol stearate reference standard. However, in all studies that have looked at the detection of steroid esters in animal tissues following administration of commercially available steroid ester preparations, no endogenous concentrations of the esters were detected in the pre-dose or untreated samples (for example, Marcos et al. 2004; Nielen et al. 2006; Boyer et al. 2007). Depending on the matrix in question, contamination issues also need to be considered carefully in the context of the housing history of the individual animals. The detection of intact steroid esters in hair has attracted the most attention in the literature for this purpose and has already found use in some European labs as a confirmatory technique for detecting natural steroid abuse (Bruno Le Bizec, personal observation 2007). The detection of steroid esters in hair, however, has not been comprehensively reviewed in this survey as the scope was limited to naturally occurring steroids.

Summary and consideration of areas for future research

The occurrence of steroids related to testosterone, nandrolone and boldenone in bovine, ovine and porcine matrices, as well as the occurrence of steroids related to nandrolone and boldenone in equine, caprine and cervine matrices, have been reviewed.

Several specific questions have been raised during this review, including some unusually high steroid concentrations under specific physiological conditions and a relevant dearth of information regarding some steroids in different species (i.e., boldenone/nandrolone). Furthermore, continuously improving standards of analytical detection, as well as artificial selection within certain animal populations, give rise to a continual evolution of steroid concentrations and suggest constant re-evaluation of detection approaches is necessary. An ideal situation might therefore be analogous to human sports: the use of a uni/multivariate concentration threshold approach as a screening strategy (as are currently in use for some steroid/matrix/species combinations already) and the use of more laborious but definitive techniques such as carbon isotope ratio mass spectrometry for confirmation.

A major deficiency in much of the existing published literature is the lack of standardization and formal validation of experimental approach. Key articles are cited that highlight the huge variation in reported steroid concentrations that can result when samples are analysed by different laboratories under different conditions. These deficiencies are in most cases so fundamental that it is difficult to make reliable comparisons between data sets and hence it is currently impossible to recommend definitive detection strategies. Standardization of experimental approach would need, most importantly, to involve communication among researchers before experiments are conducted and methods cross-validated.

In order to standardize data aimed at producing thresholds for controlling steroid abuse, the following specific recommendations are made.

Physiologically based recommendations

- Adequate consideration should be given to the demographic of the animal population used for the study. Specifically, the population needs to be representative of that which control of abuse is aimed at, i.e. it must take into account sex, age, gestation and castration status, breed (genetic history), housing conditions, geographical region of origin, disease, medication, hydration and stress status, diet, housing, time of sampling (clock hour and sun hour) and season (and particularly the terminology surrounding season, i.e. the word 'September' may relate to a different photoperiod in one hemisphere compared with the other hemisphere so could lead to confusion when considering seasonal effects). When delineating the resulting data for the control of endogenous steroid abuse, it would seem necessary to separate animals based upon, at the very least, their age, sex, castration, gestation, disease (particularly casualty animals) and medication status otherwise the variation under these different conditions may lead to irrelevantly high thresholds for the control of steroid abuse. However, there would appear to be balance between a physiological need to separate animals and a statistical requirement for a large enough population on which to base threshold calculations.
- When considering sample collection strategies, it is very difficult to produce unilateral recommendations because different matrices are likely to be suitable in different locations, i.e. many laboratories will have access to urine or plasma, but export testing programmes may need to analyse meat. Factors that apply to all situations include the need to avoid contamination of one matrix with that from another, e.g. no faecal contamination of urine and the need for samples to be frozen as quickly as possible after collection in order to reduce degradation or the artefactual production of certain steroids. It may be necessary to consider data derived from live animals differently to dead animals, not necessarily because the physiology of a live animal would be different to a dead animal, but because 'on-farm' testing of live animals compared with 'slaughter-house' testing of dead animals may have implications for type of matrix used, the age of the animal, stress levels, etc. When sampling from dead animals, the time from slaughter to sample collection should be minimized.
- Care should be taken to distinguish between the usefulness of longitudinal and latitudinal studies. Latitudinal studies may be of most use when applying unilateral thresholds to whole populations, while longitudinal studies may be of more use in probing the individual variables that may need to be taken into account when designing the demographic of animal populations used in latitudinal studies.

Analytically based recommendations

- All methods should be subject to a full quantitative validation before use. As a minimum, the following factors should be covered and reported:
- Intra- and inter-batch precision and accuracy.

- Ability to dilute samples from outside the calibration range.
- Method selectivity.
- Method sensitivity (defining both the LOD and LOQ).
- Recovery.
- Linearity.
- Stability (of analyte solutions, extracted samples and samples stored in matrix under the appropriate conditions).
- Following validation of any new method, the laboratory should attempt, where possible, to cross-validate their method with those from other laboratories and participate in the inter-laboratory exchange of QC samples.
- Data should only be considered fully quantitative if a certified reference standard is available.
- Mass spectrometry (preferably MS/MS) should be the method of choice for definitive quantitative studies.
- Sample preparation procedures should be designed such that it can be stated whether data are derived from an analysis of the 'total' concentration of steroid, or the concentration from a particular fraction, i.e. glucuronic acid conjugates. When hydrolysing glucuronide steroid conjugates, recombinant versions of the glucuronidase enzymes should be used because heterogeneous preparations such as that from *Helix pomatia* are known to produce artefacts of certain steroids.
- Given the above quantitative validation requirements, consideration needs to be given as to whether calibration lines and QCs are best prepared in matrix, i.e. standard addition, or through alternative means, i.e. using isotope dilution or surrogate matrices. The most suitable option will depend on the steroid, matrix and species in question.
- Where possible, attempts should be made to monitor various precursors, metabolites and other compounds related to the steroids in question so that multivariate approaches based on the perturbation of 'normal' profiles can be investigated.
- Once published, the raw data from all studies should be made available to the European Union Community Reference Laboratory (CRL) so that independent statisticians are able to combine data from a number of studies and produce more workable unilateral thresholds. The current approach of reporting certain statistical parameters, but not others, makes the amalgamation of data sets difficult.

General recommendations

When publishing the results of studies, as much detail as possible regarding the aforementioned factors (including, critically, the analytical method validation results) should be reported so that comparisons can be made between different studies.

Future work

Although efforts are already underway (at HFL and LABERCA) to produce more definitive data and promote communication among the scientific community on this issue, the convening of a formal European Union working party is recommended.

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Appendix: Glossary

Barrow	castrated male pig
Boar	uncastrated (intact or entire) male pig
Bovine	of or relating to cattle
Bull	uncastrated (intact or entire) male cattle
Caprine	of or relating to goats
C18 androgens	androgenic–anabolic steroids with 18 carbons, meaning they have no 19-methyl group
CCα	Decision limit (see Council Decision 2002/657/EC)
CCβ	Detection capability (see Council Decision 2002/657/EC)
Cervine	of or relating to deer
Colt	uncastrated (intact or entire) young male horse (several age cut-off definitions in use)
Cow	female bovine that has produced a calf
CRL	European Community Reference Laboratory
DHEA	dehydroepiandrosterone

ELISA	enzyme-linked immunosorbent assay
Endogenous	present naturally in certain situations
Equine	of or relating to horses
EIA	enzyme immunoassay
ERC	Endogenous Reference Compound
Ewe	female sheep that has produced a lamb
Exoge	nousnot known to occur naturally in a particular situation
Filly	young female horse (several developmental cut-off definitions in use)
GC-C-IRMS	gas chromatography combustion isotope ratio mass spectrometry
GC-MS	gas chromatography-mass spectrometry
Gelding	castrated male horse
Gilt	young female pig (most often applied to those not having borne young)
Heifer	female bovine that has either not given birth or only had one calf
HPLC-UV	high-performance liquid chromatography with ultraviolet detection
IA	immunoassay
LC-MS^n	liquid chromatography-mass spectrometry (where *n* indicates the number of mass spectrometry stage, i.e. three for a triple quad instrument and zero, or not indicated at all, for a single quad instrument)
LOD	limit of detection
LOQ	limit of quantification
Mare	older female horse (several developmental cut-off definitions in use)
MRPL	minimum required performance limit (see Council Decision 2002/657/EC)
ND	not detected
NMP	National Monitoring Programme
Ovine	of or relating to sheep
Porcine	of or relating to pigs
Ram	uncastrated (intact or entire) male sheep
RIA	radio-immunoassay
Sow	female pig after her first litter
Stallion	uncastrated (intact or entire) older male horse (several developmental cut-off definitions in use)
Steer	castrated male cattle
Veal calf	calf intended for slaughter between 16 and 26 weeks of age (Blanchard et al. 1999)
Wether	castrated male sheep