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Neoformation of boldenone and related steroids in faeces of veal calves

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Neof ormation of boldenone and related steroids in faeces of veal

calves

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Abstract

Conflicting findings regarding the boldenone content of bovine faeces suggests it may be synthesised *de novo* in emitted faeces. We tested this hypothesis by analysing uncontaminated urine, fresh and various forms of dried faeces from 10 calves (not given boldenone) by liquid chromatography/tandem mass spectrometry for 17 α - and 17 β -boldenone (α and β BOL); 1,4-androstadiene-3,17-dione (ADD); 4-androstene-3,17-dione (AED), testosterone (T) and epitestosterone (ET). Urine contained no α BOL, β BOL or ADD. The analysed substances were variably present in the rectal faeces, and at generally higher levels in faeces scraped from skin or stall floor. In pooled rectal faeces naturally dried for 13 days, α BOL, ADD, AED and ET levels were extremely high (much higher than accounted for by increases due to drying), and β BOL and T were absent. It is concluded that *de novo* synthesis of α BOL and metabolites occurs naturally in bovine faeces and only uncontaminated urine should be analysed for illegal boldenone.

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9 **Keywords:** boldenone, veal calves, faeces, urine
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12 **Introduction**

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15 The first report of the presence of boldenone in bovine faeces, published in 1998
16 (Van Puymbroeck et al.), proposed that faeces could be analysed as an alternative to
17 urine to detect illegal dosing of cattle with this steroid. The authors attributed the
18 high concentrations of 17 α -boldenone (α BOL), 1,4-androstadiene-3,17-dione
19 (ADD) and, in some samples, of 17 β -boldenone (β BOL) present in slaughterhouse
20 faeces, to oral administration of β BOL esters or precursors. More recently, Nielen
21 et al. (2004) found α BOL (520.0 ng/g) and β BOL (21.0 ng/g) in dried faeces from
22 calves reported to be untreated; Sangiorgi et al. (2005) detected these substances in
23 rectal faeces from untreated calves, while De Brabander et al. (2004) reported low
24 concentrations of α BOL and (1- >10 ng/g) and β BOL (0.1-2.0 ng/g) in faeces
25 scraped off the skin of calves. It therefore seems that α and β BOL can be present in
26 a wide range of concentrations in faecal materials sampled outside the animals.
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47 Previous work from our group (Sgoifo Rossi et al., 2004) demonstrated that
48 deliberate contamination of calves urine with faeces can render the urine positive
49 for α BOL and occasionally β BOL. By contrast, Nielen et al. (2004) found that
50 boldenone epimers were absent from faeces collected from the rectum but present in
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7 dried faeces at the low β BOL level of 21.0 ng/g, and concluded that only massive
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9 and unlikely contamination would result in urine positivity to β BOL.
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14 The results of these analyses on various faecal samples (rectal faeces, dried faeces,
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16 faeces adhering to the animal's skin) from untreated animals, and faeces-
17
18 contaminated urine, cast doubt on the utility and reliability of measuring boldenone
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20 in faeces to provide an indication as to whether this substance is being
21
22 administered illegally to veal calves. We hypothesised that the reason for these
23
24 conflicting results might be that boldenone and related steroids may form *de novo* in
25
26 calf faeces after emission. We therefore determined these steroids and the related
27
28 substances ADD, 4-androstene-3,17-dione (AED), testosterone (T) and
29
30 epitestosterone (ET) in various faecal samples (fresh from individual animals, from
31
32 animals' skins, and pooled naturally dried samples) as well as in urine, from
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34 animals not dosed with boldenone
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42 **Materials and methods**

43 *Animals*

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45 Ten Friesian veal calves, age 135 ± 5 days, fed with milk replacer (2.8 kg /calf/day)
46
47 and corn silage (1.3 kg/calf/day) were studied. The lipid content in the milk replacer
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49 was 20% (35% coconut and 65% tallow + lard). The animals were housed in
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51 individual stalls within a single cowshed and cared for in accordance with European
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7 Union guidelines (No. 86/609/EEC) approved by the Italian Ministry of Health
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9 (L.116/92).
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11

12 13 *Materials*

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15 Analytical-grade solvents and reagents were purchased from Merck (Darmstadt,
16
17 Germany). AED, ADD, β BOL, ET, T, d3 testosterone and β -glucuronidase from
18
19 *Helix pomatia* (containing also sulfatase activity) were from Sigma-Aldrich (St
20
21 Louis, MO, USA). α BOL was synthesized by modified Mitsunobo protocol
22
23 (Dodge and Lugar III, 1996). OASIS[®] SPE columns (HLB 60 mg) were from
24
25 Waters (Millford, MA, USA). Bakerbond 500 mg SPE columns were from J.T
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27 Baker (Philipsburg, NJ, USA).
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35 *Urine collection and extraction*

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38 Urine (pH ranged from 6.8 to 7.1) was collected into long-handled container after
39
40 cleaning and trimming the sheath area and extracted according to the method
41
42 described by Sangiorgi et al.(2005) with slight modifications. Briefly urine samples
43
44 (10 ml) from each of the ten animals were spiked with trideuterated (d3)
45
46 testosterone (2 ng/ml) as internal standard, and brought to pH 4.5 with acetic acid;
47
48 100 μ l of β -glucuronidase from *Helix pomatia* extract was added, and after
49
50 overnight incubation at 37°C, the digest was solid-phase extracted (SPE) on a
51
52 column Oasis[®] HLB (60 mg) previously activated with 3 ml methanol and 3 ml
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54 water. The column was washed with 2 ml methanol:NaOH 0.01 M (60:40) followed
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7 by 2 ml purified water and the analytes eluted with methanol (2 x 2 ml). The eluate
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9 was evaporated to dryness under nitrogen at 40°C and the residue dissolved in 200
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11 µl MeOH:H₂O (50:50). 10 µl samples analysed by LC/MS/MS.
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14 15 16 *Faeces collection and extraction* 17

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19 Faecal samples were collected as follows: (a) rectal faeces from each of the ten
20
21 animals, (b) wet and dried faeces from the shed floor; (c) faeces scrapped from skin
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23 or fur of three animals; and (d) pooled rectal faeces samples from the 10 animals
24
25 and allowed to dry for 13 days in the cowshed. Samples a-c were analysed
26
27 immediately; pooled faeces were analysed at 4 and 13 days.
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31 Faeces samples (2 g) were spiked (10 ng/g) with (d3) testosterone and dissolved in
32
33 6 ml acetate buffer (pH 4.8). After sonication and vortexing, 1 ml NaOH 1M plus 6
34
35 ml diethyl ether were added and the mixture head-over-head incubated for 20 min.
36
37 The sample was then centrifuged for 15 min at 2000 g and the organic layer
38
39 collected. The ether extraction was repeated twice more. The organic phase was
40
41 evaporated under nitrogen at 40°C and the residue dissolved in 800 µl
42
43 ethanol:methanol (3:1). Isooctane (1.2 ml) and acetic acid 0.2 M (0.4 ml) were then
44
45 added. The mixture was loaded onto a C18 SPE cartridge (Bakerbond SPE 500 mg)
46
47 previously activated with methanol (5 ml) followed by water (5 ml). After washing
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49 with 3 ml H₂O:methanol (95:5) and 3 ml n-hexane, and drying under vacuum the
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51 analytes were eluted with 2 x 2ml diethyl ether. The eluate was evaporated to
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7 dryness under nitrogen at 40 °C and the residue dissolved in 200 µl methanol:H₂O
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9 (50:50). 10 µl samples were analysed by LC/MS/MS.
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11 The water content of faeces was determined after drying till no further weight loss
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13 in an oven at 100°C.
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15 16 17 18 19 *LC/MS/MS analysis*

20 The LC/MS system consisted of a ThermoFinnigan LC Surveyor (S. José CA,
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22 USA) equipped with a LCQ Deca XP Max ion trap mass spectrometer
23
24 (ThermoFinnigan, S. José, CA, USA). The mass spectrometer was operated in
25
26 positive atmospheric pressure chemical ionization (APCI) mode with capillary
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28 voltage of 31 V, capillary temperature of 220°C, discharge current of 4.5 µA,
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30 vaporizer temperature of 350°C and sheath and auxiliary gas (nitrogen) flow rates
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32 of 20 and 8 arbitrary units, respectively. Helium was used for collision induced
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34 dissociation (CID). The molecular ions (α BOL, β BOL and AED m/z 287; ADD
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36 m/z 285; and T and ET m/z 289) were precursors for CID. The most abundant
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38 product ions were identified: the associations m/z 287→173, 287→147 and
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40 287→135 for α BOL and β BOL; 285→151, 285→147 and 285→121 for ADD;
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42 287→97 and 287→109 for AED, and 289→253 and 289→271 for T and ET. The
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44 analytical column was a 150 mm x 2.1 mm i.d., 5 µm ODS Hypersil-Keystone
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46 (ThermoFinnigan, S. José, CA, USA) in a column oven at 30°C. The mobile
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48 phase was water with 0.1% acetic acid (A) and methanol (B), flow rate 0.3
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50 ml/min. Solvent (B) was kept at 65% for 1 min, increased to 90% over 7 min,
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7 held at 90% for 1 min, and then reduced to 65% in 1 min. An isocratic period of 5
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9 min followed with 65% solvent B. Quantitative analyses were carried out
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11 comparing the peak areas of analytes with those of standard solutions.
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16 The matrix calibration curves (four replications; six concentration points) were
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18 prepared by fortifying and then extracting blank samples with mixtures of the six
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20 analytes at 0.2-0.5-2.0-10-50-100 ng/ml (urine) or ng/g (faeces) levels and with I.S.
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22 at the concentration of 2.0 ng/ml in urine and 10 ng/g in faeces. For the full
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24 identification of the analytes the criteria established in the 2002/657/EC
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26 Commission Decision were followed.
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33 **Results and discussion**

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35 In urine, the calibration curve of AED was linear in the range 0.2 (LOQ) -100
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37 ng/ml, while other analytes showed linearity from 0.5 ng/ml (LOQ). In faeces the
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39 curves were always linear in the range 2.0(LOQ)-100 ng/g. The correlation
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41 coefficients (r^2) ranged between 0.93 and 0.98.
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47 Under our operating conditions the limits of detection (LODs), considered as the
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49 analyte amount giving a signal to noise ratio ≥ 3 , resulted about 0.1 ng/g in urine
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51 and 0.5 ng/g in faeces. Recoveries determined on blank samples fortified with a
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53 mixture of the six steroids at 5, 10 and 50 ng/ml (urine) or ng/g (faeces) were
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7 between 75 and 122%. No significant differences in recoveries were noted between
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9 17 –keto and 17 hydroxy steroids.
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14 α BOL, β BOL and ADD were never detected in urine samples, while AED, T and
15
16 ET were always present (Table I).
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21 [insert Table I about here]
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26 Figures 1 and 2 show the reconstructed LC/MS/MS chromatograms and the related
27
28 MS/MS spectra of the analytes more frequently found in rectal faeces and in stall
29
30 faeces, respectively, as below explained.
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35 [insert Figs. 1 and 2 about here]
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39 In rectal faeces (Table II), β BOL was present in all samples, α BOL was detected
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41 in four samples only, and at lower concentrations than β BOL. ADD and T were
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43 present in one sample each. ET was always present. AED was always absent.
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49 [insert Table II about here]
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54 Data regarding the presence of boldenone in rectal faeces are contradictory. Studies
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56 by Rijkskwaliteitsinstituut voor Land en Tuinbouwproducten (RIKILT) and
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7 Rijksinstituut voor Volksgezondheid en Milieu (RIVM), as reported by De
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9 Brabander et al. (2004), found neither α BOL nor β in rectally sampled faeces from
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11 46 untreated calves of age about 27 weeks. By contrast, Attucci et al. (2003)
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13 detected α BOL (42.4 ng/g) and β BOL traces (0.04 ng/g) in rectal faeces from an
14
15 untreated calf, although neither substance was present in faeces from the same
16
17 animal sampled five days later. Recently, Sangiorgi et al. (2004) reported α BOL
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19 and β BOL at levels of about 10 ng/g, in rectal faeces from a six month-old calf not
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21 given boldenone or precursors, but not in another animal from the same shed and
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23 fed on the same diet.
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31 We found that β BOL was always present in the rectal faeces from the animals
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33 studied. However additional rectal faecal sampling of these animals and of others
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35 housed in the same shed showed that β BOL was not always present, although it
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37 was present more often than α BOL (data not shown). Our findings therefore
38
39 suggest that both α BOL and β BOL can be present in rectal faeces from untreated
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41 animals and that this presence is highly variable. This would explain contradictory
42
43 literature findings, and furthermore indicates that analysis of rectal faecal samples is
44
45 not a reliable method for detecting illegal dosing of boldenone since positive
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47 findings, particularly of β BOL, are common in the absence of boldenone
48
49 administration. It is possible that boldenone was present in 'untreated' animals'
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51 faeces because it was given without the experimenters' knowledge. However, the
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53 results of the urine analyses exclude this in the present study (Table I): BOL
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7 epimers and their metabolites were never detected. These substances always occur
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9 in urine following oral or parenteral boldenone administration (Nielen et al., 2004;
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11 Sangiorgi et al., 2004; De Brabander et al., 2004).
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16 The results of the analyses of faeces from the skin are shown in Table III.
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21 [insert Table III about here]
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25 β BOL was present in all faeces taken from the skins of the three animals sampled.
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27 However, unlike rectal faeces, α BOL was present in most (7/9) of these samples,
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29 while ADD was present in three samples from skin, yet in only one rectal faeces
30
31 sample. ET was always present in skin faeces, while T was present in one sample.
32
33 Thus α BOL, β BOL and ADD were more often present in crusted skin samples
34
35 than in rectal faeces samples and generally at higher concentrations. Nevertheless
36
37 concentrations of α BOL (0-988 ng/g) and β BOL (2.1-482 ng/g) were much more
38
39 variable than concentrations in rectal faeces (2.6-89.0 ng/g both epimers).
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47 Table III also presents the results of the analyses wet faeces collected from the
48
49 cowshed floor and trampled by the calves (samples A, B, C and D) and of old dried
50
51 faeces taken from the side of the cowshed (samples E and F). Samples E and F were
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53 the oldest faecal material analysed, and the concentrations of most steroids analysed
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55 (the exception being β BOL) were much higher in these than in wet samples.
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9 The high steroid content of dried faeces (from skin and stall floor) cannot be
10 explained by concentration of analytes due to the water loss. The dry matter of
11 rectal faeces was 10-15% and complete water loss could result in an increase in
12 analyte concentrations by a maximum of 10 times. Since the dry matter content of
13 the samples shown in Table III ranged from 40% (A, B, C and D) to 85% (E and F),
14 only neoformation of α β BOL, ADD, AED and ET can explain the higher
15 concentrations in dried material.
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28 To assess de novo boldenone formation in faeces after emission, fresh samples from
29 each animal were pooled, naturally dried in the cowshed, and analysed after 4 and
30 13 days (Table IV).
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34 [insert Table IV about here]
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40 As expected the dry matter content increased during drying (50% at day 4 and 85%
41 at day 13) but the water loss was not sufficient to explain the overall increases in
42 steroid concentrations shown in Table IV. In particular α BOL increased by about
43 1600-fold over the 13 days. ADD, AED and ET also increased massively. These
44 findings indicate de novo synthesis. Our data also indicate that α BOL and ADD
45 could not have originated from the β BOL present in rectal faeces. Although the β
46 BOL level at day 4 increased by an amount compatible with drying, at day 13 it was
47 not detected. Yet levels of α BOL and ADD at day 13 were very much higher can
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7 be explained by conversion of the β BOL originally. It is also evident that ET and
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9 AED could not have been produced by transformation of T, since this was present
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11 initially – at very low levels – in only one sample of rectal faeces.
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16 We therefore conclude from our data that one or more precursors in rectal faeces
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18 can be biotransformed into ET, ADD, AED and α BOL under certain conditions.
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20 These same conditions do not permit the production of β BOL, which nevertheless
21
22 is usually present in rectal faeces. We propose that the anaerobic condition within
23
24 the gut allow the production of β BOL from the same precursors. Groh et al. (1993)
25
26 widely described the metabolic activities of intestinal micro-organisms on
27
28 substrates like steroids, that can be degraded via reduction of the A ring, the delta 5-
29
30 bond, the 17-keto or cleavage of the C17 side chain. In his review, for instance, a
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32 strain of *Pseudomonas* was reported to anaerobically transform cholesterol into
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34 ADD.
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43 Nevertheless at present we cannot indicate which are the precursors of BOL and
44
45 related steroids in faeces. To elucidate it, studies are in progress in our laboratory.

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47 The results of this study on rectal faeces apparently do not agree with those
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49 obtained by Van Puymbroeck et al. (1998) who found that, in faeces positive on
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51 routine screening α BOL and ADD were ubiquitously present, but β BOL was
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53 present in only 17 samples. Furthermore ADD concentrations were higher (up to
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55 632 ng/g) than α BOL (up to 240 ng/g) and β BOL (up to 24.0 ng/g) concentrations.
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7 However these authors stated that preliminary screening was performed to detect α
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9 BOL, and this suggests that the quantitative analysis was not performed
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11 immediately after faeces emission. If the faeces had been in contact with air for
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13 some time it is possible that the transformations we observed in the present study
14
15 occurred in their study. The study of Nielen et al. (2004) on untreated calves,
16
17 reported in dried faeces similar concentrations and concentration ratios (ADD 790
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19 ng/g, α BOL 520 ng/g, β BOL 21.0 ng/g) to ours.
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26 In the light of these considerations, the proposal of Van Puymbroeck et al (1998)
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28 that illegal oral treatment with boldenone precursors or esters is the only
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30 explanation for high concentrations of α BOL and ADD in cattle faeces, has to be
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32 revised.
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38 The significance of α BOL and β BOL findings bovine urine must also be re-
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40 assessed in the light of our findings on faeces. Nielen et al. (2004) suggested that
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42 urine contamination by faecal matter must be unrealistically high in order to give a
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44 positive β BOL result in urine. However this conclusion was based on the finding of
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46 21.0 ng/g β BOL in one faeces sample only. We found that faeces from skin could
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48 have a β BOL concentration of up to 482 ng/g, suggesting that contamination with a
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50 few milligrams of such material would be enough to give a positive urine result.
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52 These considerations also apply to α BOL and ADD. We calculate that 4 mg of
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54 dried faeces containing 2680 ng/g of α BOL and 13550 ng/g of ADD (Table IV)
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7 would produce detectable contamination of 10 ml of urine (1.0 ng/ml of α BOL and
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9 5.4 ng/ml of ADD). Contamination at these levels is possible when urine is
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11 collected with a container without prior cleaning and trimming of the sheath area or
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13 when a collection apron (tied round the animal) is used.
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18 However, simple transfer of steroids to urine is only one aspect of faecal
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20 contamination. The high *de novo* production of α BOL, ADD, AED, and ET
21
22 occurring in faeces after deposition is almost certainly due to microbial activity and
23
24 bacteria, as well as undigested food and catabolism products would be transferred to
25
26 urine by faecal contamination. It is therefore possible that *de novo* synthesis of these
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28 steroids also occurs in urine. This hypothesis is in agreement with the data of de la
29
30 Torre et al. (2001), which demonstrated that a *de novo* synthesis of androgen
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32 steroids (e.g. AED from dehydroepiandrosterone sulfate) can occur in urine
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34 contaminated with bacteria, so causing alterations of the urine steroid profile.
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42 Contamination of urine with faecal matter is therefore likely to result in highly
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44 variable presence of α BOL or β BOL since microbic conversion and/or *de novo*
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46 synthesis will be influenced by variable storage conditions prior to analysis.
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51 The contamination with faeces can make urine positive (via a transfer or a neo
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53 formation) to α BOL and β BOL that will be detected in their free (unconjugated)
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55 form (Sgoifo Rossi et al.,2004; Nielen et al.,2004): the detection of these steroids in
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7 their conjugated form could therefore be used to discriminate between the presence
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9 due to contamination and the presence due to treatment (Nielen et al.,2004; De
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11 Brabander et al., 2004). At the moment, however, it is not yet possible to affirm that
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13 the presence in veal urine of conjugated β BOL surely indicates an illicit treatment.
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16 It is moreover unclear whether boldenone and metabolites in rectal faeces derive
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18 from biliary excretion or anaerobic conversion of the same precursors that give rise
19
20 to them outside the animal. The second possibility would explain the occasional
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22 finding of α BOL and β BOL in rectal faeces for untreated calves, in which
23
24 testicular synthesis has been excluded (De Brabander et al., 2004). In such cases the
25
26 presence of conjugates of α BOL and β BOL in urine might be due to production in,
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28 and absorption from the enteric tract, but our results (presence of β BOL in faeces
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30 and absence in urine from the same animal) seem to contrast with this hypothesis.
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32 However factors influencing these processes are unknown and it is therefore not yet
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34 possible to correlate the faecal concentration of β BOL with the presence of α and β
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36 BOL in veal urine.
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45 Moreover, conjugated α BOL (4ng/ml), was found in bovine colostrum samples by
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47 Brambilla et al. (2005) and α and β BOL (7 ng/g and 3 ng/g, respectively) were
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49 detected in a sample of milk replacer by Nielen et al. (2004), so these steroids
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51 and/or their precursors could come from feed and be excreted in urine according to
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53 the mechanism reported by Rapp et al. (1989) with regard to 19-nortestosterone.
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7 In this contest the intake by the animals (licking activity) of dried and skin faeces
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9 containing the high concentrations of ADD, AED and BOL reported in this paper
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11 could represent a considerable source of these steroids. Further studies are
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13 necessary to understand the amount of boldeneone an/or precursor taken by this
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15 way.
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21 Finally, this study showed that large amounts of α BOL and ADD are ejected with
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23 veal calves faeces and that these steroids must be added in the list of natural
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25 hormones (T, ET and AED) present in livestock excreta reported by Lange et al.
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27 (2002). An evaluation of their fate in the environment should be carried out.
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31 32 33 **Acknowledgements**

34
35 We thank Dr. Silvana Casati of the Department of Preclinical Sciences LITA
36
37 (Laboratorio Interdisciplinare Tecnologie Avanzate) Vialba (Milan, Italy) for the
38
39 synthesis of α BOL.
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Table I. Concentrations of α BOL, β BOL, ADD, AED, T and ET in urine (ng/ml)

Animal	α BOL	β BOL	ADD	AED	T	ET
1	nd	nd	nd	0.25	1.45	3.40
2	nd	nd	nd	0.60	1.30	3.40
3	nd	nd	nd	0.59	2.15	6.40
4	nd	nd	nd	0.45	1.40	9.65
5	nd	nd	nd	0.25	6.05	17.90
6	nd	nd	nd	0.86	8.15	22.80
7	nd	nd	nd	1.12	5.45	31.4
8	nd	nd	nd	0.57	1.23	8.00
9	nd	nd	nd	0.72	2.30	7.10
10	nd	nd	nd	0.46	4.70	16.80

nd: < LOD

Table II. Concentrations (ng/g) of α BOL, β BOL, ADD, AED, T and ET in rectal faeces

Animal	α BOL	β BOL	ADD	AED	T	ET
1	nd	30.6	nd	nd	nd	47.9
2	nd	54.6	nd	nd	nd	9.5
3	5.9	29.8	nd	nd	4,4	7.5
4	nd	31.1	nd	nd	nd	6.5
5	nd	36.8	nd	nd	nd	2.4
6	nd	55.2	nd	nd	nd	17.4
7	5.1	54.9	nd	nd	nd	26.4
8	3.1	89.0	nd	nd	nd	44.1
9	2.6	35.3	nd	nd	nd	11.1
10	nd	27.6	20.9	nd	nd	2.4

nd: < LOD

Table III. Concentrations (ng/g) of α BOL, β BOL, ADD, AED, T and ET in faeces from skin and stall

Animal	Sample	α BOL	β BOL	ADD	AED	T	ET
1	skin	14.5	437	nd	nd	nd	17.0
1	skin	nd	2.1	nd	nd	nd	16.1
1	skin	32.8	268	4.3	nd	nd	120
2	skin	988	318	76.7	nd	4,5	179
2	skin	7.3	3.9	nd	nd	nd	51.8
2	skin	858	194	19.7	nd	nd	251
3	skin	nd	8.1	nd	nd	nd	16.8
3	skin	853	482	nd	nd	nd	70.5
3	skin	401	308	nd	nd	nd	71.0
A*	stall	nd	nd	14.6	nd	nd	12.6
B*	stall	nd	nd	40.5	nd	nd	7.1
C*	stall	15.3	18.6	nd	nd	nd	68.6
D*	stall	6.4	28.5	nd	nd	nd	186
E**	stall	3090	nd	1490	12070	nd	11000
F**	stall	459	nd	401	2900	nd	3770

* wet samples collected from cowshed floor; ** old dry samples collected from corner of cowshed; nd: < LOD

Table IV. Concentrations (ng/g) of α BOL, β BOL, ADD, AED, T and ET in naturally dried faeces

Sample	α BOL	β BOL	ADD	AED	T	ET
Pooled rectal faeces *	1.7	44.5	2.1	nd	0.5	17.5
Pooled faeces at day 4	51.9	102.0	15.7	34.2	nd	195.0
Poled faeces at day 13	2680	nd	13550	5790	nd	16580

- Mean of the ten values reported in Table II; nd: < LOD

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7 Figure 1 – Reconstructed LC-MS/MS chromatograms of a sample of rectal
8 faeces and related MS/MS spectra of β BOL and ET. Each chromatogram is the
9 sum of the MRM transitions reported in the text.
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16 Figure 2 – Reconstructed LC-MS/MS chromatograms of a sample of stall faeces
17 and related MS/MS spectra of ADD, α BOL, AED and ET. Each chromatogram
18 is the sum of the MRM transitions reported in the text.
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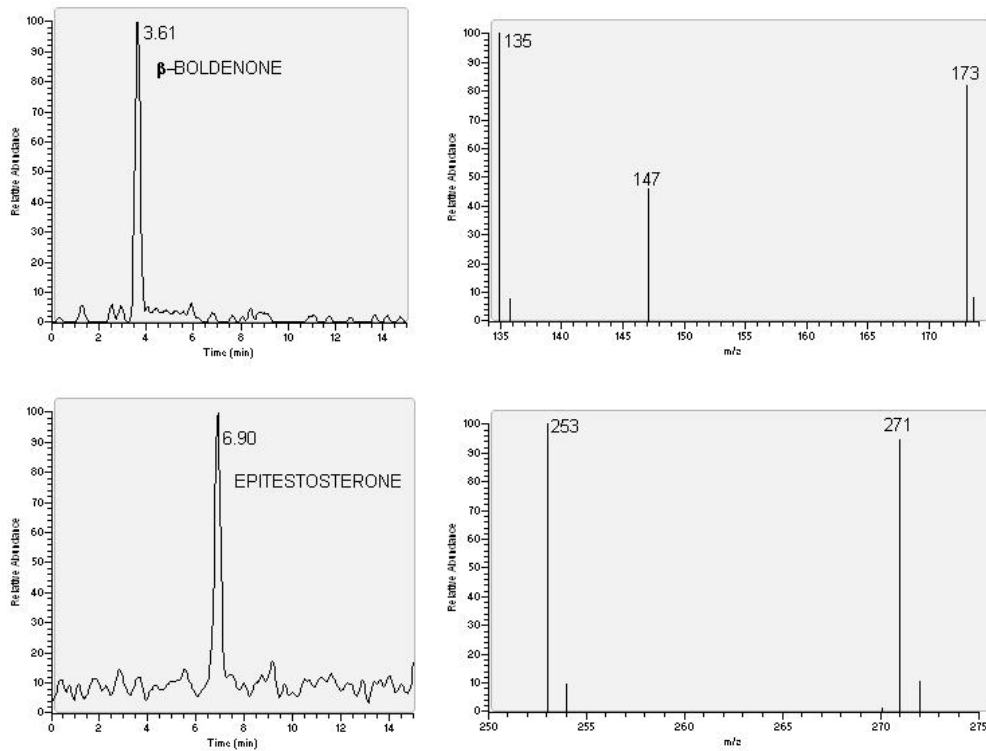


Figure 1

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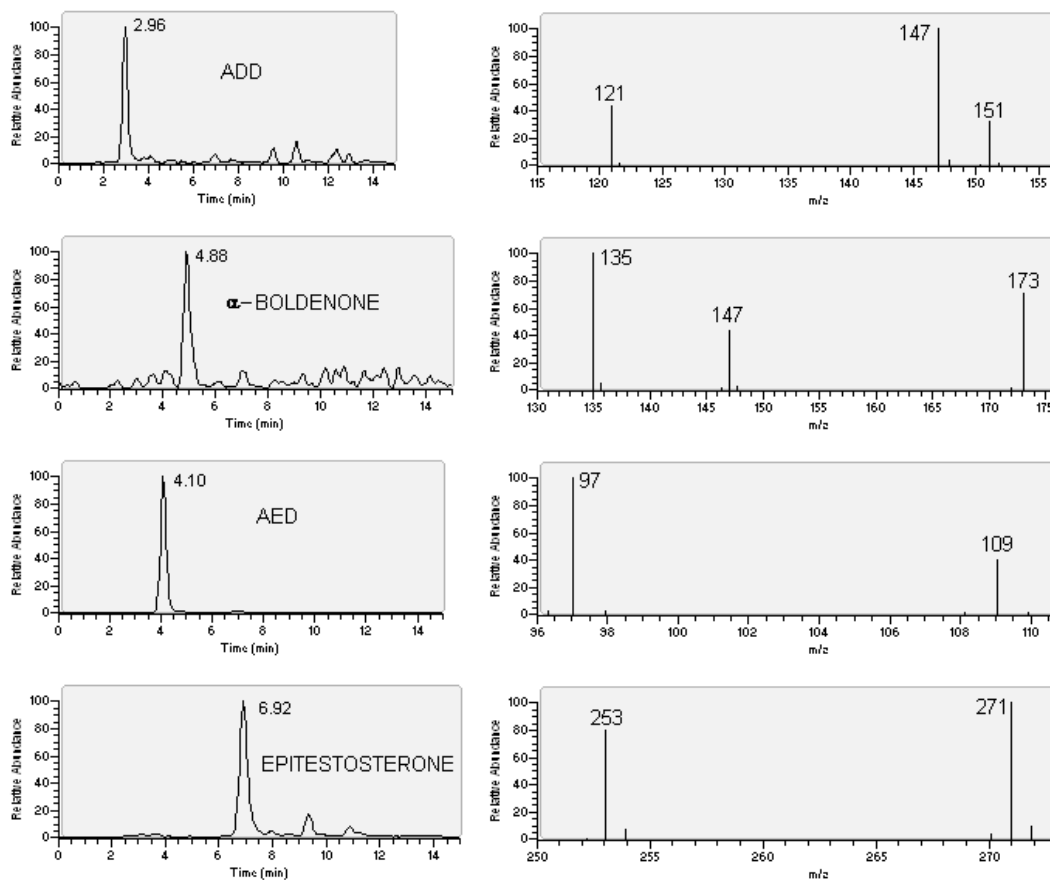


Figure 2

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