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Elena Bozzetta, Marzia Pezzolato, Cristiana Maurella, Katia Varello, Guia Benedetta Richelmi, Rosa Draisci, Carolina Ferranti, Antonio d'Angelo, Maria Caramelli

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Development of an enhanced histopathological approach to detect low-dose dexamethasone illicit treatment in veal calves

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Complete List of Authors:	Bozzetta, Elena; Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Histopathology lab. Pezzolato, Marzia; Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Histopathology lab. Maurella, Cristiana; Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta Varello, Katia; Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta Richelmi, Guia; Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta Draisci, Rosa; Istituto Superiore di Sanità Ferranti, Carolina; Istituto Superiore di Sanità D'Angelo, Antonio; Faculty of Veterinary Medicine, University of Torino Caramelli, Maria; Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta
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Food Types:	Animal products – meat
Abstract:	Dexamethasone is one of a number of synthetic corticosteroids illegally used to promote growth in food-producing animals. Since these low-level drug cocktails evade detection by currently available

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	<p>chemical methods, simple biological indicators that can aid in laboratory analysis are needed.</p> <p>In the attempt to devise an accurate biological method that could detect illicit drug treatment in food-producing animals, we characterized microscopic morphologic alterations of the thymus in veal calves administered low-dose dexamethasone versus control animals. For this purpose, 122 male calves were farmed for 6 months in controlled condition: 81 animals were orally administered dexamethasone (0.4 mg/day) for 20 days during the sixth month and the remaining 41 were kept as control. Urine samples were collected systematically during the treatment period, the suspension period and at the slaughterhouse. All animals were slaughtered 10 a day starting from 10 days after the last dexamethasone administration and the thymus was sampled for histological examination. The difference between the two animal groups was evaluated by means of a non parametric test of hypothesis.</p> <p>No residues were detected in the urines collected since the third day after the last administration, whereas morphometrical analysis of the thoracic thymus revealed a significant decrease in the cortex: medulla ratio in the treated animals ($p < 0.0005$). We can conclude that this histological approach offers encouraging prospects as a screening method to overcome current limitations in controlling growth promoter abuse.</p>



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1 Development of an enhanced histopathological approach to detect 2 low-dose dexamethasone illicit treatment in veal calves

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4 E. Bozzetta^{a*}, M. Pezzolato^a, C. Maurella^a, K. Varello^a, G.B. Richelmi^a, R. Draisci^b, C. Ferranti
5 ^b, A. D'Angelo^c, M. Caramelli^a

6 a Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna
7 148, 10154, Turin, Italy. elena.bozzetta@izsto.it; marzia.pezzolato@izsto.it;
8 cristiana.maurella@izsto.it; katia.varello@izsto.it; guida.richelmi@izsto.it;
9 maria.caramelli@izsto.it

10 b Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italia.
11 rosa.draisci@iss.it; carolina.ferranti@iss.it

12 c Faculty of Veterinary Medicine, University of Torino, Via Leonardo Da Vinci 44, 10095,
13 Grugliasco (Turin), Italy . antonio.dangelo@unito.it

15 ***Corresponding author**

16 Elena Bozzetta DVM PhD

17 SC Istopatologia e Test Rapidi

18 Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta

19 Via Bologna 148

20 10154 TORINO

21 Italy

22 Tel +39-011-2686361

23 Fax +39-011-2686362

24 e-mail:elena.bozzetta@izsto.it

27 **Abstract**

28 Dexamethasone is one of a number of synthetic corticosteroids illegally used to promote
29 growth in food-producing animals. Since these low-level drug cocktails evade detection by
30 currently available chemical methods, simple biological indicators that can aid in laboratory
31 analysis are needed. In the attempt to devise an accurate biological method that could
32 detect illicit drug treatment in food-producing animals, we characterized microscopic
33 morphologic alterations of the thymus in veal calves administered low-dose dexamethasone
34 versus control animals. For this purpose, 122 male calves were farmed for 6 months in
35 controlled condition: 81 animals were orally administered dexamethasone (0.4 mg/day) for
36 20 days during the sixth month and the remaining 41 were kept as control. Urine samples
37 were collected systematically during the treatment period, the suspension period and at the
38 slaughterhouse. All animals were slaughtered ten per day starting from 10 days after the last
39 dexamethasone administration and the thymus was sampled for histological examination.
40 The difference between the two animal groups was evaluated by means of a non-parametric
41 test of hypothesis. No residues were detected in the urines collected since the third day after
42 the last administration, whereas morphometrical analysis of the thoracic thymus revealed a
43 significant decrease in the cortex: medulla ratio in the treated animals ($p < 0.0005$). We can
44 conclude that this histological approach offers encouraging prospects as a screening method
45 to overcome current limitations in controlling growth promoter abuse.

46
47 **Key words:** illicit treatment; dexamethasone; calves; thymus; histology

49 **Introduction**

50 The illegal use of chemical growth promoters in food-producing animals constitutes a
51 potential risk for human health. Increasingly widespread is the administration of synthetic
52 corticosteroids that can mimic, block or interfere with hormone actions (Vazquez et al.,
53 2005). Furthermore, glucocorticoids may be responsible for Cushing's syndrome or other

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3 54 side-effects such as hypertension, hypokalemia, hypernatremia, metabolic alkalosis and
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5 55 connective tissue weakness. They may also induce ulcer formation or cause permanent eye
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7 56 damage by inducing central serous retinopathy. Thanks to their lipolytic effect, these
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10 57 cocktails result in the lean meat that consumers find more appealing (Brambilla et al., 1998).
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12 58 The novel synthetic compounds also exert relevant biological effects: appetite stimulants in
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14 59 cattle lead to increased feed intake and rapid weight gain (Vazquez et al., 2005).
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61 To date, the administration of synthetic hormones, such as dexamethasone in livestock, is
62 approved by the European Union (EU) only for therapeutic indications, given the proven
63 benefits of these veterinary drugs in treating inflammatory disorders. To protect consumers
64 against illegal drug use in food-producing animals, the EU set appropriate maximal residue
65 limits (MRLs) in tissues and milk intended for human consumption (Council of the European
66 Communities, 1990. Council Regulation 90/2377/EEC). Depending on the particular
67 glucocorticoid formulation, withdrawal periods of up to several weeks to prevent the
68 accumulation of illegal residues in animal products are recommended.
69

70 To expose illicit corticosteroids use, rapid and simple analytical techniques need to be
71 developed that can be applied in routine analysis laboratories targeting these substances or
72 their residues. Furthermore, to accurately detect trace residues in samples of animal origin
73 and new compounds or low-dose cocktails, novel screening tools are needed. Innovative
74 methods employ indirect markers that can determine receptor concentration (Odore et al.,
75 2006). Other methods that can aid in identifying animals treated with anabolic agents include
76 the so-called omics techniques (transcriptomic, proteomic and metabolomic), based on the
77 simultaneous detection of biomarkers predictive of administration of specific substances
78 (Gardini et al., 2006; Toffolatti et al., 2006; Reiter et al., 2007; Courant et al., 2009) .
79 Several studies on young animals have found that illegally administered glucocorticoids
80 promote a physiological reduction of the thymus identifiable as gross and microscopic lesions
81 (Groot et al., 1998; Schilt et al., 1998; Biolatti et al., 2005; Cannizzo et al., 2008). In this

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3 82 context, anatomo-histopathological examination may provide a valid tool as an indirect
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5 83 marker of illicit drug treatment. However, the growing use of low-dosage drug cocktails that
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7 84 escape chemical detection and cause only minimal tissue changes makes the detection of
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10 85 suspected alterations a difficult task for veterinary inspectors (Courtheyn et al., 2002; Biolatti
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12 86 et al., 2005; Carletti et al., 2007), with the inevitable consequence of increased risk for
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14 87 consumer health (Groot et al., 1998).

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18 89 This situation further stresses the importance of identifying chemical-induced thymic atrophy
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20 90 and differentiating it from physiological thymic involution. Due to the potential difficulties of
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22 91 differentiating those latter changes from chemical-related effects, histopathological
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25 92 examination should be carried out by comparing treated with control animals (Elmor, 2006).
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27 93 The aim of this study was to determine whether morphological changes in the thymus could
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29 94 constitute a reliable treatment parameter following administration of low-dose glucocorticoid
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31 95 for growth promotion in veal calves. To do this, we devised an accurate histopathology-
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33 96 based biological method that may prove useful in detecting glucocorticoid compound abuse
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35 97 by the meat producing industry.

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39 40 99 **Materials and Methods**

41 42 100 ***Animals and experimental design***

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44 101 A total of 122 male veal calves, aged between 15 and 35 days, were bought by local
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46 102 breeders and randomly divided in two groups homogenous for body weight and age and
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48 103 farmed in two boxes in the same condition for 6 months. Each box had its own crib, multiple
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50 104 drinking troughs, and a dedicated automated milk feeder system. To protect the animals
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52 105 against infections, all were vaccinated against IBR, Para influenza (PI3), BRSV and BVDV
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54 106 (CATTLEMASTER® 4 Pfizer Animal Health; New York, USA). Clinical controls were carried
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56 107 out daily by a veterinarian and treatments for occurring infections were performed without
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58 108 using hormonal active substances.
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3 109 All animals had free access to fresh water and were fed standard milk replacer with an
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5 110 automatic milk feeder until 4 months of age, then 0.5 kg of corn was added to the diet twice a
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7 111 day. Before administration, all feeds, milk replacer and corn were analyzed with an ELISA
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9 112 test to exclude the presence of hormonal active substances.

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11 113 During the sixth month, 81 randomly selected animals received dexamethasone 21 disodium
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13 114 phosphate (0.4 mg/day *per os*) mixed with the milk for 20 days while the remaining 41
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15 115 animals were used as controls and administered milk and placebo mixed too. The study was
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17 116 designed to achieve a statistical power of 80% with Confidential Interval 95%; an expected
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19 117 frequency of disease in the treated group of 27 % and a frequency of 5% in the control group
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21 118 were considered. On this basis a simple random and representative sample of 40 controls
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23 119 and 80 treated animals was evaluated to be consistent with a detection of an Odds Ratio of
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25 120 7.

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27 121 The trial was conducted in a blinded manner.

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29 122 The animals were slaughtered 10 a day in an EC certified slaughterhouse starting from 10
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31 123 days after the last drug administration. All experiments were carried out according to
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33 124 European Economic Community Council Directive 86/609, recognised and adopted by the
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35 125 Italian Government (D.L. 27/01/1992 no. 116).

36 126 ***Chemical analysis***

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38 127 Urine samples of 10 treated animals and 10 controls were collected when spontaneous
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40 128 urination occurred, the day before the first administration, 10 days later, and the last day of
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42 129 treatment; urine was also collected after the last treatments, 1, 2, 5 and 10 days later, and at
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44 130 the slaughterhouse. Each sample, without preservative, was divided into 6 mL aliquots and
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46 131 stored at -80 °C until use.

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49 133 ***Sample preparation***

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51 134 Urine samples (5 mL) spiked with triamcinolone acetonide-d₆ used as the internal standard
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53 135 (IS) were added with acetate buffer solution 1 M, pH=4.8 and β-glucuronidase-arylsulphatase
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55 136 enzyme solution (*Helix pomatia*). The samples were then incubated for 12 h at 37°C,

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3 137 centrifuged and purified by solid phase extraction (SPE) using an Oasis hydrophile lipophile
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5 138 balance (HLB) cartridge (60 mg, 3 mL) (Waters, Milford, Ma, USA). After elution with
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7 139 methanol, the solvent was removed at 40°C under nitrogen stream and the residue was
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10 140 dissolved in 100 µl of methanol and injected into the liquid chromatography-mass
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12 141 spectrometry/mass spectrometry (LC-MS/MS) system.

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15 16 143 *Liquid chromatography-mass spectrometry/mass spectrometry analysis*

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18 144 Analyses were carried out using a liquid chromatography system (Perkin Elmer Series 200
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20 145 Micro Pump with a PE Series 200 autosampler, PerkinElmer, Waltham, MA, USA). The
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22 146 chromatographic separations were obtained under gradient conditions at room temperature
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24
25 147 using a reversed phase HPLC column (250 mm × 4.60 mm I.D., 4 mm POLAR-RP 80 A)
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27 148 Synergi C18 (Phenomenex, Torrance, CA, USA) with a C18 guard column (4 mm × 2 mm
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29 149 I.D.) (SecurityGuard, Phenomenex). The mobile phase was composed of 1% acetic acid and
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31 150 acetonitrile and the flow rate was 0.8 mL min⁻¹.

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36 152 The API 3000 triple quadrupole mass spectrometer equipped with an atmospheric pressure
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38 153 chemical ionization (APCI) - heated nebulizer (HN) source was set in negative ionization
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40 154 mode with a source temperature of 400°C and a needle current of -3 µA; ultra pure nitrogen
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42 155 was used as curtain and collision gas, while ultra pure air was used as nebulizer and
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44 156 auxiliary gas.

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49 158 Decision limit (CC_α) and detection capability (CC_β) for dexamethasone, calculated in
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51 159 accordance with Commission Decision 2002/657/EC [16], were 0.3 ng mL⁻¹ and 0.4 ng mL⁻¹,
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53 160 respectively.

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57 58 162 ***Histopathology***

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3 164 *Sample preparation*
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5 165 After slaughtering, the central area of the thoracic thymus of each animal was sampled, fixed
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7 166 in 4% buffered formaldehyde at room temperature for 2 days, routinely processed,
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9 167 embedded in paraffin wax, sectioned at 3-5 μm and stained with hematoxylin and eosin (HE).
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11 168 Routine histology by light microscopy was performed on HE-stained paraffin sections.
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16 170 *Histopathological characterization*
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18 171 Histopathologic examination was blindly performed. The presence of fat, as a possible
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20 172 indirect indicator of the degree of gland atrophy, was evaluated and a score from 1 to 3 (mild,
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22 173 moderate, severe) was attributed to the pattern. The patterns of the optical and dyeing
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24 174 parameters were evaluated based on the histopathologist's experience rather than by
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26 175 statistical methods.
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31 177 *Morphometry*
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33 178 The thymus sections were examined at low magnification (4x) using a digital microimaging
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35 179 device (Leica DMD108 Digital microimaging device for clinical diagnostics labs) to evaluate
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37 180 cortex and medulla thickness. For each slide, five functional lobules, composed of an outer
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39 181 cortex and inner medulla and surrounded by connective tissue, were randomly selected and
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41 182 measured against a graduated line, starting and ending at the interlobular connective tissue;
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43 183 a second parallel line was drawn to measure medulla thickness.
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46 184 All data were recorded on a spreadsheet; the cortex thickness was obtained by subtracting
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48 185 the second from the first value and the cortex: medulla ratio was calculated.
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53 187 *Statistical analysis*
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55 188 The results of the microscopic observations were entered into an *ad hoc* database. Two
56
57 189 different tests of analysis were carried out: to demonstrate the difference between treated
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59 190 and control animals with regard to the cortex: medulla ratio, we performed a test of
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191 hypothesis for non parametric data (Wilcoxon rank-sum test), as the data were not normally

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3 192 distributed, assuming as the null hypothesis that there would be no difference between the
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5 193 two groups; to verify the presence of a significant difference in the degree of thymic atrophy
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7 194 between the two groups, a test for linear trend was performed and the difference between
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9 195 three increasing degrees of atrophy was expressed as odds ratios (OR).
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11 196

14 197 **Results and discussion**

16 198 ***Chemical analysis***

18 199 Dexamethasone residues ($> CC\alpha$) were detected in all the urine samples collected from the
19
20 200 treated bovines at the 10th and 20th day of treatment and the day after the last administration
21
22 201 (21st day). At the 22nd and 25th day of sampling, dexamethasone residues were found in 90%
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24 202 and 50% of the analyzed samples, respectively (Table 1). No residues were detected in the
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26 203 samples collected the day before the treatment, 10 days later, or in the samples collected
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28 204 from the urinary bladder at the slaughterhouse. These findings showed that the drug
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30 205 disappeared shortly after treatment, precluding a long-term survey.
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33 206 No dexamethasone residues were detected in the control animals; this precluded any further
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35 207 statistical analysis.
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38 208 Data were determined by averaging the urine concentration values of dexamethasone
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40 209 obtained from the treated and control animals at each time interval.
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42 210 ***Histopathological characterization***

44 211 On blinded analysis, the score of 1 and 2 (mild and moderate), indicating the presence of
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46 212 infiltrating fat, were present in both groups, whereas the score of 3 was attributed only to the
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48 213 thymus of the treated animals.
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51 214 The amount of fat infiltrating the thymic tissue was not found to be significantly associated
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53 215 with the steroidal treatment.
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55 216 Table 2 shows the trend and the chi square value for trend.
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57 217 ***Comparative morphometry***

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3 218 Microscopic examination at low magnification of the thymus glands showed a striking
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5 219 tinctorial change: the thymus of the controls was darker than that of the treated animals. At a
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7 220 higher magnification, a decrease in the cortical area in the treated animals was visible.
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9 221 Qualitative overall assessment indicated that the cortical: medullary ratio was reversed in the
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11 222 treated animals (Fig. 1) in comparison with the normal 2:1 ratio (Fig. 2).
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13 223 The two-sample Wilcoxon rank-sum (Mann-Whitney) test highlighted a significant decrease
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15 224 in the cortex: medulla ratio between the treated animals and the controls ($p=0.0005$) (Graph
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17 225 1).
18
19 226 Our findings demonstrate that low dosages of dexamethasone administered as a growth
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21 227 promoter in veal calves, according to a protocol often illegally adopted in farm practice (Biolatti
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23 228 et al., 2005), can induce morphologic changes in the thymus, resulting in a significant
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25 229 reduction in the cortex: medulla ratio. In 1998 Schilt (Schilt et al., 1998) first reported this
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27 230 finding as a valuable feature to distinguish between beclomethasone treated and untreated
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29 231 veal calves. Later Elmore (Elmor, 2006) included this parameter among criteria to distinguish
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31 232 between chemical-induced and age or stress-related thymic atrophy. Other possible causes of
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33 233 thymus atrophy, as autoimmune phenomenon and immunodeficiency syndromes, have been
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35 234 shown to affect cellular density with symmetrical reduction of thymic cortical and medullary
36
37 235 areas, as well (Jubb et al., 2007). Here we show for the first time significant values for this
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39 236 parameter. Cortical atrophy and fat infiltration has been variously reported (Groot et al., 1998;
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41 237 Biolatti et al., 2005; Cannizzo et al., 2008) as co-existing findings in dexamethasone-fed veal
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43 238 calves; in our experiment, fat infiltration could not be associated with low-dose dexamethasone
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45 239 treatment, as it was detected in both animal groups.
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241 **Conclusions**

242 Examination of illegal preparations and results of specific investigations have exposed the
243 depth and breadth of the continuing illegal use of growth promoters in livestock production
244 (Courtheyn et al., 2002). Dexamethasone and other corticosteroids are frequently used for this
245 purpose, although it has long been recognised that large doses of synthetic glucocorticoids

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3 246 reduce growth rates and lead to muscle atrophy. Once often combined with beta-agonists
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5 247 and/or anabolic steroids, more recently corticosteroids are administered alone, because even
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7 248 low doses of glucocorticoids have been found to improve feed intake, increase live weight
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10 249 gain, reduce feed conversion ratio, reduce nitrogen retention and increase water retention and
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12 250 fat content (Istasse et al., 1989). Yet precisely because of their use at low dosage, their
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14 251 detection in matrices of biological origin is far from evident.

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18 253 One way to improve the efficiency of residue testing programmes might be to apply screening
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20 254 methods that measure indirect parameters. Our findings show that histopathological
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22 255 assessment of the cortex: medulla ratio is a simple and objective parameter for the detection
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25 256 of corticosteroid abuse and suggest that it may be a useful tool in monitoring illicit drug
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27 257 treatment. No false negatives occurred with our method, even in thymus samples collected
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29 258 after the 10-day withdrawal period, which is known to limit the capability of detection by
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31 259 classical chemical analysis.

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35 261 We can conclude that efforts to improve the control of illegal use of growth promoters in cattle
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37 262 should include screening methods based on histological investigations; specifically,
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39 263 assessment of the thymus parameter (thymus morphometry) could represent a standardizable
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42 264 and reproducible method for screening purposes and following the validation it could be
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44 265 considered as a new promising approach in term of screening to evidence the diffusion of
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46 266 corticosteroids illicit treatment in farm animals in order to orientate controls by confirmatory
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49 267 methods.

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56
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60 273 **References**

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11 332 **Legends**

12 333 Fig.1: Treated animal: Thymus, HE 4x

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14 334 Fig.2: Control animal: Thymus, HE 4x
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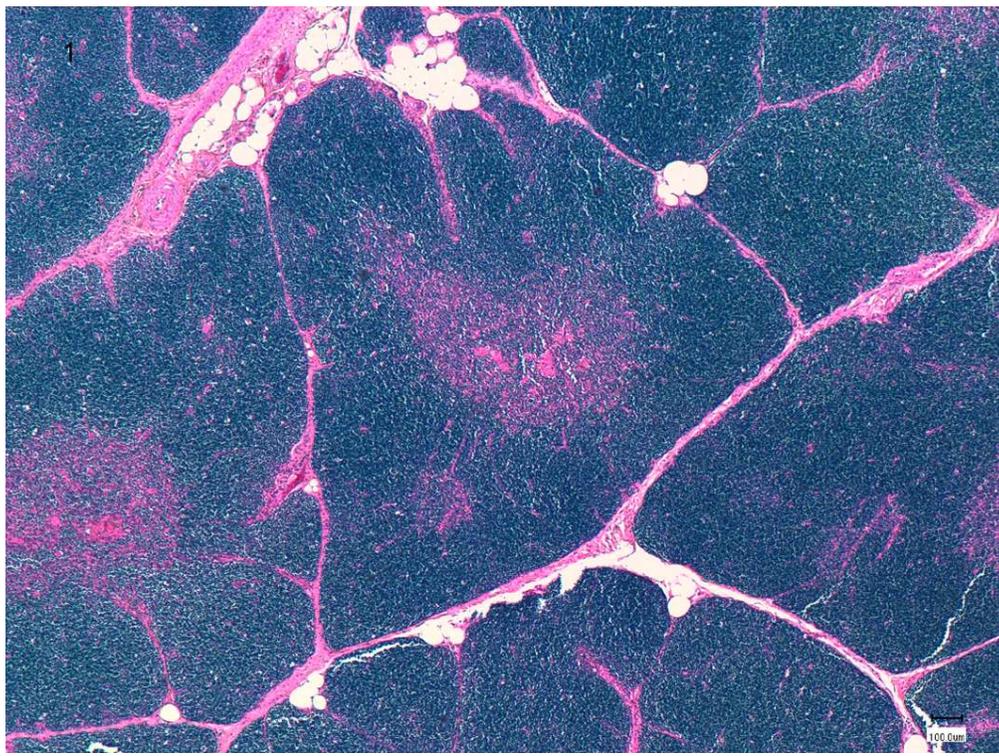
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20 336 Graph 1: Box plots representing the significant difference in the cortex: medulla ratio
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22 337 between treated and control animals.
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27 339 Table 1 Dexamethasone concentration in urine samples of treated and control bovines

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29 340 Table 2: The risk of gland atrophy is expressed as odds ratio (OR) and relative 95%

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31 341 Confidential Interval: there was no increase in infiltrating fat in treated vs. control animals.
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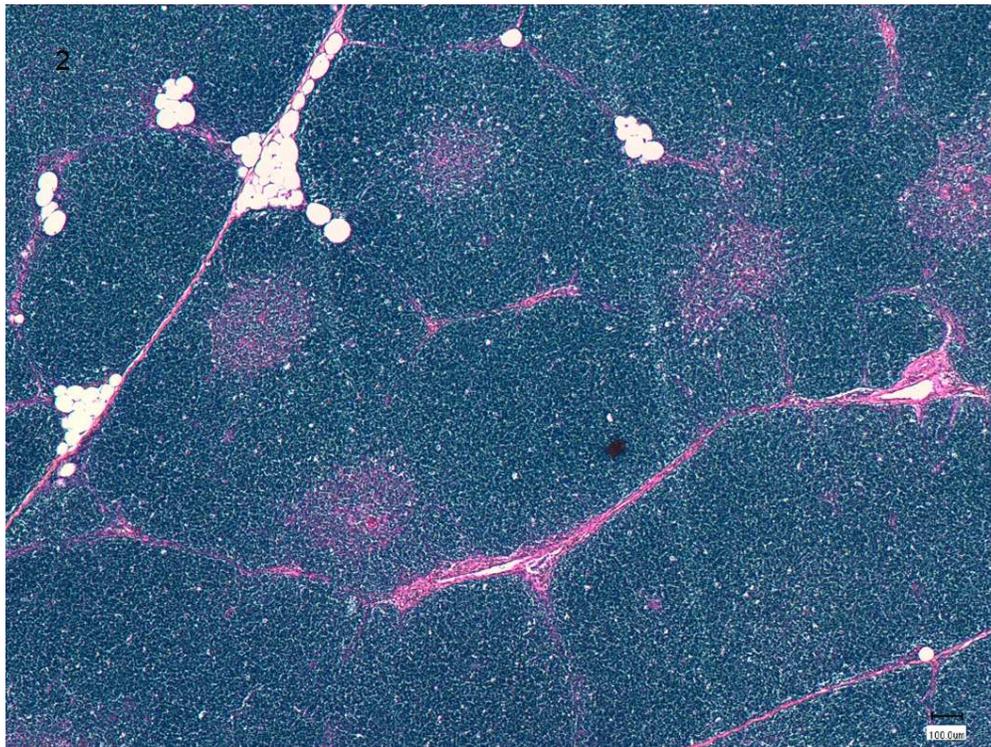
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Treated animal: Thymus, HE 4x
90x67mm (300 x 300 DPI)

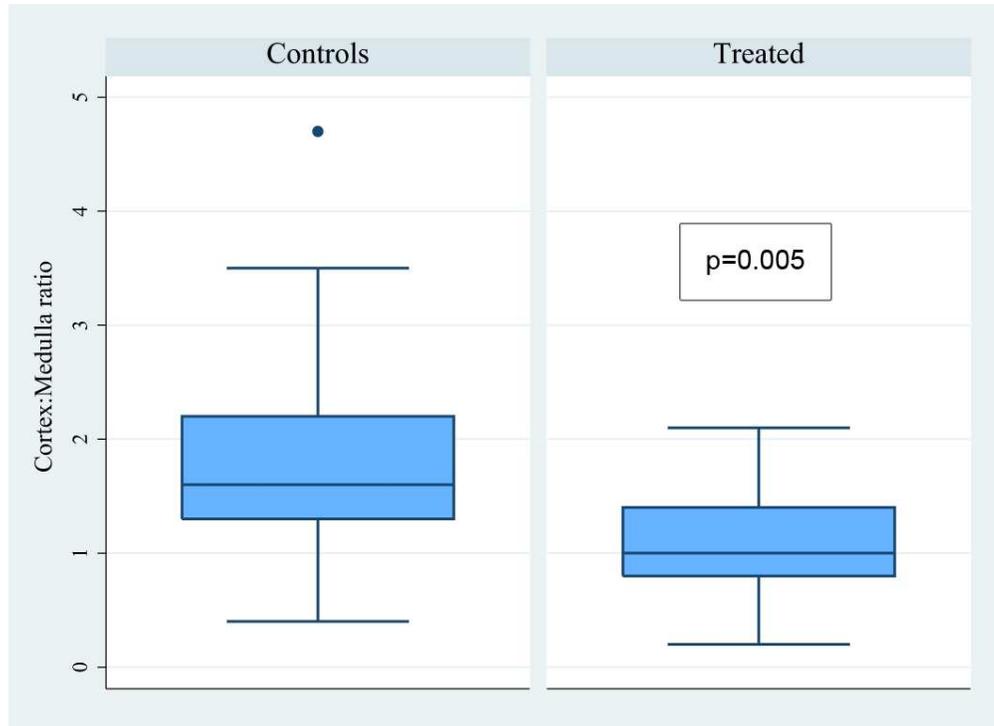
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Control animal: Thymus, HE 4x
90x67mm (300 x 300 DPI)

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Box plots representing the significant difference in the cortex: medulla ratio between treated and control animals.
90x65mm (300 x 300 DPI)

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Table 1 Dexamethasone concentration in urine samples of treated and control bovines.

DEXAMETHASONE CONCENTRATION IN URINE SAMPLES (ng mL ⁻¹)								
TREATED BOVINES								
Sampling day	0 th	10 th	20 th	21 st	22 nd	25 th	30 th	30 th slaughterhouse
Average	n.d.	2.0	2.1	1.2	1.0	0.4	n.d.	n.d.
Concentration interval	--	0.5-9.1	0.3-5.9	0.4-3.1	n.d.-2.5	n.d.-2.5	--	--
>CC α (0.3 ng mL ⁻¹)	0/7	10/10	10/10	10/10	9/10	5/10	0/10	0/10
CONTROL BOVINES								
Sampling day	0 th	10 th	20 th	21 st	22 nd	25 th	30 th	30 th slaughterhouse
Average	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Concentration interval	--	--	--	--	--	--	--	--
>CC α (0.3 ng mL ⁻¹)	0/8	0/9	0/10	0/10	0/10	0/10	0/8	0/9

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Table 2: The risk of gland atrophy is expressed as odds ratio (OR) and relative 95% Confidential Interval: there was no increase in infiltrating fat in treated vs control animals.

Fat Score	Odds Ratio	95% CI	
1	1	-	-
2	1.2	0.1	20.4
3	1.8	0.1	30.3
chi square for trend=0.16,		p=0.7	