



HAL
open science

Progesterone receptor up-regulation, a diagnostic tool for the illicit use of oestrogens in adult beef cattle

Sara Divari, Chiara Mulasso, Federica Uslenghi, Francesca Tiziana Cannizzo,
Francesca Spada, Raffaella de Maria, Nicola Brina, Bartolomeo Biolatti

► **To cite this version:**

Sara Divari, Chiara Mulasso, Federica Uslenghi, Francesca Tiziana Cannizzo, Francesca Spada, et al.. Progesterone receptor up-regulation, a diagnostic tool for the illicit use of oestrogens in adult beef cattle. Food Additives and Contaminants, 2011, pp.1. 10.1080/19440049.2011.609492. hal-00743845

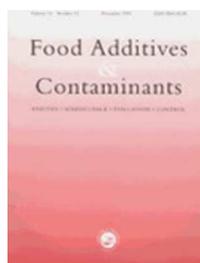
HAL Id: hal-00743845

<https://hal.science/hal-00743845>

Submitted on 21 Oct 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Progesterone receptor up-regulation, a diagnostic tool for the illicit use of oestrogens in adult beef cattle

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2011-281.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	27-Jul-2011
Complete List of Authors:	Divari, Sara; University of Turin, Dep. of Animal Pathology Mulasso, Chiara; University of Turin, Dep. of Animal Pathology Uslenghi, Federica; University of Turin, Dep. of Animal Pathology Cannizzo, Francesca Tiziana; University of Turin, Dep. of Animal Pathology Spada, Francesca; University of Turin, Dep. of Animal Pathology De Maria, Raffaella; University of Turin, Dep. of Animal Pathology Brina, Nicola; COOP Biolatti, Bartolomeo; University of Turin, Dep. of Animal Pathology
Methods/Techniques:	Screening assays, Molecular biology - PCR
Additives/Contaminants:	Hormones, Oestrogens
Food Types:	Animal products - meat
Abstract:	The monitoring of gene regulation via mRNA levels to detect anabolic sex steroid administration in cattle is a novel approach to detecting the illicit treatment of livestock in meat production. A previous study revealed that progesterone receptor (PR) gene expression levels were increased in the bulbourethral glands and prostates of 17 β -oestradiol-treated prepubertal calves, suggesting that the PR can be used as a specific molecular biomarker for oestrogen treatment. The aim of this study was to verify the specificity and applicability of the PR to detect the illegal use of 17 β -oestradiol in sexually mature beef cattle. Accessory sex glands were sampled from 42 male beef cattle that were divided into six experimental groups, including two control groups, K1 and K2. Group A cattle were treated with 17 β -oestradiol (five weekly intramuscular doses of 20 mg), and group B cattle were treated with dexamethasone (40 daily doses of 0.7 mg per os). Group C cattle received an implant of Revalor-200 (200 mg of trenbolone acetate and 20 mg of 17 β -oestradiol), and group D cattle received Revalor-200 plus dexamethasone (0.7 mg daily per os).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

	17 β -Oestradiol, either alone or in combination with other steroids, up-regulated the PR gene and protein expression, even in the absence of detectable histological changes in the accessory sex glands, confirming the high sensitivity of PR gene expression as an indirect diagnostic screening tool to detect illicit oestrogen treatment in sexually mature male bovine

SCHOLARONE™
Manuscripts

For Peer Review Only

1
2
3 1 **Progesterone receptor up-regulation, a diagnostic tool for the illicit use of**
4
5
6 2 **oestrogens in adult beef cattle**
7
8
9 3

10 4 S. Divari^a, C. Mulasso^a, F. Uslenghi^b, F.T. Cannizzo^a, F. Spada^a, R. De Maria^a, N. Brina^b, B.
11
12 5 Biolatti^{a*}
13
14
15 6

17 7 ^aDepartment of Animal Pathology, School of Veterinary Medicine, University of Turin, Grugliasco
18
19 8 (Turin), Italy ^bCOOP Italia, Casalecchio del Reno (Bologna), Italy
20
21 9

23 10 Keywords: progesterone receptor gene expression, beef cattle, 17 β -oestradiol, growth promoters
24
25
26 11

28 12 *Corresponding author: Bartolomeo.biolatti@unito.it
29
30
31 13

14

15 ABSTRACT

16 The monitoring of gene regulation via mRNA levels to detect anabolic sex steroid
17 administration in cattle is a novel approach to detecting the illicit treatment of livestock in meat
18 production. A previous study revealed that progesterone receptor (PR) gene expression levels were
19 increased in the bulbourethral glands and prostates of 17β -oestradiol-treated prepubertal calves,
20 suggesting that the PR can be used as a specific molecular biomarker for oestrogen treatment. The
21 aim of this study was to verify the specificity and applicability of the PR to detect the illegal use of
22 17β -oestradiol in sexually mature beef cattle. Accessory sex glands were sampled from 42 male
23 beef cattle that were divided into six experimental groups, including two control groups, K1 and
24 K2. Group A cattle were treated with 17β -oestradiol (five weekly intramuscular doses of 20 mg),
25 and group B cattle were treated with dexamethasone (40 daily doses of 0.7 mg per os). Group C
26 cattle received an implant of Revalor-200 (200 mg of trenbolone acetate and 20 mg of 17β -
27 oestradiol), and group D cattle received Revalor-200 plus dexamethasone (0.7 mg daily per os).
28 17β -Oestradiol, either alone or in combination with other steroids, up-regulated the PR gene and
29 protein expression, even in the absence of detectable histological changes in the accessory sex
30 glands, confirming the high sensitivity of PR gene expression as an indirect diagnostic screening
31 tool to detect illicit oestrogen treatment in sexually mature male bovine.

32

33 INTRODUCTION

34 Drugs with anabolic effects have been used to enhance feed conversion, growth rates and lean tissue
35 depositions in stock farming for several decades (Cacciatore et al. 2009). There are multiple lines of
36 evidence linking sex steroids, including testosterone and oestradiol in particular, to increased
37 muscle mass in mammals. Testosterone has been demonstrated to induce muscle hypertrophy
38 (Sinha-Hikim et al. 2003), and oestrogen has been demonstrated to stimulate muscle repair and
39 regeneration processes (Enns and Tiidus 2010). In addition to natural steroids, synthetic derivatives,
40 such as the glucocorticoid dexamethasone (DEX), are often administered in combination with
41 sexual steroids and β 2-agonists in young cattle to enhance re-partitioning effects of these drugs
42 (Abraham et al. 2004). The use of synthetic steroids is banned in the EU under Directive
43 88/146/EEC (Stephany 2001), but in ruminants, DEX can be administered legally to treat
44 inflammation, shock, stress and metabolic disease (Cantiello et al. 2009)

45
46 The detection of the illicit use of such agents is largely based on the direct detection of drug
47 residues via highly sensitive LC-MS/MS and GC-MS analyses of various matrices, such as muscle
48 tissue, blood and urine (De Brabander et al. 2007). Although mass spectrometry-based residue
49 analysis is very sensitive, it is expensive and currently unable to fully encompass the ever-
50 expanding range of growth-promoting compounds that are often administered as cocktails of several
51 low-dose drugs (Courtheyn et al. 2002). The advent of new designer drugs, together with the illicit
52 use of hormones, has highlighted the necessity of the development of new techniques to improve
53 the detection of growth promoter abuse in livestock. Bioassays have been developed as screening
54 tools for the determination of hormonal activities in a variety of matrices, such as urine (Divari et
55 al. 2010), feed (Bovee et al. 2006, Bovee et al. 2009), hair (Becue et al. 2011) and blood (Mooney
56 et al. 2009). In addition, indirect screening methods have been used to detect growth promoter use
57 in farm animals at the histopathological level (Biolatti et al 2003, Groot et al. 2007). These
58 screening methods depend on being able to identify, with relatively low costs and in a short time,

1
2
3 59 the morphological and functional changes in tissues caused by the anabolic drugs. The accessory
4
5 60 sex glands, such as the bulbourethral, prostate, major vestibular and mammary glands, have been
6
7 61 demonstrated to be very sensitive to the action of sexual hormones, even at low levels, resulting in
8
9 62 clear histological changes (Biolatti et al. 2003, Castagnaro and Poppi 2006, Biolatti 2009).
10
11 63 Recently, a novel “omics” approach has been developed (Feng et al. 2008, Schneider and Orchard
12
13 64 2011) to clarify the correlations and dependencies between molecular components in biological
14
15 65 systems. “Omics” sciences include a wide variety of fields including genomics, transcriptomics,
16
17 66 proteomics and metabolomics. In particular, transcriptomics is the study of the transcriptome, which
18
19 67 is also referred to as the expression profiling changes at specific times under specific environmental
20
21 68 conditions in a given tissue or cell population. The screening of the regulation and function of
22
23 69 anabolic sex steroids via modified gene expression levels in various tissues could be a new
24
25 70 approach for detecting treatments with unknown drugs or newly combined drug cocktails (Reiter et
26
27 71 al. 2007). The ligand-stimulated steroid hormone receptors, such as the oestrogen receptor (ER),
28
29 72 androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), and
30
31 73 mineralocorticoid receptor, form complexes with co-activators and general transcription factors as
32
33 74 well as recognise and bind hormone response elements in the regulatory regions of various
34
35 75 hormone-responsive genes. This induces the modulation of target gene transcription (Horie-Inoue et
36
37 76 al. 2006).
38
39
40
41
42
43
44

45 78 A number of novel gene expression profiles have been examined in different bovine tissues, and
46
47 79 some of these findings could be applicable in the field as routine screening methods (Toffolatti et al.
48
49 80 2006, Reiter et al. 2007, Carraro et al. 2009, Lopparelli et al. 2010, Giantin et al. 2010, Lopparelli et
50
51 81 al. 2011,). Recently, De Maria et al. (2010) investigated PR mRNA expression in bulbourethral
52
53 82 glands as a potential biomarker of illicit 17 β -oestradiol administration in veal calves and found that
54
55 83 it may be a promising new approach to improve the current residue control programmes.
56
57
58 84 Furthermore, PR gene over-expression following 17 β -oestradiol administration can be detected for
59
60

1
2
3 85 up to 7 days after the treatment is discontinued, whereas chemical methods can detect residues for
4
5 86 only up to a few hours after administration. Because PR gene over-expression has only been
6
7 87 reported in prepubertal veal calves, the aim of this work was to investigate its behaviour in the
8
9
10 88 prostate and bulbourethral glands of adult male cattle after treatment with 17β -oestradiol compared
11
12 89 to groups of animals treated with glucocorticoids or sex steroid cocktails and controls. This method
13
14 90 was primarily developed to facilitate the detection of the abuse of natural steroids, such as
15
16 91 oestradiol and testosterone, in adult animals, which is normally difficult due to the high
17
18 92 physiological production of endogenous hormones.
19

20
21 93
22
23 94 Because cytokeratins are involved in the genesis of hyperplastic and metaplastic lesions in the
24
25 95 prostate and bulbourethral glands of cattle treated with oestrogens, particularly 17β -oestradiol
26
27 96 (Lommen and Groot 1993, Groot et al. 2007, De Maria et al. 2010, Pezzolato et al 2011),
28
29 97 cytokeratin 5 (KRT5) gene expression of the accessory sex glands of experimental animals was also
30
31 98 examined to investigate the correlation between PR and KRT5 gene expression and epithelial
32
33 99 lesions in the same glands of treated animals.
34
35

36
37 100
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4 101 **MATERIALS AND METHODS**

5
6
7 102 Experimental design

8
9 103 In trial 1, 19 male Charollais beef cattle ranging from 17 to 22 months of age were divided into
10
11 104 three groups as follows: group A cattle (n=6) were administered five weekly intramuscular doses of
12
13 105 20 mg of 17 β -oestradiol, group B cattle (n=6) were administered DEX (40 doses of 0.7 mg per day
14
15 106 per os) and group K1 cattle (n=6) were untreated .
16
17

18
19 107

20
21 108 In trial 2, 24 Friesian male beef cattle ranging from 13 to 20 months of age were divided into three
22
23 109 groups as follows: group C cattle (n=8) were administered 200 mg of trenbolone acetate and 20 mg
24
25 110 of 17 β -oestradiol (Revalor-200, Intervet, USA) as a subcutaneous implant for 89 days plus DEX per
26
27 111 os (0.7 mg/day/animal) for 40 days, group D cattle (n=8) received Revalor-200 for 89 days in the
28
29 112 same manner and group K2 cattle (n=8) were untreated .
30
31

32
33 113

34 114 The animals were housed in boxes that measured 10 m \times 15 m with concrete floors lacking litter or
35
36 115 lateral partitions. All groups were housed in separate boxes and fed a concentrated diet consisting of
37
38 116 corn silage, corn, hay, and a commercial protein supplement; water was supplied ad libitum.
39
40 117 Hormone dosages were chosen according to the literature (Vanderwal et al. 1975; Meyer 2001;
41
42 118 Cannizzo et al. 2008, De Maria et al. 2010).
43
44

45
46 119

47 120 In trial 1, animals were euthanised after 7-day drug withdrawals; in trial 2, euthanasia was
48
49 121 performed 5 days after the last DEX treatment, with the implant remaining in place until euthanasia.
50
51 122 Target tissues were collected at slaughter and preserved for histological and molecular analyses.
52
53

54 123 This experiment was authorised by the Italian Ministry of Health and the Ethics Committee of the
55
56 124 University of Turin. Carcasses of treated animals were destroyed (2003/74/CE - DL 16 March 2006,
57
58 125 2 n. 158).
59
60

1
2
3 126

4
5 127 Tissue sampling and processing

6
7 128 Bulbourethral glands and prostates were collected from each animal. Tissue samples were fixed in
8
9 129 10% neutral buffered formalin at room temperature, processed and paraffin-embedded according to
10
11 130 routine histological procedures. Representative sections of each sample were stained with
12
13 131 haematoxylin–eosin (HE). Samples from all tissues were fixed in 5–10 volumes of RNAlater
14
15 132 Solution (Ambion, USA) for molecular studies and stored at 4°C overnight, after which the
16
17 133 supernatants were removed and frozen at –80°C for long-term storage.
18
19

20
21 134

22
23 135 Immunohistochemistry

24
25 136 Immunohistochemical staining of the basal cells was performed on the prostate and bulbourethral
26
27 137 sections after the inactivation of endogenous peroxidase and antigen retrieval with 10 mM citrate
28
29 138 buffer, pH 6.0 in a water bath at 98°C for 40 min. The sections were subsequently incubated with
30
31 139 the following antibodies: anti-PR monoclonal antibody (1:70) (Ab-2 clone hPRa, Thermo
32
33 140 Scientific, USA) for 60 minutes at room temperature, anti-ER α (1:50) (clone 1 D5, Dako,
34
35 141 Denmark) for 90 min at room temperature and anti-KRT5 (1:15) (clone RCK 103, Euro-
36
37 142 diagnostica, Sweden) for 90 min at room temperature. The immunostaining was visualised with the
38
39 143 HRP universal polymer (ABC Vectastain, Vector Lab, USA) by a Dako Autostainer.
40
41
42

43 144

44
45 145 Total RNA extractions and quantitative expression analyses of PR, ER α and KRT5

46
47 146 Several milligrams of each tissue were disrupted using a TissueLyser II (Qiagen, Germany) with
48
49 147 stainless steel beads in 1 mL of Trizol (Invitrogen, USA) and a DNA-free kit (Ambion, USA). RNA
50
51 148 concentrations were determined spectrophotometrically, and RNA integrities were evaluated using
52
53 149 an automated electrophoresis station (Experion Instrument, BioRad, USA). Total RNA was purified
54
55 150 from residual genomic DNA, and cDNA was synthesised from 1 μ g of total RNA using QuantiTect
56
57 151 Reverse Transcription (Qiagen, Germany). To determine the relative amounts of specific ER α and
58
59
60

1
2
3 152 KRT5 transcripts, the cDNA was subjected to quantitative polymerase chain reaction (qPCR)
4
5 153 (Kubista et al. 2006) using the IQ 5 detection system (BioRad, USA) and respective gene primers in
6
7 154 an IQ SYBR Green Supermix (BioRad, USA). Primer sequences were designed using Primer
8
9 155 Express (vers. 1.5) and Primer 3 (vers. 0.4.0) (31) (Table 1). The cyclophilin A (PPIA) gene was
10
11 156 used as a housekeeping gene control as previously reported (De Maria et al., 2010). To determine
12
13 157 the amount of PR transcripts, the cDNA was subjected to qPCR using TaqMan probes that were
14
15 158 specific for the PR and PPIA genes and labelled at the 5' ends with fluorescein and
16
17 159 hexachlorofluorescein, respectively (Table 1). The probes and relative primers were designed by the
18
19 160 software geNorm, which was available online (<http://medgen.ugent.be/genorm>).
20
21
22
23

24
25 162 The relative levels of gene expression were calculated using a relative quantification assay
26
27 163 according to the comparative C_t method ($\Delta\Delta C_t$ method) when the primer efficiencies were similar;
28
29 164 before each qPCR, we performed a validation experiment to demonstrate that the amplification
30
31 165 efficiencies of the target and reference genes were approximately equal. Then, the relative
32
33 166 abundances of each transcript that were normalised to the endogenous housekeeping gene transcript
34
35 167 (PPIA) and relative to the control sample, was recorded as $2^{-\Delta\Delta C_t}$ (fold increase), where $\Delta\Delta C_t = \Delta C_t$
36
37 168 (treated sample) - ΔC_t (control sample), and ΔC_t is the C_t of the target gene subtracted from the C_t
38
39 169 of the housekeeping gene (Livak and Schmittgen 2001, Pfaffl 2004, Wong and Medrano 2005.).
40
41
42
43

44 170

46 171 Statistical analyses

47
48 172 Statistical tests were performed using GraphPad InStat (vers. 3.05) statistical software (GraphPad
49
50 173 Inc., San Diego, CA, USA). The gene expression of PR, ER α , KRT5 was analysed by one-way
51
52 174 analysis of variance followed by the Bonferroni post hoc test; when Bartlett's test suggested that the
53
54 175 differences between the standard deviations of each group were significant, the non-parametric
55
56 176 Kruskal-Wallis test and Dunn's post hoc test were applied.
57
58
59
60

1
2
3 177 Grubbs' test was used to reveal potential outliers. A p value of < 0.05 was considered statistically
4
5 178 significant.

6
7 179

8
9 180 **RESULTS**

10 181 Gross findings and histopathology

11
12 182 Macroscopic examination of the accessory sex glands revealed no differences among the
13
14 183 experimental and control groups. In contrast, histological evaluation of group A cattle revealed
15
16 184 marked hyperplasia and metaplasia in the prostatic urothelium and similar but less severe lesions in
17
18 185 the glandular epithelium that were mostly localised to the peripheral portion of the gland, whereas
19
20 186 the epithelium of the ducts had similar features as those of the urothelium. In the bulbourethral
21
22 187 glands, diffuse hyper-secretion, cysts, hyperplasia and focal metaplasia were detected.

23
24 188 No hyperplasia or metaplasia was detected in the other experimental groups (B, C, D) or controls
25
26 189 (K1, K2) (Fig. 1), excluding one animal from group D that had focal metaplasia in its urothelium.
27
28 190 Lymphocytic infiltration in the urethral epithelium and glandular portion that was independent of
29
30 191 the hormonal treatment was observed in a few animals from all groups.

31
32 192

33
34 193 Immunohistochemistry

35
36 194 Immunohistochemical staining revealed widespread nuclear PR expression in the bulbourethral
37
38 195 gland, prostate epithelium and urothelium in all groups; however, this expression was markedly
39
40 196 increased in the animals of groups A, C and D. In group A, PR was strongly expressed in the
41
42 197 epithelial hyperplastic and metaplastic areas of the prostate and bulbourethral glands, and the positive
43
44 198 areas were mostly localised to the basal layer of the urothelium and glandular duct epithelium.
45
46 199 Animals in groups C and D exhibited diffuse PR-positive areas in both of the glands, the epithelium
47
48 200 and basal layer of the urothelium, whereas PR-positive areas were mostly apical in the glandular
49
50 201 ducts (Fig. 3).

202 In contrast, PR protein was generally weak and observed only in the periphery of the glands in the
203 control and DEX-treated animals. The nuclei of the urothelium were also diffuse and weakly
204 positive, whereas the epithelial ducts exhibited weak apical PR expression (Fig. 2).

205 Furthermore, in the cattle in groups A, C and D, PR protein expression was increased in the smooth
206 muscle cells, especially those around the urethra and ducts of the prostate.

208 ER α staining was evident in all nuclei of the excretory ducts and glandular epithelia of the prostate
209 in all animals, but ER α was more highly expressed in the urothelium than in other areas of the
210 gland. ER α staining of the bulbourethral glands was weaker than that of the prostate and was
211 observed in all animals (data not shown).

213 In both the prostate and bulbourethral glands of group A animals, KRT5 staining revealed the over-
214 expression of the cytoplasmic KRT5 protein in the hyperplastic and metaplastic epithelia. In the
215 urethra in particular, the entire urothelium was diffusely and strongly stained (Fig. 4).

216 In the prostates of animals in groups B, C and D, KRT5 positivity was limited to scattered basal
217 cells in both the glandular epithelium and urothelium, which was similar to the findings in the
218 control glands (Fig. 4). The same features were observed in the bulbourethral gland, in which the
219 basal epithelium of the excretory ducts and basal cells of the glandular acini exhibited staining
220 patterns that were similar to the control staining patterns (Fig. 4).

222 Molecular analysis

223 qPCR specific for PR, ER α , AR, GR, and KRT5 was performed to examine the expression levels of
224 the corresponding genes in the bulbourethral and prostate glands. Tables 2 and 3 reveal significant
225 up-regulation in the levels of PR, ER α , and KRT5 in both glands of group A animals that exhibited
226 histological changes. PR gene expression was also elevated in the bulbourethral glands of animals
227 in groups C and D, but the gene expression of ER α and KRT5 did not change; the same expression

1
2
3 228 profile was observed in the prostate gland, in which PR gene up-regulation was less marked. In
4
5 229 particular, the bulbourethral and prostate glands of group C animals had higher PR expression than
6
7 230 group D animals. The tested genes were not regulated by DEX in group B animals, and the
8
9 231 expression of AR and GR in particular did not change in any experimental group compared to those
10
11 232 in the controls.
12
13 233

16 234 **DISCUSSION**

18 235 As described in the introduction, the alternative indirect screening methods that are used to detect
19
20 236 hormonal treatment in farm animals are useful for direct analyses of residues. The present study
21
22 237 examined a novel strategy based on PR gene expression in adult male accessory sex glands in
23
24 238 response to 17 β -oestradiol that was administered either alone or in combination with other
25
26 239 molecules to reveal growth promoter abuse. In this experiment, in addition to the typical epithelial
27
28 240 hyperplastic and metaplastic changes that were observed in the prostate, urethra and bulbourethral
29
30 241 glands (Biolatti et al. 2003, Groot et al. 2007, De Maria et al. 2010), 17 β -oestradiol induced
31
32 242 significant PR transcriptional up-regulation in the prostate (25.07-fold) and bulbourethral glands
33
34 243 (23.42-fold). These data were confirmed by a protein expression analysis using
35
36 244 immunohistochemistry. Moreover, 17 β -oestradiol specifically induced the transcriptional up-
37
38 245 regulation of KRT5 and ER α . KRT5 is involved in the genesis of epithelial hyperplastic and
39
40 246 metaplastic lesions in accessory sex glands. No expression changes were observed in the GR and
41
42 247 AR. This is explained by the fact that 17 β -oestradiol only binds to ER α , which is one of two
43
44 248 specific ERs (α and β) that are members of the nuclear receptor super-family of ligand-regulated
45
46 249 transcription factors (Pettersson et al 2001, Paech et al 2007). The complex formed by ER α and an
47
48 250 oestrogen co-migrates from the cytosol into the nucleus, interacting with specific DNA sequences
49
50 251 that directly or indirectly activate or repress the target genes (Björnström et al 2005, Glidewell-
51
52 252 Kennedy et al 2005, Prins et al 2006, Cheskins et al 2007). The PR and ER α genes are regulated by
53
54
55
56
57
58
59
60

1
2
3 253 17 β -oestradiol (Ing NH and Tornesi MB 1997, Hess et al 2001, Luconi et al 2002). In breast cancer
4
5 254 and other oestrogen-regulated neoplasias, such as prostate cancer, the PR is a widely accepted
6
7 255 marker that indicates oestrogen action (Bonkhoff et al 2001). In the mouse model, exposure to
8
9
10 256 oestrogens induces metaplasia of the urethral epithelium, and PR over-expression (Risbridger et al
11
12 257 2001) is clearly detectable by immunohistochemistry. Therefore, PR gene expression is oestrogen-
13
14 258 dependent; in fact, group B animals treated with DEX did not exhibit any alterations in the mRNA
15
16 259 levels of the PR gene in their accessory sex glands. Furthermore, in support of the previous finding,
17
18 260 testosterone administration in veal calves did not up-regulate PR and ER α (De Maria et al 2010). It
19
20
21 261 is very likely that the innovative results of this work will be applied to the detection of PR over-
22
23 262 expression in adult male bovine, such as beef cattle, which have been treated with 17 β -oestradiol,
24
25 263 either alone or in combination with other hormones, such as androgens and glucocorticoids. In this
26
27
28 264 case, despite the low 17 β -oestradiol concentrations, PR mRNA and protein levels in the accessory
29
30 265 sex glands were significantly higher in 17 β -oestradiol-treated animals than in the untreated animals.
31
32 266 In contrast, the bulbourethral and prostate glands of the animals that were treated with the drug
33
34 267 cocktail did not exhibit any histological lesions. Consequently, KRT5 gene and protein expression
35
36 268 remained similar to those of the control. Similar results were observed for ER α . It is likely that the
37
38
39 269 presence of molecules other than 17 β -oestradiol and its low concentration in the implants
40
41 270 modulated the effects of the oestrogen hormone.
42
43
44 271

45
46 272 Immunohistochemistry with specific antibodies against the PR, ER α , and KRT5 proteins performed
47
48 273 on bulbourethral and prostate glands permitted the verification of the increased expression and
49
50 274 tissue localisations of the target proteins. The increased areas of PR positivity in the accessory sex
51
52 275 glands of animals in groups A, C, and D confirmed the gene expression data. The proteins were
53
54
55 276 expressed mainly in the urothelial basal cell layers as well as hyperplastic and metaplastic cells of
56
57 277 the glandular portion. This was more evident in group A cattle, in which histological changes were
58
59
60

1
2
3 278 observed. In the animals treated with the drug cocktail, the urethral epithelium appeared more
4
5 279 sensitive to the action of oestrogen and exhibited strongly positive regions, particularly in the basal
6
7 280 layers, whereas in the glandular ducts, PR over-expression was only observed in the epithelial cell
8
9 281 nuclei of the superficial layers and scattered groups of cells. In the bulbourethral glands, PR protein
10
11 282 staining was less evident, which was probably because the nucleus is flattened at the base of mature
12
13 283 secreting cells. In all of the groups of animals that were treated either with 17β -oestradiol alone or
14
15 284 in cocktails, the smooth muscle cells surrounding the urethra and ducts of the prostate were positive
16
17 285 for PR staining, confirming the physiological regulatory effects of progesterone on smooth cell
18
19 286 contraction that are analogous to its progestinic effects on the oviductal and uterine smooth muscle
20
21 287 cells (Schleicher et al., 1993). Unlike PR protein expression, KRT5 expression increased only in
22
23 288 hyperplastic and metaplastic cells, whereas in cattle in groups C and D, in which histological
24
25 289 changes were absent, KRT5 protein levels did not increase, which was confirmed at the mRNA
26
27 290 level. This observation leads to the conclusion that the PR is an effective biomarker for previous
28
29 291 oestrogen hormone treatment, even at low dosages and in the absence of clear target tissue
30
31 292 alterations.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

294 17β -Oestradiol induced a moderate up-regulation of $ER\alpha$ that was not sufficient to be detected by
295 immunohistochemical staining (data not shown), and the administration of other molecules in
296 combination with 17β -oestradiol inhibited the expected over-expression of its receptor in the
297 accessory sex glands. Trenbolone and DEX did not change the gene expression of AR and GR in
298 the prostate and bulbourethral glands, but the combination of DEX and Revalor-200 slightly
299 increased PR expression. An explanation for these increased mRNA levels could be related to the
300 promoter structure of the PR gene. Petz and Nardulli (2002) demonstrated that an oestrogen
301 response element half-site and two adjacent Sp1 sites in the human PR gene are involved in 17β -
302 oestradiol-mediated activation of the PR gene. As GRs also interact with Sp1-binding sites
303 indirectly via the transcription factor Sp1 to activate the transcription of the gene target (Ou et al.

1
2
3 304 2006), DEX could enhance the up-regulation of PR gene expression in combination with 17β -
4
5 305 oestradiol.

6
7 306

8
9
10 307 These data support the use of PR gene expression in accessory sex glands in a screening analysis to
11
12 308 identify animals that have been treated with drug cocktails. Moreover, this over-expression may not
13
14 309 necessarily be linked to histological lesions, and consequently, the PR mRNA levels could also be
15
16 310 utilised to support the histological test that has already been adopted for screening by the Italian
17
18 311 Ministry of Health.

19
20
21 312

22 313 **CONCLUSION**

23
24
25 314 These results demonstrate that 17β -oestradiol specifically induces an increase in the PR mRNA
26
27 315 levels in the prostate and bulbourethral glands of sexually mature adult beef cattle. PR over-
28
29 316 expression can be induced by 17β -oestradiol alone or in combination with other agents and is
30
31 317 independent of histological changes. This finding permits the application of this indirect biomarker
32
33 318 in a screening test to detect the illicit treatment of male cattle with oestrogens in the official residue-
34
35 319 monitoring programs.

36
37
38
39 320

40
41 321 The detection of oestradiol abuse by PR up-regulation is therefore a promising tool to further ensure
42
43 322 food safety and quality, considering that changes in the expression of this gene may persist for up to
44
45 323 a week after the suspension of 17β -oestradiol treatment.

46
47
48 324

49 325 **ACKNOWLEDGMENTS.**

50
51
52
53
54 326 This work was partially funded by the Regione Piemonte 2009 project “Prevenzione dell’uso di
55
56 327 anabolizzanti in zootecnia: le biotecnologie nello sviluppo di disciplinari per la qualità e salubrità
57
58 328 della carne e derivati” and the Ministero delle Politiche Agricole Alimentari and Forestali

1
2
3 329 SAFORISK project “Prevenzione dell’uso di anabolizzanti in zootecnia Creazione di Marchio a
4
5 330 difesa degli allevamenti italiani”. We would like to thank COOP ITALIA, Domenico Palmerini, and
6
7 331 Alessandra Sereno for the technical support provided and the Reference Center of Comparative
8
9 332 Pathology “Bruno Maria Zaini” of the Faculty of Veterinary Medicine.
10

11
12
13 333

14
15 334 **REFERENCES**

- 16
17 335 Abraham G, Gottschalk J, Ungemach FR. 2004. Possible role of dexamethasone in sensitizing the
18
19 336 beta-2-adrenergic receptor system in vivo in calves during con- comitant treatment with clenbuterol.
20
21 337 *Pharmacology*. 72:196–204.
22
23 338 Becue I, Bovee TF, Van Poucke C, Groot MJ, Nielen MW, Van Peteghem C. 2011. Applicability of
24
25 339 a yeast bioassay in the detection of steroid esters in hair. *Anal Bioanal Chem*. 399(3): 1031-9
26
27 340 Biolatti B, Cabassi E, Rosmini R, Groot M, Castagnaro M, Benevelli R, Gilioli G, Ghizzinardi A,
28
29 341 Giorni P, Mazzini C, Comellini F, Alberghini L, Zancanaro G, Canonizzo T, Amedeo S, Poppi L,
30
31 342 Cantoni A. 2003. Lo screening istologico nella prevenzione dell’uso di anabolizzanti nel bovino.
32
33 343 *Large Animals Review*. 2: 9-19.
34
35 344 Biolatti B. 2009. Lesioni indotte dai promotori di crescita illeciti negli animali da carne. In: Nebbia
36
37 345 *C. Residui di farmaci e contaminanti ambientali nelle produzioni animali* EdiSES ed. pp. 321-352
38
39 346 Björnström L. and Sjöberg M. 2005. Mechanisms of estrogen receptor signaling: convergence of
40
41 347 genomic and nongenomic actions on target genes. *Molecular Endocrinology* 19, 833-842
42
43 348 Bonkhoff H, Fixermer T, Hunsicker I, Remberger K. 2001. Progesterone receptor expression in
44
45 349 human prostate cancer: correlation with tumor progression. *The Prostate* 48:285-291.
46
47 350 Bovee TF, Bor G, Heskamp HH, Hoogenboom RL, Nielen MW. 2006. Validation and application
48
49 351 of a robust yeast estrogen bioassay for the screening of estrogenic activity in animal feed. *Food*
50
51 352 *Addit Contam A*. 23(6):556-68.
52
53
54
55
56
57
58
59
60

- 1
2
3 353 Bovee TF, Bor G, Heskamp HH, Lasaroms JJ, Sanders MB, Nielen MW. 2009. Validation and
4
5 354 application of a yeast bioassay for screening androgenic activity in calf urine and feed. *Anal Chim*
6
7 355 *Acta.* 637(1-2):225-34.
8
9 356 Cacciatore G, Eisenberg SWF, Situ C, Mooney MH, Delahaut P, Klarenbeek S, Huet A-C ,
10
11 357 Bergwerff AA, Elliott CT. 2009. Effect of growth-promoting 17 β -estradiol, 19-nortestosterone and
12
13 358 dexamethasone on circulating levels of nine potential biomarker candidates in veal calves.
14
15 359 *Analytica Chimica Acta.* 637: 351–359.
16
17 360 Cannizzo FT, Miniscalco B, Riondato F, Bollo E, Barbarino G, Giorgi P, Mazzini C, Biolatti B.
18
19 361 2008. Effects of anabolic and therapeutic doses of dexamethasone on thymus morphology and
20
21 362 apoptosis in veal calves. *Vet Rec.* 163:448-52.
22
23 363 Cantiello M, Giantin M, Carletti M, Lopparelli RM, Capolongo F, Lasserre F., Bollo E, Nebbia C,
24
25 364 Martin PG, Pineau T, Dacasto M. 2009. Effects of dexamethasone, administered for growth
26
27 365 promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf. *Biochem*
28
29 366 *Pharmacol.* 77:451–63
30
31 367 Carraro L, Ferraresso S, Cardazzo B, Romualdi C, Montesissa C, Gottardo F, Patarnello T,
32
33 368 Castagnaro M, Bargelloni L. 2009. Expression profiling of skeletal muscle in young bulls treated
34
35 369 with steroidal growth promoters. *Physiol Genomics.* 38(2):138-48.
36
37 370 Castagnaro M and Poppi L. 2006. Indirect Biomarkers of Illegal Anabolic Treatments: Five Years
38
39 371 of Activity Under the Microscope *Veterinary Research Communications.* 30: 105–108
40
41 372 Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, Van de Wiele M,
42
43 373 Vercammen J, De Wasch K. 2002. Recent developments in the use and abuse of growth promoters
44
45 374 *Anal. Chim. Acta.* 473: 71–82.
46
47 375 De Brabander HF, Antignac JP, Courtheyn D, Pinel G, Verheyden K, Le Bizec B, Mortier V,
48
49 376 Noppe H. 2007. Past, present and future of mass spectrometry in the analysis of residues of banned
50
51 377 substances in meat-producing animals *J. Mass Spectrom.* 42: 983–998.
52
53
54
55
56
57
58
59
60

- 1
2
3 378 De Maria R, Divari S, Spada F, Oggero C, Mulasso C, Maniscalco L, Cannizzo FT, Bianchi M,
4
5 379 Barbarino G, Brina N, Biolatti B. 2010. Progesterone receptor gene expression in the accessory sex
6
7 380 glands of veal calves. *Vet Rec.* 167(8):291-6.
8
9 381 Divari S, De Maria R, Cannizzo FT, Spada F, Mulasso C, Bovee TF, Capra P, Leporati M, Biolatti
10
11 382 B. 2010. A RIKILT yeast estrogen bioassay (REA) for estrogen residue detection in urine of calves
12
13 383 experimentally treated with 17beta-estradiol. *Food Addit Contam.* 27(1):19-28.
14
15
16 384 Enns DL and Tiidus PM. 2010. The influence of estrogen on skeletal muscle: sex matters. *Sports*
17
18 385 *Med* 40: 41-58.
19
20 386 Feng X, Liu X, Luo Q, and B-F Liu B-F. 2008. Mass spectrometry in system biology: an overview.
21
22 387 *Mass Spec Rev.* 27:635–660.
23
24 388 Giantin M, Lopparelli RM, Zancanella V, Martin PG, Polizzi A, Gallina G, Gottardo F, Montesissa
25
26 389 C, Ravarotto L, Pineau T, Dacasto M. 2010. Effects of illicit dexamethasone upon hepatic drug
27
28 390 metabolizing enzymes and related transcription factors mRNAs and their potential use as
29
30 391 biomarkers in cattle. *J Agric Food Chem.* 58:1342-9.
31
32 392 Glidewell-kenney C, Weiss J, Lee EJ, Pillai S, Ishikawa T, Ariazi EA and Jameson JL. 2005. ERE-
33
34 393 independent ER α target genes differentially expressed in human breast tumors. *Molecular and*
35
36 394 *Cellular Endocrinology* 245, 53-59
37
38 395 Groot MJ., Ossenkoppele JS, Bakker R., Pfaffl MW, Mever HH. and Nielen MW. 2007. Reference
39
40 396 histology of veal calf genital endocrine tissues: an update for screening on hormonal growth
41
42 397 promoters. *Journal of Veterinary Medicine A.* 54, 238-246
43
44 398 Hess RA, Bunick D, Bahr J. 2001 Oestrogen, its receptors and function in the male reproductive
45
46 399 tract – a review. *Molr Cell Endocrinol.* 178, 29-38
47
48 400 Horie-Inoue K, Takayama K, Bono HU, Ouchi Y, Okazaki Y, Inoue S. 2006. Identification of novel
49
50 401 steroid target genes through the combination of bioinformatics and functional analysis of hormone
51
52 402 response elements *Bioch. and Biophys. Res. Comm.* 339: 99–106
53
54
55
56
57
58
59
60

- 1
2
3 403 Ing NH, Tornesi MB. 1997. Estradiol up-regulates estrogen receptor and progesterone receptor gene
4
5 404 expression in specific ovine uterine cells. *Biol Reprod.* 56(5):1205-15.
6
7 405 Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjoback R,
8
9 406 Sjogreen B, Strombom L, Stahlberg A, Zoric N. 2006. The real-time polymerase chain reaction.
10
11 407 *Mol. Aspects Med.* 27: 95–125
12
13 408 Livak KJ and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time
14
15 409 quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods.* 25: 402-408.
16
17
18 410 Lommen A and Groot MJ. 1993. Combining two independent indirect methods as a new possibility
19
20 411 for screening the illegal use of growth promotants. *Zentralbl Veterinarmed A.* 40(4):271-82.
21
22
23 412 Lopparelli RM, Zancanella V, Giantin M, Ravarotto L, Cozzi G, Montesissa C, Dacasto M. 2010.
24
25 413 Constitutive expression of drug metabolizing enzymes and related transcription factors in cattle
26
27 414 testis and their modulation by illicit steroids. *Xenobiotica.* 40(10):670-80
28
29
30 415 Lopparelli RM, Zancanella V, Giantin M, Ravarotto L, Pozza G, Montesissa C, Dacasto M. 2011.
31
32 416 Steroidogenic enzyme gene expression profiles in the testis of cattle treated with illicit growth
33
34 417 promoters. *Steroids.* 76(5):508-16.
35
36 418 Luconi M., Forti G, Baldi E. 2002. Genomic and nongenomic effects of estrogens: molecular
37
38 419 mechanism of action and clinical implications for male reproduction. *J of Steroid Bioch and Mol*
39
40 420 *Biol* 80, 369-381
41
42
43 421 Meyer HH. 2001. Biochemistry and physiology of anabolic hormones used for improvement of
44
45 422 meat production. *APMIS.* 109: 1-8.
46
47 423 Mooney MH, Bergwerff AA, van Meeuwen JA, Lupp PB, Elliott CT. 2009. Biosensor-based
48
49 424 detection of reduced sex hormone-binding globulin binding capacities in response to growth-
50
51 425 promoter administrations. *Anal. Chim. Acta.* 637: 235–240.
52
53
54 426 Ou XM, Chen K, Shih JC. 2006. Glucocorticoid and androgen activation of monoamine oxidase A
55
56 427 is regulated differently by R1 and Sp1. *J Biol Chem.* 281:21512-25
57
58
59
60

- 1
2
3 428 Pettersson K. and Gustafsson J-A. 2001. Role of estrogen receptor β in estrogen action. Annual
4
5 429 Review of Physiology 63, 165
6
7 430 Petz LN and Nardulli AN. 2002a. Sp1 binding sites and an estrogen response element half-site are
8
9 431 involved in regulation of the human progesterone receptor A promoter. Mol Endocrinol 14: 972-985
10
11 432 Pezzolato M, Maurella C, Varello K, Meloni D, Bellino C, Borlotto L, Di Corcia D, Capra P,
12
13 433 Caramelli M, Bozzetta E. 2011. High sensitivity of a histological method in the detection of low-
14
15 434 dosage illicit treatment with 17 β -estradiol in male calves. Food Control 22:1668-1673.
16
17 435 Pfaffl MW. 2004. Quantification strategies in real-time PCR. In: A-Z of quantitative PCR (ed. S.A.
18
19 436 Bustin). International University Line (IUL). La Jolla, CA, USA, chapter 3 p. 87-112
20
21 437 Prins GS, Huang L, Birch L. and Pu Y. 2006. The role of estrogens in normal and abnormal
22
23 438 development of the prostate gland. Annals of the New York Academy of Sciences 1089:1-13
24
25 439 Reiter M, Walf AM, Christians A, Pfaffl MW, Meyer HH. 2007. Modification of mRNA expression
26
27 440 aftertreatment with anabolic agents and the usefulness for gene expression-biomarkers. Analytica
28
29 441 Chimica Acta. 586:73-81
30
31 442 Risbridger GP, Bianco JJ, Ellem SJ, McPherson SJ. 2003. Oestrogens and prostate cancer.
32
33 443 Endocrine Related Cancer 10: 187-191
34
35 444 Risbridger GP, Wang H, Frydenberg M, Cunha G. 2001. The metaplastic effects of estrogen on
36
37 445 mouse prostate epithelium: proliferation of cells with basal cell phenotype. Endocrinology 142:
38
39 446 2443-2450
40
41 447 Schleicher G, Sar M, Bidmon HJ, Stumpf WE. 1993. Progestagen nuclear binding sites in the male
42
43 448 genital tract of the mouse, studied by autoradiography. J Steroid Biochem Mol Biol. 46:389-93.
44
45 449 Schneider MV, Orchard S. 2011. Omics technologies, data and bioinformatics principles. Methods
46
47 450 Mol Biol. ;719:3-30.
48
49 451 Sinha-Hikim I, Roth SM, Lee MI and Bhasin S. 2003. Testosterone-induced muscle hypertrophy is
50
51 452 associated with an increase in satellite cell number in healthy, young men. Am J Physiol
52
53 453 Endocrinol Metab 285: E197-205.
54
55
56
57
58
59
60

- 1
2
3 454 Stephany RW. 2001. Hormones in meat: different approaches in the EU and in the USA. *APMIS*
4
5 455 103: S357–S363.
6
7 456 Toffolatti L, Rosa Gastaldo L, Patarnello T, Romualdi C, Merlanti R, Montesissa C, Poppi L,
8
9 457 Castagnaro M, Bargelloni L. 2006. Expression analysis of androgen-responsive genes in the
10
11 458 prostate of veal calves treated with anabolic hormones. *Domest Anim Endocrinol.* 30(1):38-55.
12
13 459 Vanderwal P, Berende IM, Sprietsma JE. 1975. Effects of anabolic agents on performance on
14
15 460 calves. *J of Anim. Science.* 41:978-985
16
17
18 461 Wong ML and Medrano JF. 2005. Real-time PCR for mRNA quantitation. *BioTechniques.* 39: 75-
19
20 462 85.
21
22
23 463
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 464 LEGEND4
5 465

6
7 466 Figure 1. Trial 1: Accessory sex glands of 17β -oestradiol-treated beef cattle (group A) and control
8
9
10 467 cattle (K1). The bulbourethral and prostate glands of group A animals exhibited epithelial
11
12 468 hyperplasia and metaplasia in the secreting portions (a and b) and metaplasia of the urothelium (c).
13
14 469 The bulbourethral (d) and prostate glands (e) and urethra (f) of group K1 animals did not have
15
16 470 lesions. HE (200 \times).
17

18 471

19
20 472

21
22
23 473 Figure 2. Trial 1 and Trial 2: Immunohistochemistry staining patterns of PR in the accessory sex
24
25 474 glands of experimental animals. The bulbourethral (a) and prostate glandular epithelia (b) of group
26
27 475 A cattle exhibited strong PR nuclear positivity, and PR positivity was observed, although less
28
29 476 strongly, in the same regions of the groups C (g and l) and D glands (h and m). The urothelia of
30
31 477 groups A (c), C (i) and D (n) cattle exhibited strong PR positivity, particularly in the basal layers.
32
33 478 The bulbourethral and prostate glands and urothelium of group B (d, e, and f) and control cattle (o,
34
35 479 p, and q) exhibited weak staining for the PR protein. Immunohistochemistry (200 \times).
36
37 480

38 481

39
40
41 482 Figure 3. Trial 2, group C: The epithelium of the prostate glandular duct (*) inserting into the
42
43 483 urethra (***) exhibited strong superficial PR positivity, whereas the PR protein expression in the
44
45 484 urethra was higher in the basal layers of the urothelium. Immunohistochemistry (100 \times).
46
47 485

48 486

49
50 487 Figure 4. Trial 1 and Trial 2: Immunohistochemistry of KRT5 in the accessory sex glands of the
51
52 488 experimental animals. The cytoplasm of the bulbourethral (a) and prostate (b) gland epithelial cells
53
54 489 urothelium (c) of group A cattle were diffusely and strongly positive for KRT5. In all of the other
55
56 490 groups, PR positivity was limited to scattered basal cells of the bulbourethral and prostate glands (d
57
58 491 and e: group B; g and h: group C; l and m: group D; o and p: group K1). KRT5 protein expression
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

490 in the urothelia of cattle in groups B (f), C (i), D (n), K1 (q) and K2 was principally localised to the
491 basal layer. Immunohistochemistry (200×)
492

For Peer Review Only

Table 1. Primer sequences and TaqMan probes for qPCR.

Gene	Primer	Sequence	GenBank no.
ER	Forward	5'-ACCAACCAGTGCACGATTGA-3'	NM_001001443.1
	Reverse	5'-TTCCGTATTCCGCCTTTCAT-3'	
AR	Forward	5'-CTTCTCTCCACCCGATGTGTG-3'	AY862875.1
	Reverse	5'-TCTCCATCCAGGGACCCAT-3'	
GR	Forward	5'-CCATTTCTGTTACGGTGTG-3'	AY238475
	Reverse	5'-CTGAACCGACAGGAATTGGT-3'	
KRT5	Forward	5'-GAGCCTTTGTTAGAGCAGTACATCAA-3'	NM_001008663.1
	Reverse	5'-CCTGCATATTCCTGAGCTCTGA-3'	
PR	Forward	5'-CCAGAGCCCACAGTACAGCTT-3'	AY656812
	Reverse	5'-CAGCTTCCACAGGTGAGGACA-3'	
	Taq Man	5'-CAGCCTGATGCTTCATCCCCACAG-3'	
	Probe		
PPIA	Forward	5'-GCCCCAACACAAATGGTT-3'	NM_178320
	Reverse	5'-CCCTCTTTCACCTTGCCAAAG-3'	
	Taq Man	5'-TGCTTGCCATCCAACCACTCAGTC-3'	
	Probe		

Table 2 . Fold gene expression changes of the five genes considered in the bulbourethral glands of the animal from the four experimental groups versus each control (group K1 for A and B group and K2 for C and D groups, to whom a control value of 1 was assigned)

Genes	Normalized fold expression ($2^{-\Delta\Delta Ct}$)			
	A	B	C	D
PR	23.42 ± 21.49***	0.83 ± 1.40	34.69 ± 60.82***	20.19 ± 30.08***
ER	3.09 ± 2.04***	0.8 ± 0.48	1.93 ± 1.79	2.39 ± 2.44
AR	2.01 ± 1.33	0.99 ± 0.73	2.59 ± 2.86	1.33 ± 1.15
GR	2.17 ± 1.59	0.81 ± 0.93	1.73 ± 1.69	1.30 ± 1.17
KRT5	9.94 ± 13.05**	0.84 ± 0.48	1.81 ± 1.87	1.13 ± 1.36

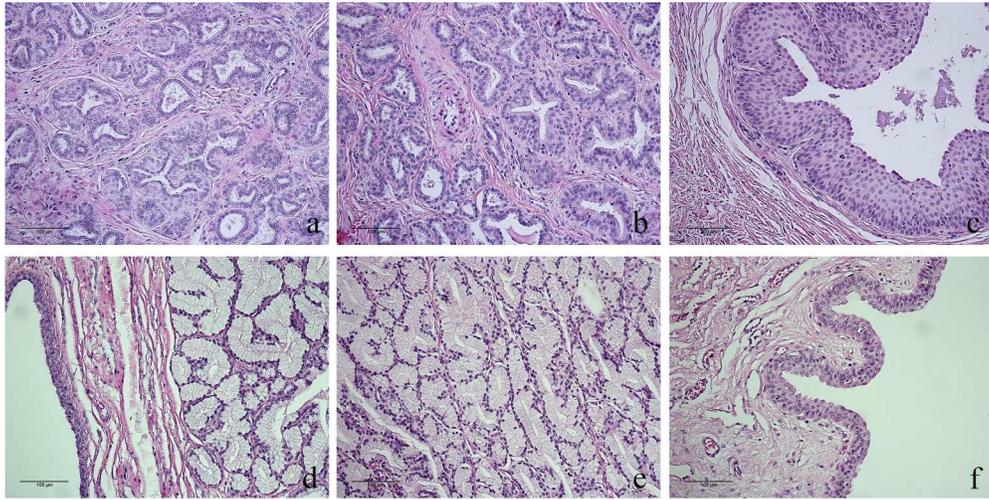
P<0.01; *P<0.001

1 Table 3 . Fold gene expression changes of the five genes considered in the prostate gland of the
 2 animal from the four experimental groups versus each control (group K1 for A and B group and K2
 3 for C and D groups, to whom a control value of 1 was assigned)

Genes	Normalized fold expression ($2^{-\Delta\Delta Ct}$)			
	A	B	C	D
PR	25.07 ± 15.90***	0.86 ± 1.07	7.11 ± 11.35*	5.38 ± 8.43*
ER	6.37 ± 6.96**	1.57 ± 1.58	1.07 ± 1.28	1.46 ± 1.96
AR	1.70 ± 0.94	1.40 ± 1.51	0.78 ± 1.06	1.15 ± 1.39
GR	1.82 ± 1.25	1.04 ± 1.58	0.24 ± 0.52	0.85 ± 1.12
KRT5	5.18 ± 6.74*	0.74 ± 0.92	1.21 ± 1.25	2.10 ± 3.33

*P<0.05; **P<0.01; ***P<0.001

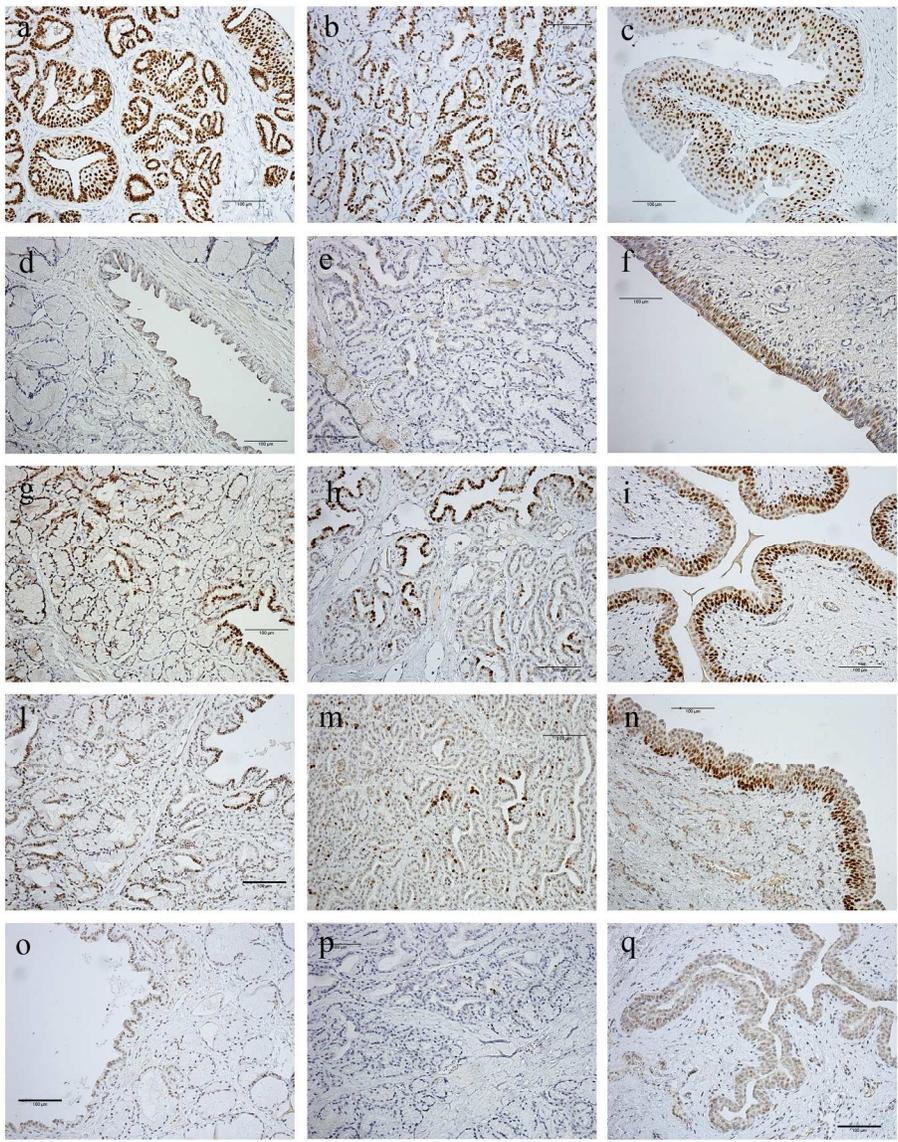
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



199x99mm (300 x 300 DPI)

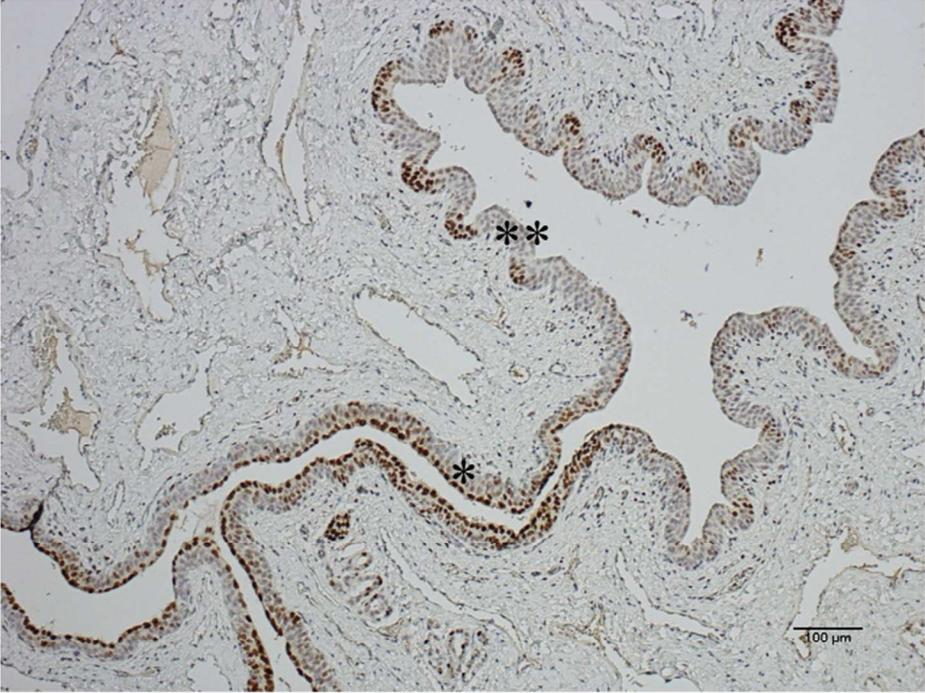
Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



180x239mm (300 x 300 DPI)

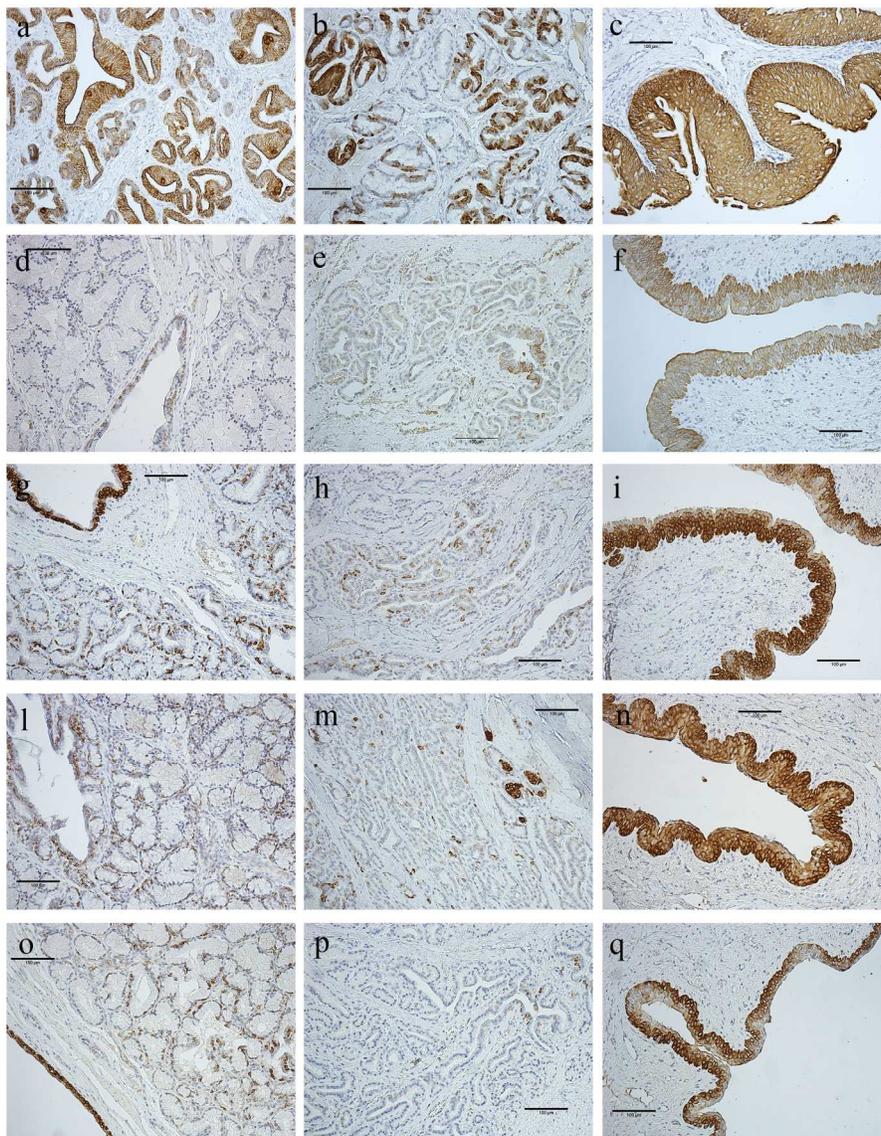
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



70x55mm (300 x 300 DPI)

Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



180x240mm (300 x 300 DPI)