Progesterone receptor gene expression in the accessory sex glands of veal calves

Progesterone receptor gene expression in the accessory sex glands of veal calves

R. De Maria, S. Divari, F. Spada, C. Oggero, C. Mulasso, L. Maniscalco, F. T. Cannizzo, M. Bianchi, G. Barbarino, N. Brina, B. Biolatti

This study investigated progesterone receptor (PR) cDNA expression in the testes, prostate and bulbourethral glands of prepubertal calves treated experimentally with high and low doses of 17b-oestradiol and with testosterone. Tissue samples were examined histologically and immunohistochemically for PR. Western blot analysis and quantitative PCR against PR was performed on cDNA and protein extracted from the same tissues. Bulbourethral glands from animals treated with low and high dosages of 17b-oestradiol had 39- and 429-fold increases of PR transcript, respectively, compared with controls. In the prostate there were 7.5- and 16-fold increases, respectively. Animals treated with testosterone showed no increases in PR transcript. The results demonstrate that 17b-oestradiol specifically induces marked overexpression of the PR gene and protein, particularly in the bulbourethral gland.

G. Barbarino, DVM,
Regione Piemonte, Settore Sanità
Animale Igiene Alimenti, Corso Stati
Uniti 1,10128 Torino, Italy
N. Brina, BAgr,
COOP Italia, Via del Lavoro 6/8,40033
Casalecchio del Reno (BO), Italy
E-mail for correspondence: raffaella.demaria@unito.it
Provenance: notcommissioned; externally peer reviewed

THE use of natural and synthetic hormones as growth promoters in animal production is banned in the European Union. Analytical meth-ods, such as liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry, are officially applied by national surveillance programmes (EC *1996,* Sabbe and others 2001). However, because these techniques seem to give unsatisfactory results (Groot and others 2007), new screening tools to reinforce veterinary control need to be developed. Histological and biotechnological approaches (Bovee and others 2005, Nielen and others 2006, Cannizzo and others 2007, 2008) can help to identify animals treated with growth promoters and improve the effectiveness of residue testing through targeted sampling of these animals (Mooney and others 2009). In this respect, genomics may be a valuable aid in elucidating the behaviour of single or multiple genes involved in steroid metabolism. Genes expressed differentially in the prostate and skeletal muscle of calves treated experimentally with combinations of growth promoters have been identified by microar-ray and quantitative PCR (qPCR) assay (Toffolatti and others 2006, Carraro and others 2009), and have been recommended as biomarkers to assist in residue monitoring.

17b-oestradiol is one of the major steroid hormones used ille-gally as growth promoters in cattle. There is increasing concern that environmental and dietary oestrogenic chemicals have adverse conse-quences for reproductive health(Golden and others 1999) by altering the normal developmentalprocess. 17b-oestradiol plays a primary role in the maintenance and function of the female and male reproductive tract, as well as in many other tissues, including bone, liver, digestive and cardiovascular tissues. A growing body of literature underscores the importance of 17b-oestradiol in regulating physi-ological processes in the male reproductive system (Chang and others 1999, Kurita and others 2000, Risbridger and others 2007) and in the development and progression of prostate cancer (Ho 2004, Singh and others 2008). The biological activity of oestrogens is mediated by binding to one of two specific oestrogen receptors (ERs), ERa and ERb. These are members of the nuclear receptor superfamily, a family of ligand-regulated transcription factors (Petterson and others 2001, Paech and others 2007). Contained in the cytosol, these receptors bind with oestrogens to form a complex that co-migrates into the nucleus and interacts with specific DNA sequences called oestrogen-responsive elements (EREs) that activate or repress target genes (Björnström and others 2005, Glidewell-Kennedy and others 2005, Prins and others 2006, Cheskis and others 2007). One of several oestrogen-regulated genes is the gene that encodes PR, which shows the typical ERE at the 5' end of the gene. It is widely accepted as a marker of oestrogenic activity in various human and mouse tissues (Risbridger and others 2001, Micevych and others 2008). PR overexpression has also been implicated in human prostate cancer, confirming the link between steroid hormone exposure and abnormal cell proliferation (Hess and others 2001, Luconi and others 2002, Sasaki and others 2002, Risbridger and others 2003). In the mouse model, exposure to oestrogens induces metaplasia of the urethral epithelium, and PR overexpression (Risbridger and others 2001) is clearly detectable by immunohistochemistry (IHC). Genes and proteins encoding PR characterised in the bovine female reproductive system show cellular behaviour and tissue distribution simi-lar to those in human beings (Schams and others 2003). The authors recently demonstrated that 17b-oestradiol induces overexpression of the PR transcript in bovine prostate stromal cells in vitro and in the prostate tissue of calves in vivo, suggesting that the PR gene could be used as a specific oestrogen-related biomarker in veterinary food inspection (De Maria and others 2009). The aim of this study was to investigate PR gene expression in the testes, prostate and bul-

bourethral glands of prepubertal calves treated experimentally with
different dosages of 17b-oestradiol and testosterone to determine whether the PR gene can be considered as a novel biomarker for the detection of oestrogen abuse in veal calves.

Materials and methods

Animals and experimental design

The study was carried out on 24 male Friesian veal calves recruited at 35 days of age. The calves were housed in boxes measuring 10 m x 15 m, with a concrete floor without litter or lateral partitions. They were tethered and f ed with liquid milk replacer twice a day (dry matter [DM] 0.95 per cent, crude protein [CP] 0.23 per cent, ether extract [EE] 0.21 per cent, ash 0.6 per cent, cellulose 0.001 per cent, vitamin A 25,000 iu/ kg, vitamin C 50 mg/kg, copper 5 mg/kg, vitamin D_3 5000 iu/kg, vita-min E 80 mg/kg). The amount of f eed was increased gradually up to 16 l/calf/day; after one month, 0.5 kg of barley straw (DM 0.90 per cent, CP 0.02 per cent, EE 0.01 per cent, ash 0.06 per cent, crude fibre 0.41 per cent) was added to the diet, as indicated by European Commission (97/182/EC) recommendations. At approximately 140 days of age, the calves were randomly assigned to one of four experimental groups and received intramuscular injections as follows: group A (n=6) received 10 mg/week of 17b-oestradiol, repeated four times; group B (n=6) received 35 mg/week of 17b-oestradiol, repeated six times; group C (n=6) received 175 mg/week of testosterone, repeated six times; and group D (n=6) animals were used as controls.

The dosages were chosen according to indications from the litera-ture (Vanderwal and others 1975, Meyer and others 2001 , Cannizzo

TABLE 1: PCR primers and TaqMan probes for qPCR

Gene (GenBank accession number)	Sequence (5′-3′)
PR (AY656812)	
Forward primer	CCAGAGCCCACAGTACAGCTT
Reverse primer	CAGCTTCCACAGGTGAGGACA
TaqMan probe	CAGCCTGATGCTTCATCCCCACAG
Cyclophilin (NM_178320)	
Forward primer	GCCCCAACACAAATGGTTCC
Reverse primer	CCCTCTTTCACCTTGCCAAAG
TaqMan probe	TGCTTGCCATCCAACCACTCAGTC
ERα (NM_001001443.1)	
Forward primer	ACCAACCAGTGCACGATTGA
Reverse primer	TTCCGTATTCCGCCTTTCAT

and others 2008, De Maria and others 2009). The animals were eutha-nased seven days after the last treatment. The experiment was author-ised by the Italian Ministry of Health and the Ethics Committee of the University of Turin. The carcases of treated animals were appro-priately destroyed.

Tissue sampling and processing

The bulbourethral glands, prostate and testes were collected from each animal. The tissue samples were fixed in 10 per cent neutral buff-ered formalin at room temperature, processed and paraffin-embedded according to routine histological procedures. Representative sections of each sample were stained with haematoxylin and eosin. Samples from all tissues were fixed in RNAlater (Ambion) for the molecular studies.

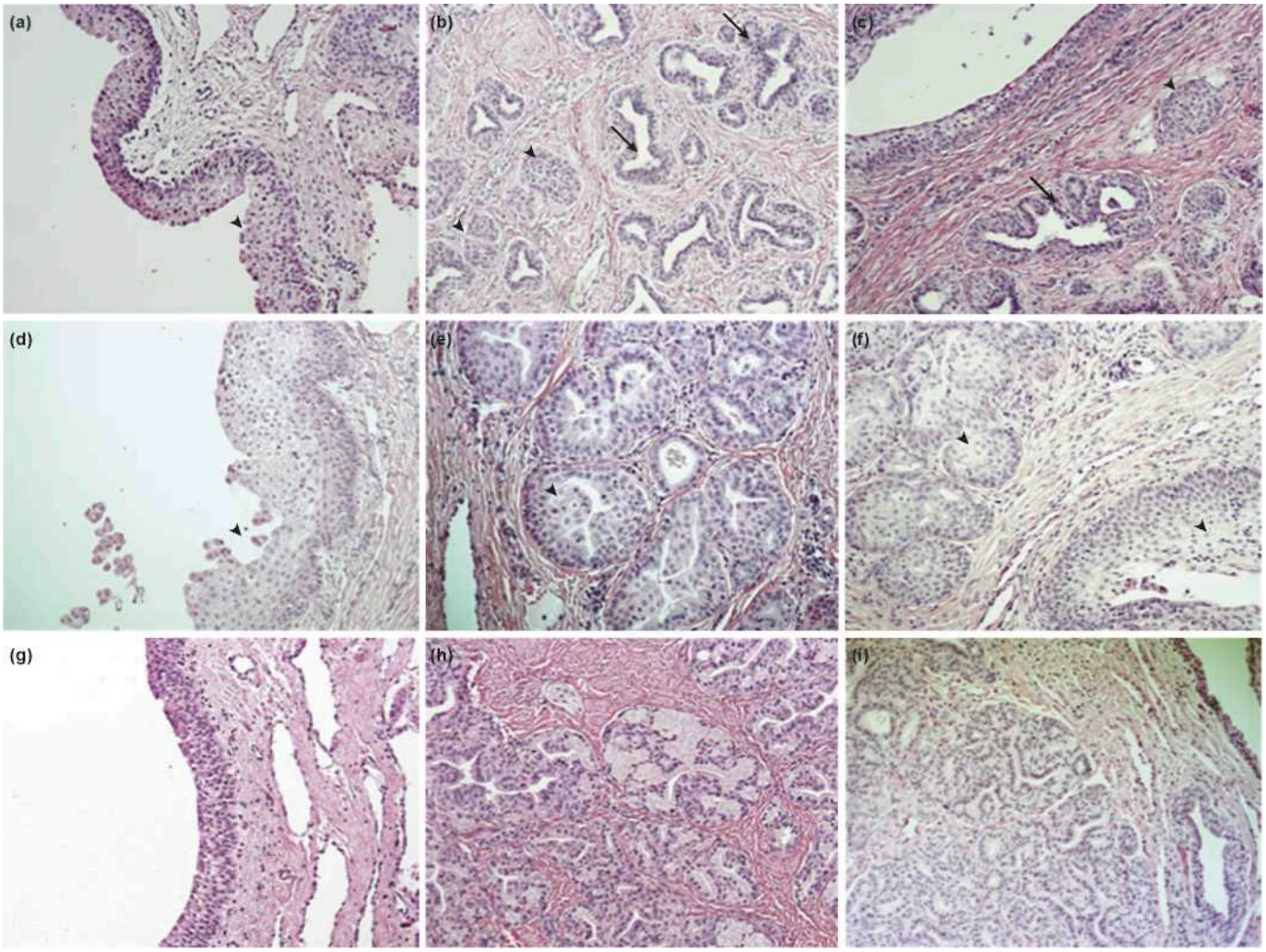

FIG 1 : Histological findings in the accessory sex glands of male calves treated with different doses of 17b-oestradiol (groups A and B), testosterone (group C) or untreated controls (group D). (a) Urethra, (b) glandular epithelium of the prostate, and (c) bulbourethral gland of a calf from group A, showing areas of epithelial hyperplasia (arrows) and metaplasia (arrowheads). (d) Urethra, (e) glandular epithelium of the prostate, and (f) bulbourethral glands of a calf from group B, with severe squamous metaplasia (arrowheads) involving most of the glands. (g) Urethra, (h) glandular epithelium of the prostate, and (i) bulbourethral glands of a control calf (group D), showing no visible lesions

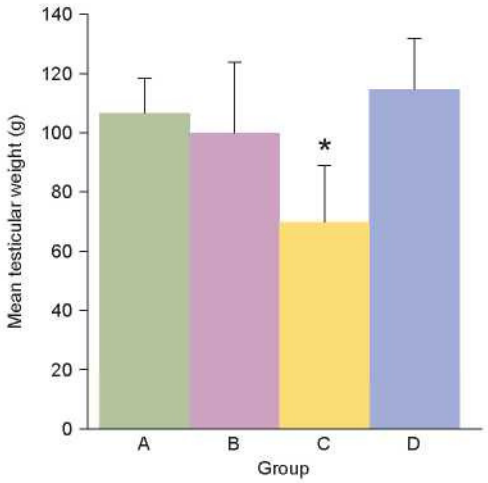

FIG2: Mean (sd) testicular weight in groups of calves treated with different doses of 17b-oestradiol (groups A and B), testosterone (group C) or untreated controls (group D). * Significant reduction versus controls (P<0.05)

Immunohistochemistry

The tissues from the bulbourethral glands, prostate and testes of all four groups were examined by IHC. Immunolocalisation of PR was performed using an anti-PR monoclonal antibody (Ab-2 clone hPRa; Thermo Scientific). Briefly, sections (4 *[ira)* were deparaffin-ised and rehydrated. Endogenous peroxidase activity was blocked by incubating the sections in 3 per cent hydrogen peroxide for 15 minutes. After repeated rinsing with PBS, the sections were immersed in citrate buffer (0.005M, pH 6.0) and heated in a water bath at 98°C for 45 minutes. The samples were incubated with primary antibody at a dilution of 1:50 for 120 minutes at room temperature. Immunostaining was visualised using the EnVision kit (DAKO) containing secondary antibody labelled with horse-radish peroxidase. Diaminobenzidine-hydrogen peroxide solution (DakoCytomation) was used as chromogen and applied for five minutes. The slides were then rinsed in distilled water to stop the reaction. After washing, the slides were counterstained with haematoxylin, dehydrated and mounted with a cover slip. Bovine mammary gland tissue was used as a positive control for the reaction (data not shown).

Total RNA extraction and quantitative expression of PR by qPCR

Total RNA from the prostate gland, testes and bulbourethral glands was extracted using the Trizol method (Invitrogen) and any residual genomic DNA was removed with a DNA-free kit (Ambion). RNA concentration and integrity was evaluated using an automated elec-trophoresis system (Experion Instrument; Bio-Rad). cDNA was synthesised with 400 ng of total RNA using ImProm-II Reverse Transcriptase (Promega) and random primers (Promega). To determine the amount of specific PR transcript, cDNA was subjected to qPCR by means of the IQ 5 detection system (Bio-Rad), using TaqMan probes specific for PR and the cyclophilin A gene labelled at the 5' end with fluorescein and hexachlorofluorescein, respec-tively (Table 1). Gene expression relative to the transcript specific for ERa was also evaluated in all samples. Primers and TaqMan probe sequences were designed using Primer Express Version 1.5 (Applied Biosystems) (Table 1). The cyclophilin A gene was used as a housekeeping gene according to literature data (Bustin 2002) and by means of the software geNorm available online (http://medgen. ugent.be/genorm). The amount of relative gene expression was cal-culated using a relative quantification assay corresponding to the comparative cycle threshold (Ct) method: the amount of target, normalised to an endogenous housekeeping gene (cyclophilin A gene) and relative to the calibrator (control sample), was then given by 2^{AACt} (fold increase), where AACt=ACt(sample) - ACt(control) and ACt is the Ct of the target gene subtracted from the Ct of the housekeeping gene.

Tissue extraction and Western blot analysis

One gram each of bulbourethral gland, prostate and testicular tissue was transferred into five volumes of PBS containing phenylmeth-anesulphonylfluoride and protease inhibitors. The mixture was homogenised in an ice bath with Ultra-Turrax equipment for one minute and kept in ice water for one hour. Afterwards, SDS was added to a final concentration of 5 per cent and the samples were boiled for 10 minutes. After centrifugation for 10 minutes at 3500 *g,* the concentration of protein in the supernatant was determined using a BCA protein assay kit (Pierce).

From each sample, 30 lLLg of total protein underwent acrylamide electrophoresis through 8 per cent gel at under-reducing conditions (for one hour at 190 V) and was transferred on to nitrocellulose mem-branes (Bio-Rad). To block non-specific sites, the membranes were

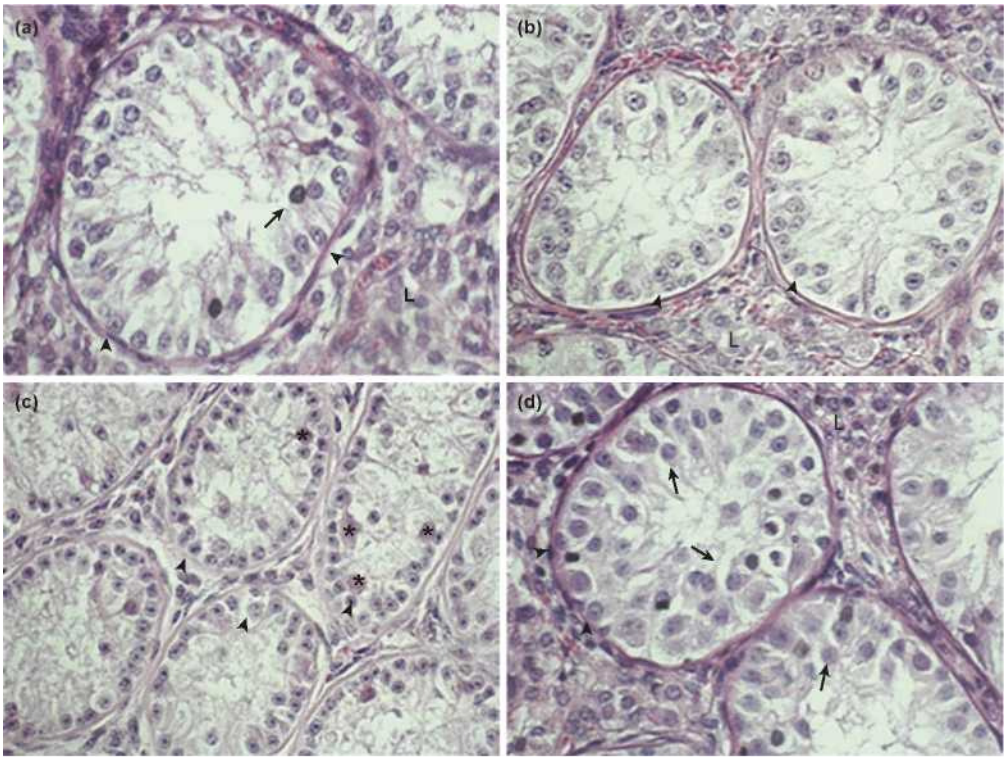

FIG 3: Histological findings in the testes. (a) Seminiferous tubules, germ cell types confined to spermatogonia (arrowheads) and primary spermatocytes (arrows) in a sample from a calf in group A. (b) Only spermatogonia (arrowheads) were present and germ cell lines show severe degeneration characterised by hydropic degeneration of spermatogonia, spermatocytes and Sertoli cells in a sample from a calf in group B. (c) Spermatogonia (arrowheads) and many degenerated cells (asterisks) in the lumen of the reduced seminiferous tubules in a sample from a calf in group C. There is reduced interstitium and volume of Leydig cells (L) in all three compared with a sample from a calf in the control group D (d), which has a normal pattern of germ cell differentiation. Haematoxylin and eosin. x 600

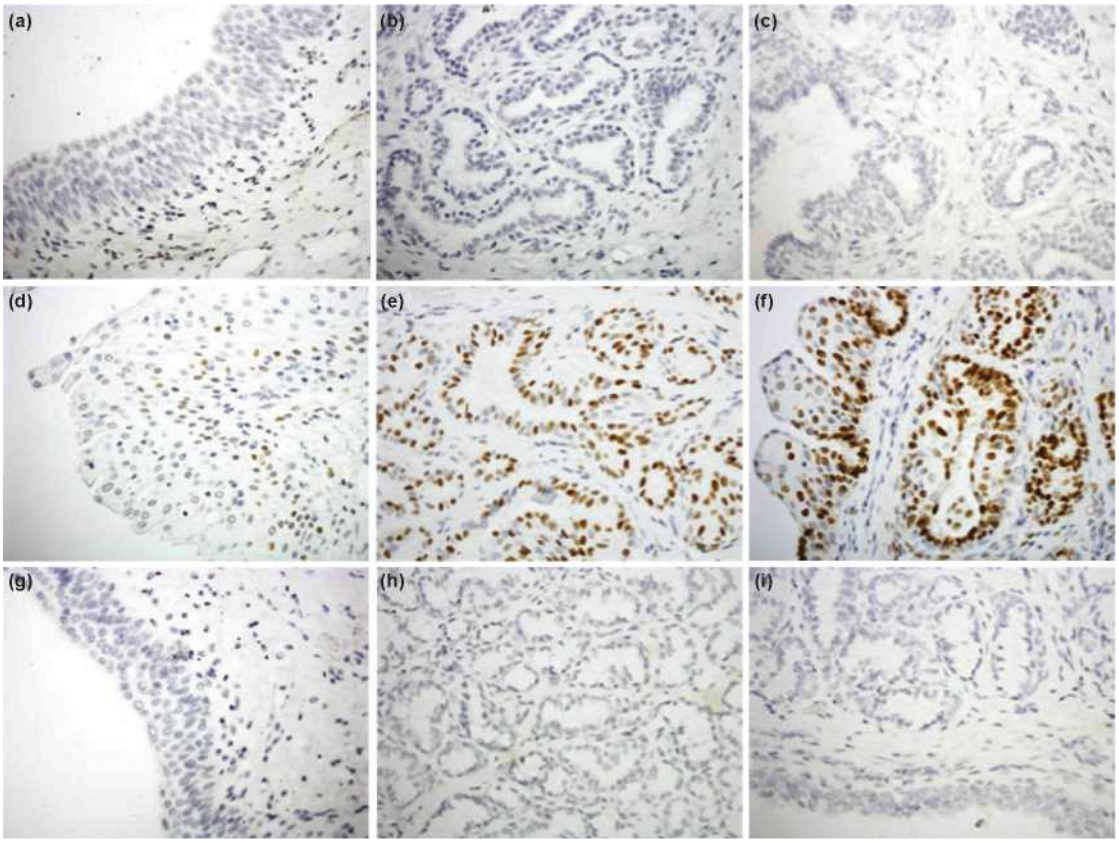

FIG4: Immunohistochemistry staining patterns of progesterone receptor (PR) in the accessory sex glands of male calves. Samplesfrom the prostate and bulbourethral glands of group A (a, b, c) show no positivityto PR. Slight positivity is visible in the basal cell nuclei of the urothelium of group B (d), and strong positive staining is evident in glandular epithelium of the prostate and bulbourethral glands from group B (e, f). Samples from the prostate and bulbourethral glands from control group D (g, h, i) show no positivityto PR. Immunohistochemistry. x400

incubated in PBS with 0.05 per cent Tween-20 (PBS-T) with 1 per cent non-fat dried milk for one hour at room temperature. The mem-branes were then incubated in PBS-T containing primary antibody against PR (Ab-2 clone hPRa; Thermo Scientific) at a dilution of 1:200 overnight at 4°C. After washing in PBS-T, the membranes were incubated with secondary anti-mouse horseradish peroxidase-linked antibody diluted to 1:1000 in PBS-T for one hour.

Finally, the membranes were washed in PBS-T for 30 minutes and incubated in enhanced chemiluminescence reagents (Super Signal West Pico Mouse IgG Detection Kit; Thermo Scientific) for 30 seconds or one minute, and exposed to CL-XPosure Film (Thermo Scientific). Tubulin was used as a housekeeping protein (data not shown).

Statisticalanalysis

Statistical tests were performed using GraphPad 3.05 statistical software. The data set of testis weight was initially subjected to the Kruskal-Wallis test (non-parametric analysis of variance) followed by post-hoc Dunn's multiple test. PR gene expression was evaluated by comparing the ACt values of the treated groups versus the control group using the unpaired *t* test with Welch's correction. $P<0.05$ was accepted as statistically significant in all tests.

Results

Gross findings and histopathology

Prostate and bulbourethral glands

Macroscopic examination of the accessory sex glands showed no dif-ferences among the four groups. Histological evaluation of the prostate and bulbourethral glands of animals in groups A and B revealed typical extensive epithelial hyperplasia to severe squamous metaplasia in either the urothelium or the glandular epithelium (Fig 1), associated with hypersecretion and cystic dilation of ducts in two animals. Marked fibro-sis causing thickening of the interstitium was also found in the prostate of the animals in groups A and B. Group C animals demonstrated no significant lesions in the accessory sex glands. No basal cell proliferation or squamous metaplasia was observed in the controls (group D).

Testes

Macroscopic examination of the testes of the treated animals (groups A, B and C) revealed reduced testicular size and weight, which was greatest in group C (Fig 2). 17b-oestradiolinduced a slight reduction in the testicular weight of the animals in groups A and B, although the difference was not statistically significant. Testosterone propi-onate induced a significant reduction in testicular weight ($P<0.05$). The mean weight of the testes of each animal was 106 g, 99 g, 70 g and 114 g in groups A, B, C and D, respectively.

Histological evaluation of the testes from the three treated groups showed an interruption of germ cell line development (Fig 3a, b, c). The most severe impairment was observed in group C, where the germ cells were reduced to a layer of spermatogonia, along with some degenerated dark cells free in the lumen of the seminiferous tubules. There was a marked decrease in tubule size, and a reduction in the volume of the interstitium and Leydig cells (Fig 3c). The seminiferous tubules of the controls displayed a full complement of seminal cells undergoing differentiation typically present in calves of that age (Fig 3d).

Immunohistochemistry

Immunohistochemical staining demonstrated PR expression in the bulbourethral gland, prostate epithelium and urothelium from the group B animals but not in those from group A, which presented hyperplastic/metaplastic lesions (Fig 4a, b, c). Conversely, the nucleus of the glandular epithelial cells of the prostate from group B showed dark brown-staining positivity to PR (Fig 4e); positivity was stronger in the bulbourethral gland (Fig 4f) and light nuclear positivity was visible in the basal cells of the urothelium (Fig 4d). No PR expression was observed in the accessory sex glands from animals in groups C and D animals (Fig 4g, h, i). The testes of all three experimental groups were negative for PR staining.

Molecular analysis

qPCR specific for PR cDNA was performed to show the expression of PRcDNA in the bulbourethral glands, prostate and testes of each ani-mal from all four groups. The bulbourethral gland tissues from groups A and B showed a 39- and 429-fold increase in PR transcript, respectively, compared with the controls. The prostate tissues from groups A and B showed a 7.5- and 16-fold increase in PR transcript, respectively, compared with the controls. Testicular tissues from groups A, B and C showed no increase in PR transcript (Fig 5a).

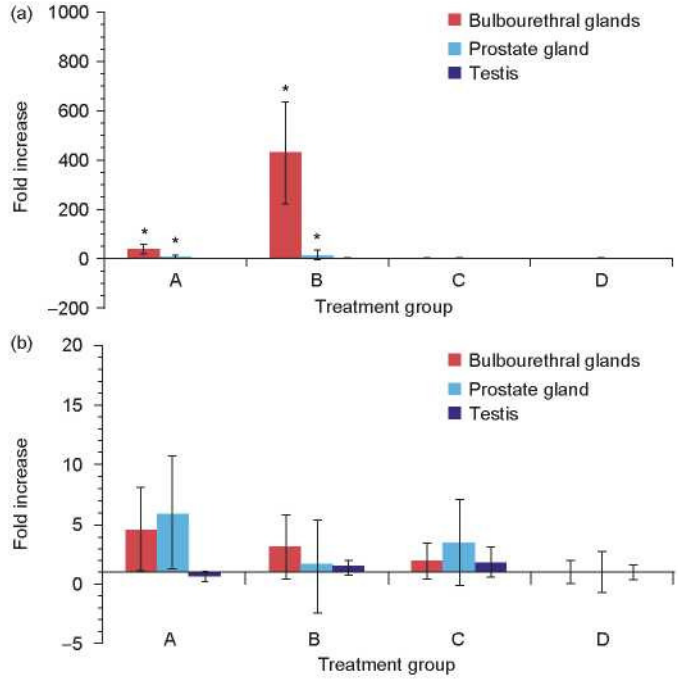

FIG 5: Quantification by real-time PCR. (a) PR gene expression and (b) ERa gene expression in the bulbourethral gland, prostate and testicular tissue from each treatment group. * Statistically significant difference ($P<0.05$) compared with controls

To better understand whether oestrogens are able to regulate ERa, its expression was evaluated by qPCRin all tissues of the experimental groups. As shown in Fig 5b, all tissues expressed ERa, and its expression did not change significantly after 17b-oestradiol or testosterone treatment.

To determine whether there was a correlation between cDNA transcript and protein synthesis of PR in the prostate and bulbourethral glands of the animals treated with 17b-oestradiol, Western blot analysis for PR was performed on tissue lysates of group B. This showed a higher PR gene expression than in the control group. Only prostate and bulbourethral gland tissues overexpressed PR protein (116 kDa), confirming the qPCR results, while the testes were negative. PR protein was recognised by the same antibody that was employed in the IHC procedure.

Discussion

The use of screening methods based on the detection of the bio-logical effects of growth promoters is a promising approach to assist in residue monitoring. The present study examined a novel detection strategy based on PR expression in the male accessory sex glands in response to 17b-oestradiol administration to reveal growth promoter abuse. In this study, besides inducing typical epi-thelial hyperplastic and metaplastic changes observed in the prostate, urethra and bulbourethral gland (Ruitenberg and others 1970, Kroes and others 1971, Hendry and others 1985) and testis atrophy (Noorafshan and others 2005), 17b-oestradiolinduced significant transcriptional upregulation of PRcDNA in the prostate (7.5- and 16-fold increases, respectively) and particularly in the bulbourethral gland (39- and 429-fold increases, respectively) in the animals from groups A and B. These results demonstrate that 17b-oestradiol specifically induced transcriptional regulation of PR cDNA in the prostate and bulbourethral glands of animals in groups A and B, while no changes in PR transcription were observed in these tissue types in animals from group C, which shows that PR is specifically upregulated by 17b-oestradiolin the prostate and the bulbourethral glands in a dose-dependent way. This was particularly evident in the bulbourethral gland, where the response was greater than in the prostate tissue.

The testes, prostate and bulbourethral glands are all oestrogen-responsive tissues, since they express ERa cDNA, but only the bulbourethral glands and prostate of these male veal calves responded to experimental treatment with 17b-oestradiol by increasing the amount of mRNA transcript of the PR gene. This biological effect could be related to the higher ratio of epithelium to interstitium in the bulbourethral gland compared with the prostate.

PR expression was found to be unchanged in the testes of the treated animals, which agrees with recently published data that demonstrated that oestrogens induce a non-genomic pathway in the testes but do not modify transcriptional profiling (Wu and others 2008).

In the present study, the high level of mRNA encoding PR was confirmed by protein expression detected by IHC and Western blot analysis. PR positivity was found only in the prostate, urethra and bulbourethral tissues from the group B animals treated with high doses of 17b-oestradiol, while the same tissues from the group A animals treated with low dosages were negative. PR positivity was stronger in the nuclei of the epithelium of the bulbourethral glands than in either the prostate or the urothelium. Western blot analysis using the same antibody as that for the IHC analysis identified a protein (116 kDa) in both the bulbourethral gland and the prostate from group B ani-mals, corresponding to isoform A of PR, which has previously been described in human and bovine mammary glands (Schams and others 2003).

The results of the present study suggest that PR could be used as a specific oestrogen-related biomarker to assist in residue monitoring specifically in prepubertal male veal calves, in which the bulbourethral gland can be considered as a specific target organ. Gene overexpression following 17b-oestradiol administration may be detected up to seven days after treatment is discontinued, while chemical methods may reveal residues only up to a few hours after administration. Further studies are needed to investigate the behaviour and expression of the PR gene in adult male cattle, as well as to study gene expression profiling in different cell populations, particularly in muscle cells and adipo-cytes, which are easily available for sampling, either from imported carcases or at the slaughterhouse.

Acknowledgements

This work was partially funded by COOP ITALIA, Regione Piemonte 2004 project, ‘Applicazione della genomica nel controllo dell'uso di anabolizzanti in zootecnia: identificazione di markers genetici innovativi’ and Ministero delle Politiche Agricole Alimentari e Forestali, project SAFORISK. The authors thank Domenico Palmerini and Alessandra Sereno for the technical support provided, and the Reference Centre of Comparative Pathology 'Bruno Maria Zaini’ of the Faculty of Veterinary Medicine, University of Torino, Italy.

References

BJÖRNSTRÖM, L. & SJÖBERG, M. (2005) Mechanisms of estrogen receptor sign-aling: convergence of genomic and nongenomic actions on target genes. *Molecular Endocrinology 19,* 833-842

BOVEE, X E H., HESKAMP, H. H., HAMERS, A. R. M., HOOGENBOOM, L. A. E & NIELEN, EIE (2005) Validation of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein, for the screening of estrogenic activity in calf

BUSTIN, S. A. (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and *problems. Journal ofMolecular Endocrinology 29,* 23-39

CANNIZZO, E T., MINISCALCO, B., RIONDATO, F., BOLLO, E., BARBARINO, G., GIORGI, P., MAZZINI, C. & BIOLATTI, B. (2008) Effects of anabolic and therapeutic doses of dexamethasone on thymus morphology and apoptosis in veal calves. *Veterinary Record 163,*448-452

CANNIZZO, E X, ZANCANARO, G., SPADA, F., MULASSO, C. & BIOLATTI, B. (2007) Pathology of the testicle and sex accessory glands following the administration of boldenone and boldione as growth promoters in veal calves. *Journal of Veterinary Medical Science 69,* 1109-1116

CARRARO, L., FERRARESSO, S., CARDAZZO, B., ROMUALDI, C., MONTESISSA, C., GOTTARDO, F., PATARNELLO, T., CASTAGNARO, M. & BARGELLONI, L. (2009) Expression profiling of skeletal muscle in young bulls treated with steroidal growth promoters. *Physiological Genomics* 38, 138-148

CHANG, SY8i PRINS, G. S. (1999) Estrogen receptor-b: implications for the prostate gland. *Prostate* 40, 115-124

CHESKIS, B.*J.,* GREGER, J. G., NAGPAL, S. & FREEDMAN, L. P. (2007) Signaling by

DE MARIA, R., DIVARI, S., GORIA, M., BOLLO, E., CANNIZZO, E T., OLIVERO, M., BARBARINO, G. & BIOLATTI, B. (2009) 17b-oestradiol-inducedgene expression in cattle prostate: biomarkers to detect illegal use of growth promoters. *Veterinary Record*

164, 459-464
EC (1996) Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repeating Directives 81/602/EEC, 88/146/EEC and 88/299/ EEC. *Official Journal of the European communities* **L125,** 3-9
GLIDEWELL-KENNEY, C., WEISS,*J.,* LEE, E.*J.,* PILLAI, S., ISHIKAWA, T., ARIAZI, E. A. & JAMESON, J. L. (2005) ERE-independent ERa target genes differentially expressed in human breast tumors. *Molecularand cellular Endocrinology* 245, 53-59
GOLDEN, A. L., MOLINE, J. M. & BAR-CHAMA, N. (1999) Male reproduction and environmental and occupational exposures: a review of epidemiologic methods. *Salud Pública De México* 41, 93-105
GROOT, M. *J.,* OSSENKOPPELE, J. S., BAKKER, R., PFAFFL, M. *W.,* MEYER, H. H. & NIELEN, M. W. (2007) Reference histology of veal calf genital and endocrine tis-sues - an update for screening on hormonal growth promoters. *Journal of Veterinary Medicine A* 54, 238-246
HENDRY, *W. J.,* III, ELLER, B. C., ORGEBIN-CRIST, M. C. & DANZO, B. J. (1985) Hormonal effects on the estrogen receptor system in the epididymis and accessory sex organs of sexually immature rabbits. *Journal of Steroid Biochemistry* 23, 39-49
HESS, R. A., BUNICK, D. & BAHR, J. (2001) Oestrogen, its receptors and function in the male reproductive tract - a review. *Molecular cell Endocrinology* 178, 29-38
HO, S. M. (2004) Estrogens and anti-estrogens: key mediators of prostate carcinogenesis and new therapeutic candidates. *Journal of cellular Biochemistry* 91, 491-503
KROES, R., BERKVENS, J. M., LOENDERSLOOT, H. J. & RUITENBERG, E. J. (1971) Oestrogen-induced changes in the genital tract of male calf. *Zentralblatt für Veterinärmedizin A* 18, 717-730
KURITA, T., LEE, K.*J.,* COOKE, P. S., TAYLOR, J. A., LUBAHN, D. B. & CUNHA, G. R. (2000) Paracrine regulation of epithelial progesterone receptor by estradiol in the mouse female reproductive tract. *Biology of Reproduction* 62, 821-830
LUCONI, M., FORTI, G. & BALDI, E. (2002) Genomic and nongenomic effects of estrogens: molecular mechanism of action and clinical implications for male reproduction. *Journal of Steroid Biochemistry and Molecular Biology* 80, 369-381
MEYER, H. H. (2001) Biochemistry and physiology of anabolic hormones used for improvement of meat production. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica* **109,** 1-8
MICEVYCH, P. & SINCHAK, K. (2008) Estradiol regulation of progesterone synthesis in the brain. *Molecularand cellularEndocrinology* **290,** 1-2
MOONEY, M. H., BERGWERFF, A. A., VAN MEEUWEN, J. A., LUPPA, P. B. & ELLIOTT, C. T. (2009) Biosensor-based detection of reduced sex hormone-binding globulin binding capacities in response to growth-promoter administrations. *Analytica chimica Acta* 637,235-240
NIELEN, M. W., BOVEE, T. F., HESKAMP, H. H., LASAROMS, J. J., SANDERS, M. B., VAN RHIJN, J. A., GROOT, M. J. & HOOGENBOOM, L. A. (2006) Screening for estrogen residues in calf urine: comparison of a validated yeast estrogen bioassay and gas chromatography-tandem mass spectrometry. *Food Additives and contaminants* 23, 1123-1131

NOORAFSHAN, A., KARBALAY-DOUST, S. & ARDEKANI, E M. (2005) High doses of nandrolone decanoate reduce volume of testis and length of seminiferous tubules in rats. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica* **113,** 122-125

PAECH, K., WEBB, P., KUIPER, G. G., NILLSSON, S., GUSTAFSSON, J. A., KUSHNER, P. J. & SCANLAN, T. S. (2007) Differential ligand activation of estrogen receptor ERa and ERb at AP1 sites. *Science* 277, 1508-1510

PETTERSSON, K. & GUSTAFSSON, J-A. (2001) Role of estrogen receptor b in estrogen action. *Annual Review of Physiology* 63, 165

PRINS, G. S., HUANG, L., BIRCH, L. & PU, Y. (2006) The role of estrogens in normal and abnormal development of the prostate gland. *Annals of the New York Academy of Sciences* 1089, 1-13

RISBRIDGER, G. P., BIANCO, J. *J.,* ELLEM, S. J. & MCPHERSON, S. J. (2003) Oestrogens and prostate cancer. *Endocrine Related cancer* 10, 187-191

RISBRIDGER, G. P., ELLEM, S. J. & MCPHERSON, S. J. (2007) Estrogen action on the prostate gland: a critical mix of endocrine and paracrine signaling. *Journal of Molecular Endocrinology* **39,** 183-188

RISBRIDGER, G. P., WANG, H., FRYDENBERG, M. & CUNHA, G. (2001) The meta-plastic effects of estrogen on mouse prostate epithelium: proliferation of cells with basal cell phenotype. *Endocrinology* 142, 2443-2450

RUITENBERG, E.*J.,* KROES, R. & BERKVENS, J. (1970) Evaluation of the 'prostate test' in checking the administration of oestrogens in the calf. *Zentralblatt für Veterinärmedizin A* 17, 351-357

SABBE, J. & VAN DERBEKEN, T. (2001) BUFALAW2001: The Illegal Use of Growth Promoters in Europe. Comparative report. Brussels, November 15 to 16, 2001

SASAKI, M., TANAKA, Y., PERINCHERY, G., DHARIA, A., KOTCHERGUINA, I., FUJIMOTO, S. & DAHIYA, R. (2002) Methylation and inactivation of estrogen, progesterone, and androgen receptors in prostate cancer. *Journal of the National cancer Institute* 94, 384-390

SCHAMS, D., KOHLENBERG, S., AMSELGRUBER, *W.,* BERISHA, B., PFAFFL, M. W. & SINOWATZ, E (2003) Expression and localisation of oestrogen and progesterone receptors in the bovine mammary gland during development, function and involution. *Journal of Endocrinology* 177, 305-317

SINGH, P. B., MATANHELIA, S. S. & MARTIN, E L. (2008) A potential paradox in prostate adenocarcinoma progression: oestrogen as the initiating driver. *European Journal of cancer* 44, 928-936

TOFFOLATTI, L., ROSA GASTALDO, L., PATARNELLO, T., ROMUALDI, C., MERLANTI, R., MONTESISSA, C., POPPI, L., CASTAGNARO, M. & BARGELLONI, L. (2006) Expression analysis of androgen-responsive genes in the prostate of veal calves treated with anabolic hormones. *Domestic Animal Endocrinology* 30,38

VANDERWAL, P., BERENDE, I. M. & SPRIETSMA, J. E. (1975) Effects of anabolic agents on performance on calves. *Journal of Animal Science* 41, 978-985

WU, F., XU, R., KIM, K., MARTIN, J. & SAFE, S. (2008) In vivo profiling of estrogen receptor/specificity protein-dependent transactivation. *Endocrinology* 49, 5696-5705