Oxytocin precursor gene expression in bovine skeletal muscle is regulated by 17β-oestradiol and dexamethasone

Food Chem., 141(4):4358-66, 2013, doi: 10.1016/j.foodchem.2013.07.029.

Oxytocin precursor gene expression in bovine skeletal muscle is regulated by 17 β-oestradiol and dexamethasone

S. Divari[3]'*, P. Pregel[3], F.T. Cannizzo[3], L. Starvaggi Cucuzza[3], N. Brina[b], B. Biolatti[3]

'Department of Veterinary Science, University of Turin, Grugliasco, Turin, Italy [b]COOP Italia, Casalecchio del Reno, Bologna, Italy

Corresponding author. Tel: +39 Oil 670 9032; fax: +39 Oil 670 9031. E-mail address: sara.divari@unito.it (S. Divari).

Growth promoter administration, in livestock, potentially poses a major threat to public health, due to the potential endocrine and carcinogenic activity of residues, accumulating in edible tissues, such as skeletal muscle. Therefore, development of new screening tests and methods for the detection of illicit treatments of food animals would be useful. In this study the serum concentrations of oxytocin peptide were measured in beef cattle receiving 17p oestradiol, dexamethasone or placebo over a period of 40 days. Changes in gene expression of oxytocin precursor in skeletal muscle were also examined in these animals. Serum analysis using an oxytocin EIA kit indicated a significant up-regulation of the biosynthesis of this nonapeptide only in cattle after 17p oestradiol, but not after dexamethasone or placebo treatment. Quantitative PCR (qPCR) analysis showed a significant overexpression of the oxytocin precursor gene by 33.5 and 13.3-fold in cattle treated with 17p oestradiol and dexamethasone, respectively, in comparison to placebo treated animals. Regulation of gene expression by some myogenic regulatory factors in skeletal muscle was also evaluated in these animal groups, confirming the activity of both growth promoters on this gene. To investigate the use of the oxytocin precursor gene as biomarker for 17p oestradiol and dexamethasone treatment in beef cattle, an absolute quantification of this gene by qPCR was developed. A standard curve was generated and developed with TaqMan® technology and optimal criterion value, sensitivity and specificity of this screening method were established through ROC analysis. This analysis suggested that the up-regulation of oxytocin precursor gene expression in skeletal muscle tissue is a valid marker for detection of illicit 17p oestradiol and/or dexamethasone use in beef cattle. This method may serve as a novel diagnostic tool in the screening phase, and, if introduced in routine testing, may significantly improve overall efficacy and success of the food screening process ordered by state authorities.

1. Introduction

In several countries, growth promoters (GPs), such as sex hormones and glucocorticoids, are still illegally used, either alone or in combination, in livestock, particularly veal calves and beef cattle, in order to improve meat production. Residues of these substances may remain in food, especially meat, representing a potential health hazard for consumers. Officially approved analysis based on GC/MS or LC/MS methodologies, although specific and sensitive, are too time-consuming and expensive to be used on a routine basis. Since they are based on the physicochemical characteristics of drugs, they show limits, including the possibility of searching only a few molecules known instead of the hundreds introduced each year in the black market. In addition, another critical point is the absence of "legal levels" for GPs in compliance testing (Stephany, 2010).

The development of novel methods (Carraro et al., 2009; Divari et al., 2011b; Lopparelli et al., 2012; Stella et al., 2011) allowing indirect detection of illegal GP administration would enhance the efficiency and success rate of food screening and safety programmes established by state authorities. In particular, the tran-scriptomic approach to skeletal muscle tissue testing could help identify biomarkers suitable to detect GP-treated animals and to understand the molecular mechanisms underlying skeletal muscle growth induced by GPs. In fact, muscle development is under tight hormonal control including modulation by glucocorticoids as well as under gene control. Androgenic and oestrogenic steroids enhance muscle growth (Sipila & Poutamo, 2003; Sipila et al., 2001). Moreover, oestrogens play a significant role by stimulating muscle repair and regenerative processes including the activation and proliferation of satellite cells (Enns & Tiidus, 2010) and increasing muscle tissue concentrations of insulin growth factor (IGF1) (Dunn et al., 2003; Johnson, White, Hathaway, Christians, & Dayton, 1998). The latter plays a predominant role in supporting normal muscle growth (Sjogren et al., 1999), however, the basic mechanism of this action is not completely understood. Although repeated administration of synthetic glucocorticoids such as dexa-methasone (DEX) at therapeutic doses induce muscle atrophy (Carraro et al., 2009), little is known about the action of low dose glucocorticoid treatment on muscle tissues.

Many proteins in skeletal muscle are regulated by GPs, but their function in this tissue is poorly understood. For example, Carraro et al. (2009) examined gene expression profiles in skeletal muscle of bulls treated with low-dose DEX as compared to controls. Results of that study showed that the expression of several encoding odorant receptors was significantly up-regulated by DEX. De Jager et al. (2011) described an unexpected induction of oxytocin precursor gene (OXT) in skeletal muscle of Brahman steers treated with a mixture of sex hormones. In addition, the concentration of circulating OXT was increased by 50-fold in steers carrying a drug implant, compared with the controls, suggesting a role of oxytocin in skeletal muscle growth. The main site of OXT gene expression and hence source of circulating oxytocin is the hypothalamus, from where the transcribed peptide is transported to the posterior pituitary and then released into the systemic circulation (Gimpl & Fahrenholz, 2001). However, the oxytocin peptide is also synthes-ised in peripheral tissues, such as uterus, *corpus luteum* (Ivell, Bathgate, Walther, & Kimura, 1998), placenta (Gimpl & Fahrenholz, 2001), epididymis (Filippi et al., 2005), human prostate (Assinder & Nicholson, 2004) and testis (Thackare, Nicholson, & Whittington, 2006). Recently, the oxytocin peptide and oxytocin receptor (OXTR) have been found also in the heart, where the hormone may modulate cardiomyocyte differentiation (Jankowski et al., 2004). However, few data are reported in the literature on OXT and OXTR gene expression or oxytocin peptide synthesis in skeletal muscle, in particular following glucocorticoid administration in cattle.

In the present study, the serum oxytocin peptide concentration was measured in beef cattle following 17p oestradiol (pE2) and DEX treatment to determine the effect of illegal GP treatment on oxytocin synthesis and release into the circulation. Furthermore, molecular effects of pE2 and DEX on gene transcription in skeletal muscle was investigated, i.e., in particular the agents' effects on the regulation of OXT gene expression and some muscle regulatory factors (MRFs). To confirm that up-regulation of OXT gene expression in skeletal muscle is a valid biomarker for detection of pE2 and/or DEX treatment in beef cattle, a method for absolute quantification of this gene by qPCR was developed.

2. Materials and methods

2.1. Experimental design

Eighteen male Charolaise beef cattle, 17-22 months old, were randomly divided into three groups: group A (n = 6) received five doses of pE2 (20 mg/week/animal, IM), group B (n = 6) was administered DEX per *os* (0.7 mg/day/animal) for 40 days, and group C (n = 6) was the

control group that received a placebo. The animals were sacrificed seven days after the last treatment (Divari et al., 2011a and Uslenghi et al., 2013). Target tissue samples were collected at the slaughterhouse and preserved for subsequent molecular analyses. The study was approved by the Italian Ministry of Health and the Ethics Committee of the University of Turin. Carcasses of treated animals were disposed.

All experiments were carried out according to European Economic Community Council Directive 86/609 (EEC, 1986), recognised and adopted by the Italian Government (D.L 27/01/1992 no. 116).

2.2. Serum oxytocin

Blood samples were collected from each animal via jugular venipuncture into vacutainers (Terumo® Venoject®, Terumo Italia, Rome, Italy). The blood sampling was performed 35 days after the first GP treatment and at the time of slaughtering. Serum was harvested after centrifugation at 2000g for 20 min and immediately stored at —80 °C until analysis. The serum oxytocin peptide concentration was measured by enzyme immunoassay methodology using the EIA kit developed by Enzo Life Sciences (Farmingdale, NY, USA). The manufacturer reports a limit of detection (LOD) for this test kit of <12 pg/ml. Oxytocin was extracted from 0.5-ml serum aliquots using a solid-phase extraction (SPE), 500 mg C-18 Sep-Pack column (Supelco®, Sigma-Aldrich, St. Louis, MO, USA) on a vacuum manifold (Supelco®). An equal volume of 0.1% trifluoroace-tic acid (TFA) was added to the serum sample, and the sample cen-trifuged at 14,000g for 15 min at 4 °C to clarify the supernatant. The samples were transferred to the Sep-Pack column previously equilibrated with 3 ml of acetonitrile, followed by 15 ml of 0.1% TFA. The column was washed with 15 ml of 0.1% TFA and the peptides were eluted using 3 ml of acetonitrile: 0.1% TFA (60:40). Then samples were collected in a glass tube and evaporated to dryness under a stream of nitrogen gas; they were reconstituted in 1 ml of assay buffer and immediately thereafter measured. The absor-bance at 405 nm was read using a Microplate Reader 680 Model (BioRad, Hercules, CA, USA) and a standard curve was created using a four parameter logistic function. Since the recovery of the peptide from the extraction process can be variable, it is important to optimise any process to obtain optimal recoveries. Extraction efficiency was determined by spiking into paired samples and determining the recovery of this known amount of added oxytocin. In this case 250 pg of oxytocin were spiked into 0.5 ml of two control serum samples which were diluted with the equal volume of 0.1% TFA and then assayed in the kit.

2.3. Tissue sampling and processing

Samples of the sternocleidomastoid muscle were harvested from each animal after slaughtering. Pieces of 150-200 mg weight of each sample were immediately frozen in liquid nitrogen and kept at —80 °C for molecular studies.

2.4. Total RNA extraction and relative quantification of OXT, OXTR, and MRF gene expression by qPCR

Total RNA from each tissue sample was extracted using TRIzol reagent (Invitrogen, LifeTechnologies, Carlsbad, CA, USA), following the manufacturer's protocol. RNA quantity was determined by UV-visible spectrophotometry and the RNA integrity was verified by an automated gel electrophoresis system (Experion Instrument, BioRad). cDNA was synthesised from 1 µg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), which included a DNase reaction, thereby following the manufacturer's protocol.

To determine the relative amounts of specific OXTR and MRFs transcripts, the cDNA was subjected to qPCR (Kubista et al., 2006) using the IQ5 detection system (BioRad) and the IQ. SYBR Green Supermix (BioRad). Primer sequences of OXTR were designed using Primer 3 (vers. 0.4.0) (Rozen & Skaletsky, 2000) (Table 1) and the oligonucleotide primers of MRFs were designed as described in Shibata et al (2006). The cyclophilin A (PPIA) gene was used as a housekeeping gene control as previously reported (De Maria et al., 2010; Uslenghi et al., 2013). To determine the amount of OXT and PPIA transcripts, cDNAs were subjected to qPCR using TaqMan probes, specific for the OXT and PPIA genes. They were labelled at the 5' end with fluorescein (FAM) for the OXT probe and hexachlorofluorescein (HEX) for the PPIA probe; the quencher utilised for PPIA was Black Hole Quencher 1 (BHQ1) whereas the quencher for the OXT probe is unknown (Table 1), since the transcript of the TaqMan® Gene Expression Assay was used (Assay ID: BtO3217196_gl; Applied Biosystems, LifeTechnol-ogies). The probes and relative primers of PPIA were designed as previously described (De Maria et al., 2010).

The levels of gene expression were calculated using a relative quantification assay based on the comparative C_q method (AAC_q method) (Bustin et al., 2009) verifying that efficiencies of target and housekeeping gene amplification were similar. Then, the relative abundances of each transcript, normalised to the endogenous housekeeping gene (PPIA) and relative to the control sample, were recorded as $2^{\sim AACq}$ (fold increase) (Livak & Schmittgen, 2001; Pfaffl, 2004; Wong & Medrano, 2005).

2.5. *Cloning of OXT and PPIA amplicons into the pDRIVE plasmid vector and generation of recDNAs calibration curves*

Each amplicon, OXT gene and housekeeping PPIA gene, was mixed with the pDRIVE vector in a ligation reaction mixture for 30 min at 16 °C using a Qiagen PCR Cloning Kit (Qiagen), and the ligation product was transformed into QIAGEN EZ Competent Cells (Qiagen). The cell-vector mixture was incubated on ice for 5 min, heat shocked for 30 s at 42 °C in a water bath, and immediately transferred to ice for 2 min. The cells were then plated onto LB agar containing kanamycin, IPTG and X-gal and incubated at 37 °C. White recombinant colonies were picked and subjected to colony PCR to confirm the presence of the OXT or PPIA gene fragment. Positive colonies were grown in LB overnight. The bacteria were harvested, and plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen, Germany), in compliance with the manufacturer's instructions.

After purification, each recombinant plasmid DNA (recDNAoxr and $recDNA_{PPIA}$) was sequenced by BMR Genomics (Padova, Italy) using M13R and M13F primers to confirm its identity. RecDNAs obtained from the cloning reaction were stored at —80 °C and they were utilised for hundreds of standard curves. The concentration of each purified recDNA was calculated by multiple optical measurements at 260 nm using an Ultrospec 3100 Pro UV/vis spectrophotometer (Amersham Biosciences, USA). The total number of recDNAoxT and $recDNA_{PPIA}$ bases was, respectively, 3964 (pDrive vector, 3851 bp; OXT, 113 bp) and 3946 (pDrive vector, 3851 bp; PPIA, 95 bp), and it was multiplied by 650 Da (the average molecular weight of one DNA base pair) to determine each single-copy molecular weight (Da). Assuming that one Dalton is $1.67 \times 10^{\sim 24}$ g, the weight (g) of each recDNA was calculated; the number obtained was then used to calculate the amplicons $\mu l^{\sim 1}$ of purified product. RecDNAs were used as starting templates in the 20 µl qPCR reaction mix, and standard curves were generated using serial dilutions of recDNAs from 10^6 to 10^2 molecules.

The absolute qPCR reaction for OXT and PPIA amplification was performed together in the same well and in a final volume of 20 µl, containing 10 µl of 2x IQ. Multiplex Powermix (BioRad), 1 µl of each 6 µM primer for PPIA, 1 µl of 20 x TaqMan® OXT Gene Expression Assay

(Applied Biosystem), 1 µl of 2 µM TaqMan® PPIA probe (see Table 1), 1 µl of template DNA and 5 µl of nuclease-free water. The PCR cycling conditions consisted of a 3-min incubation at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s.

Absolute quantification by qPCR was performed using the iQ5 Detection System (BioRad). Each sample was amplified in triplicate.

Primer and TaqMan® probe specificity was documented by the Experion Instrument. The amplification efficiency, sensitivity and linearity of the absolute qPCR were evaluated using different starting amounts of the recDNA; these parameters were calculated for OXT and PPIA fragments separately, with two different TaqMan®probes. PCR amplification efficiencies (E) were calculated from the slopes of the standard curves using the following formula:

$E\% = [io(-\text{^}^{sl}{}^{o}P^{e_r}] - 1 \quad \times 100$ (Rasmussen, 2001).

Assuming a linear relationship between the Log of the OXT and the PPIA amplicon number present in the qPCR reaction mix and the quantification cycle (Cq) value, the equation of the standard curve was used to determine the detection limit of the qPCR reactions. The total number of PCR amplification cycles was set to 35.

To confirm the precision and reproducibility of the recDNA qPCR standards curves, the variation of intra- and inter-run standard curves was tested via a covariance analysis (ANCOVA); the P and *F* values were determined to test any significant difference between slopes and intercepts of two replicates (A and B) in each qPCR assay (intra-run) and between slopes and intercept of six standard curves developed in the three different qPCR assays (qPCR assay 1, 2, and 3) (inter-run) (Smith, Nedwell, Dong, & Osborn, 2006).

2.6. Absolute quantification of OXT gene expression in skeletal muscle of experimental animals

For the absolute quantification of OXT gene expression in each sample of the experimental animals, the OXT cDNA copy number was normalised to the PPIA cDNA copy number (OXT/PPIA). qPCR experiments to determine the variability in the absolute quantification of OXT and PPIA genes in the skeletal muscle samples within a single qPCR assay (intra-assay) and between multiple qPCR assays (inter-assay) were carried out. Therefore, three independent qPCR assays were performed as illustrated above (qPCR assay 1, 2, and 3), each individual qPCR assay containing three serial dilutions of an unknown sample, repeated four times. The OXT/PPIA ratio for each sample dilution was calculated from the standard curve of each qPCR assay. Calculation of variation was based on the change of OXT/PPIA genes ratio (mean, standard deviation, and coefficient of variation, CV%).

The gene copy number of each gene was extrapolated from the recDNA standard curves. The absolute qPCR reactions were performed in a final volume of 20 µl, containing 10 µl of 2x IQ. Multiplex Powermix (BioRad, USA), 1 µl of each 6 µM PPIA primer, 1 µl of 20x TaqMan® OXT Gene Expression Assay (Applied Biosys-tem), 1 µl of 2 µM TaqMan® PPIA probe (see Table 1), 1 µl of template DNA and 5 µl of nuclease-free water. The qPCR cycling conditions were the same as for the standard curve construction experiments. Each sample was amplified in duplicate.

2.7. Criterion value, sensitivity and specificity of the OXT screening method

The efficacy of the OXT up-regulation as a potential biomarker candidate to identify pE2- and/or DEX-treated beef cattle was studied using a receiver operating characteristic (ROC) analysis. The ROC curve was developed on OXT/PPIA values obtained from all the experimental groups. Specificity, sensitivity and criterion or cut-off value of this potential biomarker were calculated (with a 95% confidence interval, CI) by means of a ROC curve (Table 5).

Sensitivity indicates the probability that a pE2- and/or DEX-treated animal (suspect) will be correctly identified (true positive), and 1 — specificity is the probability that a negative subject will be falsely identified as a suspect (false positive) (Fig. 4). To select the optimal criterion value, the likelihood ratios of positive (+LR) versus negative (-LR) outcomes were calculated, with a 95% CI.

Table 1
Primer sequences and TaqMan® probes for qPCR.

Gene (RefSeq ID)	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
OXT (NM_176855)	TaqMan® Gene Expression Assay		113
OXTR (NM_174134)	CAAGGAAGCCTCACCTTTCA	TGCACAAGTTCTTGGAAGAGG	111
MYOD1 (NM_001040478)	CGACTCGGACGCTTCCAGT	GATGCTGGACAGGCAGTCGA	180
MYF5 (NM_174116)	ACCAGCCCCACCTCAAGTTG	GCAATCCAAGCTGGATAAGGAG	150
MYOG (NM_001111325)	GTGCCCAGTGAATGCAGCTC	GTCTGTAGGGTCCGCTGGGA	110
MRF4 (NM_181811)	GGTGGACCCCTTCAGCTACAG	TGCTTGTCCCTCCTTCCTTGG	140
PPIA (NM_178320)	GCCCCAACACAAATGGTT	CCCTCTTTCACCTTGCCAAAG	95
	TaqMan®Probe HEX-TGCTTGCCATCCAACCACTCAGTC-BHQ1		

2.8. Statistical analyses

All statistical analyses were performed using GraphPad InStat (vers. 3.05) and GraphPad Prism (vers. 3.05) software (GraphPad Inc., San Diego, CA, USA). The oxytocin peptide serum concentration data obtained for the three study groups were analysed using the Kruskal-Wallis test (non-parametric one-way analysis of variance - ANOVA), followed by Dunn's Multiple Comparison post test, because Barlett's test suggested that the differences among the standard deviations of the group data were significant.

The gene expression of target genes and the absolute quantification of OXT/PPIA were analysed by ANOVA, followed by Dunnett's post hoc test, comparing both treatment groups against the control group. Grubbs' test was used to determine and exclude potential outliers.

ANCOVA was performed to compare slopes and intercepts of the six standard curves, and *P* and *F* values were calculated. Analysis of the absolute quantification variability of the unknown sample was investigated using the Kruskal-Wallis test, followed by Dunn's Multiple Comparison post test. A P value of O.05 was considered to indicate statistically significant differences.

ROC curve analysis was used to achieve the optimal relationship between sensitivity and specificity. The analysis was conducted using

GraphPad Prism (vers. 3.05) software (GraphPad Inc., San Diego, CA, USA).

3. Results

3.1. Effect offiE2 and DEX on serum concentration of oxytocin peptide

Serum oxytocin levels measured after the fifth dose of pE2 (on day 35) had increased almost 100-fold in group A (mean±SEM, 53.6 ±11.47 pg/ml) compared to the average serum oxytocin concentration measured in control group (0.53 ±0.53 pg/ml) (Fig. 1). This increase was statistically significant ($P < 0.05$), with a sample recovery of 92%. In contrast, DEX administration in group B induced no significant increase in serum levels of this neuropeptide after 35 days of treatment (3.28 ± 2.52 pg/ml), when compared to controls. At the time of slaughtering, serum oxytocin levels were equally high in all experimental groups, including controls; the average concentrations were 195.41 ± 51.43 pg/ml, 110.27 ± 35.96 pg/ml, and 181.96 ±44.70 pg/ml in groups A, B, and C, respectively.

3.2. Relative gene expression of OXT, OXTR and MRFs in skeletal muscle

Administration of pE2 and DEX induced in skeletal muscle tissue a significant overexpression of the OXT gene by 33.5-fold in group A and by 13.3-fold in group B (Table 2) compared to control group. Treatment with pE2 induced also an up-regulation of OXTR by about 3-fold (Table 2), which was not evident in animals treated with DEX. These changes in gene expression persisted in skeletal muscle for at least 7 days (time of slaughter) following discontinuation of drug treatment. Likewise MRF-mRNA levels in skeletal muscle tissue were differently regulated by pE2 and DEX treatment. In particular, the myogenic factor 4 (MRF4) was up-regulated by pE2 and DEX by about 3 and 4-fold, respectively (Table 2). Treatment with DEX also induced over-expression of myogenic factor 5 (MYF5) and myogenic differentiation 1 (MY0D1) by about 4-fold and 9-fold versus control, respectively (Table 2). For myogenin (MYOG) no significant change in gene expression was noted.

3.3. OXT and PPIA cloning

After the PCR run, two single bands of the correct length (ampli-con relative to OXT, 113 bp; amplicon relative to PPIA, 95 bp) were detected (Fig. 2). OXT and PPIA fragments were successfully cloned into two different pDrive vectors and transformed into a bacterial host. After purification, recDNA identities as OXT and PPIA were confirmed by sequencing.

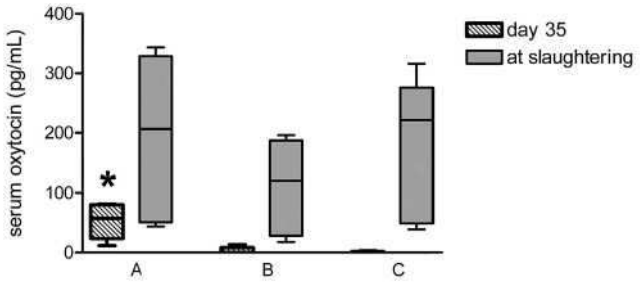

Fig. 1. The box and whiskers graph shows the serum oxytocin values (pg/ml) in experimental beef cattle. The lines at the middle represent the group medians. The boxes extend from the 25th and the 75th percentile. The error bars extend down to the lowest value and up to the highest. Blood samples were collected on day 35 after the beginning of treatment and at the time of slaughtering in group A (pE2-treated), group B (DEX-treated), and group C (control).*Signifkantly different from control group (P< 0.05).

Table 2
Fold-change in expression of the six genes analysed in the skeletal muscle of animals treated with pE2 (group A) and DEX (group B) versus control (group C to whom a control value of 1 was assigned).

Genes	Normalised fold expression ($2^{-\Delta\Delta Cq}$)	
	A	B
OXT	33.5 ± 20.3**	13.3 ± 14.5**
OXTR	3.09 ± 1.94**	1.13 ± 1.24
MYOD	0.89 ± 1.47	9.04 ± 6.30**
MYF5	2.10 ± 0.87	4.02 ± 1.27**
MYOG	1.05 ± 0.75	1.77 ± 0.51
MRF4	3.11 ± 1.75*	3.82 ± 2.29*

* $P < 0.05$.
** $P < 0.01$.

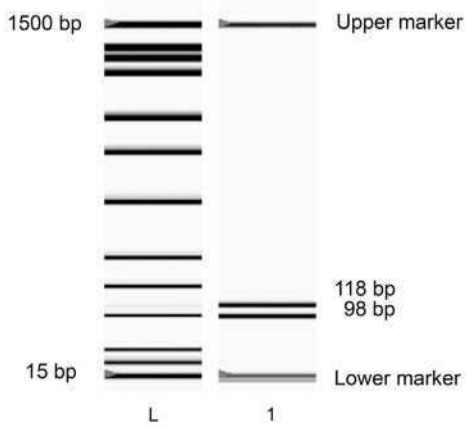

Fig. 2. A virtual gel of the automated capillary electrophoresis of the final OXT and PPIA amplicons. Lane L: molecular weight marker and lane 1: OXT and PPIA amplicons (118 bp and 98 bp, respectively). Two DNA internal markers (lower, 15 bp; higher, 1500 bp) were added to indicate peak alignments.

3.4. qPCR amplification reaction and reproducibility ofrecDNA standard curves

Standard curves have been constructed over a range of 68.8-6.88 x 10^6 molecules of OXT amplicons^l and 75.9-7.59 x 10^6 -molecules of PPIA amplicons^l. For each standard curve test linearity, r^2 value, amplification efficiency%, derived from the slope of the standard curve, and intercept value were reported in Table 3. Amplification efficiencies of the qPCR for the OXT and PPIA recD-NAs were very similar and close to 100%. A high linearity was detected in the range of 10^6-10^2 DNA molecules. The theoretical detection limits for the OXT and PPIA TaqMan® assays were calculated from the calibration curve and they were found to be similar (7-9 and 7-25, respectively). These low values indicated a great sensitivity of the qPCR protocol (Table 3).

To assess the reproducibility of absolute quantification by qPCR, it was determined whether standard curve parameters calculated with each recDNA were statistically similar. The two intra-assay standard curves in each run were highly reproducible; ANCOVA analysis of the six inter-assay standard curves revealed no significant differences between the slopes, but the P value was <0.0001 for the six elevations (Table 3).

3.5. Variability in absolute quantification of OXT and PPIA gene copy number in skeletal muscle samples

Variation in the absolute number of gene copies of OXT/PPIA amplified from skeletal muscle tissue samples was assessed. The cDNA of the same unknown sample was amplified in three independent qPCR runs at three different dilutions (1, 10^{-1}, and $1CT^2$) and the number of gene copies of OXT/PPIA was quantified using a recDNAs standard curve; the CV% range for the intra-assay was 5.88-36.34, with an uptrend at low concentrations of cDNA. The trend was similar for the inter-assay experiment, with a CV% range from 18.18 to 33.59 (Table 4).

3.6. OXT gene expression absolute quantification in skeletal muscle tissue of experimental animals

The OXT gene expression detected by absolute qPCR and the OXT gene copy number (normalised by the PPIA gene copy number) detected in each sample are reported in Fig. 3. The OXT/ PPIA ratio increased in group A (mean±SEM, 2.51 ±0.40) and group B (1.16 ±0.29) compared to group C (0.28 ±0.19). The overexpression compared to group C was statistically significant both in group A and B ($P < 0.01$). Only for one animal (number 8) in group B results were under the threshold of the optimal criterion value, whereas only one animal in the control group (number 17) showed an OXT/PPIA ratio higher than the threshold.

Table 3
Quantitative amplification parameters calculated for six standard curves relative to OXT and PPIA recDNA developed in three separate qPCR assays. The variation of intra- and inter-run standard curves was tested via an ANCOVA; the P and F values were determined to test any significant difference between the slopes and the intercept of the two replicate in each qPCR assay (intra-assay) and between the slopes and the intercept of the six standard curves developed in the three different qPCR assays (inter-assay).

OXT recDNA standard curve	qPCR run 1		qPCR assay 2		qPCR assay 3	
	A	B	A	B	A	B
Amplification efficiency %	107.6	107.9	108.6	103.2	106.0	112.4
Intercept	37.85	37.70	37.25	37.80	36.82	36.17
Slope	−3.153	−3.145	−3.131	−3.248	−3.186	−3.056
Sy.x	0.306	0.509	0.470	0.350	0.640	0.804
Detection limit	8	7	9	8	9	9
Quantification range	68.8–6.88 × 10^6	68.8–6.88 × 10^6	68.8–6.88 × 10^6	68.8–6.88 × 10^6	68.8–6.88 × 10^6	68.8–6.88 × 10^6
Test linearity. r^2	0.998	0.995	0.996	0.998	0.993	0.988
	Intra-assay 1(P; F)		Intra-assay 2 (P; F)		Intra-assay 3 (P; F)	
Slope	0.9505; 0.004069		0.3166; 1.1115		0.5184; 0.4480	
Intercept	0.6272; 0.2510		0.644; 0.2257		0.6994; 0.1571	
	Inter-assay (P; F)					
Slope	0.7802; 0.2500					
Intercept	<0.0001; 19.1627					

PPIA recDNA standard curve	qPCR assay 1		qPCR assay 2		qPCR assay 3	
	A	B	A	B	A	B
Amplification efficiency %	107.8	105.8	105.6	105.1	101.8	109.5
Intercept	38.8	39.5	38.3	38.8	38.2	37.6
Slope	−3.148	−3.191	−3.194	−3.205	−3.28	−3.113
Sy.x	0.465	0.485	0.587	0.269	0.281	0.591
Detection limit	16	25	11	15	9	7
Quantification range	75.9–7.59 × 10^6	75.9–7.59 × 10^6	75.9–7.59 × 10^6	75.9–7.59 × 10^6	75.9–7.59 × 10^6	75.9–7.59 × 10^6
Test linearity. r^2	0.996	0.996	0.994	0.999	0.999	0.994
	Intra-assay 1(P; F)		Intra-assay 2 (P; F)		Intra-assay 3 (P; F)	
Slope	0.7407; 0.1157		0.924; 0.009565		0.2062; 1.8277	
Intercept	0.05426; 4.6402		0.09273; 3.3893		0.8453; 0.03987	
	Inter-assay (P; F)					
Slope	0.9389; 0.06316					
Intercept	<0.0001; 26.9257					

Table 4
Intra-assay (test precision) and inter-assay variation (test variability) for OXT/PPIA values determinate by three different qPCR assays. Mean of OXT/PPIA values, standard deviation (s.d.), and coefficient of variation (CV%) were calculated from three different dilution of a cDNA sample.

Dilution	Inter-assay ($n = 36$)			Intra-assay ($n = 12$)								
	OXT/PPIA	s.d.	CV%	qPCR assay 1			qPCR assay 2			qPCR assay 3		
				OXT/PPIA	s.d.	CV%	OXT/PPIA	s.d.	CV%	OXT/PPIA	s.d.	CV%
1	3.60	0.65	18.18	3.94	0.94	23.73	3.36	0.43	12.90	3.50	0.51	14.53
10^{-1}	3.91	1.03	26.38	4.43	1.38	31.19	3.45	0.20	5.88	3.75	1.00	26.80
10^{-2}	2.57	0.86	33.59	3.13	1.14	36.34	2.04	0.71	34.75	2.54	0.37	14.72

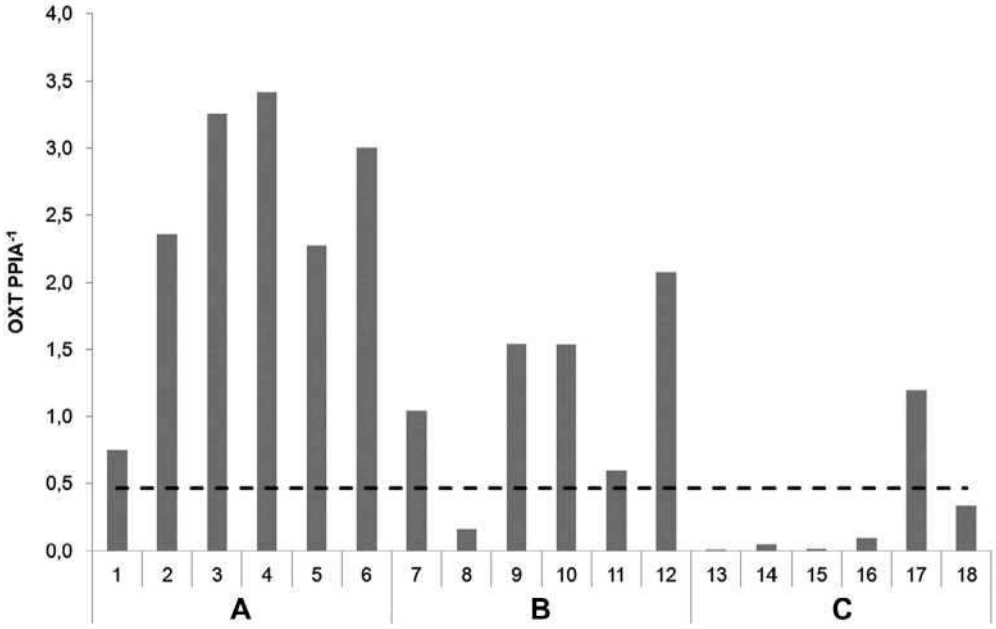

Fig. 3. Absolute qPCR data from experimental groups. OXT/PPIA ratios of each pE2-treated (group A), DEX-treated (group B) and control (C) animal. The dotted line indicates the optimal criterion value calculated by ROC curve analysis.

3.7. ROC parameters calculated on OXT screening method

The ROC curve analysis established a good diagnostic value of the OXT/PPIA gene copy number ratio in pE2- and DEX-treated beef cattle, compared to the control group (Fig. 4). The area under the curve (AUC) was 0.9306, with a 95% confidence interval (0.8072-1.054; $p<0.01$). Based on an optimal criterion value of 0.4665, the diagnostic sensitivity was 83.33%, with a specificity of 91.67% (Table 5). The positive likelihood (+LR) and negative likelihood (-LR) ratios were 10 and 0.18, respectively (Table 5). The OXT/PPIA values obtained from the experimental groups A and B were higher than the criterion value calculated by the ROC curve analysis (Fig. 3).

4. Discussion

The results of the present study in beef cattle indicate that, in accordance with previous studies by Dejager et al., 2011 and Kon-gsuwan, Knox, Allingham, Pearson, & Darlymple, 2012, pE2 administration induces a significant increase in circulating oxytocin levels after 35 days of treatment. However, a correlation between OXT-mRNA synthesis in skeletal muscle and serum oxytocin levels has not been shown. Therefore, it is probable that oestrogens act as important regulators of oxytocin production within the brain and/ or control its release into the blood stream (Chung, McCabe, & Pfaff 1991; Shughrue, Dellovade, & Merchenthaler, 2002). The OXT gene is primarily expressed in the magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei, with translated oxytocin peptide then being stored in the the posterior pituitary gland from where it is released into the systemic circulation (Gim-pl & Fahrenholz, 2001). The concentration of oxytocin in circulating blood might serve as a valid marker to detect oestradiol treatment *in vivo.* However, blood levels may be influenced by a variety of physiological stimuli, such as suckling and parturition in the female, or by certain stress factors (Gimpl & Fahrenholz, 2001). The finding that all animals at the time of slaughtering had high levels of oxytocin in their circulating blood supports the notion that the hypothalamic nuclei increase oxytocin production and release in response to the stress associated with transport, animal handling, and the slaughterhouse environment. Treatment with DEX did not induce a significant increase in oxytocin concentrations in circulating blood. Presumably, this synthetic glucocorticoid does not affect the biosynthesis of this neuropeptide within the brain, the main source for the hormone found in blood. It is also unlikely that any changes in the amount of oxytocin produced in myocytes in response to DEX would have affected oxytocin concentrations in blood, given that skeletal muscle represents overall only a very minor source for this hormone.
The up-regulation of OXT gene in skeletal muscle of both group A and group B was a particularly interesting finding of the present study. Whilst both De Jager and Kongsuwan (De Jager et al., 2011; Kongsuwan et al.2012) described OXT gene overexpression in skeletal muscle of steers and sheep treated with trenbolone acetate and an pE2 implant, our study is the first to demonstrate that sole treatment with either pE2 or DEX can induce an up-regulation of OXT gene expression in skeletal muscle of beef cattle. The mechanism of OXT gene expression induction by pE2 and DEX in skeletal muscle is not completely understood. Transcriptional activation of the gene coding for oxytocin by oestrogens does not follow the classical model of oestrogen receptor action (Koohi, Ivell, & Walther, 2005). One hypothesis involves the binding of nuclear orphan receptors oestrogen receptor-related receptor alpha (ERRa) to the oxytocin promoter binding site. The oxytocin promoter does not contain oestrogen response element (ERE); on the contrary, it possesses a high-affinity binding site for nuclear orphan

receptors (Koohi et al., 2005). The oestrogen-dependent up-regulation of the bovine oxytocin promoter was investigated in MDA-MB 231 cells. Binding of ERRa to the oxytocin promoter binding site has been demonstrated, suggesting the involvement of this nuclear orphan receptor in oestrogen-dependent up-regulation (Koohi et al., 2005). Both, ERRp and ERRy are induced and accumulate in a differentiation-dependent manner in skeletal muscle cells. The study of Wang, Myers, Dooms, Capon, and Muscat (2010) highlighted the regulatory crosstalk between ERRy and glucocorticoid receptor (GR) signalling in skeletal muscle cells, and suggested that the ERRy agonist modulates the expression of critical genes that control GR signalling and glucocorticoid sensitive gene expression. This relationship could explain the OXT up-regulation induced by DEX in skeletal muscle, but other factors, deriving from the hypo-thalamo-neurohypophyseal system, could also regulate this gene expression.

The expression of genes susceptible of change following GP treatment was determined to evaluate the molecular effects induced in skeletal muscle of beef cattle. Muscle development is under the control of genes of the MRF gene family (MY0D1, MYF5, MYOG, MRF4) and the up-regulation of these MRFs induced by hormones has been demonstrated. Although all four MRFs have similar functional properties *in vitro*, they exhibit distinct activities during embryogenesis: MYOD and MYF-5 are essential to specification of the muscle lineage, whereas MYOG and MRF4, respectively, are involved in the initial and late stages of differentiation (Perry & Rudnick, 2000; Wyzykowski, Winata, Mitin, Taparowsky, & Kon-ieczny, 2002). In the present study, pE2 and DEX induced a significant increase in MRF mRNA in skeletal muscle, which persisted after drug administration was discontinued (Table 2). Glucocorticoids may potentially affect proliferation and differentiation of myoblasts through several pathways that may differently regulate the genes of the MRF gene family. It has been reported that glucocorticoids increase myogenic repair and myoblast proliferation; in particular, low doses of glucocorticoids increase the myogenic fusion efficiency of C2C12 cells (Belanto et al., 2010).

Breton et al. (2002) described that functional OXTR is present in *in vitro* human myoblasts and they suggested that the OXT/OXTR system may be involved in the differentiation of human skeletal muscle and its regeneration. Also in the present study pE2 administration in beef cattle induced an up-regulation of OXTR gene in skeletal muscle. Furthermore, De Jager et al. (2011) demonstrated that the endogenous OXT expression was increased in fetal bovine *Longissimus dorsi* muscle during secondary myogenesis and functional differentiation of muscle, but it was not clear whether sex hormones were responsible for this overexpression.

To verify the up-regulation of the OXT gene as a biomarker for the detection of pE2 and DEX treatment in beef cattle, an absolute quantification of this gene by qPCR was optimised. The intra-assay approach indicated that the reproducibility of recDNA standard curves by qPCR was high (Table 1). The developed inter-assay showed that the six standard curves had similar slopes, but different intercept values. Quantification of unknown gene copy numbers from standard curves with the same r^2 and slope but different intercept values would result in differences in the absolute values of gene copies obtained. For this reason, for future applications of this method in meat screening, the authors would recommend to use an internal standard curve for each qPCR run to determine the OXT/ PPIA value in an unknown sample. The test precision and variability showed that the variation mostly depended on sample dilution rather than on the standard curve included in each run. Therefore, the determination of low concentrations of cDNA could be subjected to a higher error. Anyway, analysis of the variation of the absolute quantification of the unknown sample was not significant ($p = 0.3821$).

A ROC curve was produced to evaluate the most important parameters for application of this screening test. ROC curves seek to identify the rate of true positives versus false positives and thereby provide a measure of the specificity and sensitivity of any potential biomarker candidate.

These first results were obtained under experimental conditions, and they should be confirmed in-field on a greater number of animals. By the way, preliminary results showed a quite clear distinction between GP-treated and control animals, with an AUC value of 0.9306. The ROC curve data indicate that, for the chosen criterion value, only approximately 17% of the control subjects were wrongly identified and about 92% of pE2- and DEX-treated beef cattle were accurately identified. Obviously, the hormone oxytocin synthesis, storage and release and its binding to OTR may be endogenously and exogenously regulated to counteract patho-physiological states (such as sexual behaviour, diabetes, cancer, and stress) (Viero et al., 2010). Nevertheless, the OXT gene expression change in skeletal muscle could become an interesting screening test to verify the healthiness of the meat, taking into account the possibility to have false positive results. Given the reason described above and the easy application of OXT expression measurements as screening tool in state food safety laboratories, the use of this test has been patented by the University of Turin (Italy) (N. TO2012A000119).

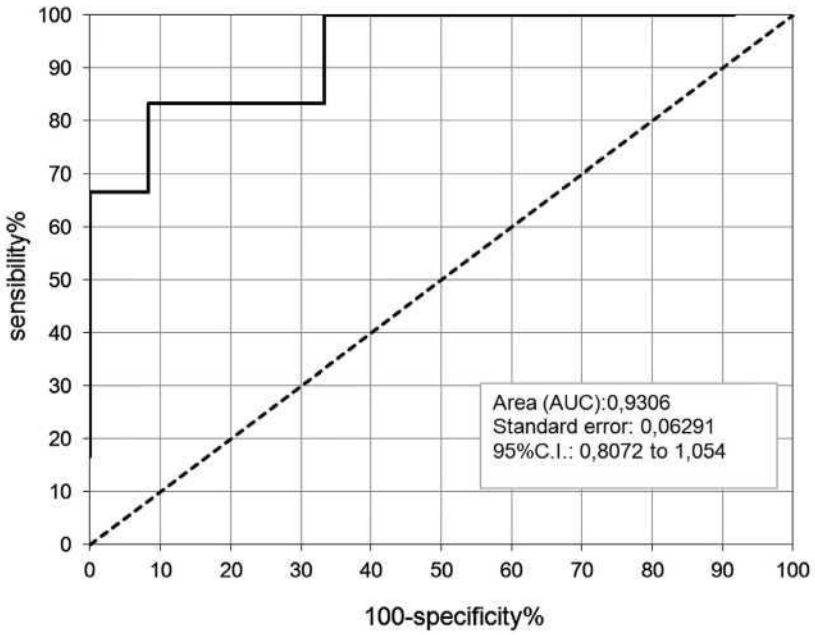

Fig. 4. ROC curve for OXT up-regulation in pE2- and DEX-treated animals versus untreated control animals. The area under the ROC curve (AUC) was 0.9306 with a 95% CI (0.8072-1.054; P> 0.01). The optimal criterion value selected was 0.4665 with 83.3% sensitivity and 91.67% specificity.

Table 5
Criterion values (express as OXT/PPIA values) and coordinates of ROC curve for OXT up-regulation as screening test (confidence interval, CI; positive likelihood ratio, +LR; negative likelihood ratio, -LR). The optimal criterion value is printed in bold.

Criterion	Sensitivity%	95% CI	Specificity%	95% CI	+LR	−LR
<0.0085	16.67	0.4211–64.12	100	73.54–100.0		0.83
<0.0285	33.33	4.327–77.72	100	73.54–100.0		0.67
<0.0695	50	11.81–88.19	100	73.54–100.0		0.50
<0.1285	66.67	22.28–95.67	100	73.54–100.0		0.33
<0.2475	66.67	22.28–95.67	91.67	61.52–99.79	8.00	0.36
<0.4665	**83.33**	**35.88–99.58**	**91.67**	**61.52–99.79**	**10.00**	**0.18**
<0.6780	83.33	35.88–99.58	83.33	51.59–97.91	5.00	0.20
<0.8990	83.33	35.88–99.58	75	42.81–94.51	3.33	0.22
<1.119	83.33	35.88–99.58	66.67	34.89–90.08	2.50	0.25
<1.366	100	54.07–100.0	66.67	34.89–90.08	3.00	
<1.541	100	54.07–100.0	58.33	27.67–84.83	2.40	
<1.809	100	54.07–100.0	50	21.09–78.91	2.00	
<2.173	100	54.07–100.0	41.67	15.17–72.33	1.71	
<2.318	100	54.07–100.0	33.33	9.925–65.11	1.50	
<2.681	100	54.07–100.0	25	5.486–57.19	1.33	
<3.129	100	54.07–100.0	16.67	2.086–48.41	1.20	
<3.338	100	54.07–100.0	8.333	0.2108–38.48	1.09	

5. Conclusions

Further studies are needed to understand the mechanisms of action of the oxytocin peptide on skeletal muscle growth. The significant overexpression of OXT gene in tissues harvested from pE2- and DEX-treated animals may help explain the anabolic effect of low-dose oestrogen and synthetic glucocorticoid administration on muscle growth. The effect of illegal GPs on the bovine transcrip-tome has drawn increasing attention of the scientific community and spurred efforts to identify biomarkers of hormone abuse in livestock. The change in OXT gene expression may well be an intriguing biomarker to discover GP abuse in adult beef cattle. The described methodology, using an indirect marker to detect illegal GP treatment, promises to significantly improve food safety control programs once introduced. Used as a screening test the method helps safeguarding the quality of meat for human consumption because significant OXT gene up-regulation was still detected at the time of slaughtering, i.e., one week after the last treatment with pE2 or DEX.

Acknowledgements

This work was partially funded by the Ministero delle Politiche Agricole Alimentari and Forestall SAFORISK project "Prevenzione dell'uso di anabolizzanti in zootecnia Creazione di Marchio a difesa degli allevamenti italiani" and Ministero della Salute in "Sviluppo di tecniche innovative (OMICS) e impiego di tecniche analitiche classiche per l'individuazione rispettivamente di biomarcatori e di residui di trattamenti illeciti di bovini destinati all'alimentazione umana". The authors are grateful to COOP ITALIA, Domenico Palmerini, and Alessandra Sereno for the technical support provided, to Prof. Laura Zarucco and Prof. Bernd Driessen for language revision, and to the "Bruno Maria Zaini" Reference Centre of Comparative Pathology, Department of Veterinary Science, University of Turin, Italy.

References

Assinder, S. J., & Nicholson, H. D. (2004). Effects of steroids on oxytocin secretion by the human prostate in vitro. *International Journal of Andrology, 27*,12-18.

Belanto, J. J., Diaz-Perez, S. V., Magyar, C. E., Maxwell, M. M., Yilmaz, Y., Topp, K., et al. (2010). Dexamethasone induces dysferlin in myoblasts and enhances their myogenic differentiation. *Neuromuscular Disorders, 20*, 111-121.

Breton, C, Haenggeli, C, Barberis, C, Heitz, F., Bader, C. R., Bernheim, L, et al. (2002). Presence of functional oxytocin receptors in cultured human myoblasts. *The Journal of Clinical Endocrinology and Metabolism, 87*,1415-1418.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Hugget, J., Kubista, M., et al. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55, 611-622.

Carraro, L, Ferraresso, S., Cardazzo, B., Romualdi, C, Montesissa, C, Gottardo, F., et al. (2009). Expression profiling of skeletal muscle in young bulls treated with steroidal growth promoters. *Physiological Genomics, 38*,138-148.

Chung, S. K., McCabe, J. T., & Pfaff, D. W. (1991). Estrogen influences on oxytocin mRNA expression in preoptic and anterior hypothalamic regions studied by in situ hybridization. *Journal of Comparative Neurology, 307*, 281-295.

De Jager, N., Hudson, N. J., Reverter, A., Wang, Y. H., Nagaraj, S. H., Cafe, L M., et al. (2011). Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fiftyfold increase in circulating oxytocin in cattle. *Physiological Genomics, 43*, 467-478.

De Maria, R., Divari, S., Spada, F., Oggero, C, Mulasso, C, Maniscalco, L, et al. (2010). Progesterone receptor gene expression in the accessory sex glands of veal calves. *Veterinary Record, 167*, 291-296.

Decreto legislativo n. 116 del 27/01/1992 (in Suppl. ordinario alia Gazz. Uff. n. 40, del 18 febbraio). Attuazione della direttiva (CEE) n. 609/86 in materia di protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici.

Divari, S., Cannizzo, F. T., Uslenghi, F., Pregel, P., Mulasso, C, Spada, F., et al. (2011a). Corticosteroid hormone receptors and prereceptors as new biomarkers of the illegal use of glucocorticoids in meat production. *Journal of Agricultural and Food Chemistry, 59*, 2120-2125.

Divari, S., Mulasso, C, Uslenghi, R, Cannizzo, F. T., Spada, R, De Maria, R., et al. (2011b). Progesterone receptor up-regulation: a diagnostic tool for the illicit use of oestrogens in adult beef cattle. *Food Additives & Contaminants Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 28*,1677-1686.

Dunn, J. D., Johnson, B. J., Kayser, J. P., Waylan, A. T., Sissom, E. K., & Drouillard, J. S. (2003). Effects of flax supplementation and a combined trenbolone acetate and estradiol implant on circulating insulin-like growth factor-I and muscle insulin-like growth factor-I messenger RNA levels in beef cattle. *Journal of Animal Science, 81*, 3028-3034.

EEC (1986). Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Communities*, L358(l), 29.

Enns, D. L, & Tiidus, P. M. (2010). The influence of estrogen on skeletal muscle: Sex matters. Sports *Medicine, 40*, 41-58.

Filippi, S., Morelli, A., Vignozzi, L, Vannelli, G. B., Marini, M., Ferruzzi, P., et al. (2005). Oxytocin mediates the estrogen-dependent contractile activity of endothelin-1 in human and rabbit epididymis. *Endocrinology, 146*, 3506-3517.

Gimpl, G., & Fahrenholz, F. (2001). The oxytocin receptor system: structure, function, and regulation. *Physiological Reviews, 81*, 629-683.

Ivell, R., Bathgate, R. A., Walther, N., & Kimura, T. (1998). The molecular basis of oxytocin and oxytocin receptor gene expression in reproductive tissues. *Advances in Experimental Medicine and Biology, 449*, 297-306.

Jankowski, M., Danalache, B., Wang, D., Bhat, P., Hajjar, F., Marcinkiewicz, M., et al. (2004). Oxytocin in cardiac ontogeny. *Proceedings of the National Academy of Sciences of the United States of America, 101*,13074-13079.

Johnson, B. J., White, M. E., Hathaway, M. R., Christians, C. J., & Dayton, W. R. (1998). Effect of a combined trenbolone acetate and estradiol implant on steady-state IGF-I mRNA concentrations in the liver of wethers and the longissimus muscle of steers. *Journal of Animal Science, 76*, 491-497.

Kongsuwan, K., Knox, M. R., Allingham, P. G., Pearson, R., & Darlymple, B. P. (2012). The effect of combination treatment with trenbolone acetate and estradiol-17p on skeletal muscle expression and plasma concentrations of oxytocin in sheep. *Domestic Animal Endocrinology, 43*, 67-73.

Koohi, M. K., Ivell, R., & Walther, N. (2005). Transcriptional activation of the oxytocin promoter by oestrogens uses a novel non-classical mechanism of oestrogen receptor action. *Journal of Neuroendocrinology, 17*,197-207.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonak, J., Lind, K., et al. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine, 27*, 95-125.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitativa PCR and the 2-AACt method. *Methods, 25*, 402-408.

Lopparelli, R. M., Giantin, M., Pozza, G., Stefani, A. L, Ravarotto, L, Montesissa, C, et al. (2012). Target gene expression signatures in neutrophils and lymphocytes from cattle administered with dexamethasone at growth promoting purposes. *Research in Veterinary Science, 93*, 226-233.

Perry, R. L, & Rudnick, M. A (2000). Molecular mechanisms regulating myogenic determination and differentiation. *Frontiers in Bioscience, 5*, D750-D767.

Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. In S. A. Bustin (Ed.), *A-Z of quantitative PCR* (pp. 87-112). International University Line (IUL), Lajolla, CA, USA: E-Publishing Inc. (chapter 3).

Rasmussen, R. P. (2001). *Rapid cycle real-time PCR, methods and applications.* Heidelberg (Germany): Springer-Verlag, Quantification on the LightCycler, p. 21-34.

Rozen, S., & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In S. Krawetz & S. Misener (Eds.), *Bioinformatics methods and protocols: Methods in molecular biology* (pp. 365-386). Totowa, NJ: Humana Press.

Shibata, M., Matsumoto, K., Aikawa, K., Muramoto, T., Fujimura, S., & Kadowaki, M. (2006). Gene expression of myostatin during development and re generation of skeletal muscle in Japanese Black Cattle. *Journal of Animal Science, 84*, 2983-2989.

Shughrue, P. J., Dellovade, T. L, & Merchenthaler, I. (2002). Estrogen modulates oxytocin gene expression in regions of the rat supraoptic and paraventricular nuclei that contain estrogen receptor-beta. *Progress in Brain Research, 139*, 15-29.

Sipila, S., & Poutamo, J. (2003). Muscle performance, sex hormones and training in peri-menopausal and post-menopausal women. *Scandinavian Journal of Medicine and Science in Sports, 13*, 19-25.

Sipila, S., Taaffe, D. R., Cheng, S., Puolakka, J., Toivanen, J., & Suominen, H. (2001). Effects of hormone replacement therapy and high-impact physical exercise on skeletal muscle in post-menopausal women: A randomized placebo-controlled study. *Qinical Science, 101*,147-157.

Sjogren, K., Liu, J. L, Blad, K., Static, S., Vidal, 0., Wallenius, V., et al. (1999). Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 7088-7092.

Smith, C. J., Nedwell, D. B., Dong, L R, & Osborn, A. M. (2006). Evaluation of quantitative polymerase reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environmental Microbiology, 8*, 804-815.

Stella, R., Biancotto, G., Krogh, M., Angeletti, R., Pozza, G., Sorgato, M. C, et al. (2011). Protein expression changes in skeletal muscle in response to growth promoter abuse in beef cattle. *Journal ofProteome Research, 10,* 2744-2757.
Stephany, R. W. (2010). Hormonal growth promoting agents in food producing animals. *Handbook of Experimental Pharmacology, 195,* 355-367.
Thackare, H., Nicholson, H. D., & Whittington, K. (2006). Oxytocin-its role in male reproduction and new potential therapeutic uses. *Human Reproduction Update, 12,* 437-448.
Uslenghi, R, Divari, S., Cannizzo, R T., De Maria, R., Spada, R, Mulasso, C, et al. (2013). Application of absolute qPCR as a screening method to detect illicit 17 *(,*-oestradiol administration in male cattle. *Food Additives & Contaminants Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 30,* 256-263.
Viero, C, Shibuya, I., Kitamura, N., Verkhratsky, A, Fujihara, H., Katoh, A., et al. (2010). REVIEW: Oxytocin: Crossing the bridge between basic science and pharmacotherapy. *CNS Neuroscience & Therapeutics,* 16, el38-el56.
Wang, S. C, Myers, S., Dooms, C, Capon, R., & Muscat, G. E. (2010). An ERRbeta/ gamma agonist modulates GRalpha expression, and glucocorticoid responsive gene expression in skeletal muscle cells. *Molecular and Cellular Endocrinology, 315,*146-152.
Wong, M. L, & Medrano, J. R (2005). Real-time PCR for mRNA quantitation. *BioTechniques, 39,* 75-85.
Wyzykowski, J. C, Winata, T. I., Mitin, N., Taparowsky, E. J., & Konieczny, S. R (2002). Identification of novel MyoD gene targets in proliferating myogenic stem cells. *Molecular and Cellular Biology, 22,* 6199-6208.