Proteomic identification of plasma proteins as markers of growth promoter abuse in cattle

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Abstract

Growth-promoting agents are continually misused for increasing animal growth and fraudulent gain in the meat industry, yet detection rates from conventional targeted testing for drug residues do not reflect this. This is because testing currently relies on direct detection of drugs or related metabolites and administrators of such compounds can take adaptive measures to avoid detection through the use of endogenous or unknown drugs, and low dose or combined mixtures. New detection methods are needed which focus on the screening of biological responses of an animal to such growth-promoting agents as it has been demonstrated that genomic, proteomic and metabolomics profiles are altered by xenobiotic intake. Therefore, an untargeted proteomics approach using comparative two-dimensional gel electrophoresis (2DE) was carried out to identify putative proteins altered in plasma after treatment with oestradiol, dexamethasone or prednisolone. Twenty-four male cattle were randomly assigned to four groups ($n = 6$) for experimental treatment over 40 days, namely a control group of non-treated cattle, and three groups administered 17β-oestradiol-3-benzoate (0.01 mg/kg, intramuscular), dexamethasone sodium phosphate (0.7 mg/day, per os) or prednisolone acetate (15 mg/day, per os), respectively. Plasma collected from each animal at day 25 post study initiation was subjected to proteomic analysis by 2DE for comparison of protein expression between treated and untreated animals. Analysis of acquired gel images revealed 22 plasma proteins which differed in expression by more than 50 % ($p < 0.05$) in treated animals compared to untreated animals. Proteins of interest underwent identification by LC–MS/MS analysis and were found to have associated roles in transport, blood coagulation, immune response and metabolism pathways. In this way, seven proteins are highlighted as novel biomarker candidates including transthyretin which is shown to be significantly increased in all treatment groups compared to control animals and potentially may find use as global markers of suspect anabolic practice.

Keywords

Bovine Anabolic Corticosteroid Proteomics 2D gel electrophoresis

Introduction

The European Union has deemed illegal the use of any substance contributing to anabolism in food-producing animals, and routine monitoring is compulsory within member states to enforce this regulation (Directive 2002/657/EC) [1]. However, the economic benefits reaped from such substance use in terms of meat yield and improved feed efficiency cannot be overlooked, and therefore their administration continues illicitly [2, 3]. Such use not only puts consumers at risk of exposure to possible carcinogenic and pharmacologically active compounds, but also raises important animal welfare issues, calling into question the integrity and quality of meat supplies. The range of compounds known to contribute to growth promotion is constantly expanding in line with advancements in detection capabilities and administration has moved to the use of low dose or combined mixtures of substances which can evade discovery including synthetic analogues and veterinary drugs or hormonal compounds that occur endogenously [2–4] such as oestradiol, testosterone and corticosteroid derivatives.

The anabolic activity of oestrogenic compounds has been claimed to be superior to androgens with enhanced growth performance of 5–15 % in cattle reported [5]. Oestradiol is typically applied as the esterified 17β- or benzoate (17βE) form via an implant or injection to avoid rapid metabolism and promote long-term activity. Having been originally prescribed as a reproductive tool in cattle, oestradiol was confirmed as having tumour-initiating and -promoting effects by the Sub-Committee on Veterinary Public Health (SCVPH) [6] and its use in Europe was prohibited (EU Directive 2003/74/EC) [7]. Glucocorticoids such as prednisolone and dexamethasone are readily available and are prescribed as anti-inflammatory agents. They are given orally to cattle and maximum residue limits (Commission Regulation 37/2010 [8]) are in place to ensure that adequate withdrawal times have elapsed prior to slaughter following therapeutic administration. Recent reports have described their illicit use in long-term low-dose regimes to improve beef tenderness [9] and increase live slaughter weight via water retention [2]. Additionally, oestradiol and glucocorticoids applied together have been reported to exhibit synergistic effects possibly via modulation of the bovine immune system [10] or through alteration of internal steroid receptors [11].

Current testing for growth promoter use relies heavily on the direct detection of drug residues and associated metabolites by mass spectrometry, yet despite advancements in technology major problems are encountered as a result of rapid drug metabolism, inadequate method sensitivity resulting from low dose formulations, and difficulties in differentiating between exogenous and endogenous forms of hormones [12]. The last issue is a major constraint in the detection of oestradiol abuse as administered esters once cleaved are structually identical to natural forms. The pharmacology of oestradiol has been studied extensively [13] and it is known to bind to receptors in muscle and other tissues at low concentrations, with indirect effects via growth hormone in the liver and insulin-like growth factor (IGF-1) in the blood stimulating protein metabolism [5]. Focusing on the discrimination between 17α- and β- metabolites of oestradiol may identify animals treated with exogenous oestradiol [14], but oestradiol levels vary depending on sex, age and breed, and threshold levels have yet to be determined. Similarly, undetectable levels of prednisolone residues following long-term controlled dosing have been reported as a result of rapid metabolism and elimination [15], and urinary concentrations of dexamethasone have been found to be below the maximum residue limit despite administration for up to 60 days [16]. It has also been suggested that endogenous prednisolone metaboilites can increase in coordination with animal stress [17, 18], and that residues may be detected in urine enhanced by faeces contamination [19], sample handling [20] and other environmental effects [21]. Under such conditions false positive results may occur, impacting on the legal confirmation of corticosteroid misuse.

Given the many issues which compromise the effective and sensitive monitoring of illegal growth promoter use, it is clear that new strategies are required to more confidently determine cases of anabolic exposure that do not solely focus on concentration levels of drugs or their metabolites which can be eliminated rapidly or are autogenously present. Screening approaches based on the concept of indirect detection have been proposed which can associate alterations to biological metabolism with the administration of compounds [12]. Some countries currently implement histological screening of the thymus as an indirect method to identify steroid misuse [22], but this requires a priori knowledge of the drug being used alongside the known observable effects. Omics-based approaches have emerged as potential powerful diagnostic tools where assessment of the genomic, proteomic or metabolomic state of an individual can reveal xenobiotic influence. In this way, screening of animals treated with growth-promoting agents yields a differing output profile to that of non-treated cattle with altered genes, proteins or metabolites used as markers indicative of treatment, thereby highlighting suspect samples. The incorporation of multiple biomarkers in such testing approaches could facilitate screening for a wide range of growth-promoting agents, reducing the number of analyses required and enabling higher sample throughput.

Many studies have revealed significant molecular changes in the tissues of treated compared to untreated animals, but the aim of the current study is to achieve compatibility with less-invasive sampling methods such as blood which can be acquired at any point of the beef-rearing process [23]. The proteome is believed to hold the most promise of biomarker discovery as it is reflective of both gene profiles and the environment relevant to the phenotypic state, and proteins of significance can be readily incorporated into novel screening techniques. Ludwig et al. [24] have demonstrated the potential of a protein biomarker assay to detect hormone treatment in the plasma of veal calves based on the differential profiles of IGF-1, IGFBP-2 and ostecoalcin after exposure to oestradiol, dexamethasone or prednisolone in comparison to untreated cattle. However another study had previously shown that IGF-1 and IGFBP-2 levels were not affected by similar hormone use [25], therefore more robust protein markers are needed. The key is to determine markers which provide a signature of a particular growth-promoting regime and can therefore indicate abuse; the difficulty is to also confirm transferability across biological variables such as breed, age and sex.

Current analysis of altered plasma protein profiles has been limited to targeted techniques focusing on individual proteins using radio-immunoassays, ELISA or mass spectrometry analysis, with minimal large-scale non-targeted profiling of changes at the proteome level. As such there are still no known protein markers capable of detecting abuse of prednisolone. Others have reported plasma proteins altered by treatment with oestradiol or dexamethasone including upregulation of IGFBP-3, ir-inhibin, osteocalcin and propeptide type III procollagen (PIIINP) and downregulation of sex hormone binding globulin (SHBG) [4, 12, 23–26] but these markers have still to be tested outside controlled experimental conditions. There have also been no proteins reported to be commonly altered by differing treatment regimens as this would significantly aid multi-marker detection capabilities.

This study aims to identify proteins altered significantly by exposure to oestradiol, dexamethasone or prednisolone in the plasma of treated animals compared to untreated cattle by comparison of the respective protein output by two-dimensional gel electrophoresis (2DE). It is anticipated that by focusing on three of the most commonly employed growth-promoting agents, robust candidates to screen for such illicit practice will be revealed. Such untargeted analysis of proteins will provide a large repertoire of candidates in plasma specifically expressed by the respective treatment groups. Differentially expressed proteins will be isolated for identification as potential biomarkers which may elucidate the systemic biological actions of such compounds. It is hoped that affected proteins

common to the three treatments will also be revealed by comparative analysis and aid development of future screening applications which prevent consumer exposure to hazardous residues or fraudulent activity in the beef industry.

Materials and methods

Chemicals and reagents

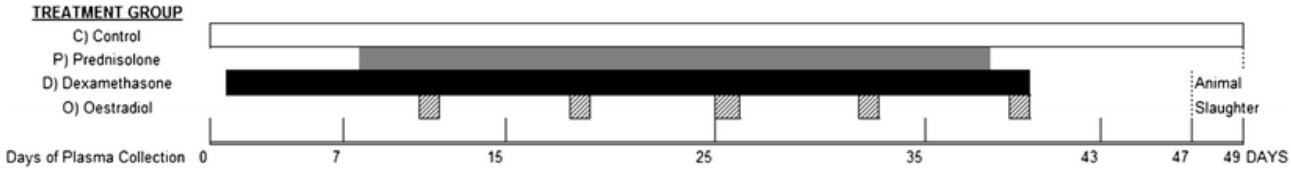
Immobiline Drystrip Cover Fluid, IPG buffer pH 4–7, Immobiline Drystrip Gels pH 4–7 13 cm, and Readysol IEF 40 % were purchased from GE Healthcare (Buckinghamshire, UK); Bradford assay reagent and Nunc® MicroWell™ were from Sigma-Aldrich (USA); sodium dodecyl sulphate (SDS), dithiothreitol (DTT), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPs), urea, and iodoacetamide were obtained from Thermo Fisher Scientific (USA); bovine serum albumin and glycine were from Apollo Scientific (Stockport, England); sequence-grade modified trypsin was purchased from Promega (USA).

Animal treatment

Plasma samples were obtained from a study authorized by the Italian Ministry of Health and bioethics committee of the University of Turin. The bovine growth promoter study cohort consisted of twenty four 17- to 22-month-old male Charolais cattle with an average weight of 600 kg. They were randomly assigned to four treatment groups: group O (n = 6) received 0.01 mg/kg intramuscular injection of 17β-oestradiol-3-benzoate (Sigma-Aldrich, Milan, Italy) weekly on days 12, 19, 26, 33 and 40; group D (n = 6) were subject to oral dosing of 0.7 mg/day dexamethasone-21-sodium phosphate (Desashock Fort Dodge Animal Health, Bologna, Italy) for 40 days; group P (n = 6) were subject to oral dosing of 15 mg/day prednisolone acetate (Novosterol, Ceva Vetem SpA, Milan, Italy) for 30 days beginning on day 8; group C (n = 6) were untreated control animals. Oral dosing consisted of one capsule containing compound per application using a drenching gun whilst injection was in the neck. The steroid dosage and administration route were chosen to reflect current suspicious farming practice conveyed in the literature [2, 26, 27]. Control animals were not given a supplementary placebo so the non-treated state, and subsequent proteome profile, would reflect the methods taken at the farm level. All participants were kept in separate housing and fed a diet of silage, corn and hay alongside a commercial protein supplement and water. Animals were slaughtered after the end of the treatment period. Blood was collected via the jugular vein on days 0, 7, 25, 35 and 43 and at slaughter using EDTA tubes for plasma preparations, which were then centrifuged at 2000 g for 20 min prior to storage at −20 °C. A timeline representing the study dosage and duration of sample collection is shown in Fig. 1. Plasma samples taken at day 25 and characteristic to half way through the study were chosen for initial analysis.

Fig. 1

Administration timeline of animal groups. Charolais cattle were assigned to four treatment groups (n = 6). Group C were untreated control animals given no placebo supplement; group P received 15 mg prednisolone acetate daily by oral capsule for 30 days; group D received 0.7 mg dexamethasone-21-sodium phosphate daily by oral capsule for 40 days; group O received 0.01 mg of 17β-oestradiol-3-benzoate per kilogram by weekly intramuscular injection beginning at day 12 through to day 40. The cattle were then slaughtered after a 6-day withdrawal period



Sample preparation

Plasma samples were thawed and subject to centrifugation at 10,000 *g* for 10 min before handling. Samples were prepared in duplicate with depletion of high molecular weight components via DTT reduction [28] by addition of 10 µl of 500 mM DTT to 90 µl plasma, mixed by vortexing followed by incubation at room temperature (25 °C) for 2.5 h. After centrifugation at 15,000 *g* for 30 min, supernatant was concentrated by drying under nitrogen and resuspended in 50 µl rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPs).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis of the depleted plasma samples was conducted following the workflow outlined in Fig. 2. The protein concentration in each plasma sample was determined via the Bradford assay [29] standardized to bovine serum albumin both before and after depletion. Thus 450 µg of each depleted sample was prepared in a volume of 250 µl rehydration buffer plus 10 µl IPG buffer solution (1 % IPG pH 4–7; 72.8 µg DTT; 0.1 % Bromophenol Blue). Four samples in duplicate were selected from each treatment group and randomly assigned to batches of 12 for subsequent IEF applied to Immobiline™ Drystrip (GE Healthcare, UK) 13 cm non-linear polyacrylamide gels pH 4–7 for rehydration overnight in DryStrip Cover Fluid (GE Healthcare) at room temperature. Rehydrated strips were transferred to a Multiphor II Electrophoresis System (GE Healthcare) flatbed system for simultaneous focusing carried out on a gradient voltage of 300 V increasing to 3500 V within 90 min and maintained for a further 4.5 h with temperature controlled to 20 °C. Focused strips were stored at −80 °C and reduced in DTT (1 % w/v) in equilibration solution followed by alkylation with iodoacetamide (2.5 % w/v) prior to second dimension separation on laboratory cast 18 cm × 16 cm 12.5 % SDS PAGE gels with temperature cooling at 25 °C in Laemmli buffer (250 mM Tris base; 1.92 M glycine; 1 % (w/v) SDS). Equilibrated strips were inserted horizontally on top of the pre-cast gel set in 50 % agarose sealing solution and current was applied at a flow rate of 15 mA per gel for 15 min, elevated to 30 mA per gel for 3.75 h. Gels were incubated in fixing solution (50 % MeOH, 3 % OPPA) overnight with subsequent washing by incubating for 20 min in ddH_2O three times before transferring to stain solution (34 % MeOH, 15 % ammonium sulphate, 2 % PPA) for 1 h followed by addition

of Colloidal Blue G powder for 120 h [30] on an orbital shaker. Destaining was carried out using ddH_2O for 24 h on an orbital shaker with several water changes. Gels were scanned using an Epson Perfection v700 Scanner with Silver Fast launcher v 6.6 and image capture set at 16-bit greyscale, 400 dpi in TIFF format. Each individual gel was bound with acetate in the presence of ddH_2O to prevent desiccation or contamination and stored at +4 °C until subsequent MS analysis.

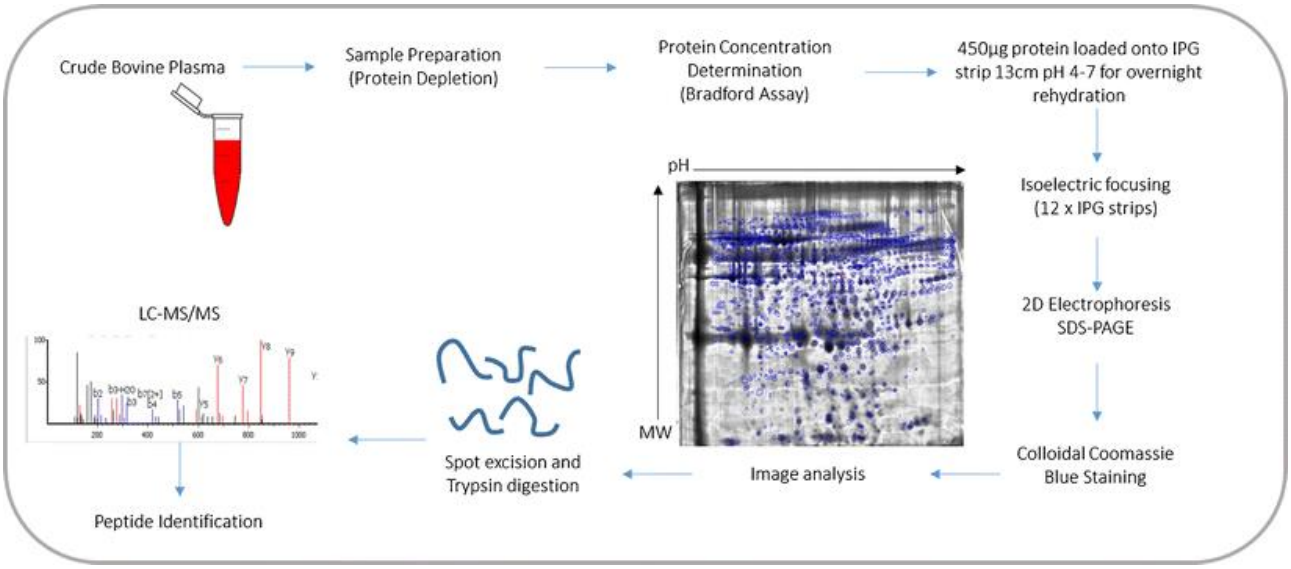


Fig. 2

Optimized proteomics method workflow. Plasma collected at day 25 was prepared by DTT reduction; protein concentration was determined via Bradford assay and samples analysed in duplicate with depleted plasma loaded onto Immobiline Drystrip (IPG) gel pH 4–7 in the first dimension and 12.5 % SDS-PAGE lab-cast gels in the second dimension. Staining with Coomassie blue yields spots representing various proteins isoforms mapped against pH and MW; spots of interest were excised from the gel, subjected to trypsin digestion and identified via LC–MS/MS analysis against the *Bos taurus* database

Image analysis

Analysis of the final SDS-PAGE gel output for each plasma sample in duplicate was conducted using Redfin Solo software (Ludesi, Sweden) where a reference gel was selected to laterally align all images to each other and generate a fusion image for advanced spot detection. Coomassie stained spots were quantified on normalized volume (% volume of each spot in comparison to total spot volume).

Statistical analysis

Output spot volumes were assessed for normalisation and linear distribution using SPSSv21 (IBM, UK) by regression analysis with plotted histograms and homoscedasticity confirmed by observation of related Q–Q plots. Results were filtered to exclude normalised spot volume of less than 200 as

inadequate for screening alongside those less than 100 % detectable across all gels. The respective fold change was determined to be greater than 50 % to be considered significantly different to the untreated cohort and a comparison was made of each treatment group to the untreated by Student's *t* test analysis. Only those spots showing a significant increase or decrease in spot volume ($p < 0.05$) compared to the control animals were considered reliable protein candidates requiring further analysis. To confirm reliability of sample quality, Student's *t* test was also used to compare the difference in spot volumes between the duplicate gels, and ANOVA was conducted to compare spot volumes expressed between the randomized batches of samples to confirm that no significant differences could be attributed to a specific day or run of samples.

Protein digestion

Selected protein spots were excised from a single reference gel and subjected to trypsin digestion according to a protocol adapted from Shevchenko et al. [31] with destaining of Coomassie spots in 100 mM ammonium bicarbonate/acetonitrile (1:1 v/v) solution followed by dehydration in acetonitrile to concentrate. Trypsin was added at a concentration of 13 ng/μl in stock solution to 40 μl volume and gel pellets incubated on ice. After 4 h, 20 μl of 100 mM ammonium bicarbonate was added for digestion at 37 °C overnight (20 h). To extract the peptide digestion products, samples were centrifuged at 7500 *g* for 2 min, the collected supernatant was transferred to a fresh Eppendorf tube and 100 μl extraction buffer (5 % formic acid, 67 % acetonitrile) was added to the digest. Gel extracts were incubated at 37 °C with shaking for 30 min, spun down again by centrifugation (7500 *g*) and pooled with the supernatant. Sample digests were air-dried in a MiVac Quattro Concentrator at 40 °C overnight (16 h) and stored at −20 °C for further LC–MS/MS analysis.

Peptide purification

Air-dried peptides were reconstituted in 20 μl equilibration solution (0.1 % TFA) prior to purification on C18 ZipTip® (Merck Millipore, Germany). A fresh ZipTip was used for each sample and was prepared by first aspirating with 10 μl wetting solution (100 % ACN) two times followed by 10 μl in equilibration solution (0.1 % TFA) twice. Uptake of 10 μl of sample ten times ensured adequate C18 binding, followed by washing in 10 μl washing solution (0.1 % TFA). Peptides were then eluted by uptake of 5 μl elution solution (0.1 % TFA, 60 % acetonitrile) and dispensed into a clean vial. This process was repeated five times and resulting aliquots pooled. Purified peptides were then concentrated in a MiVac Quattro concentrator (Genevac Ltd, UK) operating at 60 °C for approximately 8 h to remove residual acetonitrile.

Protein identification

Digested peptides were analysed on a Thermo Scientific Q Exactive mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Purified samples were resuspended in 0.1 % formic acid and loaded onto Biobasic Picotip Emitter (120 mm length, 75 μm ID) packed with Reprocil Pur C18 (1.9 μm) reversed-phase media and separated by an increasing acetonitrile gradient over 30 min at a flow rate of 250 nl/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220 °C with a potential of 2000 V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data-dependent switching mode. A high resolution (70,000) MS scan (350–1600 Da) was performed using the Q Exactive to select the 15 most intense ions prior to MS/MS analysis using high-energy collision dissociation. Results were searched against the *Bos taurus* subset of the Uniprot Swissprot database

using the PEAKS Studio Version 6 (Bioinformatics Software Inc.), for peptides cleaved by trypsin. Each peptide used for protein identification met specific Peaks parameters to include only peptide scores that corresponded to a false discovery rate (FDR) of ≤1 % accepted from the Peaks database search. Peaks results were filtered using an average local confidence (ALC) of ≥65 %, a total local confidence of (TLC) of ≥6 and peptide score ($-10\lg P$) of ≥15.

Results

The aim of the study was to separate plasma proteins by the aid of 2DE in order to achieve a bovine protein profile characteristic to each animal group so as to compare and identify proteins specifically expressed/not expressed as a result of growth-promoting treatments.

Assessment of plasma protein concentration

Sample pre-treatment prior to 2DE was assessed using a volume of pooled plasma generated from all bovine samples to be analysed. This showed that successful 2DE resolution was achieved when using a DTT reduction method to suppress the expression of interfering high molecular weight proteins. The protein concentration of each individual sample to be assessed ($n = 24$) was measured before and after DTT reduction. Crude plasma gave an average protein concentration of 55.7 mg/ml and this was reduced to yield 11.5 mg/ml protein per sample following DTT treatment. For reproducibility purposes, all plasma samples ($n = 24$) prepared in duplicate were compared to ensure similar yield after DTT treatment and replicates showing greater than 25 % coefficient variation in protein content were excluded from further analysis. Consequently, 16 plasma samples representing four animals from each treatment group prepared in duplicate and producing a total of 32 gels were chosen for continued comparison by 2DE.

Assessment of proteins detected from the output gel

A total of 450 μg protein of each depleted plasma sample was loaded onto IPG strips for isoelectric focusing followed by SDS-PAGE. Coomassie blue staining resulted in gels showing good spot resolution with over 700 spots detected in pH range 4–7 and MW 10–200 kDa as shown in Fig. 3. Gel image analysis via Ludesi Redfin software enabled advanced spot detection, normalisation of spot volume, alongside statistical comparison between groups. The spot output on every gel was checked by visual inspection and manually edited to ensure correct spot matching and omission of smeared or distorted gel regions. A representative gel from the control group was then selected to geometrically align all images using anchors to map the respective spots and generate a single composite image. Verification and adjustment of spot borders was carried out and detectable spots were then assigned a volume in each image with an algorithm applied to normalize the expression volumes across the replicate gels. From initial detection of approximately 1033 spots, only 731 of these were present on every gel and therefore considered reliable protein fragments. The spot volume was also set at a minimum of 200 to enable adequate detection; this reduced the spot output to 491. The duplicate gels were first compared to ensure reproducibility and showed no significant difference in spot volume when comparing the two replicates via Student's *t* test. Since the gels were also ran across three different batches, variation of spot volumes expressed between the batches was compared via ANOVA with just two spots showing fluctuating spot volume and were subsequently excluded. The duplicate gel images were then combined and a comparison was made between each treated animal group ($n = 4$) against the untreated

cohort ($n = 4$) by t test analysis. Only those spots showing greater than 50 % difference in spot volume from the corresponding untreated spot were considered relevant i.e. a fold change of greater than 1.5 or less than 0.5. This resulted in 162 spot differences between dexamethasone and prednisolone compared to control spot output and 175 showing greater than 50 % difference between oestradiol and control animals. However, these were then compared via Student's t test for significance ($p < 0.05$) and this reduced the candidate spots to 12 highlighted as significantly altered by treatment with oestradiol, 12 spots significantly altered after dexamethasone exposure and six protein spots significant to prednisolone administration. Seven of these spots were common between treated groups so 23 candidate proteins (Table 1) were finally selected as potential biomarkers of growth promoter treatment.

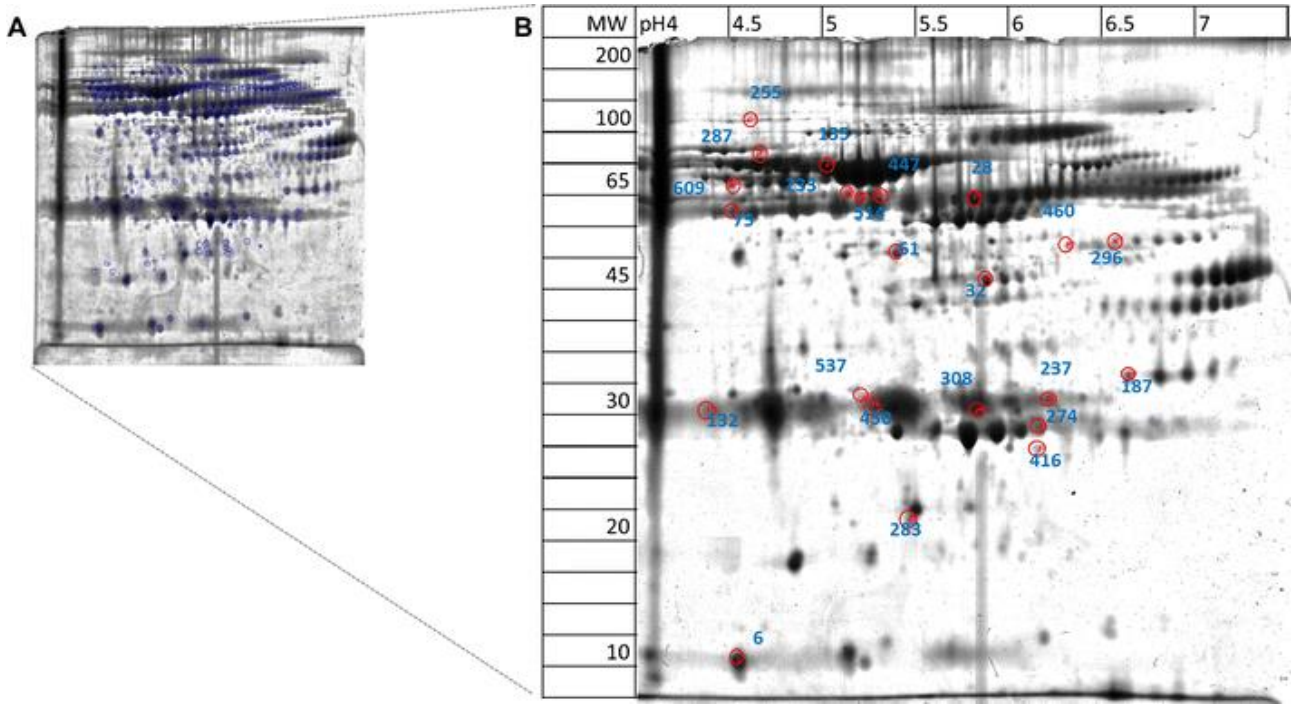


Fig. 3

Isolation of protein candidates. A representative 2DE gel of bovine plasma is shown (**a**) with the protein spots visually edited and detected by Ludesi Redfin software across all samples analysed. A total of 731 potential proteins are highlighted in *blue* and zooming in (**b**) reveals the corresponding 2DE map under enhanced contrast. The expression volume of each spot was assessed by comparison between treated and control animals by t test ($p < 0.05$), and those spots showing a fold change of <0.5 or >1.5, culminating in 23 spots of interest as displayed in *red*

Table 1

Spots of interest (as highlighted in Fig. 3): assessment by Student's *t* test of the treated animals compared to control animals resulted in 23 spots showing significant ($p < 0.05$) increase/decrease in spot volume

Spot ID	Untreated spot volume	Treatment Group	Fold change from untreated	Significance of change (*p* value)
6	2475	Oestradiol	4.13	0.0165
		Dexamethasone	4.15	0.0081
		Prednisolone	3.30	0.0116
28	4199	Oestradiol	2.02	NS
		Dexamethasone	2.06	0.0168
		Prednisolone	1.63	0.0178
32	896	Oestradiol	2.34	NS
		Dexamethasone	2.93	0.0253
		Prednisolone	2.40	NS
61	1440	Oestradiol	0.31	0.0452
		Dexamethasone	0.81	NS
		Prednisolone	1.01	NS
75	3475	Oestradiol	2.34	0.0248
		Dexamethasone	1.02	NS
		Prednisolone	1.42	NS
132	8114	Oestradiol	0.24	0.0227
		Dexamethasone	0.25	0.0269
		Prednisolone	0.30	0.0338
135	4799	Oestradiol	1.35	NS
		Dexamethasone	1.54	0.0005
		Prednisolone	1.10	NS
187	1344	Oestradiol	0.44	0.0001
		Dexamethasone	1.06	NS
		Prednisolone	0.89	NS
233	369	Oestradiol	3.77	0.0091
		Dexamethasone	3.29	0.0206
		Prednisolone	1.58	NS
237	1085	Oestradiol	1.15	NS
		Dexamethasone	0.42	0.0177
		Prednisolone	1.23	NS
255	478	Oestradiol	0.49	NS
		Dexamethasone	0.73	NS
		Prednisolone	0.35	0.0007

Spot ID	Untreated spot volume	Treatment Group	Fold change from untreated	Significance of change (*p* value)
274	3469	Oestradiol	1.86	0.0451
		Dexamethasone	0.85	NS
		Prednisolone	0.86	NS
283	783	Oestradiol	2.49	0.0115
		Dexamethasone	0.64	NS
		Prednisolone	1.36	NS
287	633	Oestradiol	0.53	0.0001
		Dexamethasone	1.03	NS
		Prednisolone	0.38	0.0001
296	285	Oestradiol	0.72	NS
		Dexamethasone	1.62	0.0068
		Prednisolone	1.11	NS
308	1641	Oestradiol	1.94	0.0404
		Dexamethasone	0.87	NS
		Prednisolone	1.52	NS
416	238	Oestradiol	2.30	0.0006
		Dexamethasone	1.45	NS
		Prednisolone	1.41	NS
447	736	Oestradiol	2.34	NS
		Dexamethasone	2.64	0.0004
		Prednisolone	1.54	NS
450	1008	Oestradiol	1.14	NS
		Dexamethasone	1.99	0.0072
		Prednisolone	1.16	NS
460	321	Oestradiol	0.98	NS
		Dexamethasone	1.98	0.0100
		Prednisolone	0.98	NS
514	598	Oestradiol	2.19	0.0178
		Dexamethasone	3.44	0.0006
		Prednisolone	1.71	NS
537	1055	Oestradiol	0.58	0.0325
		Dexamethasone	1.11	NS
		Prednisolone	0.41	0.0154
609	235	Oestradiol	1.84	0.0073
		Dexamethasone	1.48	NS
		Prednisolone	1.66	NS

NS not significant ($p > 0.05$)

Identification of proteins by liquid chromatography mass spectrometry

Spots of interest were excised from a representative gel and, after analysis by LC–MS/MS, data was processed using Peaks software for identification against the Uniprot *Bos taurus* database (Table 2). The peptide mass fingerprints were filtered to ensure tryptic peptide specificity with chemical modification fixed for carbamidomethylation and variable modifications for oxidation and deamidation with a maximum of two missed cleavage sites. The percentage coverage of the identified peptides ranged from 2 to 81 % most likely due to post-translational modifications of the protein which can occur. However, the ratio of unique peptides to the number of peptides identified was 73–100 %, showing excellent homology to *Bos taurus*, with the exception of peroxiredoxin-1 showing 40 % uniqueness (two out of five peptides); however, it is a very small protein and the peptide spectrum match was highly significant at −10lg*P* of 124.15. The −10lg*P* of the matched spots ranged from 44.26 to 306.83, which is equivalent to a *p* value of 5×10^{-2} and 6×10^{-4}, respectively, confirming accurate peptide spectrum assignment.

Table 2

LC–MS identification of protein fragments: spots were excised from a 2DE gel and digested peptides were analysed on Thermo Scientific Q Exactive mass spectrometer connected to Dionex Ultimate 3000 RSLC system

Spot ID	Estimated MW	Estimated p*I*	Average mass kDa	Theoretical p*I*	Accession number	−10lg *P*	% coverage	Number of peptides	Number unique	PTM	Identity
6	7490	4.75	15,727 *	5.91*	O46375	306.83	81	30	30	Y	Transthyretin
28	56,438	6.32	46,104	5.98	P34955	209.72	33	14	14	Y	Alpha-1-antiproteinase
32	42,822	6.22	43,018	5.29	Q32PJ2	194.07	38	16	15	Y	Apolipoprotein A-IV
61	43,363	5.89	37,995	5.63	P23805	327.03	44	32	25	Y	Conglutinin
75	57,336	4.83	53,340	4.36	P02676	166.06	18	8	8	Y	Fibrinogen beta chain
132	27,722	4.74	19,883 *	4.83	P02754	117.82	15	3	3	N	Beta-lactoglobulin
135	65,663	5.50	69,294	5.6	P02769	263.79	58	45	44	Y	Serum albumin
609	63,396	4.96				216.61	22	15	14		
187	24,666	7.44	22,210	7.80	Q5E947	124.15	18	5	2	Y	Peroxiredoxin-1

Spot ID	Estimated MW	Estimated p*I*	Average mass kDa	Theoretical p*I*	Accession number	−10lg *P*	% coverage	Number of peptides	Number unique	PTM	Identity
233	62,603	5.55	52,209	7.10*	Q3SZV7	233.81	34	22	22	Y	Haemopexin
237	29,650	6.61	26,562	6.42	F1MZ96	94.18	14	3	3	N	Uncharacterized protein
255	129,081	4.97	99,551*	5.17	P56652	94.37	2	2	2	Y	Inter-alpha-trypsin inhibitor heavy chain H3
274	25,777	6.63	22,393	5.98	Q3T149	44.26	4	1	1	N	Heat shock protein beta-1
283	17,069	5.68	15,954	5.52	P02070	226.3	70	15	11	Y	Haemoglobin subunit beta
287	67,228	5.04	77,753	6.50*	Q29443	319.36	45	42	38	Y	Serotransferrin
296	45,590	7.00	38,348	6.50	Q2KIF2	101.53	20	8	7	Y	Leucine-rich alpha-2-glycoprotein 1
308	26,887	6.19	30,276	5.63	P15497	228.7	45	19	19	Y	Apolipoprotein A-I
416	20,944	6.49	15,184	8.19*	P01966	75.72	17	3	3	N	Haemoglobin subunit alpha
447	57,560	5.69	50,244	5.46	P12799	209.19	27	12	12	Y	Fibrinogen gamma-B chain
450	25,915	5.73	21,069	5.44	P18902	145.76	17	3	3	Y	Retinol-binding protein 4
460	45,761	6.75	44,471	7.06	P33433	107.34	8	3	3	Y	Histidine-rich glycoprotein
514	56,663	5.64	53,342	5.19	Q3MHN5	71.44	3	2	2	Y	Vitamin D-binding protein
537	27,304	5.71	23,182	5.67	Q3SZR3	107.16	18	3	3	Y	Alpha-1-acid glycoprotein

Spectra were imported into Peaks Studio and analysed against *Bos taurus* library for identification. Assignments were made by matching the MW and p*I* to that estimated from the gel position

*Denotes those showing >20 % difference in MW and p*I* from the estimated value

Assignment of identities to potential biomarker candidates

Putative identifications of the spots of interest were made on the basis of assignment of the corresponding molecular weight (MW) and isoelectric point (p*I*) of the protein spots. The average mass output and theoretical p*I* were determined from Expasy p*I* calculator (http://web.expasy.org/compute_pi/) and criteria for a protein match was set to be within 20 % of the estimated MW and p*I*. Four of the proteins showed greater than 20 % deviation in p*I* from that estimated and the MW differed for three proteins between Expasy and that estimated from the gel position; however, this is known to occur during 2DE as a result of the presence of different isoforms generated by the varying post-translational modifications. Two spots (135 and 609) were both identified as corresponding to fragments of serum albumin which is a high molecular weight protein commonly found across the plasma 2D gel map (see Electronic Supplementary Material (ESM) Fig. S1) and the remaining 21 spots were successfully identified as proteins specific to treated animals as depicted in Fig. 4.

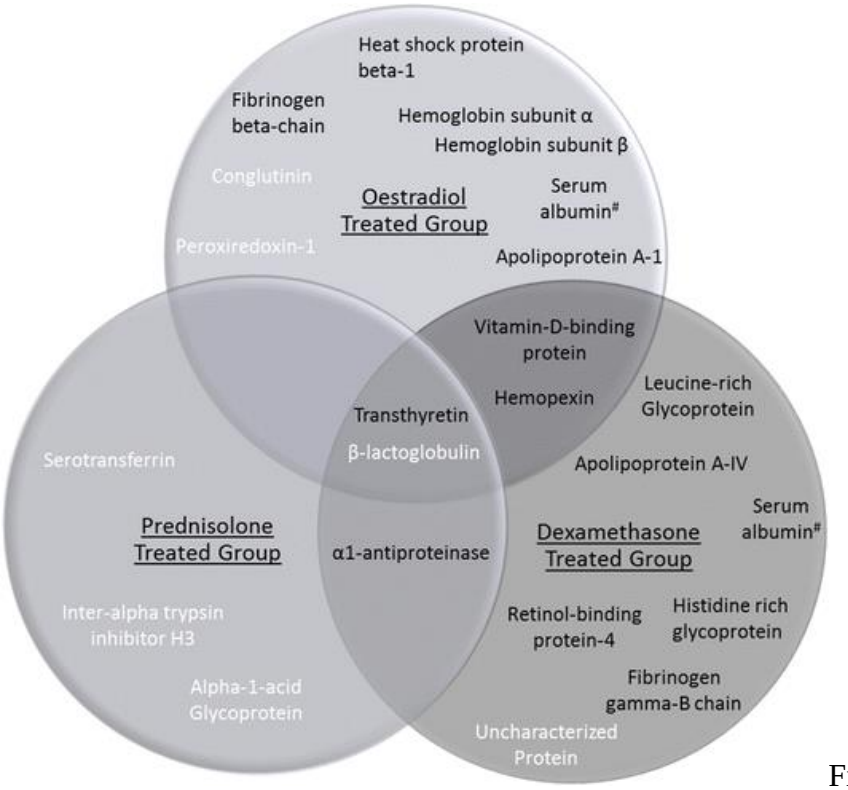


Fig. 4

Determination of proteins common to more than one type of treatment. Comparison of the respective treatment groups reveals two proteins altered by all treatments, two common to both oestradiol and dexamethasone treatment and one common to glucocorticoid treatment. Those in *white font* were found at decreased levels and those in *black font* showed increased levels compared to control animals. In addition, oestradiol treatment showed alteration of eight other proteins, dexamethasone significantly altered a further seven markers and prednisolone showed decreased levels of three proteins. #Denotes alteration of different fragments of the same protein

Discussion

The molecular mechanisms and effects of an array of growth-promoting agents have long been studied and the phenotypic results of their use in cattle are obvious through enhanced feed efficiency and muscle accretion. The objective of this study was to examine plasma at the proteome level using 2DE with the aim of identifying specific protein constituents which reflect exposure to exogenously administered growth-promoting compounds. Whilst there have been recent advancements made in gel-free proteomics techniques, 2DE remains one of the most commonly employed approaches of proteomic analysis [32]. This is mainly due to the simplicity of the top-down approach, whereby the entire profile of proteins can be screened to yield intact isolates that can be identified by downstream analysis. Bottom-up approaches rely on protease efficiency to separate peptide mixtures alongside compatible enrichment techniques based on known sequence information, whereas 2DE displays protein fragments in a quantitative array separated by MW and isoelectric point, retaining additional information such as relative post-translational modifications. The majority of plasma proteins are observable within the IPG range pH 4–7 and sample preparative steps in this study were optimized to ensure high resolution 2DE images were obtained, yielding more than 700 reproducible protein isoforms from each plasma sample analysed.

Plasma samples from four non-treated versus 12 growth-promoter-treated animals were assessed comparatively by 2DE to unveil oscillations in the blood protein profile after administration of oestradiol, dexamethasone and prednisolone. Plasma samples collected at day 25 were assessed as being representative of the period halfway through the glucocorticoid treatment and 6 days post administration of oestradiol, with a view to selecting robust markers with potential for assessment at the farm level. Proteomic analysis using 2DE is a non-targeted analytical approach and it is preferable to limit sample preparative steps to minimise loss of potential proteins of interest. However, with plasma it is necessary to suppress interference from high molecular weight proteins such as albumin, globulin and fibrinogen to ensure effective resolution of a wider range of constituent proteins. Comparison of a range of plasma preparative procedures concluded that the selected approach utilising sample reduction by DTT [30] depleted 70–80 % of the high molecular weight protein content, whilst enhancing the resolution of low molecular weight protein gel spots (data not shown).

Comparison of plasma proteins found to be differentially expressed in growth-promoter-treated groups relative to levels in non-treated control animals (Fig. 4) revealed two proteins (transthyretin and β-lactoglobulin) to be altered within all treatment groups, and three additional proteins (haemopexin, vitamin D-binding protein and α-1-antiproteinase) elevated by both dexamethasone and oestradiol treatments, or dexamethasone and prednisolone treatments for the latter; the expression levels of these five proteins are shown in Fig. 5. Plasma albumin was also found to be altered by oestradiol and dexamethasone treatment, but within different gel spots representing distinct fragments of the same protein which can occur through post-translational modifications and is reflected by multiple protein isoforms on the same 2D gel. Such variance is reflected in the differences observed between predicted and acquired gel outputs (MW/p*I*) for a range of identified proteins (transthyretin, β-lactoglobulin, haemopexin, inter-α-trypsin inhibitor H3, serotransferrin and haemoglobin subunit-α) (Table 2). Through identifying proteins significantly altered by various growth promoter regimes, it is possible to associate mechanisms responsible for the effects initiated by their administration, and assess their suitability as markers for use as potential drug abuse screening tools. Gene ontology data of identified proteins (Table 3) reveals primary

involvement in transport, blood coagulation, immune response and metabolism pathways. Proteins corresponding to typical plasma constituents such as fibrinogen and haemoglobin subunits were found at increased levels after growth promoter exposure whilst serotransferrin was suppressed by dexamethasone exposure. This is in agreement with other studies [33, 34] and may be relevant to altered hepatic metabolism, but the specific role in anabolic instigation is unclear.

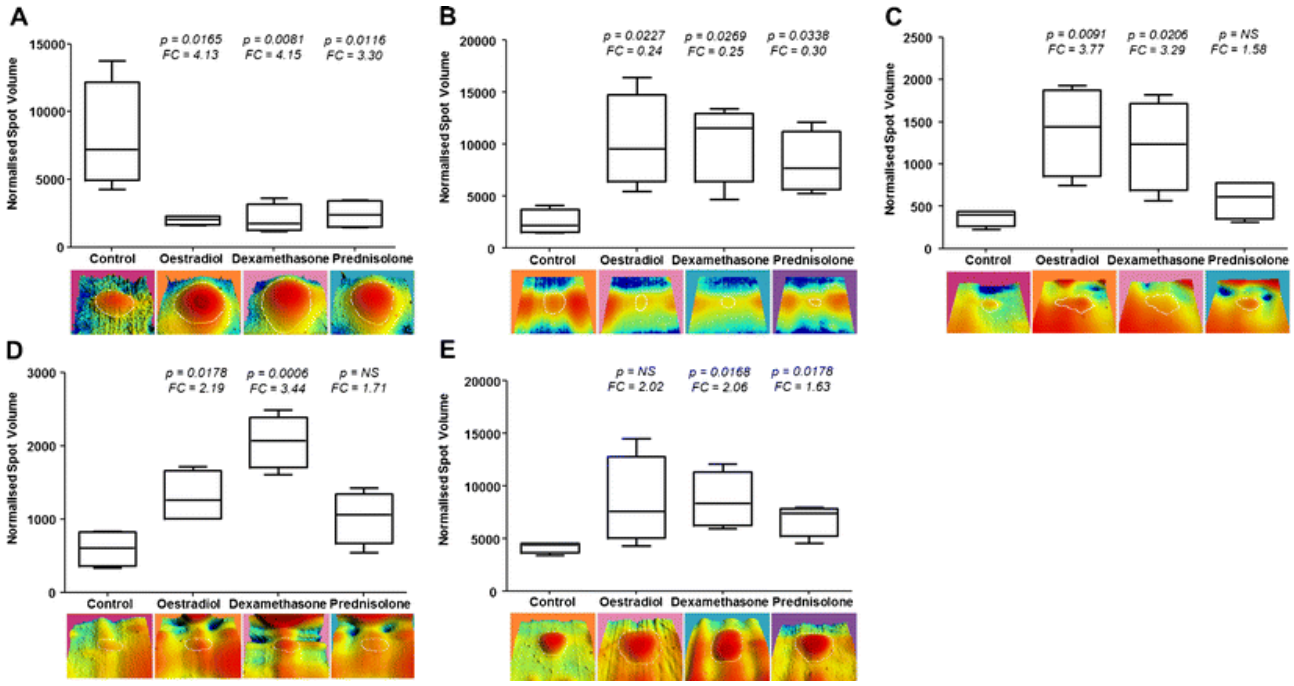


Fig. 5

Expression levels of those proteins commonly altered by growth-promoting treatment. The normalised spot volume detected across all of the gels is displayed in the box and whisker plots to show the profiles of **a** transthyretin, **b** beta-lactoglobulin, **c** haemopexin, **d** vitamin D-binding protein, **e** alpha-1-antiproteinase alongside the respective fold change and significance (*p* value) assessed via *t* test against untreated spot volume. *NS* not significant ($p > 0.05$). A representative spot is shown from each treatment group at the same scale with enhanced colour imaging for visual comparison

Table 3

Classification of biological function of identified proteins

Treatment group	Protein	Gene ontology (Uniprot)		
		Biological process	***Cellular component***	***Molecular function***
Oestradiol (O)	Conglutinin	Multi-functional	Collagen	Mannose binding
	Fibrinogen beta chain	Blood coagulation	Secreted	Fibrinogen complex
	Peroxiredoxin-1	Multi-functional	Cytoplasm	Antioxidant
	Apolipoprotein A-I	Metabolism	Secreted	Binding; transport
	Haemoglobin subunit alpha	Transport	Haemoglobin complex	Haeme-binding
	Heat shock protein beta-1	Stress response	Cytoplasm	Chaperone
	Haemoglobin subunit beta	Transport	Haemoglobin complex	Haeme-binding
	Leucine-rich alpha-glycoprotein	Fat differentiation	Extracellular space	Adipocytes
	Retinol-binding protein 4	Transport	Secreted	Retinol-binding
Dexamethasone (D)	Histidine-rich glycoprotein	Blood coagulation	Secreted	Immunoglobulin binding
	Apolipoprotein A-IV	Transport	Secreted	Lipid binding
	Fibrinogen gamma-B chain	Blood coagulation	Secreted	Fibrinogen complex
	Uncharacterized protein	Homologous to IGK protein (immunoglobulin kappa locus)		
Prednisolone (P)	Inter-alpha-trypsin inhibitor H3	Metabolism	Secreted	Protease inhibitor
	Alpha-1-acid glycoprotein	Acute phase response	Secreted	Lipocalin
	Serotransferrin precursor	Transport	Secreted	Iron binding
D & P	Alpha-1-antiproteinase	Blood coagulation	Secreted	Protease inhibitor
	Haemopexin	Transport	Secreted	Metal-ion binding
O & D	Vitamin D-binding protein	Transport	Secreted	Vitamin transporter
	Serum albumin precursor	Transport	Secreted	Binding
O, D & P	Transthyretin	Transport	Secreted	Hormone
	Beta-lactoglobulin	Transport	Secreted	Retinol-binding

The biological process represented by each identified protein was determined from the Uniprot gene ontology database revealing the respective subcellular location of expression as well as the projected molecular function. Identified proteins show main roles as typical blood constituents, acute phase reactants or transport/binding proteins

As corticosteroids are anti-inflammatory agents, the increase in α-1-antiproteinase after both prednisolone and dexamethasone treatment reflects its role as an acute phase protein and has been shown to be upregulated by dexamethasone exposure in previous studies [34, 35]. Similarly, increased plasma expression of α-1-acid glycoprotein and inter-α-trypsin inhibitor H3 has been found following prednisolone administration [36, 37]. Heat shock protein β-1 is also known to participate in the acute phase response and increased plasma levels during oestradiol treatment were shown in the current study, with increased levels in skeletal muscle due to dexamethasone reported elsewhere [38]. However, levels of acute phase proteins are known to vary depending on the health of an animal, and for that reason may not be suitable indicators of growth promoter use. Additionally, decreased levels of conglutinin and peroxiredoxin found after exposure to oestradiol in this study reflect a suppressed immune response [39] which also varies with animal health status.

The role of lipid and vitamin binding is considered of particular interest since seven of the proteins altered by growth promoter treatment were identified as important regulators of lipids and vitamin interaction and transport. These include apolipoprotein A-I, increased by oestradiol treatment; apolipoprotein A-IV, leucine-rich α-2-glycoprotein-1 and retinol-binding protein-4, increased after dexamethasone exposure; vitamin D-binding protein and transthyretin, increased after both oestradiol and dexamethasone treatments and decreased β-lactoglobulin exhibited by all treatment groups. Apolipoproteins AI and AIV are important mediators of lipid metabolism where they form protein complexes for efficient transport of cholesterol with deficiency known to impair endogenous steroid production [40] and they have previously been found at increased levels after growth promoter administration [41, 42]. Interestingly, leucine-rich α-2-glycoprotein-1 is already adopted as a marker of recombinant human growth hormone use in horses [43] where it is also found at increased levels.

Transthyretin, notably increased in expression within all treated animals, is a low abundant plasma protein responsible for transporting thyroxine (T4) from the liver. T4 is a thyroid hormone shown to increase during periods of increased food intake and subsequent weight gain in cattle, and is correlated to an increase in growth hormone and IGF-1 levels [44]. Alterations to transthyretin levels have not previously been reported in bovines administered growth-promoting agents, but have been elevated in humans following growth hormone administration [45] and the strong association with protein synthesis and metabolism suggests potential as a viable candidate biomarker. Transthyretin is often associated with retinol-binding protein-4 [46] which was also found to be increased by dexamethasone exposure in this study. β-Lactoglobulin binds plasma retinol during lipid metabolism [47] and was found at depressed levels in all treated animals but as a dietary compound [48] may not be an appropriate biomarker. Vitamin D-binding protein was found at increased levels in both dexamethasone and oestradiol treatment groups in this study and is

associated with bone formation [49] which is concurrent with other studies which have shown alteration of proteins responsible for bone formation such as osteocalcin [24] and PIIINP [22].

Conclusion

This study set out to identify proteins expressed in bovine plasma as markers of oestradiol, dexamethasone or prednisolone exposure to aid future applications of novel screening approaches to detect illegal administration. Despite its challenges, 2DE remains a valuable comparative analysis method capable of displaying an array of protein isoforms and a total of 22 proteins were found to be differentially expressed in plasma from treated relative to non-treated animals. Seven of the protein markers revealed possess primary influence on the growth hormone axis through lipid and vitamin metabolism, whereas the activity of the others is associated with common blood regulatory mechanisms or the acute phase immune reaction pathways, which are dependent on animal health status thereby limiting their potential as appropriate markers. Transthyretin was identified as being commonly increased within all animal treatment groups and such a common or global marker, providing a signature of abuse irrespective of the type of drugs used, would be of significant benefit to screening methodologies. For the first time plasma proteins significantly altered following prednisolone exposure in bovines are reported with downregulated levels of serotransferrin, inter-α-trypsin inhibitor H3 and α-1-acid glycoprotein. Additionally, α-1-antiproteinase was increased in both dexamethasone- and prednisolone-treated animals, whilst vitamin D-binding protein and haemopexin levels were increased after exposure to oestradiol and dexamethasone. Prior to implementation in targeted screening approaches, further validation will be required to analyse protein levels at time-points post administration to determine the detectable range as well as inclusion of additional animal cohorts of differing breed, sex and age. The potential for compounds when used in combination to interfere with the protein profile response when agents are administered individually is an important consideration in the context of the eventual application of screening methods based on protein marker profiles and must also be given consideration. Whilst it is anticipated that future screening would incorporate a range of markers relevant to various drugs of abuse, the potential of transthyretin, retinol-binding protein-4, apolipoproteins A-I and A-IV, vitamin D-binding protein, haemopexin and leucine-rich α-2-glycoprotein-1 as novel protein markers of growth promoter treatments has been demonstrated in this study.

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