agriculture

MDPI

Article

Assessment of the Physiological Values and the Reference Histological Profile Related to Sex Steroids in Veal Calves

Marzia Pezzolato [1], Elena Biasibetti [1,*], Marilena Gili [1], Cristiana Maurella [1], Alessandro Benedetto [1], Marianna Marturella [1], Federica Ostorero [1], Giancarlo Bozzo [2], Claudio Bellino [3], Antonio D'angelo [3], Flaminia Valentini [3], Francesca Roberti [4], Maurizio Fiori [5], Paolo Stacchini [5,†] and Elena Bozzetta [1,†]

1 Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, 10154 Torino, Italy
2 Department of Veterinary Medicine, University of Bari 'Aldo Moro', 70010 Bari, Italy
3 Dipartimento di Scienze Veterinarie, Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Torino, Italy
4 Direzione Generale per L'igiene e la Sicurezza Degli Alimenti e la Nutrizione, Ufficio 8 Ministero della Salute, Via G. Ribotta, 5, 00144 Roma, Italy
5 Dipartimento Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria, Istituto Superiore di Sanit Viale Regina Elena, 299, 00161 Rome, Italy
* Correspondence: elena.biasibetti@izsto.it
† These authors contributed equally to this work.

Abstract: Among forbidden substances included in the European Union legislation, endogenous steroids constitute a challenge in the framework of veterinary Official Monitoring Plans. They can be naturally present in body fluids at variable levels depending on the species, sex and age of the animals. Considering the significant advances achieved in breeding conditions and in the selection of producing traits in meat cattle, the aim of this study was to verify by analytical method, in veal calves housed under controlled conditions, if the level of natural steroids hormones assumed as physiological are still actual. The second aim of the study was to verify if the normal histological pattern of growth promoters in target organs is influenced accordingly. Bovine male sex organs are currently analysed in the frame of the Italian histological plan to monitor illicit treatments trend, highlighting microscopic, induced alterations. The levels of 17β-estradiol and progesterone residues resulted under the Limit of Quantitation of the approved official methods and the level of testosterone resulted below the level stated in the Italian Ministerial Decree in force. Male target organs appeared within the limits of the standard histological features.

Keywords: calves; natural steroids hormones; histology; LC-MS/MS

check for updates

Citation: Pezzolato, M.; Biasibetti, E.; Gili, M.; Maurella, C.; Benedetto, A.; Marturella, M.; Ostorero, F.; Bozzo, G.; Bellino, C.; D'angelo, A.; et al. Assessment of the Physiological Values and the Reference Histological Profile Related to Sex Steroids in Veal Calves. *Agriculture* **2023**, *13*, 1145. https://doi.org/10.3390/agriculture13061145

Academic Editors: Olga Witkowska-Piłaszewicz, Bartosz Pawliński and Michal Skibniewski

Received: 31 March 2023
Revised: 12 May 2023
Accepted: 26 May 2023
Published: 29 May 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Growth-promoting practices are still encountered within the European Union (EU), despite Directive 88/146/EC published in the late 1980s totally banning the use of growth promoters in livestock production [1]. Contemporarily, to comply with the ban on all hormonal active growth promoters ("hormones") in livestock production, mandatory monitoring and surveillance programs, based on screening and confirmatory analysis, were implemented in European Countries through the Council Directive 96/23/EC [2] then replaced by Regulation (EU) 2017/625 of the European Parliament and of the Council from December 2022 [3]. Directive (EU) 2017/625 applies to food control and introduces new national surveillance programs for the monitoring of residues of prohibited substances.

In the frame of a risk-based control plan, each Member State developed effective methods to control anabolic agents' misuse, thus verifying the agreement between the EU legislation on the use of pharmacologically active substances and the food-producing animal sector. Council Directive 96/23/EC required that Member States implement a national residue monitoring plan for specific groups of residues specified in its Annexes I

and II, in agreement with the sampling strategy and sampling frequency laid down in Annexes III and IV [2]. All cited aspects are still enclosed in CDR (EU) 2022/1644, which replaces Dir. 96/23.

The presence of residues of unauthorized substances such as growth hormones may represent a health risk for consumers [4]. Among anabolic agents, sex hormones exert the most potent effect by significantly improving feed conversion rate, weight gain and muscle growth, thus assuming particular interest for breeders [5].

Regarding their chemical nature, they can be divided into two sub-groups: steroids natural to the body (endogenous steroids such us 17β-estradiol, progesterone and 17β-testosterone) and steroids foreign to the body (synthetic steroids). The serum concentration of the molecules of the first group varies according to the physiological status, and the age of the animal and they could also be influenced by the presence of specific bacteria or enzyme; from a control point of view these molecules are difficult to manage; their synthetic counterpart, on the other hand, not being produced physiologically and not being admitted, must be absent at serum level [6].

For endogenous steroids, due to their natural origin, there is no established European maximum residue limit (MRL) but only recommended minimum method performance requirement (MMPRs) for control purposes [6]. However, in Italy, the illegal use of natural hormones is established by comparing the concentrations detected in animals with the reference physiologic values reported in the Ministerial Decree of 14 November 1996, broken down by hormone and by animal production category. 17β-estradiol limit is 0.040 μg L^{-1} for all categories (males and females <6 months and >6 months); progesterone limit is 1 μg L^{-1} for males and females <6 month, 1.5 μg L^{-1} for males > 6 months and 14 μg L^{-1} for females >6 months); 17β- testosterone limit is 10 μg L^{-1} for males < 6 months; 0.5 μg L^{-1} for females <6 month and >6 months and 30 μg L^{-1} for males [7]. Italian legislation limits are well below the EU MMPR, pointing to a stricter attitude of the national legislator on residues.

Considering that in the last decade, breeding practices have dramatically changed, in particular for the calf category, we need to consider whether these limits can be still actual or have to be revaluated. Changes in breeding concern, in particular feed ingredients, feeding methods and a portion of the milk replacer solid feeds (mixture of roughage and concentrate); moreover, the slaughtering categories established on the basis of the achievement of a weight of about 240 kg and the slaughtering age, raised from six to eight months, were redefined. These changes were introduced to meet consumer expectations and comply with stringent regulations on animal welfare [8].

Moreover, extensive genetic selection allowed us to obtain an important increase in production traits in both meat and dairy cattle [9].

To date, research has mainly focused on setting up methods to identify illicit treatments with synthetic hormones, while the natural hormonal profiles essential to highlight the misuse of natural hormones were poorly investigated due to the lack of discriminatory power of the current screening methods even if this last focus has the attention of the Institutions and the reference scientific bodies (European and Nationals Laboratories). Therefore, a survey to revise and assess physiological levels of natural sex hormones in meat-producing animals could significantly contribute to clarifying when their concentrations in body fluids can be related to a suspected illicit administration.

The aim of this study was to collect and update reference data for natural concentrations of endogenous hormones in veal calves, performing a lifetime-controlled animal experiment, to verify if concentrations of natural steroids hormones reported in the national and European Regulation are still actual.

The second aim was to describe the normal histological patterns of sex hormones' target organs in males and in females. Male sex target organs were further analysed in order to update the histological profile adopted as "normal" in the Italian histological plan to monitor illicit hormonal treatment [10].

Contemporarily also the accuracy of the expression of progesterone receptor antibody as a marker used to highlight sex hormone abuse in male calves was evaluated.

2. Materials and Methods

The study was conducted according to animal welfare considerations and regulations, and the protocol of breeding was approved by the Italian Ministry of Health (242/2020-PR) and the Bioethics Commission of the University of Turin and Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta.

2.1. Study Design and Sample Size

To set up an observational study and to calculate the range of normality, the mean values and two standard deviations for progesterone, estrogen and testosterone a lifetime-controlled animal experiment was set up. The lowest number of animals was recruited following available guidelines and also considering the size of the farm, the welfare of the animals bred and the costs. The number of animals has been significantly reduced according to Friedrichs K.R. et al., 2012 while assuming to save and being able to compare the data with those already present in the literature and in the Ministerial Decree of 14 November 1996 and possibly with other future ones [11]. Therefore, a total of 30 (15 male and 15 female) Holstein Fresian calves coming from local markets were enrolled at the approximate age of 15 to 60 days of life on arrival at the farm and weight of 40–60 kg.

2.2. Animals' Management

Upon arrival all calves were weighed using a digital weighing scale and a complete physical examination was performed; clinical parameters such as rectal temperature, cough, nasal discharge, ocular discharge or ear position were evaluated. Animals displaying evidence of disease, injury and dehydration, and those presenting an initial unhealthy status were not included in the study.

The animals were randomly divided into four groups matched for body weight, age and sex, and farmed under the same conditions for 8 months. Each box had its own crib and multiple drinking troughs.

The animals included in the experimental trial were housed under controlled conditions, faithfully reproducing the zootechnical practices of veal industry farm.

To protect the animals against infections, all were vaccinated against infectious bovine rhinotracheitis, parainfluenza-3 virus and bovine respiratory syncytial virus. Clinical controls were carried out daily by a veterinarian. In case of occurrence of disease, a therapeutic protocol was established in compliance with the production category, the drug legislation and the objectives of the experimental trial. Each treatment was followed by a variable monitoring period to verify the therapeutic efficacy and the presence of any undesirable effect. Animals were slaughtered before 8 months or at approximate 240 kg weight, according to the practice of veal industry farms.

2.3. Blood Sampling

Blood was collected from the jugular vein of each calf into a sterile blood-collection tube; serum obtained by centrifugation at 2000× *g* for 20 min was stored at −20 °C until analysis. Blood parameters such as total protein and haematocrit were measured. Sampling started at two months of age and was carried out every month at a slaughterhouse.

A timeline representing the study and duration of sample collection is shown in Figure 1.

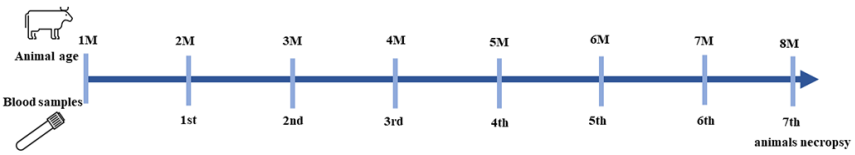

Figure 1. A timeline of study. Timeline of the study, duration, and time of sample collection.

2.4. Chemicals and Reagents

Acetonitrile, methanol, and ammonium fluoride were of analytical or High Performance Liquid Chromatography (HPLC) grade quality and were supplied by Sigma-Aldrich (St. Louis, MO, USA). Molecularly imprinting polymers (MIPs) Solid phase extraction (SPE) cartridges Affinmip Estrogens (100 mg, 3 mL) were purchased from Affinsep (Petit-Couronne, France). 17β-estradiol, 17β-estradiol-d4, 17β-testosterone, 17β-testosterone-d3, progesterone and progesterone-d9 were supplied by Sigma-Aldrich (St. Louis, MO, USA). The stock standard solutions of each analyte and internal standard (ISTD) were prepared in methanol at the concentration of 2 μg L^{-1} μg/mL and stored at −20 °C in the dark; solutions were stable for 2 years. Suitable working standard solutions in methanol were obtained by appropriate dilution of the corresponding stock solutions and stored at −20 °C.

2.5. Blood Sample Preparation

Blood serum (2 mL) was spiked with internal standard spiking solution (ISTD) and diluted with 7 mL water. The sample was loaded on AFFINIMIP® SPE (0.5 drop/s), previously conditioned with 3 mL acetonitrile and 3 mL water; after washing with 2 × 3 mL water and 3 mL water/acetonitrile (6:4), the cartridge was dried; analytes were eluted with 3 mL methanol. After evaporation to dryness in nitrogen stream at 50 °C, the residue was dissolved in 0.1 mL methanol/water (1:1) mixture.

2.6. LC-MS/MS Analysis

LC analysis was carried out through an HPLC system Exion (Sciex, Framingham, MA, USA). The analytes were detected in ESI positive (17β-testosterone and progesterone) and negative (17β-estradiol) MRM mode. Chromatographic separation was performed on a Waters XSelect HSS T3 XP (3 × 100 mm; 2.5 μm) column, kept in a column oven at 40 °C, using gradient elution with ammonium fluoride (-NH_4F) 0.2 mM in water (A) and ammonium fluoride 0.2 mM in methanol (B). 17b-estradiol-d4, 17b-testosterone-d3 and progesterone-d9 were used as internal standards.

The mass spectrometer was a QTRAP 6500+ (Sciex, Framingham, MA, USA). Injection volume was 25 μL and flow rate was 0.3 mL/min, with an overall run time of 20 min. The gradient profile was as follows: 0–1 min 50% (B); 1–6 min linear increase up to 95% (B); 6–14 min 95% (B); 14–18 min ramp linearly to 50% (B); 18–20 min 50% (B). At least 3 product ions were monitored for each analyte. The multiple reaction monitoring (MRM) conditions are given in Table 1.

The method was validated both for screening and quantification purposes according to Commission Decision 2002/657/EC at the following concentration range: 0.020–0.080 μgL^{-1} for 17β-estradiol, 0.25–10 μg L^{-1} for 17β-testosterone and 0.5–20 μg L^{-1} for progesterone [3].

2.7. Tissue Sampling and Processing

At the slaughterhouse from male animals' prostate and bulbourethral glands were sampled, while female animals' mammary glands, Bartholin's gland and ovaries.

All tissues were fixed in 4% neutral buffered formaldehyde at room temperature for about three days, routinely processed, embedded in paraffin wax and then sectioned in 3–5 μm slices.

2.7.1. Histology

Slides were hydrated, routinely stained with haematoxylin and eosin (HE), dehydrated, cleared and mounted. All samples were prepared and analysed in blind by three pathologists.

The sections were observed under an optical microscope and the degrees of maturation of the tissues and the pictures other than the physiological ones were described.

Specifically, Prostate, bulbourethral glands in males and Bartholin's glands in females were analysed in order to identify microscopic changes, in particular parameter analysed

was absence/presence of squamous metaplasia graded from mild to severe of glandular tissue and ducts [12].

The mammary gland tissue was analysed and the grade of tissue maturation such as presence of glandular and ducts recorded. Concretions were checked too.

The ovaries were analysed in order to verify the grade of follicle development.

Table 1. MS/MS parameters (DP, declustering potential; EP: entrance potential; CE: collision energy; CXP: Collision Cell Exit Potential).

Analytes	DP	EP	Parent Ion	Product Ion	CE	CXP
17β-estradiol	−100	−10	271.2	**145.0**	−50	−12
				183.1	−54	
				143.0	−67	
17β-estradiol-d4	−130	−10	275.2	**147.0**	−53	−12
				187.0	−54	
				145.0	−65	
17β-testosterone	74	10	289.1	**97.2**	29	12
				109.0	33	
				79.1	70	
17β-testosterone-d3	90	10	292.2	**256.4**	25	12
				109	32	
				97.2	29	
Progesterone	103	10	315.2	**97.1**	26	12
				109.1	30	
				297.4	22	
Progesterone-d9	80	10	324.3	**100.1**	29	12
				113.1	33	
				306.4	25	

2.7.2. Immunohistochemistry

Immunohistochemical analysis for progesterone receptor was performed on paraffin sections (3 +/− 2 µm) from all prostate and bulbourethral glands samples. Slides were deparaffinised and rehydrated in graded alcohols. Heat-induced (97 °C) citrate buffer (pH 6) antigen retrieval was applied in a water bath for 30 min. Slides were then incubated with 3% hydrogen peroxide for 30 min at room temperature to inhibit endogenous peroxidase activity. Sections of prostate and bulbourethral glands were incubated with mouse monoclonal anti-progesterone receptor (PR) antibody (clone hPRa 2, Thermo Fisher Scientific, Fremont, CA, USA) diluted 1:50 for 60 min at room temperature.

The EnVision System Kit (Dako, Glostrup, Denmark) was used for identification purposes. Diaminobenzidine-hydrogen peroxide solution (Dako) was used as chromogen and applied for 4 min. Sections were slightly counterstained with hematoxylin, dehydrated, cleared and mounted. The specificity of the staining was assessed by the omission of primary antibodies.

3. Results

The animals of this study remained in good health; mean body weight at slaughter was 232 kg.

3.1. LC-MS/MS Method for the Quantitative Determination of Sex Steroids in Blood Serum

The developed method was successfully validated both for screening and quantitative confirmatory purposes according to the Commission Decision 2002/657/EC criteria at the following concentrations range: 0.020–0.080 µg L^{-1} for 17β-estradiol, 0.25–10 µg L^{-1} for 17β-testosterone and 0.5–20 µg L^{-1} for progesterone. The method met the criteria set out in Commission Decision 2002/657/EC for the purpose of confirmation in terms of retention time and ion ratio in the whole range of its application [13]. Moreover, the method resulted

fit for purpose according to new criteria required by CDR Reg 808/21 [14], which replaces CD 2002/657/EC.

The developed method is specific and sensitive, suitable for measuring the natural level of sex hormones in bovine serum. During specificity evaluation, none of the 20 blank serum samples analysed resulted affected by matrix interference in selected retention time windows. For each analyte, trueness ranges between 95–110%, intermediate repeatability always stays below 18%; decision limits (CCα) were, respectively, 0.043 µg/L for 17β-estradiol, 11.4 µg/L (male) or 0.55 µg/L (female) for testosterone and 1.49 µg/L (male) or 1.98 µg/L (female) for progesterone.

In residue studies, in addition to the appropriate techniques of detection, no less important is the proper preparation of the sample. It is essential to use repeatable sample preparation procedures in order to minimise the differences between the samples in the course of analysis.

The developed method allows achieving the simultaneous extraction of the three natural sex hormones from blood serum, avoiding two different sample treatment procedures. Ammonium fluoride in the aqueous phase allowed to obtain good sensitivity for all analytes without the need to use ammonium hydroxide or an APCI ionization mode.

Molecularly imprinting polymers (MIPs) cartridges as solid-phase extraction improve the method selectivity and the specificity [15]: this kind of sorbent is made by a polymerisation process to create a three-dimensional network that recognises the shape and the functional group positions of a template molecule.

A small number of stages of the procedure eliminates the problems associated with the recovery and shortens the sample preparation time. In addition, an internal standard used in this method reduced all the variation and losses during sample preparation before instrumental analysis. Moreover, the application of the stable isotope-labelled internal standard is one way to compensate for the matrix effect. Matrix effect (ME), such as enhancement or suppression of analytical signal frequently occurs in the chemical analysis of biological samples measured by LC-MS/MS: this is related to the alteration in ionisation efficiency in the ionisation source due to the presence of co-eluting, in particular, endogenous substances present in the sample and remaining in the final extract.

3.2. Blood Serum Sample Analysis

Of the 210 serum samples collected during the study, 26 samples were not suitable for analysis, due to scarce volume collected or hemolysis; 184 serum samples were analysed by the multiresidue screening method. In all the samples, both 17b-estradiol and progesterone were not detected above the limit of quantification (LOQ) value.

In 66 male serum samples, 17β-testosterone was detected at concentration levels above the LOQ value, and the results of the confirmatory analysis for 17β-testosterone were reported in Table 2. The data obtained were in accordance with previous studies [16,17], and confirm that the values in calves were below the limits indicated in the national legislation [7].

Histology

The histological features observed are summarised below.

The bulbourethral glands of male animals showed peripheral mucinous epithelium and central more immature epithelium, and only slight secretion was highlighted, as normal findings for the animals age (Figure 2).

Bartholin's glands showed normal ducts and gland epithelium lined by cuboidal to the transitional epithelium in all animals (Figure 3).

The mammary gland tissue of all the females consisted of an immature duct system and the stromal tissue appeared immature. Few developed alveoli were observed, while the ducts and lobes of the glands were smaller in respect of what observed in the gland in its active phase. Furthermore, neither secretion nor concretions were detected in all preparations (Figure 3).

Table 2. 17β- testosterone levels (μg L^{-1}) in male bovine serum, NC sample not collected.

Animal	Age (Days)	Testosterone Level (μg L^{-1})	Age (Days)	Testosterone Level (μg L^{-1})	Age (Days)	Testosterone Level (μg L^{-1})	Age (Days)	Testosterone Level (μg L^{-1})	Age (Days)	Testosterone Level (μg L^{-1})	Age (Days)	Testosterone Level (μg L^{-1})	Age (Days)	Testosterone Level (μg L^{-1})
1	56	-	84	-	109	-	138	1.20	165	0.64	192	2.90	219	2.90
2	57	0.56	85	0.26	110	-	139	0.69	166	0.59	193	2.90	220	7.80
3	57	0.50	85	0.29	110	-	139	0.50	166	3.20	193	2.50	220	2.50
4	57	0.45	85	0.75	110	0.37	139	3.00	166	10.0	193	8.80	213	1.70
5	57	-	85	0.53	110	0.25	139	1.60	166	0.75	193	2.70	220	9.00
6	58	-	86	-	111	0.71	140	0.25	167	7.90	194	8.10	214	5.40
7	60	-	88	0.56	113	0.58	142	1.50	169	0.65	196	3.90	210	0.80
8	60	-	88	-	113	-	142	1.00	169	0.75	196	5.10	210	1.80
9	60	-	88	-	113	-	142	0.26	169	0.77	NC	NC	NC	NC
10	66	-	94	-	119	-	148	0.31	175	1.17	NC	NC	NC	NC
11	67	-	95	-	120	-	149	0.82	176	-	203	5.10	NC	NC
12	68	0.56	96	0.43	121	0.25	150	0.64	177	5.90	204	0.37	211	0.54
13	70	-	98	0.27	123	0.25	152	0.48	179	0.60	206	1.90	NC	NC
14	73	-	101	-	126	-	155	-	182	-	209	-	NC	NC
15	74	-	102	0.29	127	-	156	0.75	183	0.51	210	1.80	NC	NC

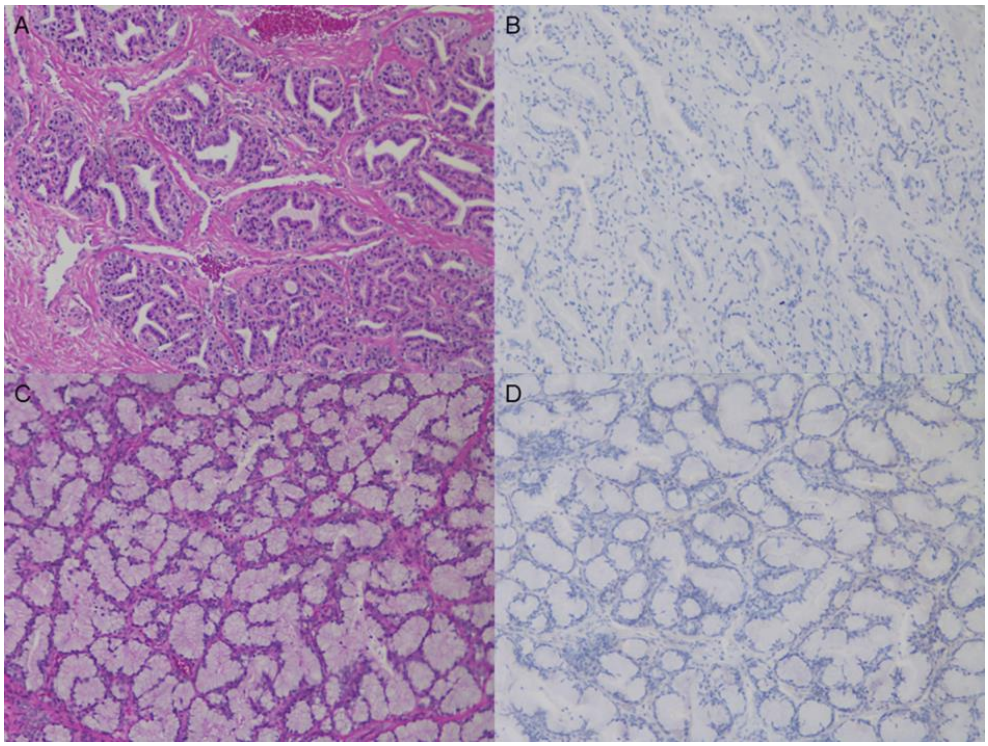

Figure 2. Histological pictures of prostate and bulbo-urethral glands. (**A**–**C**) normal glandular tissue of the prostate and bulbo-urethral glands (HE; 10x). (**B**–**D**) absence of immunopositivity to PR in the prostate and bulbo-urethral gland (10x).

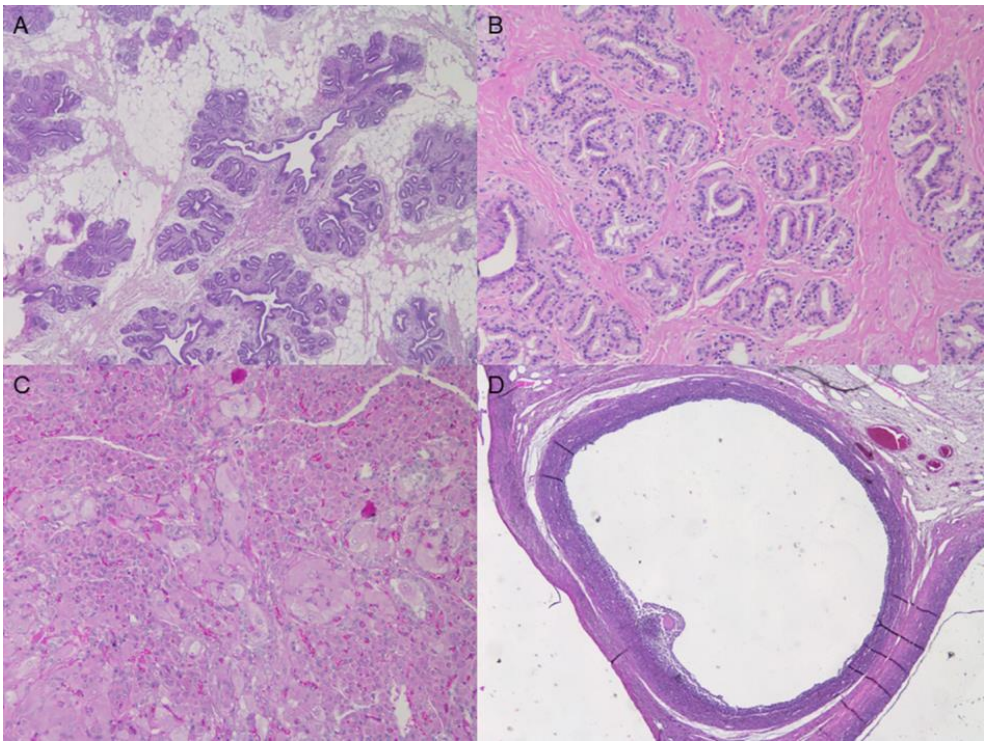

Figure 3. Histological pictures of mammary gland, Bartholin gland and ovary. (**A**) mammary gland tissue characterised by an immature duct system (EE; 2x); (**B**) normal histology of the Bartholin gland (EE; 10x); (**C**) ovaries with developing follicles (primary and secondary) (EE; 10x); (**D**) ovaries with atretic follicles (EE; 2x).

The ovaries showed growing follicles (primary and secondary) and atretic follicles. No corpora lutea were seen (Figure 3).

3.3. Immunohistochemistry

Immunohistochemistry for progesterone receptor was negative, no brown precipitate was detected in all prostate and bulbourethral glands analysed (Figure 2).

4. Discussion

Among forbidden substances included in the EU legislation, endogenous sex steroids constitute a challenge in the framework of veterinary official monitoring plans. Indeed, these molecules are naturally present at low and variable concentrations depending on the animal's race, sex and age.

A study aimed at collecting data on the physiological levels of sex hormones in veal calves, reared in controlled conditions according to the normal practice applied in Europe, was completed to assess the validity of threshold levels reported in legislation in force and to update histological characterisation of sex hormones target organs. In compliance with the European Union legislation, the enrolled animals were slaughtered within eight months of age (category V) and below 240 kg of live weight.

Regarding sex hormones determination, the first systematic list of analytical methods for residue control was published by European Community in 1994 [6]. However, the more recent advances in MS spectrometry have further allowed simultaneous quantitative determination of all-natural sex hormones.

Indeed, the developed method allowed to achieve the simultaneous extraction of the main three natural sex hormones from blood serum, avoiding different sample treatment procedures. Matrix effect, variation and losses during sample preparation are minimised by using the stable isotope-labelled internal standard (SIDA approach). The use of the AFFINIMIP® cartridges allows for a very good clean up. Furthermore, ammonium fluoride as a mobile phase provides an enhancement in the estradiol signal, the analyte with the lowest limits. The developed method satisfied therefore the criteria set in CDR 808/21 [14] for its confirmation purpose, considering recorded retention times and ion ratio in the whole range of its application. The natural level of sex hormones in bovine serum can be effectively detected and then quantified by the reported fit-for-purpose validated method.

As recently suggested by the European Union Reference Laboratory of Wageningen Food Safety Research (EURL WFSR) for Natural Growth Promoting substances [18], further investigations should be focused on unequivocally differentiating sex endogenous hormones from molecules derived from an illicit treatment. The two main approaches described in this Reflection Paper are the direct detection of steroid esters (prodrugs) by LC_MS/MS and isotopic ratio mass spectrometry (GC-IRMS), respectively [19–24]. Cited confirmation methods will need to be applied in future field surveys.

Residue data of natural sex hormones obtained in the study are confirmed below those reported in the national legislation [7], confirming that limits reported in the Italian regulation are consistent with the objective of ensuring consumers' health and the trustworthiness of the meat production chain. However, the results obtained suggest that the levels of the current MMPR applied at the EU level should be reconsidered to more effectively highlight illicit treatments and protect consumers' health.

Moreover, the histological investigations performed on target organs showed microscopic findings within the limits of normal standards, as described in previous results obtained from our [25,26] and other studies [27,28].

In addition, no PR expression was observed by immunochemistry in the accessory sex glands sampled from male animals [29,30], confirming the specificity of the PR biomarker for detecting estrogenic treatment. This result confirms PR as a reliable biomarker in detecting estrogen illicit treatment. The gene encoding for progesterone receptor (PR) and its translated protein are widely accepted as a marker of oestrogenic effects in different human and murine tissues; this evidence was indeed verified in

the reproductive system of male cattle. Successively PR was further confirmed as a specific biomarker for oestrogen treatment in male cattle (category V) by both validated immunohistochemical and molecular methods [29,30].

Based on the above evidence, none of the animals could have been s classified as suspect positive based on the screening procedure for sex hormones adopted for the Italian Histological Plan [10], thus showing its reliability.

Our results highlight that new breeding cycles and feeding protocols of calves imposed on farmers to become more efficient and at the same time sustainable in production as well as maintaining high standards of animal welfare and reducing the use of antibiotics does nott interfere with the specificity of the criteria adopted in the Italian histological monitoring plan to suspect sex hormones related treatment.

This evidence is also in line with results already published, showing that the presence of phytoestrogen in feeds could not cause microscopic alteration referable to illicit treatment in the target tissues [31].

Finally, microscopic results are in agreement with the levels of 17β-estradiol and progesterone residues retrieved.

Author Contributions: Conceptualization, M.P., F.R., P.S. and E.B. (Elena Bozzetta); methodology, E.B. (Elena Biasibetti), M.G., A.B. and C.B.; software, C.M.; validation, C.M., M.G. and C.B.; formal analysis, C.M., M.G., M.M., F.O. and M.F.; investigation, C.B., A.D., F.V. and A.B.; resources, E.B. (Elena Bozzetta); data curation, C.M.; writing—original draft preparation, M.P. and E.B. (Elena Biasibetti); writing—review and editing, A.B., C.M., M.G., M.F., G.B. and E.B. (Elena Bozzetta); visualization, A.D., A.B., G.B., P.S. and E.B. (Elena Biasibetti); supervision, F.R., P.S. and E.B. (Elena Bozzetta); project administration, P.S. and E.B. (Elena Bozzetta); funding acquisition, F.R., P.S. and E.B. (Elena Bozzetta). All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Italian Ministry of Health (project number: J11F19000480001 and J11J22000500001).

Institutional Review Board Statement: The study was conducted in accordance with animal welfare considerations and regulations, and the protocol of breeding was approved by the Italian Ministry of Health (242/2020-PR) and the Bioethics Commission of University of Turin and Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. *Council Directive 88/146/EEC*; Council Directive 88/146/EEC of 7 March 1988 Prohibiting the Use in Livestock (Arming of Certain Substances Having a Hormonal Action. Official Journal of the European Communities: Luxembourg, 1988; Volume L70, pp. 16–18.
2. *Council Directive 96/23/EC of 29 April 1996*; Council Directive 96/23/EC of 29 April 1996 on Measures to Monitor Certain Substances and Residues Thereof in Live Animals and Animal Products and Repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. Official Journal of the European Communities: Luxembourg, 1996; pp. 10–32.
3. EU. *Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on Official Controls and Other Official Activities Performed to Ensure the Application of Food and Feed Law, Rules on Animal Health and Welfare, Plant Health and Plant*; EU: Maastricht, The Netherlands; Official Journal of the European Communities: Luxembourg, 2017; pp. 1–142.
4. Mooney, M.H.; Situ, C.; Cacciatore, G.; Hutchinson, T.; Elliott, C.; Bergwerff, A.A. Plasma biomarker profiling in the detection of growth promoter use in calves. *Biomarkers* **2008**, *13*, 246–256. [CrossRef] [PubMed]
5. McGrath, T.F.; Van Meeuwen, J.A.; Massart, A.C.; De Pauw, E.; Delahaut, P.; Buijs, J.; Bergwerff, A.A.; Elliott, C.T.; Mooney, M.H. Effect-based proteomic detection of growth promoter abuse. *Anal. Bioanal. Chem.* **2013**, *405*, 1171–1179. [CrossRef] [PubMed]
6. Heitzman, R.J. *Veterinary Drug Residues: Residues in Food-producing Animals and Their Products—Reference Materials and Methods*; WileyBlackwell: Hoboken, NJ, USA, 1994.
7. Decreto Ministeriale. *Decreto Ministeriale 14 Novembre 1996. Determinazione dei Livelli Fisiologici Massimi Degli Ormoni Sessuali di Natura Endogena Estradiolo 17 beta, Progesterone e Testosterone nel Siero o nel Plasma di Sangue Bovino*; Gazzetta Ufficiale: Trieste, Italy, 1997.
8. Rutherford, N.H.; Lively, F.O.; Arnott, G. A Review of Beef Production Systems for the Sustainable Use of Surplus Male Dairy-Origin Calves Within the UK. *Front. Vet. Sci.* **2021**, *8*, 635497. [CrossRef] [PubMed]

9. Doyle, J.L.; Berry, D.P.; Veerkamp, R.F.; Carthy, T.R.; Evans, R.D.; Walsh, S.W.; Purfield, D.C. Genomic regions associated with muscularity in beef cattle differ in five contrasting cattle breeds. *Genet. Sel. Evol.* **2020**, *52*, 2. [CrossRef] [PubMed]
10. Pezzolato, M.; Baioni, E.; Maurella, C.; Benedetto, A.; Biasibetti, E.; Bozzetta, E. The Italian strategy to fight illegal treatment with growth promoters: Results of the 2017–2019 histological monitoring plan. *Ital. J. Food Saf.* **2022**, *10*, 9775. [CrossRef]
11. Friedrichs, K.R.; Harr, K.E.; Freeman, K.P.; Szladovits, B.; Walton, R.M.; Barnhart, K.F.; Blanco-Chavez, J. ASVCP reference interval guidelines: Determination of de novo reference intervals in veterinary species and other related topics. *Vet. Clin. Pathol.* **2012**, *41*, 441–453. [CrossRef]
12. Pezzolato, M.; Richelmi, G.B.; Maurella, C.; Pitardi, D.; Varello, K.; Caramelli, M.; Bozzetta, E. Histopathology as a simple and reliable method to detect 17β-oestradiol illegal treatment in male calves. *Food Addit. Contam.—Part A* **2013**, *30*, 1096–1099. [CrossRef]
13. Commission Decision. *2002/657/EC Commission Decision of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results*; Official Journal of the European Communities: Luxembourg, 2002.
14. European Commission. *Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the Performance of Analytical Methods for Residues of Pharmacologically Active Substances Used in Food-Producing Animals and on the Interpretation of Results as Well as on the Methods to be Used for Sampling and Repealing Decisions 2002/657/EC and 98/179/EC (Text with EEA Relevance)*; European Commission: Brussels, Belgium, 2021; Volume 180, pp. 84–109.
15. Lucci, P.; Núñez, O.; Galceran, M.T. Solid-phase extraction using molecularly imprinted polymer for selective extraction of natural and synthetic estrogens from aqueous samples. *J. Chromatogr. A* **2011**, *1218*, 4828–4833. [CrossRef]
16. Scarth, J.; Akre, C.; van Ginkel, L.; le Bizec, B.; de Brabander, H.; Korth, W.; Points, J.; Teale, P.; Kay, J. Presence and metabolism of endogenous androgenic-anabolic steroid hormones in meat-producing animals: A review. *Food Addit. Contam.—Part A* **2009**, *26*, 640–671. [CrossRef]
17. Woźniak, B.; Witek, S.; Matraszek-Zuchowska, I.; Kłopot, A.; Posyniak, A. Levels of the natural hormones 17β-oestradiol and testosterone in serum of cattle: Results from population studies in Poland. *J. Vet. Res.* **2016**, *60*, 461–466. [CrossRef]
18. Arrizabalaga Larranaga, A.; Groot, M.J.; Blokland, M.H.; Barbu, I.M.; Smits, N.G.E.; Sterk, S.S. *EURL Reflection Paper 2.0: Natural Growth Promoting Substances in Biological Samples: Presence-and Formation-of Hormones and Other Growth Promoting Substances in Food Producing Animals*; Wageningen Food Safety Research: Wageningen, The Netherlands, 2022.
19. Hooijerink, H.; Lommen, A.; Mulder, P.P.J.; Van Rhijn, J.A.; Nielen, M.W.F. Liquid chromatography-electrospray ionisation-mass spectrometry based method for the determination of estradiol benzoate in hair of cattle. *Anal. Chim. Acta* **2005**, *529*, 167–172. [CrossRef]
20. Rambaud, L.; Bichon, E.; Cesbron, N.; André, F.; Le Bizec, B. Study of 17β-estradiol-3-benzoate, 17α-methyltestosterone and medroxyprogesterone acetate fixation in bovine hair. *Anal. Chim. Acta* **2005**, *532*, 165–176. [CrossRef]
21. Gray, B.P.; Viljanto, M.; Bright, J.; Pearce, C.; Maynard, S. Investigations into the feasibility of routine ultra high performance liquid chromatography-tandem mass spectrometry analysis of equine hair samples for detecting the misuse of anabolic steroids, anabolic steroid esters and related compounds. *Anal. Chim. Acta* **2013**, *787*, 163–172. [CrossRef] [PubMed]
22. Buisson, C.; Hebestreit, M.; Weigert, A.P.; Heinrich, K.; Fry, H.; Flenker, U.; Banneke, S.; Prevost, S.; Andre, F.; Schaenzer, W.; et al. Application of stable carbon isotope analysis to the detection of 17β-estradiol administration to cattle. *J. Chromatogr. A* **2005**, *1093*, 69–80. [CrossRef] [PubMed]
23. Ferchaud, V.; Le Bizec, B.; Monteau, F.; Andre, F. Determination of the exogenous character of testosterone in bovine urine by gas chromatography-combustion-isotope ratio mass spectrometry. *Analyst* **1998**, *123*, 2617–2620. [CrossRef] [PubMed]
24. Janssens, G.; Courtheyn, D.; Mangelinckx, S.; Prévost, S.; Bichon, E.; Monteau, F.; De Poorter, G.; De Kimpe, N.; Le Bizec, B. Use of isotope ratio mass spectrometry to differentiate between endogenous steroids and synthetic homologues in cattle: A review. *Anal. Chim. Acta* **2013**, *772*, 1–15. [CrossRef]
25. Pezzolato, M.; Botta, M.; Baioni, E.; Richelmi, G.B.; Pitardi, D.; Varello, K.; Caramelli, M.; Bozzetta, E. Confirmation of the progesterone receptor as an efficient marker of treatment with 17β-estradiol in veal calves. *Food Addit. Contam.—Part A* **2015**, *33*, 60–65. [CrossRef]
26. Benedetto, A.; Pezzolato, M.; Beltramo, C.; Audino, V.; Ingravalle, F.; Pillitteri, C.; Foschini, S.; Peletto, S.; Bozzetta, E. Real-time PCR assay for detecting illicit steroid administration in veal calves allows reliable biomarker profiling of formalin-fixed, paraffin-embedded (FFPE) archival tissue samples. *Food Chem.* **2020**, *312*, 126061. [CrossRef]
27. Groot, M.J.; Ossenkoppele, J.S.; Bakker, R.; Pfaffl, M.W.; Meyer, H.H.D.; Nielen, M.W.F. Reference histology of veal calf genital and endocrine tissues—An update for screening on hormonal growth promoters. *J. Vet. Med. Ser. A Physiol. Pathol. Clin. Med.* **2007**, *54*, 238–246. [CrossRef]
28. Vascellari, M.; Katia, C.; Annalisa, S.; Giancarlo, B.; Letizia, M.; Roberto, S.; Giandomenico, P.; Franco, M. Evaluation of thymus morphology and serum cortisol concentration as indirect biomarkers to detect low-dose dexamethasone illegal treatment in beef cattle. *BMC Vet. Res.* **2012**, *8*, 129. [CrossRef]
29. De Maria, R.; Divari, S.; Goria, M.; Bollo, E.; Cannizzo, F.T.; Olivero, M.; Barbarino, G.; Biolatti, B. 17β-oestradiol-induced gene expression in cattle prostate: Biomarkers to detect illegal use of growth promoters. *Vet. Rec.* **2009**, *164*, 459–464. [CrossRef] [PubMed]

30. De Maria, R.; Divari, S.; Spada, F.; Oggero, C.; Mulasso, C.; Maniscalco, L.; Cannizzo, F.T.; Bianchi, M.; Barbarino, G.; Brina, N.; et al. Progesterone receptor gene expression in the accessory sex glands of veal calves. *Vet. Rec.* **2010**, *167*, 291–296. [CrossRef] [PubMed]
31. Groot, M.J. Effects of phyto-oestrogens on veal calf prostate histology. *Vet. Res. Commun.* **2006**, *30*, 587–598. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.