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Determination of endogenous and exogenous corticosteroids in bovine urine and effect of fighting stress during the "Batailles des Reins" on their biosynthesis

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Abstract

Natural corticosteroids include two families of substances: mineralocorticoids and glucocorticoids. Several drugs of similar structure and biological activity have been synthesized and are currently used in the clinical practice. Beside legal pharmacological treatments, these drugs have been consistently misused in animal breeding. One of the most abused corticosteroids is prednisolone. For many years, prednisolone has been considered of exclusive synthetic origin, but nowadays a debate about its possible endogenous production is under way. Several studies have been addressed to ascertain the potential relationship between stressful conditions, such as transportation and slaughtering, and endogenous production of prednisolone. In order to verify further the effect of stressful conditions, our laboratory analysed urine samples collected from the cows participating to the “Batailles des Reines” (a traditional contest based on ritual and spontaneous fights of pregnant cows), to verify if an endogenous prednisolone production may occur in these animals. We developed and validated a LC-MS/MS method for the simultaneous determination of cortisol, cortisone, prednisolone and five of its metabolites. The method was applied to the analysis of urine samples collected from “Batailles des Reines” competitions in 2012 and 2013. All these samples had been previously analysed within an anti-doping control program and tested compliant to all screenings.

Keywords: prednisolone; stress; bovine urine; cortisol; cortisone.

Introduction

Natural corticosteroids are a class of steroid hormones synthesized in the adrenal cortex from cholesterol and include two families of substances: mineralocorticoids and glucocorticoids. Mineralocorticoids influence the electrolyte-water balance, while glucocorticoids act on carbohydrate and protein metabolism (Courtheyn et al., 2002; Savu et al., 1996). Several further synthetic corticosteroids have been produced so far to be used in the clinical practice. In synthetic corticosteroids an increased pharmacological activity is obtained by introducing small modifications in the chemical backbone of physiological glucocorticoids. Such modifications result in both prolonged therapeutic effects and a several-fold increase in pharmacological potency, particularly in the anti-inflammatory action. These features, combined with the absence of a parallel increase of sodium-retaining effects, enhance the suitability of synthetic glucocorticoids for therapeutic purposes (Ferguson and Hoenig, 1995). A number of commercial preparations are currently available for administration to cattle, covering a wide range of therapeutic applications, including primary ketosis, disorders of tendons and the musculoskeletal system, allergic reactions, skin diseases, and shock (McDonald et al., 2007). Besides legal treatments, glucocorticoids may be illicitly administered shortly before the animals' sale, to mask various pathologies, as in the case of old cows at the end of their productive cycle. Another common law infringement is the administration of intra-mammary

glucocorticoid infusion without applying an appropriate withdrawal time.

One of the most utilized corticosteroids is prednisolone (Chiesa et al., 2016, 2014), which has been considered for years as an exclusively exogenous substance. In 2008, the official veterinary controls of Regione Lombardia, a high positivity rate for prednisolone (82% of all non-compliances) in the urine samples collected in slaughterhouses, whereas the urine samples collected in farms from living animals did not give any positivity to either prednisolone or prednisone (Regione Lombardia, Unità Organizzativa Veterinaria-Struttura Controllo degli Alimenti di Origine Animale, 2008). The European Commission reported that 0.14% of the bovine urine samples officially tested in 2010 were non-compliant for prednisolone (De Clercq et al., 2013). From these results, it was hypothesised that the stress evoked by handling the animals before their slaughter resulted in the endogenous production of prednisolone and/or prednisone to a level that could be detected using the current analytical methods. In 2011, Pompa and coworkers treated three dairy cows with tetracosactide hexaacetate, a synthetic analogue of adrenocorticotropic hormone, able to simulate stress. The animals were slaughtered at the end of the study and the results indicated that prednisolone could be only occasionally detected in the non-treated animals, but was consistently found in the urine of pharmacologically stressed cows (the concentrations ranged from 1.01 to 4.08 ng/mL). The stress condition was also confirmed by unusually high urinary cortisol and

cortisone levels in urine, typically detected at concentrations of hundreds ng/mL. The results of this preliminary study did not reveal the specific metabolic pathway responsible for prednisolone biosynthesis, but suggested that a mechanism of endogenous production exists (Bertocchi et al., 2013). Still in 2011 and 2013, other studies found prednisolone residues in urine samples collected from control bovines especially at the slaughterhouse, together with high levels of hydrocortisone and cortisone (Ferranti et al., 2013, 2011).

In 2012, Vincenti et al. conducted a field survey on urine samples collected from 131 guaranteed untreated cows and analysed for to verify the possible occurrence of prednisolone and prednisone, and also for determined cortisol concentrations. None of the examined samples exhibited prednisolone or prednisone levels higher than the CCα limit (0.70 ng/mL and 0.66 ng/mL respectively), therefore resulting officially compliant for both analytes. Trace amounts of prednisolone, estimated in the range 0.1-0.3 ng/mL, were found in only 7 samples from cows also showing high urinary cortisol level, possibly resulting from stressful conditions (Vincenti et al., 2012).

In accordance with the European Union Reference Laboratory (de Rijke et al., 2014), the Italian Ministry of Health enacted a new disposition that considers a bovine urine sample noncompliant only when the prednisolone concentration exceeds 5.0 ng/mL (Department of Public and Veterinary Health, 2012). This threshold appears to be largely conservative in avoiding false non-compliant results: a study from our laboratory demonstrated that the urine of

beef cattle treated with low doses of prednisolone acetate for extended periods of time, as occurs in growth-promoting illegal treatments, may contain prednisolone at 1 ng/mL or even below even during the administration period (Cannizzo et al., 2011).

In order to obtain better insight into the metabolic fate of prednisolone, our laboratory evaluated the possible presence of prednisolone metabolites in the urine of treated and untreated beef cattle (Leporati et al., 2013). We found that 20β-dihydroprednisolone is a major urinary prednisolone metabolite in beef cattle experimentally treated with low dosages of prednisolone acetate according to a growth-promoting schedule. The complete metabolic urinary excretion profile of prednisolone was also characterized after intramuscular (i.m.) administration to healthy finishing bulls and cows using a therapeutic schedule, in which three other prednisolone metabolites and one prednisone metabolite were found (Nebbia et al., 2014). All these results indicate that the merely quantitative testing currently adopted for prednisolone (5 ng/mL cut-off for the parent drug in urine) is not adequate to ascertain the illicit administration of prednisolone to cattle, according to both a growth-promoting schedule and a single high-dose treatment. Similar conclusions were obtained by Famele et al. (Famele et al., 2015). Besides the effect of stress, the hypothesis of a conversion of the natural cortisol into prednisolone during inappropriate sample storage conditions, i.e., in the presence of faecal microbiota, was investigated through studies on long-term stability of natural and synthetic glucocorticoids in livestock urine

(De Clercq et al., 2013) and feaces (Arioli et al., 2010; De Clercq et al., 2014). Recently, a metabolic fingerprinting approach proved to be a powerful tool to classify unknown bovine urine samples that tested positive for prednisolone, while providing information about the stress status of the animal (De Clercq et al. 2015).

Whereas stressful conditions (i.e. transport and slaughter imminence) proved to generate a dramatic increase of cortisol and cortisone urinary concentrations (Bertocchi et al. 2013; De Clercq et al. 2015; Capra 2016), we decided to evaluate the effects of a different source of stress, namely the fight between cows participating to the “Batailles des Reines”, on the endogenous production of cortisol, cortisone, and, possibly, prednisolone, in these animals.

The “Batailles des Reines” (http://www.amisdesreines.it/) is a traditional event, typically taking place in the alpine regions of Valle d’Aosta (Italy), Valais (Switzerland), and Haute-Savois (France), in which couples of pregnant cows spontaneously fight against each other, by joining their head and pushing, until one of the two backs down from the contest and is eliminated. Each “bataille” takes place between two “Reines”, namely the most combative representatives of the herd, who struggles for dominance, especially during the summer period when they instinctively compete for the best mountain pastures. During the course of the "Bataille de Reines", the cows are not forced to struggle by their breeders, who remains mere spectators. Competition is fair and totally bloodless: the animal instinctively fight against an equal opponent.

Materials and methods

Chemicals, reagents and standard solutions

Acetonitrile, methanol, ethyl acetate, acetic acid glacial, ammonium acetate, prednisolone, prednisone, cortisol and cortisone were supplied by Sigma Aldrich Srl (Milan, Italy) and were all of analytical grade. The analytical standards 20α-dihydroprednisolone, 20β-dihydroprednisolone, 6β-hydroxyprednisolone and 20β-dihydroprednisone were from Steraloids (Newport, RI, USA). The internal standards (ISTDs) prednisolone D6, cortisol D2 and cortisone D2 were from CDN Isotopes (Pointe-Claire, QC, Canada). β-glucuronidase/aryl-sulfatase was from Roche Diagnostics (Mannheim, Germany). Sodium hydroxide and hydrochloric acid were from Carlo Erba Reagenti (Milan, Italy). Ultrapure water was obtained by a Milli-Q Millipore system (Bedford, MA, U.S.A.). Stock standard solutions of analytes and ISTDs were prepared in acetonitrile at a concentration of 200 ng/mL and stored at −20°C in the dark.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Chromatographic separations were performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), including a vacuum degasser, a binary pump, an autosampler and a column thermostat. The liquid chromatograph was equipped with a Waters (Milford, MA, USA) X-Select HSS T3 (2.5 µm, 3.0 x 100

mm) column and a Phenomenex (Castel Maggiore, BO, Italy) SecurityGuard 4.0 mm × 2.0 mm precolumn. The column was kept in a column oven at 23 °C. The chromatographic run was carried out by a binary mobile phase of a 0.1% v/v aqueous acetic acid solution and acetonitrile, using the following program: isocratic with 20% acetonitrile for 2 min; linear gradient from 20% to 40% in 10 min; isocratic with 40% acetonitrile for 6 min; linear gradient from 40% to 70% in 1 min; isocratic with 70% acetonitrile for 2 min; total run time 21 min. The injection volume was 10 µL, while the flow-rate was 0.2 mL/min. The LC was interfaced to an Applied Biosystems API 4000 triple–quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), operating in electrospray ionization (ESI) – negative ionization mode. The other MS parameters were set as follows: curtain gas: 30 psi; nebulizer gas: 40 psi; probe temperature: 300 °C; IS voltage: -4200 V; gas for collisional activation: N_2 at 2 psi. Ion acquisition was operated at unit mass resolution in the selected reaction monitoring (SRM) mode, using the transitions from the acetate adduct ion of each analyte (precursor ion) to the fragment ions indicated in Supplementary Material.

Sample preparation

After centrifugation at 3500 rpm for 5 min, 5.0 mL of urine was transferred into 30 mL glass tubes and 100 µL of the ISTDs solution at 0.1 µg/mL was added. 1.0 mL of aqueous ammonium acetate solution 1.1 M pH=4.8 was added and the pH was checked out and

adjusted to 5.0 with HCl 1 M, if required. Then 20 µL of β-glucuronidase/arylsulfatase solution, obtained by 1:20 dilution of the enzyme in deionized water, was added and the enzymatic deconjugation was carried out for 2 h at 37 °C. The sample mixture was cooled at room temperature and loaded onto a Strata-X 33µm, 60 mg x 3 mL SPE column (Phenomenex, Castel Maggiore (BO), Italy), previously conditioned with 3 mL of methanol and 3 mL of aqueous ammonium acetate solution 0.15 M pH=4.8. After sample loading, the column was washed with 3 mL of deionized water, 6 mL of a methanol:sodium hydroxide 0.02 M (30:70 v/v) mixture and 3 mL of deionized water. The analytes were eluted with 4 mL of ethyl acetate. The resulting solution was evaporated to dryness under a gentle stream of nitrogen and mild heating (50 °C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was dissolved in 100 µL of 0.1% acetic acid aqueous solution and acetonitrile (80:20 v/v) mixture and transferred into the analytical vials for LC–MS/MS analysis (injection volume = 10 µL).

Analytical method validation

A first set of experiments on prednisolone, prednisone, 20α-dihydroprednisolone, 20β-dihydroprednisolone, 20β-dihydroprednisone and 6β-hydroxyprednisolone used blank bovine urine preliminarily tested as negative to these molecules. A pool of 20 urines of female animals of “Valdostana” breed, aged between one and three years, were used. Since cortisol and cortisone are endogenous corticosteroids, they are commonly present in blank

urine samples, therefore their validation experiments were conducted on a second set of samples, collected from animals treated with prednisolone acetate. A pool was obtained from the urines of six Friesian non-lactating cows at the end of their productive cycle. These samples were available from another work (Nebbia et al., 2014) and they were obtained in adherence to Italian regulations and guidelines for the care and use of experimental animals. The study was approved by the Ministry of Health and the local Committee for Animal Welfare. The treatment with this synthetic corticosteroid suppressed the production of endogenous glucocorticoids, making the collected urine negative for cortisol and cortisone. Recently published practical guidelines were followed for reporting analytical calibration results (Olivieri, 2015). Also the guiding principles expressed in the Commission Decision 2002/657/EC were considered (Commission Decision 2002/657/EC). Positive identification of the analytes was expressed by the recognition of 4 identification points, namely the SRM transitions and retention times listed in Supplementary Material. Specificity, linearity, precision, trueness, limit of detection (LOD), limit of quantification (LOQ) and ruggedness were evaluated.

Creatinine detection

Urinary creatinine was measured using a creatinine assay by ARCHITECT C8000 System (Abbott, Abbott Park, IL, USA). The creatinine assay is based upon the reaction between creatinine and sodium picrate to form a creatinine-picrate complex. The rate of

increase in absorbance at 500 nm due to the formation of this complex is directly proportional to the concentration of creatinine in the sample. Since the creatinine kit is proposed for the quantitation of creatinine in human serum, plasma or urine and not for bovine urine, in a previous experimentation the creatinine level was measured in ten samples of bovine urine by means of both the creatinine kit and a quantitative LC-MS/MS method. Strong correlation (Pearson correlation r = 0.9755) and no significant differences were found between the two sets of results. A p-value of 0.00236 was obtained, allowing us to reject the null hypothesis and accept the alternative hypothesis (i.e. there is actually a positive correlation between the different methods).

Chemical analysis of real samples

The analytical method was applied to the urine samples collected for anti-doping control from the winner cows (three weight categories) for each eliminating round and finals during the 2012 and 2013 “Batailles des Reines” tournaments (about 20 eliminating rounds plus one final event each year). Since at the final round participated the winners of the eliminatory rounds, for three animals each year urine samples were collected in two distinct events resulting in a total of 114 samples for 108 animals. Unfortunately, for 24 samples the urine volume was not sufficient for the creatinine analysis. Therefore, creatinine was measured only on 90 samples. Animals arrived on the site of competition around 9:00 a.m. from their locations on the mountains (eliminating rounds)

in order to undergo the registration operations. The cows taking part in the “Bataille des Reines” events were brought to the contest field and were allowed to rest before and after each fight. In the eliminating round, from which most of the urine samples arose, the cows generally walked to the contest field coming from the mountain pastures where they spend the summer season. Before each of the matches, and between them, the cows were separated from one another and allowed to rest. On average, the three best-classified cows, from which urine samples were collected, competed in about 3-5 matches. Only in the final event, more complex operations were necessary to transport the animals (about 120-140 cows competed in each category in the final event, but only the winners were sampled). In general, sampled animals equally competed for 3-5 matches each. In general, the competitions started around 1:30 p.m. and went on until late-afternoon. Urine sampling was performed with care to prevent faecal contamination by a licensed veterinarian under conditions of natural micturition at the end of the challenge after watering. After collection, the urine specimens were immediately stored at -20°C pending their analysis, which were carried out within one week. Beside the present method, all samples had been previously analysed within an anti-doping control program and tested negative to all target analytes. The samples underwent two freeze-thaw cycles, one for antidoping analysis and one for corticosteroids analysis.

Data analysis

In Table 2 descriptive statistics data relevant to creatinine, cortisol and cortisone concentration are summarized. Prednisolone was found in one sample only, while no prednisone metabolites were detected in the analysed samples. Results on the 90 urine samples collected during “Batailles des Reines” tournaments (group A) are compared with those collected in previous works investigating 6 tethered cows reared in traditional farms, without traces of prednisolone, in the following reproductive status: early pregnancy (4 months) n=2, late pregnancy (8 months) n=2, oestral phase n=1, anoestrus n=1 (group B) (Vincenti et al., 2012), and with 6 cows, aged between 4 and 6 years, whose urines were collected after slaughter (group C) (Capra, 2016). While urine samples from group A were necessarily collected in the afternoon, after competition, those from groups B and C, i.e. under programmed experiments, were collected in the morning (group B) or at the slaughterhouse (group C). The lack of uniformity in the urine withdrawal timing represents an unavoidable constraint, that did not allowed us to evaluate the changes of cortisol and cortisone levels due to the circadian rhythm.

Missing values, corresponding to concentration levels below the limit of detection (LOD) of the analytical method, were replaced with a value equal to half of the validated LOD, as a rule-of-thumb useful for statistical purposes. Cortisol and cortisone concentrations for each sample were standardized according to the creatinine levels, to compensate for the degree of urine dilution. In particular, cortisol and cortisone values were multiplied by a correction factor equal to the ratio between the average creatinine level (calculated

from all animals participating to the "Batailles des Reines" tournaments) and the creatinine value for each cow. Then, base-10 logarithm transformation was performed on the collected data. The creatinine, cortisol, and cortisone data values for all animals are reported in the Supplementary Material. Boxplots, Gaussian and Kernel density estimation (KDE) plots were evaluated to identify possible outliers and compare the data distributions. Then, t-tests together with one-way ANOVA and Tukey's tests were performed to compare the corrected cortisol and cortisone mean values for the different groups of animals. All calculations were performed with R software version 3.2.2 (R Core Team, 2015).

Results

Method development

In the course of method development, liquid-liquid extraction was compared with SPE extraction, the latter yielding cleaner extracts and consequent better sensitivity. Three solvents were tested for liquid-liquid extraction: ethyl acetate, tert-butyl-methyl ether and diethyl ether. The best recovery results were obtained with ethyl acetate (46%), but much lower than those obtained with SPE (87%). The chromatographic run was optimized so as to assure adequate separation of the 20α- and 20β-dihydroprednisolone isomers (see Figure 2). Calculated resolution is 1.0933 (Snyder et al., 2010). No other compounds with the same MS-transitions was affected by separation problems. Positive and negative ionization modes were

compared in ESI-MS setting: the latter proved to provide better sensitivity for the entire set of target analytes. The ion $[M+acetate]^-$ ion was selected as the precursor for all the analytes.

Validation

Occurrence of possible interferences from endogenous substances was tested by the analysis of twenty blank urine samples as described above; no interfering substances were found. Linear matrix calibration curves were built for each analyte (0, 0.4, 0.8, 1.2, 2.5, 5 ng/mL for prednisolone, cortisol and cortisone, 0, 0.3, 0.5, 1, 2, 5 ng/mL for 6β-hydroxyprednisolone and prednisone and 0, 1, 2, 5, 8, 10 ng/mL for 20β-dihydroprednisolone, 20α-dihydroprednisolone and 20β-dihydroprednisone), with two replicates for each level. Since IUPAC discourages the correlation coefficient (R) as an indicator of linearity in the correlation between concentration and signal (Danzer and Curriet, 1998), we reported the experimental F value, corresponding to the ratio of residual variance to squared pure error, and the tabulated critical F for comparison in order to test linearity (lack-of-fit test). This test is the best linearity indicator, as recommended by IUPAC. The lack-of-fit test was passed for all the analytes in the concentration ranges considered (see Table 1), with F-values below 1.4 for the main analytes (cortisol, cortisone, prednisone, prednisolone). LOD and LOQ were calculated as reported by Olivieri (Olivieri, 2015) and ranged between 0.13 ng/mL to 0.69 ng/mL and to 0.39 ng/mL to 2.08 ng/mL respectively (see Table 1).

Precision and trueness were evaluated at three levels and were estimated both intra-day (n=6) and inter-day (n=18). An ANOVA test was set for each validation level. Intra-day precision, expressed by the experimental coefficients of variation, ranged between 2.9% and 16%, while inter-day precision was between 5.3% and 19%. Quite similarly, limited bias from true values were recorded (from –6.2% to +7.8% for intra-day trueness and from -5.9% to + 6.7% for inter-day trueness).

Ruggedness test was conducted by introducing slight variations (±10% maximum) to previously selected analytical parameters and observing the resulting changes in term of quantitative response on blank urine samples spiked at the lowest validation level (see Supplementary Materials). A Youden approach was used, in order to minimize the number of experiments required. It was found that some factors influenced the final results for 6β-hydroxyprednisolone and the critical factors were identified using a t-test. They are the methanol percentage in SPE washing mixture - methanol:sodium hydroxide 0.02 M (30:70 v/v) - and its volume.

The urine extracts were stable for six days at least, if stored under appropriate conditions, as proved by the negligible differences (i.e., lower than the limit of repeatability of the method) in the absolute concentration values determined at day 0 and day 6 for all the target analytes. Complete validation data are reported in the Supplementary Materials.

Real samples

Cortisol and cortisone were found above the LOQ value in all the urine samples, with only one exception. In 2012 and 2013 sample collections, the average concentrations measured for cortisol were 8.65 ± 5.24 ng/mL and 8.00 ± 5.11 ng/mL respectively, and for cortisone 4.90 ± 3.09 ng/mL and 4.96 ± 3.14 ng/mL, respectively. It was observed no significant difference in the comparison between the two years (two-tailed t-test, $p<0.01$).

Prednisolone was found in only one sample, at a concentration of 1.45 ng/mL. In this sample, both cortisol and cortisone were found at the highest concentration among all 114 urines, i.e. 35.5 and 18.1 ng/mL, respectively. Trace amounts of prednisolone were also found in three other samples (at estimated concentrations of 0.35, 0.12 and 0.10 ng/mL, respectively, all values were under the LOQ). In the same samples, cortisol and cortisone concentrations were above the average, but not excedingely high (14.3 and 7.78 ng/mL, 11.6 and 10.8 ng/mL, 10.3 and 9.55 ng/mL, respectively). Some other samples exhibited higher cortisol and cortisone concentrations, but no prednisolone was found. In no samples, any of prednisolone metabolites were detected, not even at trace level, unlike what was observed in the urine samples of bovines treated with prednisolone (Leporati et al., 2013; Nebbia et al., 2014).

Descriptive Statistics

Minimum, maximum, quartiles and the median values of creatinine, cortisol and cortisone values were calculated for the 3 groups of cows (A-C). Data are reported in Table 2 (a-c). Creatinine detection

was possible only on 90 samples out of 114 because insufficient volume was available for the remaining 24 urines.

Boxplots and distribution plots relevant to cortisol (Figure 1a-b) and cortisone (Figure 1c-d) values were calculated. In particular, Figure 1(b-d) shows both Gaussian and KDE distribution plots (red and green lines, respectively) for the 90 urine samples of the cows participating to the "Batailles des Reines" tournaments (group A, blue histograms), together with their mean, standard deviation and CV% values. Cortisol and cortisone levels of the 6 tethered cows reared in traditional farms (group B) and of slaughtered animals (group C), are reported too. Although boxplots show overlaps among the groups, appreciable difference exists among the central quartiles for the three groups, whose significance was tested to evaluate whether the populations can be distinguished by the target analytes. Furthermore, 5 out of 6 animals of groups B and C showed largely increased cortisol and cortisone values when they were transported to the slaughterhouse and had to wait before their slaughter (i.e. animals 2, 3, 4, 5 and 6). Conversely, animal#1 showed an opposite trend.

Discussion

The appraisal of cortisol, cortisone, and prednisolone as potential biomarkers for assessing the occurrence of fighting-generated stress requires the consideration under which the cows taking part in the various "Bataille des Reines" events are brought to the contest field and are allowed to rest before and after each fight (see "Material

and methods – Chemical analysis of real samples). The natural conditions under which the cows walk to the contest field and rest before competing made us conclude that transport and environmental conditions are not likely to add a significant contribution (bias) to the stress originated by the fight itself, at least for the eliminating rounds.

By comparing the global analytical results of the present investigation with those collected in a previous study (Vincenti et al., 2012), it can be observed that the stress generated by the struggle during each "Bataille", and measured by means of cortisol and cortisone concentrations, is significantly higher (t-test, p value < 0.05) than that commonly experienced by the cows living in a farm (Figure 1, groups A and B).

On the other hand, the comparison of the same data with those collected in another more recent study (Capra, 2016), show that the fighting stress produced in the "Batailles" is significantly lower (t-test, p value < 0.05) than that experienced by the animals that are transported to the slaughterhouse and wait before their killing (Figure 1, groups A and C). Furthermore, one-way ANOVA and Tukey's tests were performed on the collected data with the aim of comparing the analytical results of this study with those from the previous studies, involving groups of cows living in a farm or slaughtered. At first, one-way ANOVA test was performed and p-values equal to 7.39×10^{-6} and 3.36×10^{-5} for cortisol and cortisone, respectively, were observed, thus revealing that there was at least one group significantly different from the other ones. Secondly,

Tukey's test was employed with the aim of investigating such differences more accurately. As it is reported in Figure 3(a-b), all groups proved to be significantly different as no confidence intervals including the zero value were observed. In this case, Tukey's test proved that all the tested groups of cows were significantly different as p-values lower than 0.05 were obtained for all the pairwise comparisons of both cortisol (Figure 3a) and cortisone (Figure 3b), with the unique exception of the cortisone levels from samples collected during "Batailles des Reines" tournaments (A) and those from the cows reared in loose housing farms (B), that shows a p-value slightly higher than 0.05 (i.e. 0.067), possibly because cortisone is a somewhat less efficient stress biomarker than cortisol.

Conclusions

The repeated fighting and environmental conditions produced in the "Batailles des Reines" events assuredly generates relatively high concentrations of cortisol and cortisone in the urines of the struggling cows, together with occasional presence of traces of prednisolone. On this basis, it is apparent that a significant stress is induced in the "Batailles des Reines" fighting cows, with respect to the general living conditions of the cows reared in loose housing farms. However, the fighting stress of the "Batailles des Reines" is considerably lower than that induced in the bovines transported to the slaughterhouse, which is extremely high indeed (De Clercq et al. 2015).

While the occasional presence of prednisolone in the urine of the competing cows can be somehow associated with the fighting stress, its rare occurrence (3%) make prednisolone an unreliable biomarker for stress. The differentiated and random production of prednisolone in stressed animals might be attributed to both the intensity and the source of stressful conditions.

The presence of prednisolone in the urine of the fighting cows was in no cases associated with the concurrent presence of its metabolites, particularly 20β-dihydroprednisolone, which was by contrast observed at concentrations often exceeding the parent compound when exogenous prednisolone was administered, both at high (therapeutic) and low (mimicking illicit purposes) dose (Leporati et al. 2013, Nebbia et al. 2014). Simultaneously, suppression of cortisol and cortisone was documented in these studies.

While the official cut-off of 5.0 ng/mL for prednisolone avoids false positive results due to endogenous production under stressful conditions, it does not appear to be adequate to ascertain the illicit repeated low-dose administration of prednisolone typical of a growth–promoting schedule.

For these reasons, a more biologically-oriented strategy involving the simultaneous determination of urinary cortisol, cortisone, prednisolone, prednisone, and 20β-dihydroprednisolone is likely to represent an effective approach for surveillance purposes and consumer's protection.

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Figure captions

Figure 1. Boxplots, histograms (only for group A) Gaussian (red line, only for group A), KDE (green line, only for group A) plots relevant to cortisol (a-b) and cortisone (c-d) values of the different groups of cows, where (A) represents the animals from "Batailles des Reines" tournaments (n = 90), (B) indicates the tethered cows reared in traditional farms, without traces of prednisolone (n = 6) (Vincenti et al., 2012) and the slaughtered ones (C), whose urines were collected after the death (n = 6) (Capra, 2016). Cortisol and cortisone values of the 6 animals from groups B and C are indicated by circles and arranged below the plots relevant to the urine samples from group A.

Figure 2. Chromatographic separation of the two isomers 20α- (peak B) and 20β- (peak A) dihydroprednisolone.

Figure 3. Tukey's test plot relevant to the 95% family-wise confidence level of all the pairwise comparisons (reported on the Y-axis) relevant to the cortisol (a) and cortisone (b) concentration of the different groups of cows, where (A) represents the animals from "Batailles des Reines" tournaments (n = 90), (B) indicates the tethered cows reared in traditional farms, without traces of prednisolone (n = 6) (Vincenti et al., 2012) and the slaughtered ones (C), whose urines were collected after the death (n = 6) (Capra, 2016).

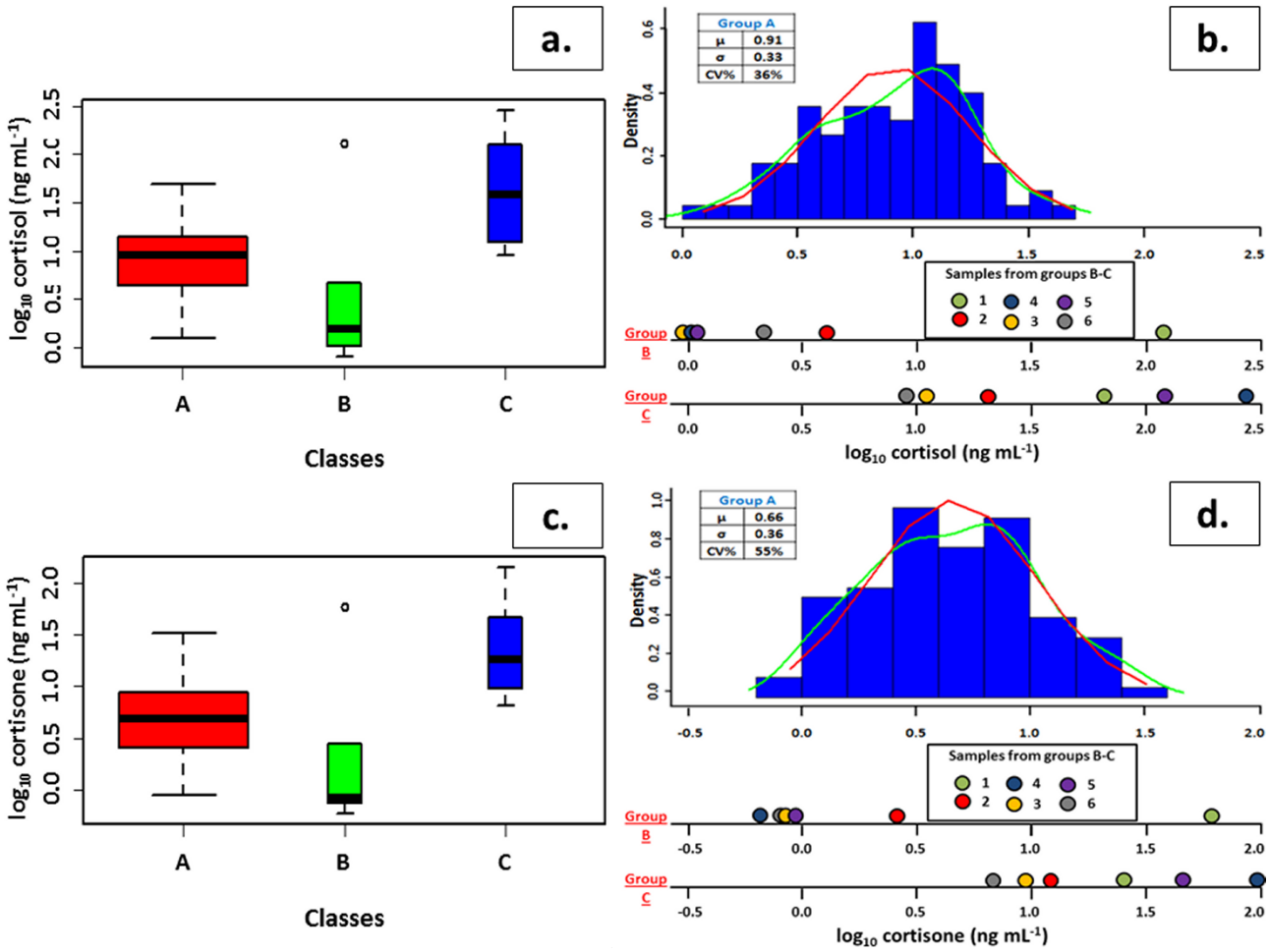


Figure 1

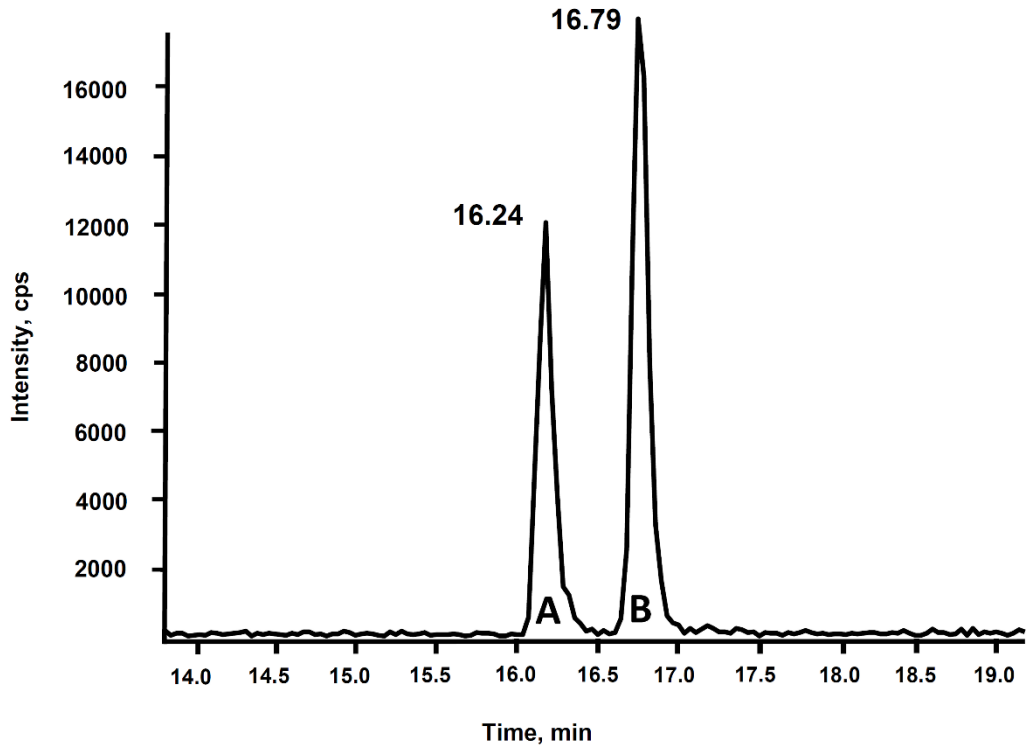


Figure 2

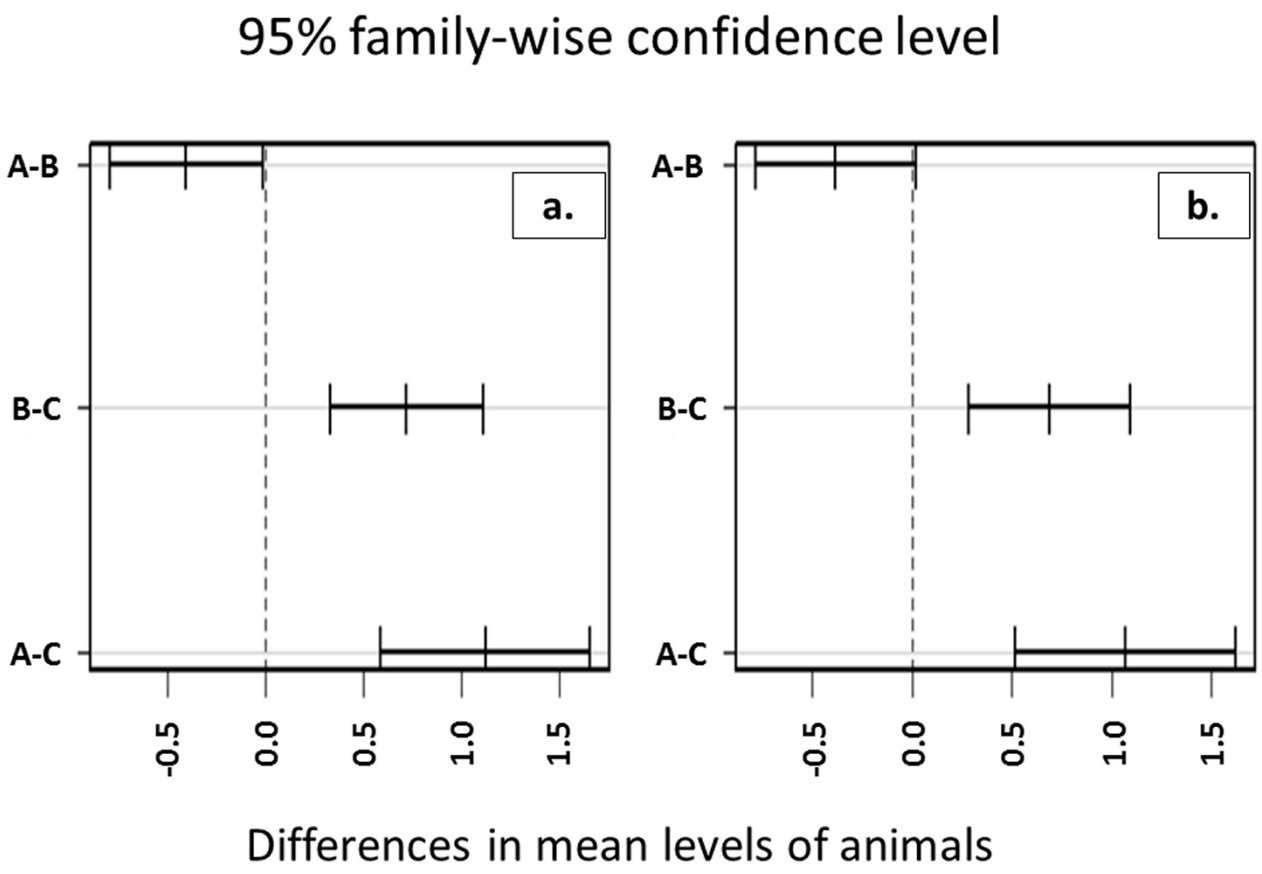


Figure 3

	Spiked urine			LOD	LOQ
Analyte	**Linearity range (ng mL^{-1})**	**F_{tab}**	**F_{exp}**	**(ng mL^{-1})**	**(ng mL^{-1})**
6β-hydroxyprednisolone	0 – 5.0	4.060	0.800	0.17	0.51
20α-dihydroprednisolone	0 – 10		3.230	0.57	1.72
20β-dihydroprednisolone	0 – 10		3.230	0.69	2.08
20β-dihydroprednisone	0 – 7.6		2.345	0.67	2.04
Prednisolone	0 – 5.0		0.800	0.14	0.44
Prednisone	0 – 5.0		0.772	0.16	0.49
Cortisol	0 – 5.0		1.327	0.14	0.42
Cortisone	0 – 5.0		1.254	0.13	0.39

Table 1. Calibration curves obtained from spiked urine samples (quantitative determinations) with corresponding linearity test results, LOD and LOQ values (where F_{tab} and F_{exp} are the tabulated critical and the experimental F values, respectively, LOD is the limit of detection and LOQ is the limit of quantitation).

Table 2a.

Cows	Minimum	1st Quartile	Median	3rd Quartile	Maximum
A	61	151	214	291	750
B	19	34	60	111	168
C	14	62	107	198	248

Table 2b.

Cows	Minimum	1st Quartile	Median	3rd Quartile	Maximum
A	1.23	4.38	9.05	14.1	48.8
B	0.80	1.03	1.63	3.93	128
C	9.11	14.8	44.0	112	291

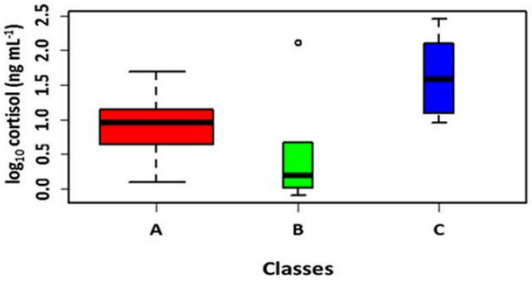
Table 2c.

Cows	Min	1st Quartile	Median	3rd Quartile	Max
A	0.90	2.54	4.86	8.61	32.6
B	0.59	0.75	0.84	2.31	58.9
C	6.42	10.3	19.4	41.2	141

Table 2. Descriptive statistics relevant to the urinary creatinine (2a), cortisol (2b) and cortisone (2c) values of cows from "Batailles des Reines" tournaments (A), the tethered cows reared in traditional farms, without traces of prednisolone (B) and the slaughtered ones (C). Cortisol and cortisone values were multiplied by a corrected factor according to the creatinine data in order to standardize the different dilution degrees of the urine samples.

Determination of endogenous and exogenous corticosteroids in bovine urine and effect of fighting stress during the "Batailles des Reins" on their biosynthesis

A – cows from "Batailles des Reines" tournaments;
B – tethered cows reared in traditional farms;
C – slaughtered cows, whose urines were collected after death.



Graphical abstract

Highlights

- The fighting stress is considerably lower than that induced by slaughtering
- Prednisolone presence in one urine sample is correlated with high level of stress
- Concurrence of prednisolone with high cortisol level rules out its exogenous origin