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Measuring corticosterone concentrations in broiler muscle: Analytical validation of an enzyme immuno assay kit and relationships with total plasma concentrations

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ABSTRACT

Corticosterone (**CCS**) concentration in chicken skeletal muscle could be a potential Animal-Based Measure that could give ex-post indications of adaptive responses to different farming systems and management practices. The aim of the present trial was to measure CCS concentrations in chicken skeletal muscle and assess their relationship with total plasma CCS concentrations. Blood and muscle samples were recovered respectively at the beginning and the end of an industrial slaughter line from 48 identified female broilers aged 84 d. Muscle juice samples were obtained by high-speed centrifugation, and analytical validation of a commercial CCS EIA kit has been carried out. Regarding repeatability (precision), intra-assay coefficient of variation (CV, $n = 12$ replicates) resulted 4.2 %. Inter-assay CV (2 replicates in $n = 4$ assay sessions) was 11.9 %. The line resulting from serial dilutions of a pooled muscle juice sample has been compared with the calibrators line for parallelism and with the expected values line for linearity. The slopes of the obtained and calibrators lines were not different ($F = 4.082$, $P = 0.090$). A strong correlation was found between the observed and expected concentrations (Pearson correlation coefficient, $r = 0.991$, $P < 0.001$). The mean RR (recovery rate, accuracy) of different quantities of hormone added to the pooled muscle juice was 90.0 ± 1.6 %. The mean CCS concentration in muscle juice samples was 455.1 ± 46.8 pg/mL (range from 47.5 to 1,340.0 pg/mL). The mean total plasma CCS concentration was 11.2 ± 0.4 ng/mL (range 7.0 to 19.5 ng/mL). The mean ratio of individual muscle:plasma CCS concentration was 0.040 ± 0.004, ranging from 0.005 to 0.133. Muscle and plasma CCS levels were moderately correlated ($r = 0.324$, $P = 0.025$). It is concluded that this EIA kit can provide reliable results for measuring CCS concentrations in juice obtained by high-speed centrifugation of chicken muscle, directly without previous extraction. Such a method can be a further tool for the assessment of animal physiological responses, stress, and welfare.

Introduction

There is a growing need for reliable and sound tools to monitor animal welfare and investigate stress-related conditions in livestock. Among the physiological parameters, glucocorticoid hormones (**GC**), mainly corticosterone (**CCS**) in birds, are considered very valuable and have been used for decades to evaluate animal adaptive responses, stress, and welfare (Nielsen et al., 2023). Glucocorticoids are the end product of the activation of the hypothalamic–pituitary–adrenal (**HPA**) axis, the main endocrine system involved in General Adaptation Syndrome (Mormède et al., 2007). About 90-95 % of circulating GC in blood is bound to plasma proteins, and only the unbound free fraction is available for tissues, interacts with the receptors and exerts biological activity (Mendel, 1989). In several avian wild species, it has been estimated that between 0.2 and 18 % of total CCS can be unbound to plasma proteins (Fokidis et al., 2009). Blood is the traditional matrix for measuring CCS, as their concentrations rapidly reflect adrenal gland secretion and can increase within minutes of exposure to a stressor—-even in broilers (Chloupek et al., 2011). In contrast, alternative matrices such as droppings and feathers provide retrospective insights into HPA axis activity over longer periods—hours and weeks, respectively (Galosi et al., 2024). Muscle tissue may also represent a promising non-invasive

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matrix for assessing cumulative stress. Cortisol concentrations have previously been measured in pig skeletal muscle (Shaw et al., 1995; Choi et al., 2012), where they were associated with various meat quality traits. To our knowledge, no studies have investigated CCS concentration in broiler muscle, and no validated laboratory methods are currently available for its determination in this matrix. Such an approach could provide valuable retrospective insights into systemic stress exposure and adaptive responses to different farming systems and management practices. Moreover, the use of muscle tissue may represent a more practical and feasible alternative to blood sampling, which at abattoirs during exsanguination often poses logistical challenges, particularly in accurately matching each blood sample to the corresponding carcass.

The aim of the present trial was to validate an EIA kit for CCS concentration in chicken skeletal muscle. Then, CCS concentrations found in muscle juice obtained by high-speed centrifugation have been related to total CCS concentrations in blood plasma samples recovered from the same animals at slaughtering.

Materials and methods

Animals and sampling

Blood and muscle samples were collected at an industrial slaughterhouse in two sampling sessions from 48 female broilers, aged 84 d, coming from the same organic farm and shed. The time from loading the animals to the beginning of the slaughtering procedures was approximately 8 h, including 145 min of road transport. Twenty-four broilers (commercial strain Hubbard Red JA/F) were sampled in February, and the mean live weight of the whole batch was 3.7 Kg. A second lot of 24 broilers (Ranger Classic/F) was sampled in May, from a batch with a mean live weight of 3.2 Kg. The chickens were manually removed from the cages, suspended on the shackle line supplied with breast support and stunned by an electrified water bath (THF - Version 4′, 400 V, Bayle SA, La Fouillouse, France) according to EC Regulation 1099/2009. A virtual identification of the birds was performed as ten empty shackles were left between subjects so that marking could be done without handling. Blood samples were collected by dripping from hanging animals immediately after jugulation in labelled 8 mL tubes containing K2EDTA (Greiner Bio-One GREI455040-1200), and centrifuged (2500 g for 15 min) within 2 h. Recovered plasma was aliquoted in 1.5 mL Eppendorf polypropylene tubes, immediately frozen, and stored at −20°C until assayed.

The identified breasts, isolated from the carcasses after cooling in the tunnel for 1.5 h, were individually placed in plastic bags and transported to the laboratory at 2°C within 40 min. A cube of about 2 to 3 cm of side was taken by the muscle *pectoralis minor* from each breast and immediately frozen at −20°C. Afterward, 4 ± 1 g of thawed muscle samples were centrifuged at 100,000 g for 30 min at 4°C (Optima L-80 XP, Beckman Coulter Inc. BREA, California, USA) (Shaw et al., 1995; Choi et al., 2012). The resulting liquid fraction (muscle juice, mainly sarcoplasmic fluid) was collected in 1.5 mL Eppendorf tubes and was frozen at −20°C until assay, within a few months. Concentrations were expressed as pg/mL.

The EIA kit

The Corticosterone EIA kit (Arbor Assays, Ann Arbor, MI, USA, Cat. #K014, Lot #24CS045a) employs a competitive-binding method, described by the producer as species independent, multi-format with improved sensitivity, and validated for different sample matrices (serum, EDTA and heparin plasma, saliva, urine, dried fecal extracts, and tissue culture media). Serum and plasma samples need to be treated with the supplied Dissociation Reagent, which looses hormone bound to blood proteins, to obtain the total CCS concentration in serum or plasma.

The kit offers two standard (calibrators) curve ranges, depending on the anticipated sensitivity needed for samples, using calibrators and sample volumes of 50 µL or 100 µL. Calibration curves built by 9 standards give ranges from 10,000 to 39 pg/mL or 5,000 to 20 pg/mL for the 50 µL or 100 µL format of sample size, respectively.

As reported in the kit leaflet, sensitivity was determined as 20.90 pg/mL for 50 µL and 14.35 pg/mL for 100 µL sample size. The limit of Detection was determined as 17.5 pg/mL for 50 µL and 7.7 pg/mL for 100 µL sample size.

Assays and analytical validation for muscle samples

Assays were carried out following the manufacturer's instructions. Microplates were read at 450 nm (Tecan Infinite 200 Pro, Tecan Austria GmbH, Grödig, Austria). Preliminary dilution tests of samples have been performed in order to obtain values with the closest percentage of binding to 50 % (optical density % B/B0), i.e., around the middle of the calibration curve. For plasma samples, the 50 µL format with samples dilution 15x (10 µL of sample + 10 µL dissociation reagent + 130 µL assay buffer) has been utilized. The intra-assay coefficient of variation (CV, $n = 6$ replicates of the same plasma sample) was 4.1 %, and the inter-assay CV ($n = 2$ assays) was 8.0 %.

For muscle juice samples, the 100 µL sample size format (higher sensitivity) was chosen, with an optimal dilution of samples 1:1 with assay buffer. A pool of muscle juice samples was obtained by mixing several individual samples. Precision has been evaluated by repeatability, measuring the intra-assay CV ($n = 12$ replicates in the same assay) and the inter-assay CV (2 replicates of the same sample in $n = 4$ assays). A dilution test has been performed mixing different amounts of assay buffer ($n = 7$) with the pooled muscle juice sample. The resulting line has been compared with the calibrators line for parallelism and with the line of the expected values for linearity. Accuracy has been measured by recovery rates (RR, observed concentration/expected concentration x 100) of known quantities of hormone ($n = 5$) added to the pooled sample, i.e., adding different small amounts of the highest calibrator with a concentration of 10,000 pg/mL.

Statistical analysis

Descriptive statistics were used to present data as means, ranges (minimum and maximum), and standard error of the mean (SEM). Diagnostic charts and the Shapiro-Wilk test were used to check assumptions and outliers. Parallelism between the calibrators line and that observed by sample dilutions was assessed. Semi-logarithmic (Y-axis) linear regression models were generated, and the slopes within the common range of both datasets were compared using the Analysis of Covariance. The F-value and corresponding two-tailed P-value, testing the null hypothesis that the slopes are all identical (i.e., the lines are parallel), were reported, as well as the coefficient of determination (R^2).

Linearity was assessed by performing linear regression analysis, plotting expected concentrations on the x-axis and observed concentrations on the y-axis. The degree of linearity was evaluated using the coefficient of determination (R^2) and the slope of the regression line, with a slope of 1 indicating perfect linearity. Finally, the runs test, which verifies the null hypothesis that the data points are randomly distributed above and below the regression line, was used to determine if the data significantly differed from a straight line.

Regarding the CCS concentration data obtained from muscle and plasma, a logarithmic transformation was first applied to improve the normality of their distribution. Subsequently, Pearson's correlation coefficient (r) was used to verify the relationship between the two concentrations. The strength of the correlation was interpreted as poor if $r < 0.3$, medium if $0.3 \leq r < 0.5$, and large if $r \geq 0.5$.

All analyses were performed using GraphPad Prism (version 8.0 for Windows, GraphPad Software, Boston, Massachusetts, USA), with the significance level set at 0.05.

Results and discussion

Analytical assay validation was carried out for muscle juice samples. Regarding repeatability (precision), intra-assay CV ($n = 12$ replicates) resulted 4.2 %. Inter-assay CV (2 replicates in $n = 4$ assay sessions) was 11.9 %. The models for the observed and calibrators lines both showed a good fit (observed: $R^2 = 0.999$; calibrators: $R^2 = 1.000$), and their slopes were not significantly different ($F = 4.082$, $P = 0.090$) (Fig. 1A). These results indicated the parallelism between the calibrators line and that observed suggesting that the assay can reliably quantify analyte in different samples. Moreover, the parameters of the regression line ($R^2 = 0.982$, slope = 0.972) and the runs test ($P = 0.200$) confirmed the linearity between observed and expected values (Fig. 1B) and then that the method is valid for quantification (at least) within our range of values. Mean (± SEM) RR (accuracy) of different quantities of hormone added to the pooled muscle juice was 90.0 ± 1.6 % (Table 1). The features of the CCS assay in muscle juice samples resulted fully satisfying in terms of both precision and accuracy. To our knowledge, this is the first report about measuring CCS in chicken muscle and in muscle by EIA.

Table 1
Recovery Rates (RR) of added corticosterone hormone to a pooled sample of muscle juice.

Volumes (μL)		Concentrations (pg/mL)		RR (%)
Pooled sample	Calibrator 10,000 pg/mL	Observed	Expected	
290	10	638.8	666.2	95.9
280	20	906.3	1051.1	86.2
270	30	1205	1339.8	89.9
250	50	1779	2013.3	88.4
240	60	2064	2302.0	89.7

The quantification of CCS in muscle can be considered as an indicator of the endocrine status of the animal, but also as a possible indirect marker of meat quality. The application of this method can also extend to the field of ethological studies, correlating physiological stress and behavior. The analysis of CCS in muscle tissue collected post-slaughter could offer a complementary method to investigate retrospectively stress or welfare conditions in animals in combination with behavioral data collected on the farm.

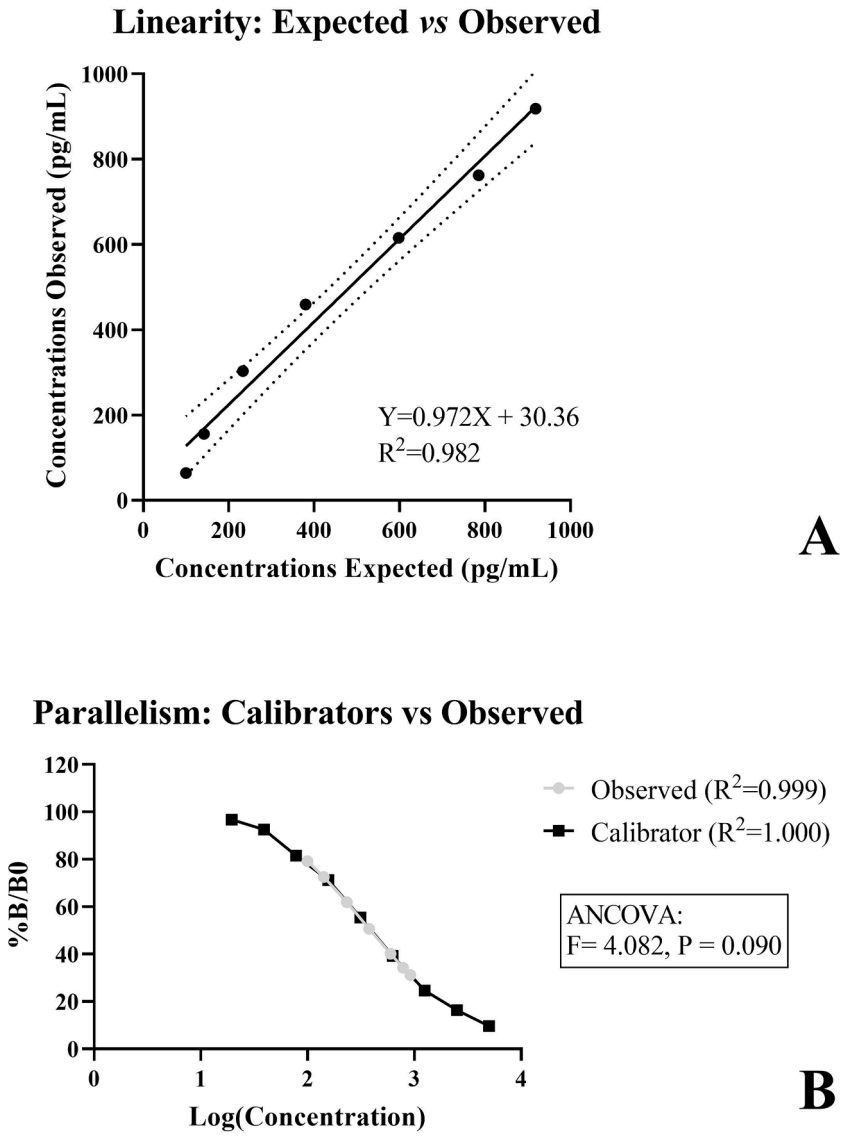

Fig. 1. Assessment of parallelism between the calibrator curve and observed samples (the curves demonstrate a good fit (R^2: coefficient of determination) and overlapping slopes (ANCOVA: Analysis of Covariance), supporting the validity of parallelism between calibrators and test samples) and linearity assessment (comparison between expected and observed analyte concentrations across the calibration range; the equation of the regression line and the R^2 are also showed confirming the method's linearity over the tested range; Panel B).

The mean CCS concentration in muscle juice samples was 455.1 ± 46.8 pg/mL, with a range (min to max) of 47.5 to 1,340 pg/mL. The mean total plasma CCS concentration was 11.16 ± 0.39 ng/mL, ranging from 7 to 19.53 ng/mL. The mean ratio of individual muscle:plasma CCS concentration was 0.04 ± 0.004, with a range of 0.005 to 0.133. New knowledge about CCS concentrations in blood and muscle samples collected from the same animal at slaughtering has been obtained. A moderate but statistically significant correlation was observed between CCS concentrations in muscle and plasma ($r = 0.324$, $P = 0.025$), in agreement with the relationships previously reported in pigs (Shaw et al., 1995). In birds, it is important to consider that plasma binding protein capacity and affinity can vary in different situations, as in response to stressful stimuli, so that the ratios among total, bound, and free GC blood levels can dynamically change. Therefore, total GC concentrations in blood do not always correspond to the biologically active hormone (i.e., the free fraction), whereas plasma free hormone concentration could be a better indicator of physiology and behavior (Malisch and Breuner, 2010). Unfortunately, blood-free GC assays using common methods are not easily feasible and precise. In mammals, good estimates of the blood-free hormone levels can be obtained by assaying concentrations in saliva or urine (Mormède et al., 2007). Analogously, concentrations found in muscle likely depend on the free fraction of the circulating hormone, as in other tissues (Breuner et al., 2013). The ratio muscle:plasma CCS found in the present trial (from 0.4 to 13.3 %) is lower than values of 18 to 24 %, previously reported for pigs (Shaw et al., 1995), whereas may be congruent with the expected free:total CCS ratio in avian blood (Fokidis et al., 2009). However, the time frame of CCS transfer from blood to skeletal muscle should be defined by biological validation procedure in order to verify whether and when changes in blood levels could match those in skeletal muscle. From the concentration ranges (and the other variability indicator) observed, it is noteworthy that muscle levels appear much more variable between individual animals than those in plasma, the maximum value in muscle being almost thirty-fold higher than the minimum, vs. less than three-fold for plasma.

It is concluded that this EIA kit can provide reliable results for measuring CCS concentrations in juice obtained from chicken muscle directly without previous extraction. Such a method can be a further tool for the assessment of animal physiological responses, stress, and welfare. Considering that this is the first trial reporting the measurement of CCS in chicken muscle by EIA, the data presented here may constitute an important basis for further studies. Future research could be addressed to investigate the relationships between muscle CCS levels and meat quality indicators.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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