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Measuring feather and plasma corticosterone in male broiler chickens: uplc–ms/ms methodology and feather growth dynamics

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Abstract

Background Plasma corticosterone (CORTp) is widely used to assess acute stress in chickens, while feather corticosterone (CORTf) has been investigated as a marker of cumulative corticosterone (CORT) exposure during feather growth. The objectives of this study were: (1) to validate an Ultra Performance Liquid Chromatography coupled to Tandem Mass Spectrometry (UPLC-MS) to evaluate measurements of CORTp and of CORTf in different type of feathers (dorsal, wing, and tail) in broiler chickens; (2) to determine at different ages whether higher exposures to chronic exogeneous administration of CORT increases CORTf and CORTp; (3) to evaluate the correlation between CORTf and CORTp; (4) assess the effects of increased CORT exposure on body weight (BW) and feather morphology, exploring whether structural changes might influence CORT deposition. A UPLC-MS/MS method previously established for CORTp quantification was revalidated for application in the present study, and a new method for CORTf quantification was developed and fully validated. Sixty broiler chickens received incrementally larger daily oral doses (0–5 mg/kg) of CORT from day 1 to 42 to model prolonged elevated circulating CORT. At days 14, 28, and 42, plasma and feathers were sampled and analysed to quantify CORTp and CORTf. BW was recorded daily, and wing feather morphology was assessed macroscopically at 42 days. Data analysis included linear regression, polynomial contrasts, Pearson correlations, and log transformation for feather data.

Results The UPLC-MS/MS methods were validated for quantifying both CORTp and CORTf. By 42 days of age, CORTf increased proportionally to the higher CORT exposure levels across all feather types, and CORTp likewise showed a significant rise at this age. A moderate positive correlation was found between CORTp and CORTf at 42 days. Results showed a negative linear relationship between CORT exposure dosage and BW, as well as with wing feather growth; rachis length was notably shorter in birds exposed to higher CORT doses. Although oral dosing does not replicate the dynamic endocrine profile of a natural stress response, our findings show that CORTf partially reflects circulating CORT during feather growth, though not in a dose-dependent manner, and that prolonged elevation of CORT impairs growth and feather development in broilers.

Keywords Validation, Stress hormone, UPLC-MS/MS, Poultry, Feather structure, Body weight

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Introduction

Corticosterone (CORT) is the primary glucocorticoid in birds and a widely used indicator of hypothalamic–pituitary–adrenal (HPA) axis activity in stress research. Quantification of CORT in poultry has most commonly been performed using immunoassay kits such as ELISA [1–4]. While these assays offer high sensitivity, cross-reactivity and matrix effects can impair accuracy [2]. LC-MS and UPLC-MS methods have shown excellent performance in poultry matrices [5–7], with UPLC-MS offering shorter analysis times, better peak shape, and reduced matrix interference [8].

Stress situations activate the HPA axis: the hypothalamus releases corticotropin-releasing hormone and arginine vasotocin, which prompt the anterior pituitary to secrete ACTH. ACTH travels to the adrenal glands, stimulating adrenocortical cells to convert cholesterol into pregnenolone and CORT, which enters the bloodstream. Plasma corticosterone (CORTp) is widely used as an indicator of acute stress [5, 9]. Key limitations concern the measurement approach and the context of collection. Specifically, uncertainties remain regarding assay validation for chicken plasma [10], and CORTp values may be affected by handling during sampling, diurnal rhythms, and the short plasma half-life of CORT [11]. Together, these factors highlight the need for careful standardization and interpretation of CORTp measurements.

When in the bloodstream, some CORT is free (active) and some binds CBG/albumin. The free hormone reaches tissues, including growing feather follicles. During the feather's anagen phase, the vascularized feather pulp exposes developing keratin to circulating CORT, which incorporate circulating hormone into the keratin structure of the feather calamus [6]. In feathers, CORT reflects systemic CORT exposure during the feather's growth period presenting the potential to be used as a retrospective biomarker of systemic exposure during feather formation [12]. Feather type (e.g., dorsal, wing, or tail feathers) may influence CORTf concentrations, as CORT deposition is linked to feather growth. Given that feather types differ in their growth trajectories, the incorporation of circulating CORT can vary according to the bird's age and the specific feather type [13]. Moreover, high CORT exposure has been associated with alterations in feathers structure that could influence CORT incorporation [14]. In this matrix, some questions remains: since feather growth is closely linked to body weight gain [15] and CORT can impair growth [16], elevated CORT could affect feather development and subsequent CORT deposition. Also, the relationship between CORTp and CORTf in broilers is not well established, and correlations may depend on feather type, growth stage, and time of sampling [17].

Therefore, the objectives of this study were to: (1) validate an UPLC-MS method to evaluate measurements of CORTp and in different types of feathers (dorsal, wing, and tail) in broiler chickens; (2) determine at different ages whether higher exposures to chronic exogeneous administration of CORT increases CORTf and CORTp; (3) evaluate the correlation between CORTf and CORTp; (4) assess the effects of increased CORT exposure on body weight (BW) and feather morphology, exploring whether structural changes might influence CORT deposition.

Materials and methods

Animal experiment

Birds and facilities

A total of 60 one-day-old male Ross 308 chicks were purchased from a commercial hatchery for broilers in Belgium (Vervaeke-Belavi, Tielt, Belgium). Birds were allocated to 6 floor pens, with 10 broilers per pen. Each bird was individually identified upon placement using sequentially numbered plastic neck tags, attached with loop fasteners applied using a fine-needle plastic tag applicator (tagging gun). The tags were lightweight, non-abrasive, and checked daily to ensure comfort, readability, and secure placement.

Access to feed and water was provided *ad libitum*. All birds received the same commercial diet according to the breed guidelines: starter (days 0 to 14), grower (days 15 to 34), and finisher (days 35 to 42) feed. The birds were kept on a 23 l:1D light schedule at 30 lx for the first 7 days, followed by a 16 l:8D schedule at 20 lx from day 7 to day 42. During the first week, the temperature was maintained at 34 °C. It was then gradually reduced by approximately 1 °C every two days until reaching 22 °C, which was subsequently maintained. This temperature was then sustained throughout the remaining three weeks. A veterinarian inspected the chickens at least once daily to ensure health and welfare of the birds. Feed and water availability were checked, as well as ambient temperature, relative humidity, air quality (ammonia and dust), litter condition (moisture and caking), and lighting (intensity and photoperiod). The veterinarian also assessed general body condition, potential clinical signs, and any abnormal or stereotypic behaviors, including signs of pain or prolonged inactivity. All observations were documented in the farm records. No birds required culling at any point during the study. At 42 days of age, chickens were humanely killed via an injection of sodium pentobarbital, with the dosage calculated according to their BW. Birds were individually and carefully restrained, and the injection was administered into the brachial vein by an experienced veterinarian.

Experimental design and treatments

The treatment was applied to individual chickens, making the chicken the experimental unit. The experiment used an incomplete block design in which the pen was considered a block. Each pen housed birds from two of the six treatment groups, in order to control for potential behavioral influences across the range of doses. For instance, the first pen housed 5 birds from the control group (T0) and 5 birds from Treatment 1 (T1), the second pen contained 5 birds from T1 and 5 birds from Treatment 2 (T2), and so forth. This design minimized the impact of extreme dose variations within a single pen on the stress measurements of broilers (Supplementary Fig. 1). Cardboard dividers were placed between pens to reduce stress-related behavioral changes spreading from one pen to another.

Six treatment groups were used in total, with each group consisting of 10 birds. The treatments corresponded to increasing CORT concentration administered in a linear progression of 0, 1, 2, 3, 4, and 5 mg/kg of BW, labeled as T0, T1, T2, T3, T4, and T5, respectively. Exogenous administration of CORT, particularly via oral gavage, bypasses the HPA axis regulation, potentially producing circulating concentrations that differ in both magnitude and temporal profile from those observed under natural conditions. The doses used in this study were selected to create a controlled, stepwise elevation of CORT exposure.

Oral corticosterone Gavage

From day 1 to 42, all birds were individually weighed daily between 9 and 10 AM from pen one to six to calculate the CORT dosage according to their respective treatment. To dose the birds, an Excel spreadsheet was used to quickly calculate the required CORT amount to be administered to each bird depending on its BW. E.g.: for the group receiving the maximum dose (5 mg/kg), the dose per bird was computed by proportional calculation (dose = 5 mg × BW[kg]). Birds were handled in batches of five: placed in a box, weighed on a scale positioned next to the pen, dosed, and immediately returned to their pens. The entire procedure took maximum 10 min per pen. Birds in the T0 group received sham gavage to serve as a control. CORT was daily administered orally to the chickens using a syringe fitted with a soft-tipped applicator to gently deliver the solution directly into the crop. Birds were carefully restrained by hand to minimize stress, ensuring precise dosing and preventing aspiration. The stock solution consisted of 10 mg of CORT (Sigma-Aldrich, St Louis, MO, USA) mixed with 1 mL of ethanol and diluted 1:1 in water (v/v; 5 mg/mL). Fresh batches of stock solution were prepared daily. The control group received 1 mL of ethanol diluted 1:1 in water.

Sample collection

Blood samples were collected at 1 PM on days 14, 28, and 42. On day 42, birds received the last oral gavage of CORT between 9 and 10 PM and were killed at 1 PM, with blood immediately collected in the same order as CORT administration to evaluate plasma CORT measurements. For killing and blood collection, chickens were individually restrained, and the entire procedure was completed within a maximum of two minutes to minimize stress. Chickens were killed with sodiumpentobarbital (Euthanimal 20%, sodiumpentobarbital, Alfasan, Woerden, The Netherlands). Blood volumes of 1 mL on days 14 and 28, and 42 were collected from each bird via the jugular vein. Blood samples were collected in K3EDTA tubes (13 × 75 mm, non-ridge Vacuette, Greiner Bio-One, Kremsmünster, Austria). Immediately after collection, plasma was separated via centrifugation (3,000 x g, 10 min, 4 °C), transferred to Eppendorf cups and plasma was stored at – 20 °C until analysis.

On days 14 and 28 dorsal feathers, from the area under the neck and between the wings, were collected by pulling to determine CORTf. On day 42, after killing, dorsal, wing (the first primary feather from both the right and left wings), and tail (from the middle part of the tail) feathers were collected by pulling for analysis. Only dorsal feathers were sampled at three time points to evaluate changes in CORT concentration over time. Wing and tail feathers, however, were only sampled once to minimize pain and stress, and because they regrow much more slowly than dorsal feathers.

Dorsal feathers and blood were collected at days 14, 28, and 42 to evaluate how CORT could be detected at different ages and how it changes over time. The study was therefore divided into three equal 14-day periods from start to end of the trial. This design allowed us to assess age-related detection in a standardized way. Since CORT was daily provided that would maybe induce constant high measurements of CORT in blood, it was then explored if it would have a correlation between CORTp and CORTf.

Although additional stress was reduced by sampling only dorsal feathers (rather than three feather types at three ages) and by keeping weighing quick yet careful, handling and sampling inevitably induce some stress. To minimize bias from these additional stressors, all procedures were performed uniformly and only by the same two experienced veterinarians.

Prior to CORT analysis, wing feathers were photographed using a high resolution digital camera with a 12-megapixel sensor. The photographs were analyzed using Image J software (National Institutes of Health, Bethesda, USA). To ensure accuracy in data collection, each parameter was evaluated in duplicate by a single blinded researcher. The parameters measures were based

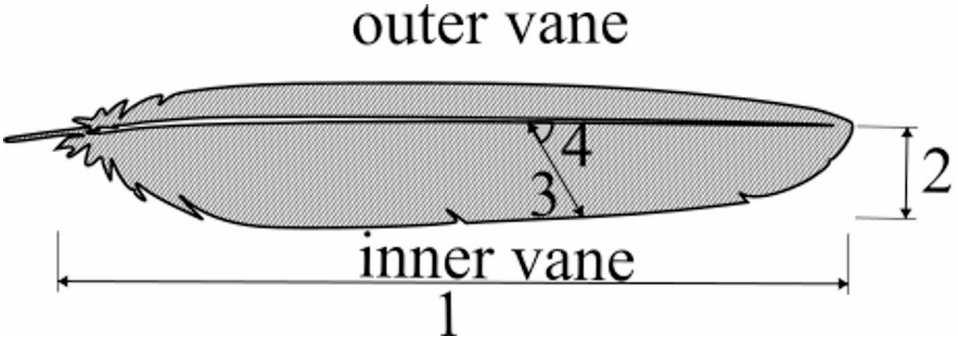

Fig. 1 Feather measurements: rachis length (1), depth (2), barb length (3), and barb angle (4)

on previous studies (Bachmann et al., 2007; Fiorini et al., 2022): length of the rachis (which includes the calamus), depth of both the outer and inner vanes, length of the barbs of outer and inner vanes (from the rachis to the fringes), and angle formed between the barbs and the rachis on both the inner and outer vanes (Fig. 1).

UPLC-MS/MS analysis

The UPLC system consisted of an Acquity H-Class Quaternary Solvent Manager and an Acquity Flow-Through Needle Sample Manager (Waters, Milford, USA). Chromatographic separation was performed using an Acquity UPLC® BEH C18 column (1.7 µm, 50 × 2.1 mm i.d.) in combination with a precolumn of the same type (VanGuard™; 5 × 2.1 mm i.d.), both from Waters. Gradient elution was carried out using a mobile phase of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B) at a flow rate of 0.3 mL/min, following this gradient profile: 0.0 min: 80% A/20% B; 0.0–3.0 min: linear gradient to 20% A/80% B; 3.0–4.4 min: 20% A/80% B; 4.4–4.5 min: linear gradient to 80% A/20% B; 4.5–6.0 min: 80% A/20% B. The column temperature was maintained at 40 °C, while the autosampler was set at 10 °C. The total chromatographic run time was 6.0 min, with the UPLC effluent being diverted via a valve from 2.0 to 3.5 min to a Xevo Triple Quadrupole – Sensitivity® triple quadrupole mass spectrometer (Waters), equipped with an electrospray ionization (ESI) ion source operating in positive ionization mode.

The UPLC-MS/MS analysis was controlled using MassLynx software (v4.1), which was also used for subsequent data processing. Operating conditions for the ESI source in positive ionization mode were optimized by direct infusion of all individual components in combination with the mobile phase at 50% A/50% B, delivered at a flow rate of 0.3 mL/min. The following tuning parameters were applied for the detection of all components: capillary voltage, 3 kV; cone voltage, 45 V; source offset, 50 V; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow, 800 l/h; cone gas flow, 150 l/h; collision gas flow, 0.2 mL/min; ion energy 1, 0.5; ion energy 2, 1.5; low mass 1 and low mass 2 resolution, 2.8; high mass 1 and high mass 2 resolution, 14.5.

Table 1 Tandem mass spectrometry (MS/MS) detection parameters for corticosterone and its internal standard

Component	Precursor Ion (m/z) [1]	Product Ions (m/z)	Collision Energy(CE, eV) [2]
Corticosterone	347.23	Quantifier: 121.15	23
		Qualifier: 97.06	25
Corticosterone-d8 (IS)	355.32	100.06	25

[1] m/z = mass-to-charge ratio

[2] CE = Collision Energy (eV) used in fragmentation

Components were detected in Tandem Mass Spectrometry (MS/MS) mode using component-specific Multiple Reaction Monitoring transitions (Table 1). For CORT, two specific product ions were monitored: a quantifier ion for quantification and a qualifier ion for identification, based on the ion ratio of both product ions. Since CORT is an endogenous compound, the obtained quantification results were corrected for its endogenous measurements present in the blank plasma/feather used for the construction of the calibration curve. The calibration curve was plotted as the response (peak area of the analyte/peak area of IS) against the analyte concentration. The endogenous concentration (Cendo) was determined as the negative intersection with the x-axis of the calibration curve, calculated as Cendo = b/a, where a represents the slope and b the intercept of the calibration curve.

Method validation

The UPLC-MS/MS method for the analysis of CORTp plasma and CORTf was validated according to EC (2002/657/EC) (EC, 2002) and VICH (GL49) guidelines. For chicken plasma, linearity was assessed over a concentration range of 0.5–20 ng/mL, with six calibrator measurements: 0.5, 1, 2, 5, 10, and 20 ng/mL. Accuracy and precision were evaluated at low (0.5 ng/mL) and high (10 ng/mL) concentration measurements, each with $n = 6$ replicates, with 0.5 ng/mL established as the lower limit

of quantification for the method. Since this was an existing method in other species (Pereira et al., 2009; Liu et al., 2022), a validation experiment was conducted over one day, covering the same range (0.5–20 ng/mL), with accuracy and precision evaluated at 1 ng/mL and 10 ng/mL, each with $n = 6$ replicates.

For chicken feathers, a newly developed and optimized method was validated using an extended validation scheme to accommodate a higher concentration range (5–500 ng/g), as applicable to the analyzed samples. Linearity was assessed over two distinct concentration ranges. In the low concentration range (0.5–20 ng/g), six calibrator measurements were included: 0.5, 1, 2, 5, 10, and 20 ng/g. Accuracy and precision were evaluated at 0.5 ng/g and 10 ng/g, each with $n = 6$ replicates, with 0.5 ng/g established as the lower limit of quantification for the method. Alternatively, a high concentration calibration curve (5–500 ng/g) was also assessed, with calibrator measurements at 5, 10, 25, 50, 100, 250, and 500 ng/g. In this range, accuracy and precision were evaluated at 25 ng/g and 250 ng/g, each with $n = 6$ replicates, with measurements repeated across three independent validation days to ensure method robustness. The analytical methodology as such was validated using the preparation of matrix-matched samples, obtained by spiking "blanco" matrix with different concentration measurements of CORT, to evaluate validation parameters such as linearity, accuracy, and precision, over three different days (VICH GL49). This is a generally accepted approach. In this way, it was demonstrated that the analytical method allowed for a reliable and reproducible measurement of CORT in the biological matrix.

Additionally, different injection volumes were tested to improve coverage across the full concentration range of the samples. The standard 5 µL injection volume was used, alongside lower injection volumes of 1 µL and 0.5 µL, to enhance detection capabilities and accommodate varying CORT concentrations. Also, all injections were performed in duplicate to ensure reproducibility and accuracy of the measurements. The results of the method validation experiments conducted on chicken plasma and feathers were fully compliant with the acceptance criteria outlined in the EC (2002/657/EC) and VICH (GL49) guidelines.

Corticosterone analysis

Chemicals and reagents

The CORT analytical standard was obtained from Sigma-Aldrich (Merck, Overijse, Belgium), while the CORT-d8 analytical standard, used as an internal standard (IS), was sourced from Toronto Research Chemicals (Toronto, Canada). Individual stock solutions of both components were prepared at 1 mg/mL in methanol and stored at ≤ −15 °C. For sample analysis, working solutions were prepared by appropriately diluting the stock solutions in methanol and were stored at 2–8 °C. A 100 ng/mL CORT-d8 working solution was used for plasma sample preparation, while a 1,000 ng/mL solution was used for feather samples. CORT working solutions in the concentration ranges of 2–80 ng/mL (feathers), 20–1,000 ng/mL (plasma, low and high ranges), and 5–200 ng/mL (plasma sample analysis) were used for spiking calibrator and quality control (QC) samples. In a general way, an analytical method will be validated in the range relevant for the samples to be analyzed. In this case, this is a lower concentration range for plasma samples, close to the instrumental detection limit, and a higher concentration range for the feather samples, given the higher measurements in this matrix for the samples of the animal trial.

Methanol, used for stock and working solution preparation as well as for sample extraction, and acetonitrile, used in sample extraction, were both of High-Performance Liquid Chromatography grade (Fisher Scientific, Filter Service, Eupen, Belgium). Milli-Q grade water, produced in-house using a Milli-Q-SP water purification system (Sigma-Aldrich), was used for the preparation of the aqueous mobile phase component and for sample preparation. Acetonitrile, used as the organic mobile phase component, and formic acid, used as a mobile phase additive, were of Ultra-Low Contaminant/Mass Spectrometry grade (Biosolve, Valkenswaard, the Netherlands). Diethyl ether and 37% fuming hydrochloric acid (HCl), both used in sample extraction, were of pro analysis grade (high-purity for analytical use, Emsure®, Merck). n-Hexane, also used in sample preparation, was of HiPerSolv CHROMANORM® quality (suitable for chromatography) and was obtained from VWR (Leuven, Belgium).

Plasma

A 250 µL plasma sample was quantitatively transferred into a 15 mL plastic centrifuge tube, ensuring the complete and accurate transfer of the entire sample without loss. Subsquently, 25 µL of a 100 ng/mL CORT-d8 IS working solution and 25 µL of methanol were added. The mixture was then vortexed to ensure homogeneity. The latter volume was replaced with 25 µL of a working solution of CORT at varying concentrations for calibrator and QC sample preparation. For the preparation of matrix-matched calibrator and QC samples, as well as blank samples, plasma from healthy chicken was used. Plasma was obtained from 10 healthy broiler chickens that were not included in the experimental design. Blank samples were not spiked with IS or analyte; instead, 50 µL of methanol was added before they were subjected to the sample preparation procedure.

Extraction was performed using liquid-liquid extraction with diethyl ether. For this, 3 mL of ether was added to a 15 mL centrifuge tube, which was then placed on a

roller mixer (SRT2, Stuart Scientific, Novolab, Geraardsbergen, Belgium) at 33 rpm for 20 min. The sample was then centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatant was transferred to a 15 mL glass tube and dried under a gentle stream of nitrogen at 40 °C using a Pierce Reacti-Therm III™ Heating Module and ReactiVap™ III Module (Rockford, USA). The extract was first redissolved in 125 µL of methanol and thoroughly vortex-mixed. Subsequently, 125 µL of water was added, followed by an additional vortex mixing step. The final extract was passed through a Nylon filter (0.22 µm, 13 mm) (Merck), collected in a plastic conical vial, and a 5 µL aliquot was injected onto the UPLC-MS/MS system. Plasma CORT concentrations were expressed in ng/mL.

Feathers

The feathers were ground for 3 min at 3,000 rpm using a mechanical grinder. A 100 mg sample of the ground feathers was accurately weighed and placed in a 15 mL plastic centrifuge tube. Then, 25 µL of a 1,000 ng/mL CORT-d8 IS working solution and 25 µL of methanol were added, followed by vortex mixing. The latter volume was then replaced with 25 µL of a working solution of CORT at varying concentrations for calibrator and QC sample preparation. For the preparation of matrix-matched calibrator, QC, and control samples, ground feather material from healthy chickens was used. Blank samples were not spiked with IS or analyte; instead, 50 µL of methanol was added before they were subjected to the sample preparation procedure.

For extraction, 4 mL of acetonitrile was added to a 15 mL centrifuge tube, which was then placed on an overhead shaker at 80 rpm for 1 h (IKA® Trayster Digital, Staufen, Germany). The sample was subsequently centrifuged at 4,000 rpm for 10 min at 4 °C. The supernatant was transferred to a 15 mL glass tube and dried under a gentle stream of nitrogen at 40 °C. The extract was first redissolved in 50 µL of methanol, followed by vortex mixing. Then, 950 µL of a 0.1 N HCl solution in water was added, followed by an additional vortex mixing step. The extract was passed through a Nylon filter (13 mm, 0.22 µm, Millipore, Merck) and applied directly for further clean-up using a hydrophilic-lipophilic balance solid-phase extraction cartridge (1 cc, 30 mg, Waters), which was preconditioned with 1 mL of methanol and 1 mL of a 0.1 N HCl solution in water.

The sample was allowed to pass slowly through the cartridge, followed by successive rinsing with 1 mL of a 0.1 N HCl solution in water, 1 mL of a 0.1 N HCl solution in a water/methanol mixture (1:1, v/v), and 1 mL of hexane. Vacuum was then applied to dry the cartridge. Elution was performed using 2 × 750 µL of methanol, and the eluate was collected in a 15 mL glass tube, then dried under a gentle stream of nitrogen at 40 °C. The extract was first redissolved in 100 µL of methanol, followed by thorough vortex mixing. Subsequently, 100 µL of water was added, followed by an additional vortex mixing step. The final extract was transferred to a plastic conical vial, and a 5 µL aliquot was injected onto the UPLC-MS/MS system. Feather CORT concentrations were expressed as ng/g of feather material.

Data analysis

Data analysis was conducted using RStudio. The experimental unit in this study was the individual chicken. Data were analyzed based on administered doses of CORT relative to BW (relative dose). The relative dose (mg CORT/kg BW) was considered an ordered discrete factor and was assessed with least square linear regression followed by a post-hoc polynomial contrast analysis to assess the relation between estimated means. Pearson correlations were used for comparing the response variables CORTp (ng/mL) and CORTf (ng/g). Wing feather morphology was evaluated using log-transformed data to adjust for heteroscedasticity and create a linear response, with BW as a continuous covariate and dose as a discrete fixed factor.

Results

Validation

The validation results for the existing analysis method for CORT quantification in plasma are presented in Table 2.

Table 2 Validation results for the calibration model y = ax + b with 1/x weighting (correlation coefficient (r) and goodness-of-fit coefficient (GoF) of corticosterone in chicken plasma, at the different injection volumes involved at sample analysis

Injection volume (µL)	Evaluation occasion	a	b	*r*	GoF (%)
5	Day 1	0.01335	0.02207	0.9996	5.3
	Day 2	0.01374	0.01638	0.9997	6.8
	Day 3	0.01420	0.03256	0.9998	3.7
1	Day 1	0.01343	0.01908	0.9996	2.4
	Day 2	0.01373	0.01446	0.9998	6.9
	Day 3	0.01372	0.03752	0.9998	3.2
0.5	Day 1	0.01307	0.02824	0.9998	3.7
	Day 2	0.01369	0.01967	0.9997	4.5
	Day 3	0.01375	0.02915	0.9996	7.3

Table 3 Results of accuracy and precision evaluation of corticosterone in chicken feather at 25 and 250 ng/g measurements, mean values ± SD are given of $n = 6$ replicates, as well as accuracy (% deviation from nominal value), and precision (as % RSD)

Injection volume (µL)	Evaluation occasion	Level 1: 25 ng/g	Level 2: 250 ng/g
5	Day 1	25.3 ± 1.2 (acc + 1.3%) RSD 4.8%	257.4 ± 10.4 (acc + 3.0%) RSD 4.0%
	Day 2	25.4 ± 0.9 (acc + 1.7%)0 RSD 3.4%	251.6 ± 7.8 (acc + 0.6%) RSD 3.1%
	Day 3	26.0 ± 1.4 (acc + 5.5%) RSD 3.8%	266.9 ± 17.8 (acc + 6.7%) RSD 6.8%
1	Day 1	24.7 ± 0.9 (acc − 1.3%) RSD 3.7%	258.1 ± 8.5 (acc + 3.2%) RSD 3.3%
	Day 2	24.9 ± 0.5 (acc − 0.4%) RSD 2.1%	250.7 ± 12.5 (acc + 0.3%) RSD 5.0%
	Day 3	25.6 ± 1.0 (acc + 2.2%) RSD 2.2%	267.1 ± 19.1 (acc + 6.8%) RSD 7.1%
0.5	Day 1	25.2 ± 1.3 (acc + 0.8%) RSD 5.0%	253.4 ± 6.8 (acc + 1.4%) RSD 2.7%
	Day 2	24.9 ± 0.7 (acc − 0.2%) RSD 2.7%	253.2 ± 12.5 (acc + 1.3%) RSD 4.9%
	Day 3	25.4 ± 1.3 (acc + 1.7%) RSD 5.1%	266.4 ± 15.7 (acc + 6.6%) RSD 5.9%

Table 4 Mean corticosterone concentration (mean ± sd) in plasma (ng/mL) and feathers (ng/g) from dorsal, wing, and tail regions of broiler chickens administered with increasing corticosterone doses (0, 1, 2, 3, 4, and 5 mg/kg) from 1 to 42 days old

	Treatment[1]						
	0 mg/kg	1 mg/kg	2 mg/kg	3 mg/kg	4 mg/kg	5 mg/kg	*P*-value
Plasma							
D14	15.1±20.0[2]	33.5±43.5	12.8±11.8	6.1±2.7	11.9±5.5	14.0±9.5	0.358
D28	11.9±10.8	10.1±11.4	5.0±2.9	12.9±7.6	14.4±11.2	13.1±4.5	0.217
D42	9.2±20.6^{c}	16.5±16.6^{bc}	41.0±30.7^{bc}	31.0±7.6^{abc}	53.0±26.8^{ab}	73.0±50.8^{a}	L <0.001
Dorsal feather							
D14	9,629±8,370^{b}	6,642±3,236^{b}	11,307±4,557^{b}	13,714±3,942^{b}	31,979±21,653^{a}	33,376±18,111^{a}	Q: 0.034
D28	937±1,034^{c}	623±304^{c}	1,165±1,108^{c}	1,836±1,061^{cb}	6,402±5,979^{ab}	8,243±6,301^{a}	Q: 0.013
D42	774±308^{b}	1,159±750^{b}	1,705±789^{b}	2,034±689^{b}	8,017±6,422^{a}	6,866±1,635^{a}	L: <0.001
Wing feather							
D42	987±333^{b}	1,667±1,091^{b}	6,199±4,798^{ab}	4,511±2,024^{a}	7,799±1,154^{a}	8,126±3,404^{a}	L: <0.001
Tail feather							
D42	1,978±864^{b}	3,097±3,191^{b}	5,434±2,838^{b}	6,307±2,298^{b}	15,338±5,984^{a}	22,791±9,804^{a}	Q: <0.001

[1]Means were estimated with linear least square regression and post-hoc polynomial contrasts with, if significant, the best relation represented by *L* Linear or *Q* Quadratic

[2]Values within the same row followed by different letters (a, b, c) are significantly different ($P < 0.05$)

The method showed linearity across the concentration range of 0.5 to 20 ng/mL, with $r > 0.99$ (0.9989) and a goodness-of-fit coefficient (GoF) coefficient below 10% (5.3%). Accuracy and precision met the acceptance criteria at both evaluated concentrations: 1 ng/mL and 10 ng/mL. Accuracy was + 13.0% and + 4.7% at 1 ng/mL and 10 ng/mL, respectively, falling within the acceptable ranges of − 50% to + 20% (for 1 ng/mL) and − 20% to + 10% (for 10 ng/mL). Precision, expressed as RSD, was 7.8% and 6.6% at 1 ng/mL and 10 ng/mL, respectively, remaining below the maximum allowed RSD of 25% and 15%.

On each validation day, linearity was observed ($r > 0.99$ and GoF < 10%) in chicken feathers, as shown in Table 3. Furthermore, it was demonstrated that the injection volume could be reduced from the standard 5 µL to 1 µL and 0.5 µL without compromising the validation results. At 25 and 250 ng/g, and on each validation day, accuracy remained within the acceptable range of − 20% to + 10%. Precision also met the required criteria, with relative standard deviations (RSDs) below the maximum allowable limits (15% at 25 ng/g and 10% at 250 ng/g).

Corticosterone concentration in feathers and plasma

Mean CORTf and CORTp concentrations (dorsal, wing and tail regions) are presented in Table 44. At day 14 and 28, CORTp was not significantly correlated to

administered doses of CORT adjusted for BW (Fig. 2). However, by day 42, a moderate linear increase (R^2 = 0.34) of CORTp was observed ($p < 0.01$).

Dorsal CORTf concentrations exhibited a quadratic response at days 14 and 28 ($p < 0.001$) with a moderate positive correlation (R^2 = 0.39 and R^2 = 0.40, respectively). At day 42, a linear response to the daily relative administered CORT doses adjusted for BW was observed ($p < 0.001$, R^2 = 0.41; Fig. 3). Temporal (age-related) variation within each dose group was assessed for dorsal CORTf and CORTp. CORTf declined with age in all groups. CORTp was relatively stable in T0 and low

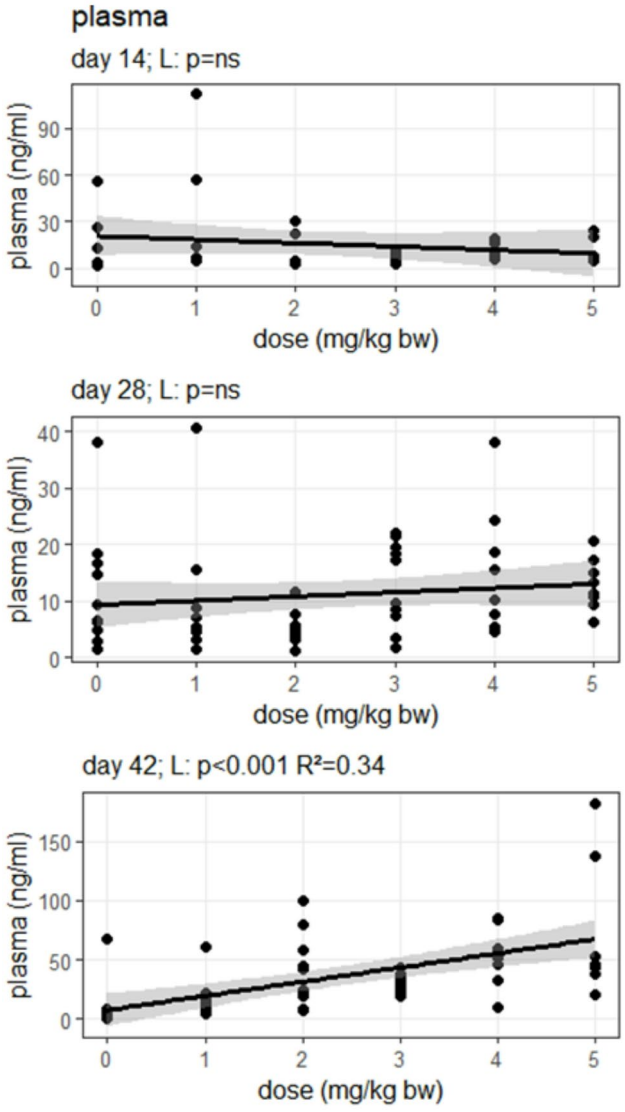

Fig. 2 Corticosterone concentration in plasma. Relation between daily relative administered dose corticosterone (mg/kg of body weight) (left side) on plasma concentration (ng/mL) at day 14, 28 and 42. Relative doses were analyzed with least square regression followed by post-hoc polynomial contrast analysis. The best relation between means is represented by either L response. The shaded areas represent the standard error

doses through D28, then rose by D42, with pronounced increases at higher doses. Wing CORTf concentrations exhibited a moderate positive linear response (R^2 = 0.36, $p < 0.001$) to administered CORT doses adjusted for BW. In contrast, tail CORTf followed a strong quadratic response (R^2 = 0.72, $p < 0.001$).

No significant correlation between CORTp and CORTf from dorsal feathers was observed on day 14 ($p = 0.670$) nor 28 ($p = 0.220$) (Fig. 4). However, at 42 days, CORTp measurements show a significant moderate and dose-dependent relationship with CORTf measurements, with the strongest correlation observed in tail feathers ($R = 0.58$, $p < 0.001$), followed by dorsal feathers ($R = 0.45$, $p < 0.001$) and wing feathers ($R = 0.45$, $p = 0.05$).

Body weight and wing feather structure

The administration of linearly increasing doses of CORT, adjusted for BW with equal increments resulted in a significant linear decrease in final BW ($p < 0.05$). Chickens that received the highest dose (5 mg/kg BW) had a final BW nearly 50% lower compared to the control group (0 mg/kg). The mean cumulative total dose administered exhibited a logarithmic response with the administered doses of CORT adjusted for BW across all stages of the rearing period.

Regarding morphological structure of wing feathers (Table 5), increasing administered doses of CORT were associated with a decrease in rachis length ($p < 0.01$). The rachis was significantly shorter in wing feathers of chickens that received 5 mg/kg of CORT compared to those that received 0–3 mg/kg ($p < 0.001$). However, no differences were observed in the length, depth, or angle of the inner and outer vanes across treatments.

Discussion

The UPLC-MS validation demonstrated a strong correlation between CORT concentration and analytical response in both plasma and feather samples, with high linearity and goodness-of-fit values. The validation outcomes for accuracy and precision in chicken feather were not affected by decreasing the injection volume. This indicates that highly concentrated CORT sample extracts can be injected at lower volumes to ensure the CORT peak remains within the linear range, without compromising quantitative reliability. In feathers, the calibration model remained stable across different injection volumes, showing minimal slope and intercept variation. Accuracy and precision were consistent across CORT measurements.

CORTp results reflected both the pharmacokinetics of oral dosing and age-related differences in metabolism. At 14 and 28 days, no significant differences in plasma CORT were observed across increasing dose groups, likely because CORT's short half-life (about 22 min; [11])

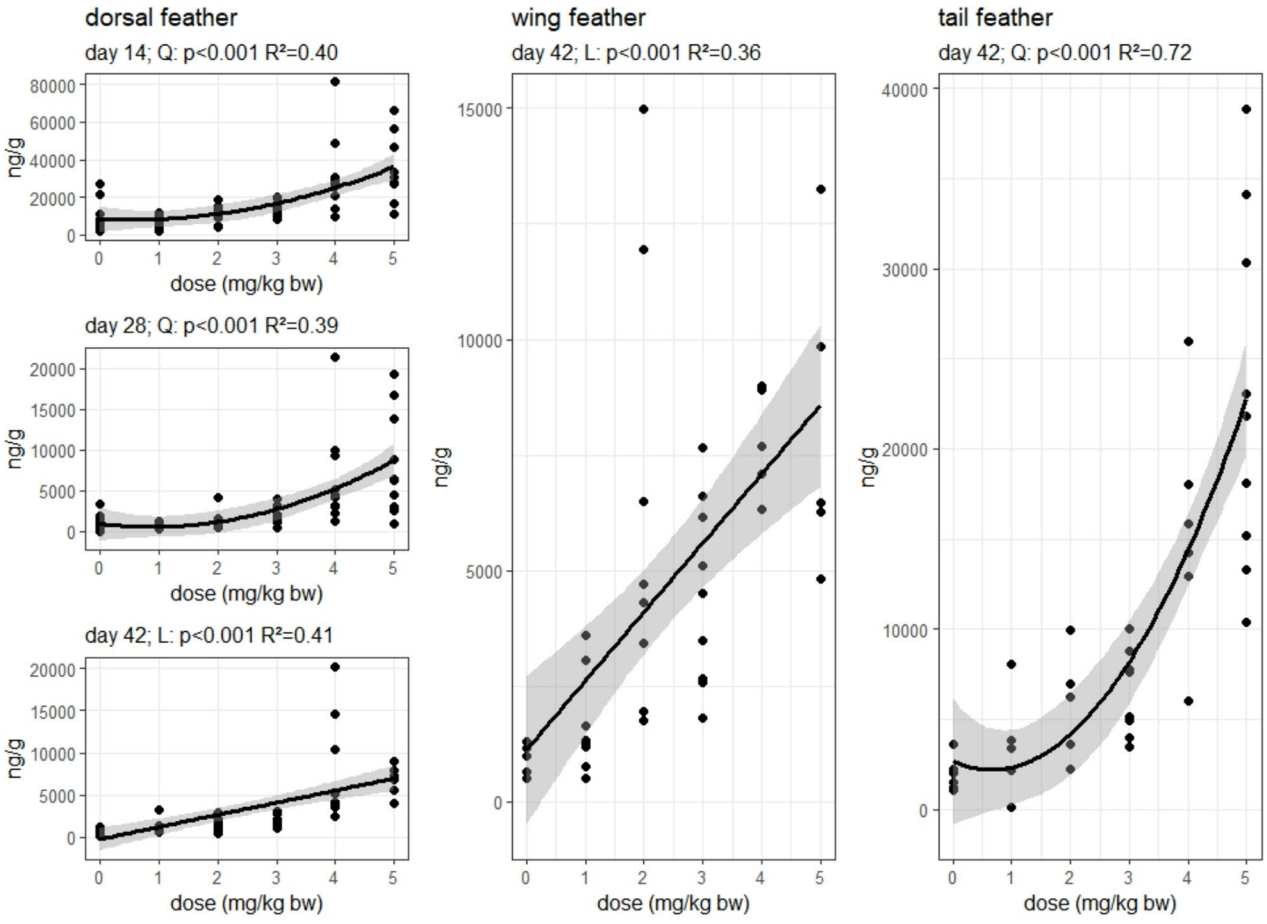

Fig. 3 Corticosterone concentration in dorsal, wing and tail feathers. Relation between daily relative administered dose corticosterone (mg/kg body weight) and feather concentration (ng/g) across feather types (dorsal, wing and tail) at day 14, 28 (dorsal feather) and 42 (all feather types). Data was analyzed with least square regression followed by post-hoc polynomial contrast analysis. The best relation between means is represented by either L or Q response. The shaded areas represent the standard error

led to clearance before the afternoon sampling. By day 42, however, higher doses may have exceeded metabolic clearance capacity (as described for other glucocorticoids in experimental models by Boudinot et al., 1986), resulting in detectable, moderately dose-dependent increases in CORTp that correlated with CORTf in the three feather types, indicating that this relationship may be sensitive to both sampling time relative to dosing and feather growth stage.

Although exogenous CORT exposure was reflected in CORTf across ages (in dorsal feather) and feather types (wing, tail, and dorsal), and in CORTp at 42 days, the correlations were only moderate. At 42 days, CORTf in all three feather types showed a moderate positive relationship with the administered CORT dose, suggesting proportional incorporation during feather growth (Jenni-Eiermann et al., 2014). However, CORTf deposition is also influenced by feather growth dynamics and structural characteristics, which may affect the amount of hormone deposited per unit of feather mass (Romero & Fairhurst, 2016). Tail feathers, had higher baseline CORTf even in control birds, consistent with reports that incorporation varies among feather tracts and may be influenced by growth duration or vascularization [13]. Early dorsal feathers (14 days) showed higher CORTf values under exposure, possibly due to differences in feather development stage and the transition from fluff to contour feathers [18] at this age. However, the temporal decline in dorsal CORTf raises questions about whether broilers habituate to chronic dosing, or whether CORT accumulates less efficiently in this feather matrix.

The effect of increased CORT exposure was reflected in both BW gain and feather macroscopic structure. Although ethanol dilution could contribute to reduced growth at higher doses [19], high circulating CORT measurements are known to negatively impact productivity [20]. Feather morphology at the macroscopic level was also affected by high CORT doses, with rachis length significantly reduced in birds receiving the highest doses. Feather growth is regulated by complex molecular

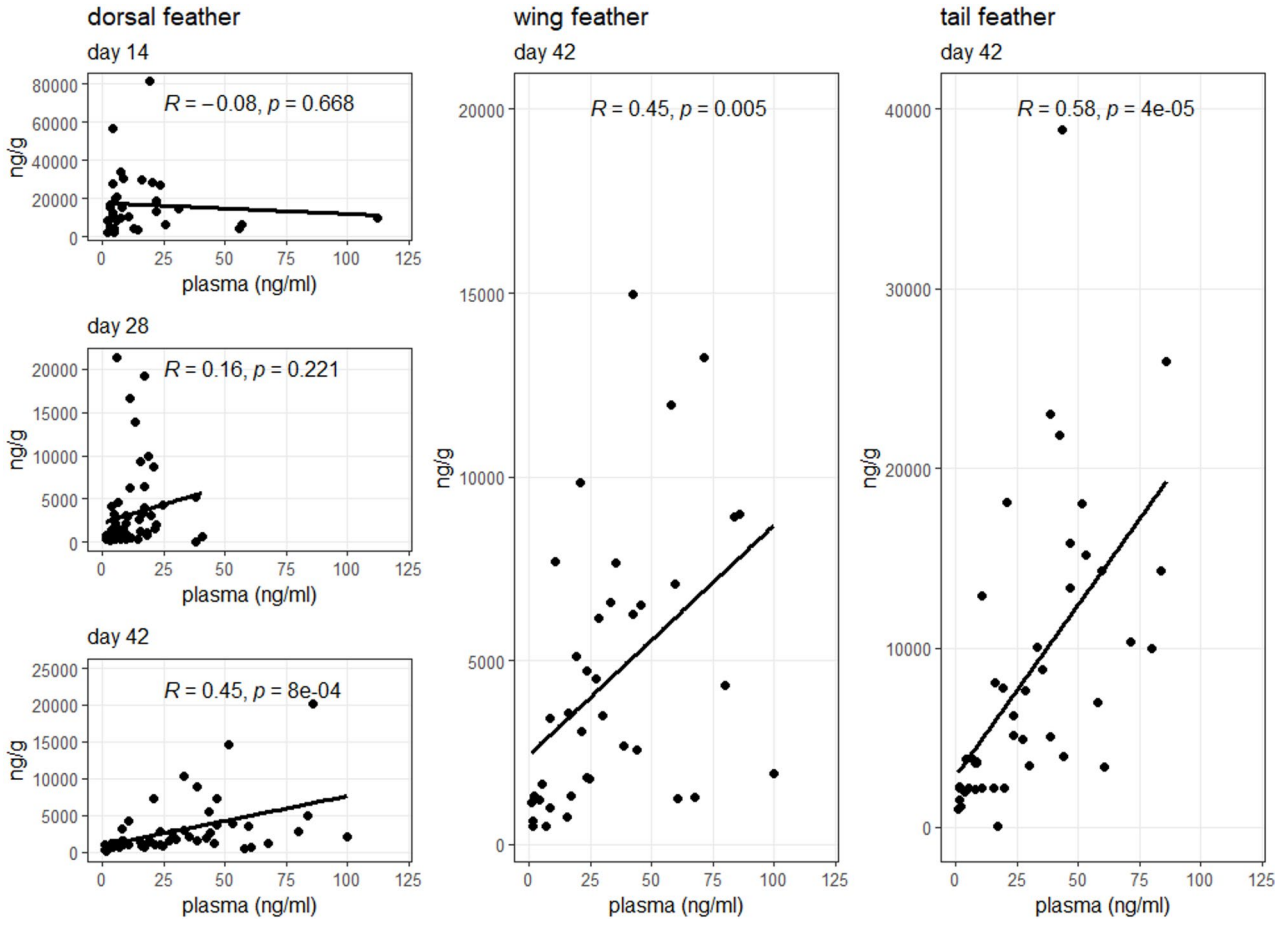

Fig. 4 Correlation between corticosterone concentrations in feathers and plasma of broiler chickens administered corticosterone orally on a daily basis. Pearson correlation between plasma (ng/mL) and feather concentrations (ng/g) across feather types (dorsal, wing and tail) at day 14, 28 (dorsal feather) and 42 (all feather types)

Table 5 Effects of increasing corticosterone doses (0, 1, 2, 3, 4, and 5 mg/kg) and body weight (BW) included as covariate on wing feather morphology in 42-day-old broiler chickens

Trait	Corticosterone Dose (mg/kg)[1]						*P*-value	
	0	1	2	3	4	5	BW	*t*-teste
Length of Rachis (mm)	111±14[a,2]	112±13[a]	111±9[a]	112±5[a]	101±6[ab]	92±8[b]	0.051	<0.001
Inner Vane								
Length (mm)	17.0±1.1	17.3±1.9	16.6±1.6	16.2±1.0	15.6±1.0	15.4±1.2	0.005	0.984
Depth (mm)	13.0±1.0	12.0±1.7	12.2±1.2	12.8±1.2	12.0±0.9	11.6±1.1	0.661	0.373
Angle (°)	39.4±4.1	36.8±4.0	38.5±2.3	39.8±4.0	39.6±3.6	38.9±3.9	0.493	0.589
Outer Vane								
Length (mm)	12.8±0.8	13.1±1.6	12.7±1.2	13.4±1.0	12.9±1.4	11.7±1.13	0.151	0.080
Depth (mm)	9.9±1.0	9.4±1.8	9.3±1.6	10.6±1.4	9.3±1.01	8.6±1.3	0.595	0.183
Angle (°)	40.5±5.3	38.2±6.1	37.8±3.6	40.9±4.4	39.0±3.2	39.8±3.4	0.620	0.774

[1]Data was analyzed with least square linear regression with body weight included as covariate

[2]Values within the same row followed by different letters (a, b, c) are significantly different ($P < 0.05$)

pathways, and disruptions (whether from nutritional, hormonal, or environmental sources) can alter both structure and CORT deposition [21]. However, in the present study only macroscopic traits of wing feathers (rachis length and vane depth) were assessed and did not examine feather microstructure that could further explain alterations in morphology. It is also important to note that BW was included as a covariate in the analysis of feather morphology. Because CORT functions not only as a stress hormone but also as a metabolic regulator (Zaytsoff et al., 2019), the feather changes observed may arise for two reasons: either from a direct effect of

circulating CORT on the growing feather, or indirectly from CORT-induced reductions in BW, since slower growth can also alter feather development.

This study has several important limitations that should be considered when interpreting the findings. First, the UPLC-MS/MS method was validated only for male broiler plasma and feathers under controlled conditions, without assessment of matrix effects, sample stability, or comparison with the commonly used ELISA method. These analytical limitations restrict the broader applicability and comparability of the method.

CORT was administered by oral gavage, a route that bypasses the natural regulation of the HPA axis. Oral CORT administration does not stimulate the HPA axis; rather, it elevates circulating CORT directly and suppresses endogenous secretion via negative feedback [10]. This approach should therefore be interpreted as a controlled elevation of systemic CORT exposure rather than a replication of a physiological stress response. Consequently, the magnitude and temporal profile of circulating CORT induced by this method may differ from endocrine patterns observed under natural stressors, and the findings should not be directly extrapolated to physiological stress responses.

In addition, repeated handling procedures (such as weighing, gavage, blood sampling, and feather plucking) constituted additional stressors that may have elevated baseline CORT measurements in both control and treated birds; notably, elevated CORT was detected even in controls. Feather type also introduced potential bias because different feather types grow at different rates.

Conclusion

The LC–MS/MS method used in the current study enables CORT quantification with high sensitivity and reproducibility. This study demonstrates that chronic oral administration of CORT is partially reflected in both feather and plasma measurements in male broiler chickens, with moderate dose-dependent relationships most evident at 42 days of age. CORTf increased with administered doses across feather types, although patterns differed according to feather growth stage and tract. CORTp showed a linear response only at later ages, highlighting the importance of sampling timing relative to dosing and developmental physiology. Elevated exogenous CORT exposure impaired BW gain and altered macroscopic wing feather morphology.

However, interpretation of these findings is constrained by methodological limitations, including HPA-axis suppression due to oral dosing, potential confounding from repeated handling, heterogeneity in feather growth dynamics, and analytical constraints of the UPLC–MS/MS method. Taken together, these results suggest that CORTf may serve as a supportive biomarker of chronic stress in broilers, provided that differences in feather type, growth rate, and structure are taken into account. Methodological refinements and further validation under conditions that more closely mimic natural stress physiology are still required before CORTf can be broadly applied in poultry welfare assessment.

Abbreviations

BW	Body Weight
Cendo	Endogenous Concentration
CORT	Corticosterone
CORTf	Feather Corticosterone
CORTp	Plasma Corticosterone
HCl	Hydrochloric Acid
HPA	Hypothalamic–pituitary–adrenal
IS	Internal Standard
QC	Quality Control
UPLC–MS/MS	Ultra-Performance Liquid Chromatography coupled to Tandem Mass Spectrometry

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-05275-w.

Supplementary Material 1: Supplementary Fig. 1: Distribution of birds from the six treatment groups (T0–T5) across floor pens in the incomplete block design. Treatments consisted of oral corticosterone administration at the following doses: T0–0 mg/kg, T1–1 mg/kg, T2–2 mg/kg, T3–3 mg/kg, T4–4 mg/kg, and T5–5 mg/kg. Each pen housed 10 birds from two different treatment groups, with 5 birds from each group

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Authors' contributions

GA, FT, and PS conceived and designed the study. PS conducted the experiments. KB, MC and SVP performed data analysis. PS and MC drafted the manuscript. CL, FT, AD, WV, MDG, and GA provided critical revisions and intellectual input.

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Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All the experiments and manipulations involving animals were approved by the Ethics Committee of Poulpharm Bvba (Approval Number LA 1400564) on November 17, 2022. All experiments were performed in accordance with the guidelines and regulations of the Ethical Committee; this study complied with ARRIVE guidelines.

Competing interests

The authors declare no competing interests.

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