Feather pulp: a novel substrate useful for proton nuclear magnetic resonance spectroscopy metabolomics and biomarker discovery

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ABSTRACT Noninvasive biomarkers of stress that are predictive of poultry health are needed. Feather pulp is highly vascularized and represents a potential source of biomarkers that has not been extensively explored. We investigated the feasibility and use of feather pulp for novel biomarker discovery using ^{1}H-Nuclear Magnetic Resonance Spectroscopy (**NMR**)-based metabolomics. To this end, high quality NMR metabolomic spectra were obtained from chicken feather pulp extracted using either ultrafiltration (**UF**) or Bligh-Dyer methanol-chloroform (**BD**) methods. In total, 121 and 160 metabolites were identified using the UF and BD extraction methods, respectively, with 71 of these common to both methods. The metabolome of feather pulp differed in broiler breeders that were 1-, 23-, and 45-wk-of-age. Moreover, feather pulp was more difficult to obtain from older birds, indicating that age must be considered when targeting feather pulp as a source of biomarkers. The metabolomic profile of feather pulp obtained from 12-day-old broilers administered corticosterone differed from control birds, indicating that the metabolome of feather pulp was sensitive to induced physiological stress. A comparative examination of feather pulp and serum in broilers revealed that the feather pulp metabolome differed from that of serum but provided more information. The study findings show that metabolite biomarkers in chicken feather pulp may allow producers to effectively monitor stress, and to objectively develop and evaluate on-farm mitigations, including practices that reduce stress and enhance bird health.

Key words: proton nuclear magnetic resonance spectroscopy, metabolomics, broiler chicken, feather pulp, biomarker

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INTRODUCTION

Physiological stress is known to have a substantial impact on bird health (Dai et al., 2009; Scanes, 2016; Jastrebski et al., 2017; Chowdhury, 2019; Zaytsoff et al., 2019), but is also very difficult to quantify (Romero and Romero, 2002). Stress has been shown to predispose chickens to a variety of important diseases incited by pathogens, including necrotic enteritis (Zaytsoff et al., 2020; Zaytsoff et al., 2022), colibacillosis (Maryvonne Dho-Moulin, 1999; Rodriguez-Siek et al., 2005; Guabiraba-Brito and Schouler, 2015; Poulsen et al., 2017), and salmonellosis (Whiley and Ross, 2015; Antunes et al., 2016), due at least in part to a suppression of the immune system (Hangalapura et al., 2004; Zhao et al., 2014; Zaytsoff et al., 2020). As such, there is an increasing need to identify reliable and noninvasive biomarkers of stress (Isaksson et al., 2005; Bouwer et al., 2021). Ideally, a biomarker should be sensitive and provide an early indication of the stress status of birds before there are any outward signs of distress (Ferreira et al., 2010). An effective biomarker of stress would be beneficial to the chicken industry, as it would allow for early detection and the implementation of mitigation strategies before stress can negatively affect bird health and production.

When birds experience a stressor, it stimulates the hypothalamic-pituitary-adrenal (**HPA**) axis, which results in varying behavioral, physiological, and metabolic changes that help birds cope with the stressor (Breuner and Orchinik, 2002; Cockrem, 2007). In chickens, exposure to a stressor stimulates production of the glucocorticoid hormone, corticosterone (**CORT**)

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(Cockrem, 2007; Lattin et al., 2011), and CORT levels in blood are often used as a biomarker of stress (Harris et al., 2016; Harris et al., 2017). However, there are limitations associated with using CORT as a biomarker in blood (Beuving and Vonder, 1977; Etches, 1979; Rosner, 1990; Breuner and Orchinik, 2001; De Jong et al., 2001; Breuner and Orchinik, 2002; Romero and Reed, 2005; Romero et al., 2006; Mormède et al., 2007; Bortolotti et al., 2009; Scanes, 2016; Weimer et al., 2018). Some groups have attempted to identify alternative biomarkers of stress in broiler chickens, primarily targeting immune markers or mRNA transcripts measured in blood or tissues (Chen et al., 2015; Giannuzzi et al., 2021; Gou et al., 2021); however, they require destructive sampling, making them problematic for tracking stress within production settings. There is an imminent need for the discovery of biomarkers in novel substrates (e.g., tissue or biofluid) to effectively measure stress and subsequently monitor bird health on farm.

Several alternative substrates, such as feces and feathers, can be obtained with minimal to no handling of the birds, and these substrates provide additional possibilities for biomarker discovery (Matysik et al., 2016; Greene et al., 2019; Will et al., 2019). Although not focused on biomarker discovery, research has previously examined feather pulp for immune markers of infection (Erf and Ramachandran, 2016). In this regard, the immune response in pulp was found to be similar to that observed in other tissues, such as wattles and wing webs. The use of feces for stress biomarker discovery has also garnered some interest (Dehnhard et al., 2003; Rettenbacher et al., 2004; Rettenbacher et al., 2006; Alm et al., 2014); however, feces are the accumulated result of both microbial and host metabolism, which are altered within the digestive system. Thus, the relationship between stress and metabolites in feces is not straightforward nor fully understood (Mormède et al., 2007). Feathers are not subjected to the same metabolome altering processes as feces and they are often used for detecting pathogens that incite important diseases such as West Nile virus (Docherty, 2004), Marek's disease virus (Cortes, et al., 2011), and avian leukosis virus (Zavala et al., 2002). Ample research measuring CORT as a biomarker of stress in whole feathers has been conducted; however, interpretation of CORT concentrations in feathers can be difficult because the exact mechanism by which CORT is incorporated into feathers is currently unclear (Breuner and Orchinik, 2002; Romero et al., 2006; Jenni−Eiermann et al., 2015; Harris et al., 2017), and CORT concentrations within different feathers from the same individual vary (Bortolotti et al., 2009; Harris et al., 2016). Moreover, the relationship between feather CORT and blood CORT concentrations is not straightforward (Bortolotti et al., 2008; Lattin et al., 2011; Koren et al., 2012; Fairhurst et al., 2013; Hõrak et al., 2013; Berk et al., 2016; Weimer et al., 2018), and the relationship between feather CORT and stress is also nebulous (Romero et al., 2005; Desrochers et al., 2009; Lattin et al., 2011; Hõrak et al., 2013 James et al., 2018; Arrazola and Torrey, 2019).

Metabolomics is the study of endogenous small molecular weight compounds, or metabolites, present in a biological substance, and allows for the analysis of all the metabolic responses of living systems (Karu et al., 2018) to external stimuli, such as stressors. ^{1}H-nuclear magnetic resonance (**NMR**) spectroscopy-based metabolomics is used to characterize the metabolome of various biological tissues and fluids. NMR spectroscopy is a nondestructive and highly reproducible technique (Markley et al., 2017; Nicholson et al., 1999; Wishart, 2008). Recent research has explored the use of NMR to elucidate the metabolomes of different chicken samples including eggs, breast muscle, liver, kidney, and feces for model development (Le Roy et al., 2016), as well as to identify biomarkers of health (Abasht et al., 2016; Beauclercq et al., 2018; Zaytsoff et al., 2019; Brown et al., 2021; Inglis et al., 2021). However, limited research to date has focused on identifying novel biomarkers of stress that are correlated with bird health, and to our knowledge, none have examined metabolomic biomarkers in feather pulp. Given the high degree of vascularization in feather pulp, we hypothesize that the metabolome of the feather pulp will be comparable to the metabolome of blood, and that metabolites will be altered by a physiological stressor, as well as by other biological factors, such as age. To test these hypotheses the following objectives were established: 1) determine if NMR spectra can be produced from chicken feather pulp, and the best metabolite extraction method for NMR-based metabolomic studies; 2) ascertain if the feather pulp metabolome is affected by bird age; 3) determine if the feather pulp metabolome differs in birds in which stress is induced; and 4) compare the feather pulp and blood metabolomes.

MATERIALS AND METHODS

Ethics Statement

The studies involving broilers were conducted in the Lethbridge Research and Development Centre (**LeRDC**) small animal facility, and were carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the LeRDC Animal Care Committee (Animal Use Protocol Review #2010) before commencement of the research. For analysis of the metabolome of feather pulp obtained from broiler breeder chickens of different ages, birds were euthanized on farm by a producer following industry standards for animal euthanization; researchers had no contact with living broiler breeder chickens.

Broiler Breeder Birds for Age Analysis

Mixed sex Ross 308FF broiler breeder chickens (n = 14) were obtained from a broiler breeder farm located near Lethbridge, AB. Chickens examined were 1-wk-old (n = 6), 23-wk-old (n = 4), and 45-wk-old (n = 4).

Stress Induction in Broiler Birds

The study was designed as a factorial experiment with two levels of stress arranged as a completely randomized design. The two stress treatments were: 1) control birds provided a basal diet (n = 6); and 2) birds provided the basal diet containing 15 mg kg^{-1} CORT to induce physiological stress (n = 6), a model of physiological stress commonly used in broiler chickens (Post et al., 2003; Virden et al., 2007; Shini et al., 2009). Each bird was treated as a replicate, and the replicates were conducted on three separate occasions (i.e., runs) to ensure independence (2 replicate birds per run).

Mixed sex Ross 308FF broiler chicks (n = 12) were obtained from a local hatchery on the day they hatched (d 0). The chicks originated from a single broiler breeder farm from adults of known health status. Upon arrival at the LeRDC small animal facility, chicks were randomly assigned to the control (n = 6) or CORT (n = 6) treatment, and placed in individually ventilated cages (**IVC**) (Techniplast, Montreal, QC, Canada) on autoclaved wood shavings (United Farmers of Alberta Co-operative Ltd., Lethbridge, AB, Canada) at a stocking density of 2 birds per IVC. Birds had ad libitum access to a non-medicated starter diet (Hi-Pro Feeds, Lethbridge, AB, Canada) and water. Birds were maintained on a 12:12 h light:dark cycle. The ambient temperature in the animal room was 30°C for the first 2 d, 28°C for the next 2 d, and 26°C thereafter. On d 5, chickens assigned to the CORT treatment were provided feed containing CORT and were maintained on the CORT-amended diet until the end of the experiment (d 12).

Sample Collection

Broiler Breeders Animals from the three age groups were obtained from a commercial broiler breeder farm located near Lethbridge, AB. Animals were euthanized by the producer following industry standards, and immediately after euthanization, 5 to 10 of the largest feathers from the right wing of each bird were removed; the number of feathers collected per bird depended on its size, with fewer feathers taken from larger birds. A composite feather pulp sample from each bird was obtained by squeezing the calamus of the feathers into a 1.5 mL centrifuge tube (Zavala et al., 2002), and the collected feather pulp was mixed to ensure uniformity. Within min of collection, feather pulp samples were snap frozen in liquid nitrogen, transported to the laboratory on dry ice, and stored at −80°C until processed.

Broilers Within the LeRDC small animal facility, birds were anesthetized with isoflurane (5% isoflurane; 1 L O_2 min^{-1}) and blood was collected via intracardiac puncture into serum separator tubes (BD, Mississauga, ON). Under anesthesia, birds were euthanized by cervical dislocation and feather pulp samples were collected, mixed, frozen, and stored as described above.

Processing of Samples Obtained From Broiler Breeders of Different Ages

In preparation for metabolomic analysis, feather pulp samples were thawed on ice, and samples were divided into 2 aliquots of approximately 150 mg each. One aliquot was used for ultrafiltration (**UF**) and the other for Bligh-Dyer (**BD**) methanol-chloroform extraction allowing for direct comparison of identical metabolomes via the 2 methods.

Ultrafiltration Amicon Ultra 0.5 mL centrifugal filters with a molecular weight cut-off of 3 kDa (MilliporeSigma, Oakville, ON, Canada) were used. Each filter was washed by adding 500 μL of Millipore water (MilliporeSigma) to the filter and centrifuging at 14,000 × g for 5 min. This washing step was repeated 10 times in order ensure that all glycerol in the filter had been removed (de Graaf and Behar, 2003; Tiziani et al., 2008). Metabolomics buffer (0.125 M KH_2PO_4, 0.5 M K_2HPO_4, 0.00375 M NaN_3, and 0.375 M KF; pH 7.4) was added to the feather pulp samples at a 2:1 volume to mass ratio, and samples were homogenized using a Bullet Blender tissue homogenizer (Next Advance, Troy, NY) with 150 mg of 2-mm-diameter zirconium oxide beads (Next Advance) on setting 8 for 5 min. Samples were then centrifuged at 14,000 × g for 5 min, and 365 μL of the supernatant was added to a pre-washed filter along with 135 μL of metabolomics buffer. The filters were then centrifuged at 14,000 × g for 30 min at 4°C, and the filtrate was collected.

Bligh-Dyer Methanol-Chloroform Extraction A 275 μL subsample of the homogenized feather pulp was combined with 687.5 μL of methanol and 343.8 μL of chloroform in a 2 mL tube. The sample was vortexed, placed at −20°C for 15 min to precipitate proteins, and then centrifuged at 15,300 × g for 15 min at 4°C. The supernatant was decanted into a new tube containing equal volumes (343.8 μL) of chloroform and deionized water. This mixture was vortexed and then centrifuged at 6,700 × g for 5 min at 4°C. Following centrifugation, 1 mL of the top layer containing the water-soluble metabolites was pipetted into a 2 mL tube, and placed in a nitrogen gas flow box for 5 to 6 d to dry. Once dry, samples were rehydrated in 360 μL of Millipore water (MilliporeSigma).

Processing of Samples Obtained From Broiler Chickens

To obtain serum, blood in separator tubes (BD) was left at room temperature for 1 h, centrifuged at 1,000 × g for 2 min, and the serum decanted and stored at −80°C. Feather pulp and serum samples was processed via UF as described above, with the exception that the serum was not homogenized, and 150 μL of non-

homogenized serum sample was added directly to a pre-washed filter along with 350 μL of metabolomics buffer.

Sample Preparation for NMR Spectroscopy

A 140 μL aliquot of deuterium oxide containing 0.05% v/v trimethylsilylpropanoic acid (**TSP**) and 200 μL of metabolomics buffer was added to 360 μL of sample extracted using the BD method and/or by UF (total volume of 700 μL); the TSP was used as a chemical shift reference for NMR spectroscopy. The solution was vortexed at high setting, and then centrifuged at 12,000 × g for 5 min at 4°C to pellet any remaining particulate matter. A 550 μL aliquot of the supernatant was placed into a 5 mm NMR tube, and each sample was run on a 700 MHz Bruker Avance III HD spectrometer (Bruker, Milton, ON) for spectral data collection.

NMR Data Acquisition and Processing

Spectra were obtained using the Bruker 1-D NOESY gradient water suppression pulse sequence 'noesygppr1d' with 10 ms mixing time. Each sample was run for 512 scans to a total acquisition size of 128 k, a spectral window of 20.5 ppm, a transmitter offset of 4.7 ppm, and a recycle delay of 4 s. All measurements were recorded using a Bruker triple resonance TBO-Z probe at room temperature. The above acquisition parameters correspond to an approximate data collection time of 1 h per sample. The Bruker automation program "pulsecal" was used on each sample before data acquisition to guarantee that the 90-degree pulse was calibrated correctly, ensuring quantitative and comparable data across samples (Paxman et al., 2018). The spectra were zero filled to 256 k, automatically phased, baseline corrected, and line-broadened by 0.3 Hz (Kiss et al., 2016). Spectra were then exported to MATLAB (MathWorks, Natick, MA) as ASCII files where they underwent Dynamic Adaptive Binning (Anderson et al., 2011), followed by manual inspection and correction. The dataset was then normalized to the total metabolome, excluding the region containing the water peak, and pareto scaled and log-transformed.

Statistical Analysis

Metabolomics analysis was performed using MATLAB (MathWorks) and the MetaboanalystR R package (Chong and Xia, 2018). Spectral bins were subjected to both univariate and multivariate analysis to determine which bins were significantly altered among independent variables (e.g., bird age, stress vs. no stress, and feather pulp vs. serum). All bins were determined to be non-parametric using a Shapiro-Wilk test, and subsequently tested for significance ($P \leq 0.050$) using a univariate Mann-Whitney (MW) U test. MATLAB was also used to calculate the percent difference of the bins. MetaboanalystR was used to carry out the principal component analysis (**PCA**) and orthogonal partial least squares discriminant analysis (**OPLS-DA**). Metabolites were identified using the Chenomx 8.2 NMR Suite (Chenomx Inc., Edmonton, AB).

RESULTS AND DISCUSSION

NMR Spectra Were Obtained From Feather Pulp

A large number of metabolites were observed in feather pulp following extraction by both the UF and BD methods (Figure 1). In total, 210 metabolites were identified in the NMR spectra. For samples extracted using the UF and BD methods, 121 and 160 metabolites were identified, respectively, with 71 metabolites common to both methods (Supplementary Table S1, Table S2). In total, 50 and 89 metabolites were unique to the

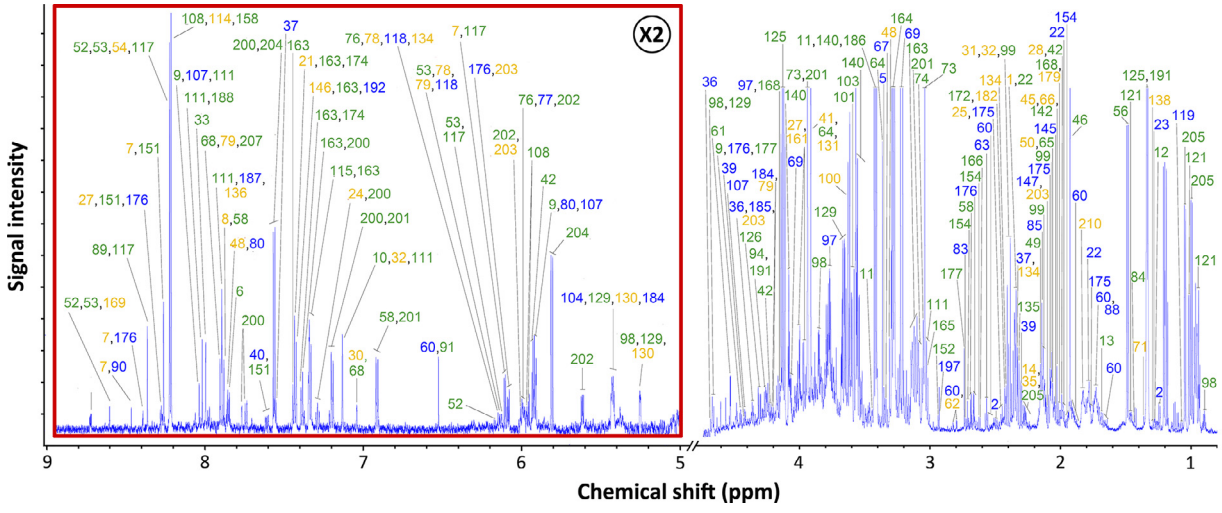

Figure 1. Nuclear magnetic resonance spectroscopy spectra showing average water-soluble metabolites in chicken feather pulp. The numbering in the figure corresponds to metabolites in Tables S1 and S2. Metabolite numbers presented in blue and yellow were only identified using ultrafiltration and Bligh-Dyer extraction, respectively. Metabolite numbers presented in green were found using both extraction methods. The spectrum has been split at the water peak and the vertical scale of the spectra plot indicated as "2X" has been increased to better illustrate the metabolites that are present.

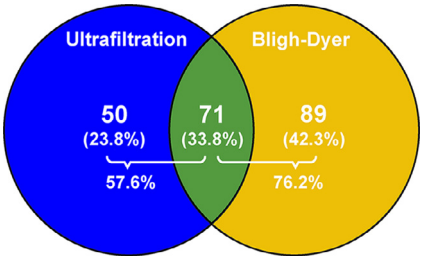

Figure 2. Venn diagram showing the numbers of identified metabolites unique and in common to the ultrafiltration and Bligh-Dyer methanol-chloroform extraction methods.

UF and BD methods, respectively (Figure 2). In contrast, the human serum metabolome was able to quantify 49 metabolites by NMR (Psychogios et al., 2011), and only 37 metabolites were previously detected in chicken plasma by NMR (Le Roy et al., 2016). This indicates that feather pulp has a greater potential for biomarker discovery, when compared to blood, for the poultry sector. Having more metabolites observed in feather pulp extracted using the BD method relative to samples extracted using UF suggests that the BD method may be better suited for untargeted metabolomics studies of feather pulp. Relative to UF, the spectra of feather pulp extracted using the BD method contained a larger number of metabolites involved in the citric acid cycle, such as succinate, cis-aconitate, oxaloacetate, and citrate. In addition, more of the metabolites identified with the BD method are involved in the beta-Alanine (L-aspartate, carnosine, beta-alanyl-N(pi)-methyl-L-histidine, uracil, and L-histidine), phenylalanine (L-tyrosine, 2-hydroxyphenylacetate, and L-phenylalanine), and pyruvate (oxaloacetate, pyruvate, (S)-lactate, acetate, and fumarate) metabolism pathways. Conversely, more metabolites involved in the arginine and proline metabolism (guanidinoacetate, creatine, agmatine, hydroxyproline, L-proline, L-glutamate, phosphocreatine, and homocarnosine), the ascorbate and aldarate metabolism (ascorbate, myo-inositol, and UDP-glucose), and the galactose metabolism (sucrose, UDP-glucose, glycerol, D-sorbitol, and myo-inositol) pathways were observed in the spectra of samples extracted by UF. Although the BD extraction method resulted in the detection of the most metabolites from feather pulp, the value of the metabolites as biomarkers is far more important than metabolite number, particularly as a relatively large number of metabolites were detected for both extraction methods. For targeted metabolomic analyses, care should be taken to make sure the extraction method used is best suited for the metabolites and pathways of interest.

Citric acid often experiences selective signal loss when processed using UF (de Graaf and Behar, 2003), which is consistent with the findings of the current study in that citric acid was only identified in BD-processed samples. Isopropanol, serine, and choline were previously found to be present at higher concentrations in human blood processed using UF as compared to samples processed using BD (McHugh et al., 2018), which is consistent with our findings of feather pulp. Other NMR studies have shown that UF removes methionine (Daykin et al., 2002), acetate (Snytnikova et al., 2019), and leucine (Tiziani et al., 2008; Snytnikova et al., 2019) from blood samples, whereas BD does not. Methionine, acetate, and leucine were not identified by either UF or BD in feather pulp in the current study, and other studies have observed that UF does not preferentially retain acetate, alanine, lactate, or valine (Kriat et al., 1992). Despite some inconsistency within the literature on the relative metabolite extraction merits of the UF and BD methods, UF is often recommended for the extraction of metabolites from many types of samples (Tiziani et al., 2008; Psychogios et al., 2011) as it provides a good signal-to-noise ratio, exhibits excellent reproducibility without undesirable evaporation of volatile metabolites, and has minimal loss of metabolites due to dissolution in various solvents, or due to hydrolysis caused by the need to neutralize the solution (Tiziani et al., 2008). From a logistics perspective, UF is the quickest of the 2 extraction methods that we evaluated, with the least amount of manipulation of the sample. A downside of UF is the cost, as the filters can be prohibitively expensive (≈$5 USD/filter). There is also the possibility that the filters can break or become clogged, which would require re-prepping, or possibly, total loss of the sample. Another issue that can arise with UF is incomplete filtration, where proteins filtered out of the sample become concentrated on the membrane surface and inside the pores due to adsorption, and block other metabolites from passing through the filter (Singh, 2015). Also, during filtration, impermeable macromolecules accumulate on the surface of the membrane, and this can cause both concentration polarization and membrane fouling, which prevents metabolites that are smaller than the molecular weight cut-off from passing through the filter (Singh, 2015).

One potential limitation of the BD extraction method is the occurrence of baseline distortions due to the incomplete removal of macromolecules in samples that are high in proteins, fats, and/or fiber such as feces (Brown et al., 2022). We did not observe substantive baseline distortions for feather pulp samples extracted with the BD method. The BD method can remove some fat and protein in the sample (Bligh and Dyer, 1959; Wu et al., 2008), and the insignificant baseline distortions that were observed in the current study suggests that the method was able to sufficiently remove problematic macromolecules that were present in feather pulp. The effectiveness of the BD method for extracting feather pulp metabolites from different feather types and feathers from different species remains to be determined. Moreover, as the BD method requires more manipulation of the sample than UF, it can result in a decrease in reproducibility and the possible loss of volatile metabolites during the drying step, as well as losses due to dissolution in methanol and chloroform (Tiziani et al., 2008). Salient advantages of the BD method are the generation of fractions for analysis of both water-soluble and water-insoluble metabolites (Lin et al., 2007), it is much less expensive than UF, and the BD method extracted a larger number of metabolites

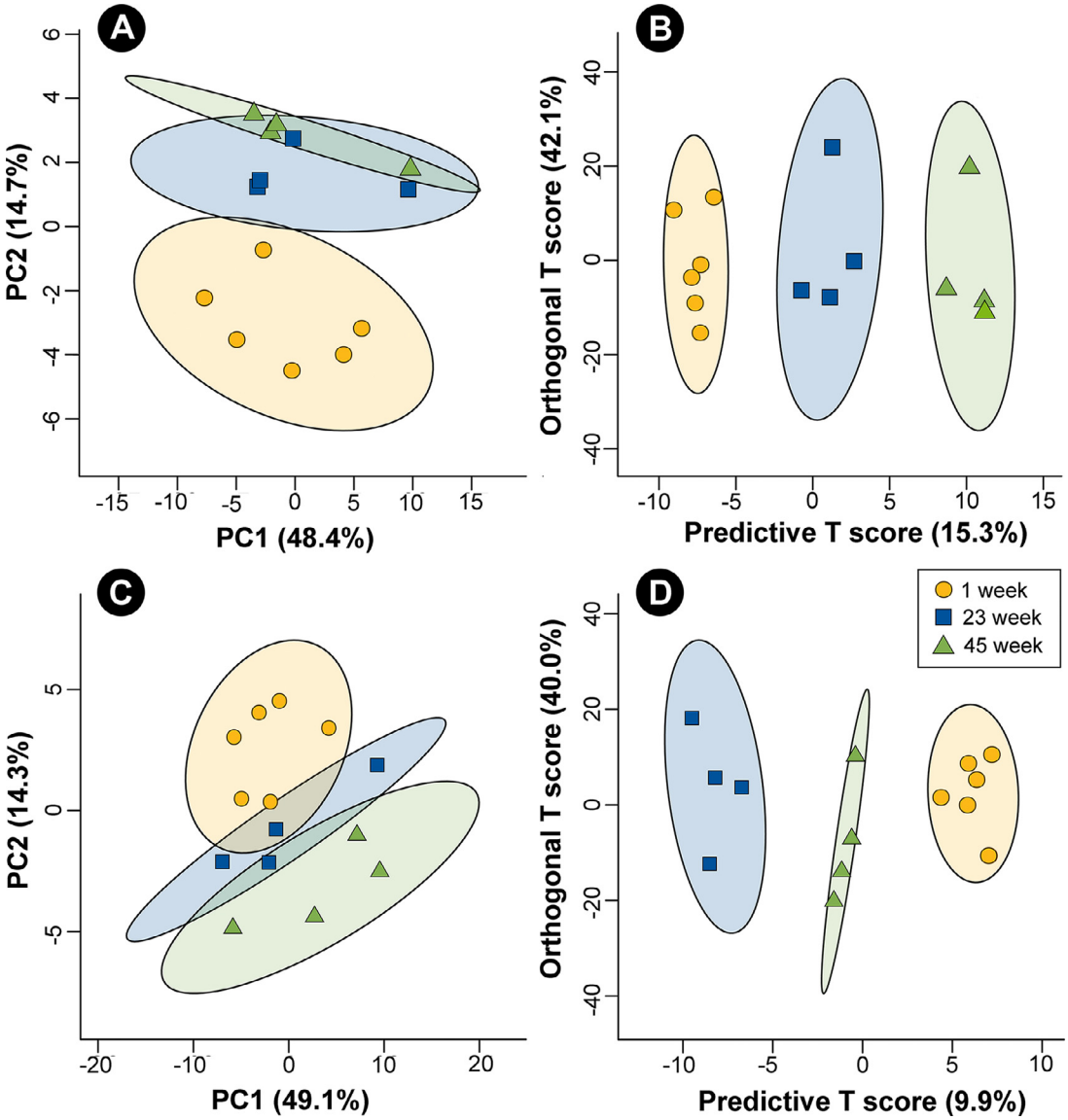

Figure 3. Principal component analysis (PCA) score plots of feather pulp from 1-wk-old, 23-wk-old, and 45-wk-old birds processed using the: (A) ultrafiltration; and (C) Bligh-Dyer methods. The x- and y-axes in the PCAs show principal components one and two, respectively, with the number in brackets indicating the percent variance explained by each component. Orthogonal partial least squares determinant analysis (OPLS-DA) score plots of feather pulp from 1-wk-old, 23-wk-old, and 45-wk-old birds processed using: (B) ultrafiltration ($Q^2 = 0.804$, $P < 0.001$, $R^2 = 0.982$, $P < 0.001$); and (D) Bligh-Dyer ($Q^2 = 0.673$, $P < 0.050$, $R^2 = 0.980$, $P = 0.342$). The x- and y-axis in the OPLS-DA analyses represent the predictive (between group separation) and orthogonal (within group variation) components of the data, respectively. Each marker represents one bird, and the shaded ellipses represent the 95% confidence intervals for each group. All metabolite bins were plotted.

from feather pulp than UF. For targeted metabolomic analyses, the variability caused by the chosen extraction method should be considered to ensure that the targeted metabolites are not adversely affected by the method. As the effectiveness of extraction methods may be differently affected by the substrate extracted, comparative validation of methods should be conducted in advance.

Bird Age Influences Metabolite Profiles in Feather Pulp

To ascertain the impact of age on the chicken feather pulp metabolome, broiler breeder chickens at 3 ages were compared (i.e., 1-, 23-, and 45-wk-of-age). The PCA score plots from both the UF and BD methods show unsupervised separation of metabolite bins among the 3 age groups, with some confidence interval overlap observed for the BD method (Figures 3A and 3C). For samples extracted using UF, OPLS-DA analysis provided a good model fit for the 3 age groups ($Q^2 = 0.804$, $P < 0.001$, $R^2 = 0.982$, $P < 0.001$), and supervised group separation of metabolite bins among them (Figure 3B). For samples extracted using the BD method, OPLS-DA analysis also showed supervised group separation, but a poorer model fit among the 3 age groups ($Q^2 = 0.673$, $P < 0.050$, $R^2 = 0.980$, $P = 0.342$; Figure 3D) relative to the UF method. Thus, the BD method delivered better unsupervised results, whereas the UF method provided better supervised results. For metabolomics studies, unsupervised separation is preferred as it is not prone to model overfitting (Szymanska et al., 2012), further suggesting that the BD method may be better suited for untargeted metabolomic studies of feather pulp.

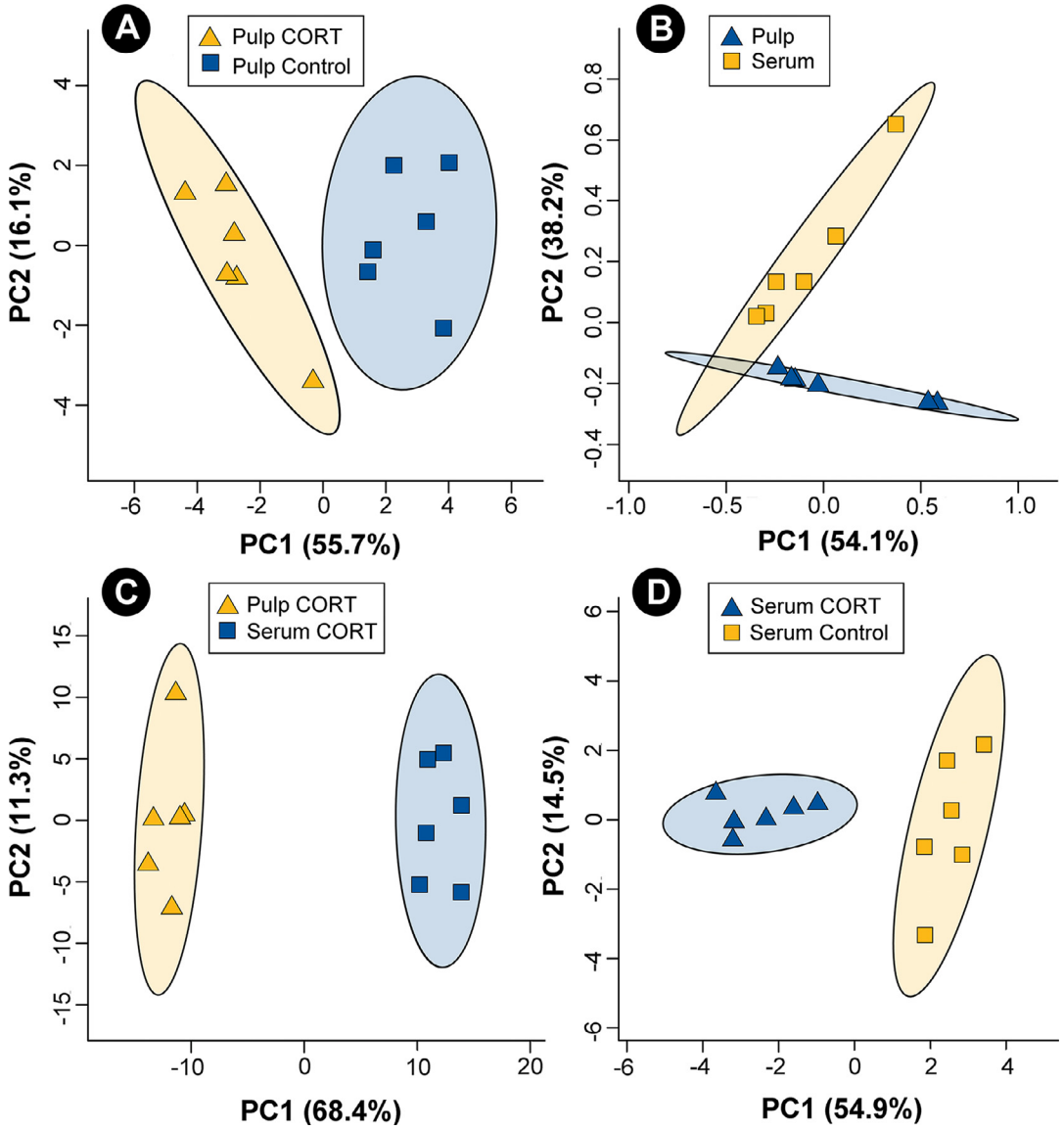

Figure 4. Principal component analysis score plots for the following comparisons: (A) feather pulp after 8 d of corticosterone (CORT) administration vs. control feather pulp; (B) control feather pulp vs. control serum; (C) feather pulp with CORT vs. serum with CORT; and (D) serum with CORT vs. control serum. Each marker represents one bird, and the shaded ellipses represent the 95% confidence interval for each group. The x- and y-axes show principal components one and two, respectively, with the number in brackets indicating the percent variance explained by each component. Bins that were determined to be significant ($P \leq 0.050$) via univariate Mann-Whitney U test were plotted in A and D, whereas all bins were plotted in B and C.

Regardless of extraction method used, the different metabolomes in feather pulp obtained from broiler breeders of different ages indicates that the feather pulp metabolome is highly sensitive to biological factors.

The Feather Pulp Metabolome is Altered in Birds Administered Corticosterone

To explore the utility of using feather pulp as a substrate for measuring the impacts of physiological stress on the birds, CORT was administered to broiler chickens in feed at a dose of 15 mg of CORT per kg of feed. Previous research has shown that CORT administered to chickens in water or feed induces a defined physiological stress response (Post et al., 2003; Zaytsoff et al., 2019; Zaytsoff et al., 2020), including conspicuous impacts on the metabolome of the liver, kidney, and breast muscle of chickens (Zaytsoff et al., 2019; Brown et al., 2021). We observed that the feather pulp metabolome was altered in 12-day-old birds that were administered CORT relative to unstressed control birds, as indicated by the unsupervised separation observed in the PCA score plot (Figure 4A).

Several metabolites were altered in feather pulp due to CORT administration as compared to the control treatment (Table 1). Interestingly, CORT was quantifiable by NMR in the feather pulp of birds that were administered the glucocorticoid, but was not detected in serum. In addition, many of the identified amino acids, including tyrosine, threonine, glycine, and phenylalanine, were down regulated in the feather pulp of birds administered CORT relative to the control treatment (Table 1). Previous work has shown a reduction in feather growth rates (Patterson et al., 2015; Berk et al., 2016) and feather quality (Desrochers et al., 2009; Lattin et al.,

Table 1. Metabolite concentrations that were altered ($P \leq 0.041$) in the feather pulp of birds administered corticosterone (CORT) for 8 d relative to control treatment birds not administered CORT.

Mann-Whitney *P*-value	Metabolite	Difference (%)
0.002	L-Tyrosine	−63.0
0.008	Threonine	−23.8
0.009	2-Hydroxy-2-methylbutyric acid	−35.7
0.009	Ethanol	88.9
0.015	Creatine	43.9
0.015	Glycine	−25.4
0.015	L-Cysteine	15.1
0.015	L-Phenylalanine	−33.5
0.019	Glutamate	20.1
0.023	Ribose	−11.8
0.026	1,3,7 - Trimethyluric acid	25.9
0.026	Corticosterone	95.8
0.026	L-Homoserine	−16.2
0.026	N1-Acetylspermine	27.9
0.041	1,9- Dimethyluric acid	19.1
0.041	3,7-Dimethyluric acid	19.0
0.041	D-Lysine	18.9
0.041	Myoinositol	−9.2
0.041	N-Methyl-D-aspartic acid	−22.1

Significance was determined using an unsupervised univariate Mann-Whitney U test.

A positive and negative percent difference indicates an increase and decrease in birds administered CORT, respectively.

2011; Arrazola and Torrey, 2019) due to stress. The primary protein in feathers is β-keratin (Stettenheim, 2000; Sharma et al., 2017), which comprises ≈80% of the crude protein of feathers (Tesfaye et al., 2017b). Thus, the decrease in amino acids that we observed in feather pulp from birds administered CORT and experiencing physiological stress could be an important contributor to a reduction in feather growth and quality. Cysteine, a precursor to glutathione, was upregulated in feather pulp of birds administered CORT as compared to the control treatment. Glutathione is a well-known antioxidant that protects cells from oxidative stress (Lu, 2013; Alanazi et al., 2015), and it is possible that the observed increase in cysteine levels observed in CORT treatment birds was caused by increased reactive oxidative species that are commonly associated with a stress response (Lin et al., 2004). β-keratin is high in cysteine residues, which form disulfide bridges providing strength and rigidity (Tesfaye et al., 2017a). It is thus plausible that the higher concentration of free cysteine that was observed in CORT-treated birds is due to reduced incorporation of this amino acid into keratin, thereby adversely affecting feather growth and quality. Future studies investigating the use of feather pulp as a biomarker of stress and bird health should consider metabolites and metabolomic pathways related to feather biosynthesis.

The Feather Pulp Metabolome is Not Reflective of the Serum Metabolome

The metabolome of feather pulp of 12-day-old broiler chickens was examined relative to the serum metabolome to ascertain if feather pulp is a good surrogate for serum. Despite being highly vascularized during growth (Bortolotti et al., 2009), unsupervised analyses indicated that the metabolome of feather pulp and serum obtained from control birds differed (Figure 4B). This is contrary to the study hypothesis that the feather pulp metabolome is reflective of the serum metabolome. To examine this further, the metabolome of feather pulp was compared to the metabolome of serum of birds administered CORT. A distinct unsupervised separation between the metabolomes of these two treatments was observed (Figure 4C). Moreover, the total percentage variance explained by Principal Component 1 (**PC1**) increased between feather pulp and serum of birds administered CORT. Importantly, the relative scale of the variance along PC1 was increased by almost 20-fold as compared to the variation between control pulp and serum of birds not administered CORT, indicating that instead of making the pulp and serum metabolomes more similar, the administration of CORT made them more dissimilar.

To further examine the effects of stress, the serum metabolome after CORT treatment was compared to control treatment broilers, and the observed differences were then compared with those in feather pulp. A distinct unsupervised separation between the control and CORT treatment serum metabolomes was observed (Figure 4D). In serum, all of the identified amino acids were downregulated except for proline (Table 2). Three of these, tyrosine, threonine, and phenylalanine, were also altered in feather pulp due to CORT administration (Table 1). It has previously been shown that under conditions of stress, collagen is broken down to make more proline available for downstream cell cycle arrest, autophagy, and apoptosis (Phang et al., 2010), which have been shown to increase under some stress conditions (Arimoto et al., 2008). Proline is also a main substrate required to produce glutamate, which is also involved in the body's response to oxidative stress (Dryer, 2015; Shen et al., 2019). We observed that

Table 2. Metabolite concentrations that were altered ($P \leq 0.027$) in the serum of birds administered corticosterone (CORT) for 8 d relative to control treatment birds not administered CORT.

Mann-Whitney *P*-value	Metabolite	Difference (%)
0.002	Mannose	17.9
0.004	Citramalic acid	−37.5
0.009	D-Glucose	−23.0
0.010	Traumatic acid	26.8
0.015	4-Aminobutyrate	39.3
0.015	4-Hydroxyproline	−22.8
0.015	D-Alanine	−41.3
0.015	L-Phenylalanine	−27.9
0.018	DL-2-Aminooctanoic acid	21.7
0.020	L-Threonine	−30.7
0.020	L-Tyrosine	−61.5
0.023	2-Hydroxy-2-methylbutyric acid	18.8
0.026	Malonic acid	−56.9
0.026	Proline	18.8
0.027	Citric acid	−21.8

Significance was determined using an unsupervised univariate Mann-Whitney U test.

A positive and negative percent difference indicates an increase and decrease in birds administered CORT, respectively.

hydroxyproline was altered in serum of birds administered CORT as compared to the control treatment, and this metabolite is involved in the synthesis of glycine, which is an essential amino acid for chickens (Wu et al., 2010). Glycine is also involved in redox regulation (Phang et al., 2010).

Although feather pulp was not a good surrogate for serum, the high number of metabolites detected in feather pulp, coupled with superior sampling logistics and the clear separation observed between the control and CORT treatments, indicates that feather pulp may be a good, and possibly superior, alternative to serum for biomarker discovery.

CONCLUSIONS

The metabolomics research that has been conducted in chickens to date is often focused on the quality of the chicken meat produced (Zhang et al., 2009; Beauclercq et al., 2016; Lytou et al., 2018; Aggrey et al., 2019; Xiao et al., 2019), and the discovery of biomarkers that are predictive of bird health is in its infancy. We observed that NMR-based metabolomics was able to identify a large number of metabolites in feather pulp. Moreover, we showed that chicken feather pulp is an easily obtained sample that is sensitive to biological and experimental variations. Two different extraction methods were used, and both the UF and BD extraction methods produced excellent spectra with both overlapping and unique metabolites. The choice of extraction method for feather pulp will depend on the goals of the research. In this regard, if a larger number of metabolites are desired, such as the case in an untargeted analysis, the BD extraction method may be the most appropriate method. In the case of targeted analysis, the extraction method must be carefully chosen based on the biochemical pathways of interest. We hypothesized that the feather pulp metabolome would be very similar to the serum metabolome, as feather pulp is highly vascularized during growth. Contrary to our hypothesis, the feather pulp metabolome differed significantly from the serum metabolome, and this difference was maintained in birds administered CORT as a physiological stressor. Although feather pulp was not a good proxy for the metabolome of serum, more metabolites were detected in feather pulp relative to serum. Furthermore, the metabolome of feather pulp differed across both broiler age and in birds experiencing physiological stress. The ease of feather pulp collection coupled with its sensitivity to biological factors suggests that feather pulp may be a good target to ascertain the impacts of factors important for monitoring and optimizing bird health, including within production settings.

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DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

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REFERENCES

Abasht, B., M. F. Mutryn, R. D. Michalek, and W. R. Lee. 2016. Oxidative stress and metabolic perturbations in wooden breast disorder in chickens. PLoS One 11:e0153750.

Aggrey, S. E., M. C. Milfort, A. L. Fuller, J. Yuan, and R. Rekaya. 2019. Effect of host genotype and *Eimeria acervulina* infection on the metabolome of meat-type chickens. PLoS One 14: e0223417.

Alanazi, A. M., G. A. E. Mostafa, and A. A. Al-Badr. 2015. Glutathione. Pages 43−158 in Profiles of Drug Substances, Excipients, and Related Methodology. Academic Press, Waltham, MA.

Alm, M., L. Holm, R. Tauson, and H. Wall. 2014. Corticosterone metabolites in laying hen droppings − effects of fiber enrichment, genotype, and daily variations. Poult. Sci. 93:2615–2621.

Anderson, P. E., D. A. Mahle, T. E. Doom, N. V. Reo, N. J. Delraso, and M. L. Raymer. 2011. Dynamic adaptive binning: an improved quantification technique for NMR spectroscopic data. Metabolomics 7:179–190.

Antunes, P., J. Mourão, J. Campos, and L. Peixe. 2016. Salmonellosis: the role of poultry meat. Clin. Microbiol. Infect. 22:110–121.

Arimoto, K., H. Fukuda, S. Imajoh-Ohmi, H. Saito, and M. Takekawa. 2008. Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. Nat. Cell Biol. 10:1324–1332.

Arrazola, A., and S. Torrey. 2019. The development of fault bars in domestic chickens (*Gallus gallus domesticus*) increases with acute stressors and individual propensity: implications for animal welfare. Anim. Welfare 28:279–286.

Beauclercq, S., L. Nadal-Desbarats, C. Hennequet-Antier, A. Collin, S. Tesseraud, M. Bourin, E. Le Bihan-Duval, and C. Berri. 2016. Serum and muscle metabolomics for the prediction of ultimate pH, a key factor for chicken-meat quality. J. Proteome Res. 15:1168–1178.

Beauclercq, S., L. Nadal-Desbarats, C. Hennequet-Antier, I. Gabriel, S. Tesseraud, F. Calenge, E. Le Bihan-Duval, and

S. Mignon-Grasteau. 2018. Relationships between digestive efficiency and metabolomic profiles of serum and intestinal contents in chickens. Sci. Rep. 8:6678–6689.
Berk, S. A., J. R. Mcgettrick, W. K. Hansen, and C. W. Breuner. 2016. Methodological considerations for measuring glucocorticoid metabolites in feathers. Conserv. Physiol. 4:1–11.
Beuving, G., and G. M. Vonder. 1977. Daily rhythm of corticosterone in laying hens and the influence of egg laying. J. Reprod. Fertil. 51:169–173.
Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917.
Bortolotti, G. R., T. Marchant, J. Blas, and S. Cabezas. 2009. Tracking stress: localization, deposition and stability of corticosterone in feathers. J. Exp. Biol. 212:1477–1482.
Bortolotti, G. R., T. A. Marchant, J. Blas, and T. German. 2008. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. Funct. Ecol. 22:494–500.
Bouwer, M., C. A. Ngcamphalala, A. Ganswindt, and A. E. Mckechnie. 2021. Validation of a non-invasive technique for quantifying a stress-associated biomarker in a southern African hornbill. J. Ornithol. 162:615–619.
Breuner, C. W., and M. Orchinik. 2001. Seasonal regulation of membrane and intracellular corticosteroid receptors in the house sparrow brain. J. Neuroendocrinol. 13:412–420.
Breuner, C. W., and M. Orchinik. 2002. Plasma binding proteins as mediators of corticosteroid action in vertebrates. J. Endocrinol. 175:99–112.
Brown, C. L. J., H. Scott, C. Mulik, A. S. Freund, M. P. Opyr, G. A. S. Metz, G. D. Inglis, and T. Montina. 2022. Fecal ^{1}H-NMR metabolomics: a comparison of sample preparation methods for NMR and novel in silico baseline correction. Metabolites 12:148.
Brown, C. L. J., S. J. M. Zaytsoff, T. Montina, and G. D. Inglis. 2021. Corticosterone-mediated physiological stress alters liver, kidney, and breast muscle metabolomic profiles in chickens. Animals 11:3056–3073.
Chen, J., G. Tellez, J. D. Richards, and J. Escobar. 2015. Identification of potential biomarkers for gut barrier failure in broiler chickens. Front. Vet. Sci. 2:1–10.
Chong, J., and J. Xia. 2018. MetaboAnalystR: an R package for flexible and reproducible analysis of metabolomics data. Bioinformatics 34:4313–4314.
Chowdhury, V. S. 2019. Heat stress biomarker amino acids and neuropeptide afford thermotolerance in chicks. J. Poult. Sci. 56:1–11.
Cockrem, J. F. 2007. Stress, corticosterone responses and avian personalities. J. Ornithol. 148:169–178.
Cortes, A. L., E. R. Montiel, S. Lemiere, and I. M. Gimeno. 2011. Comparison of blood and feather pulp samples for the diagnosis of Marek's disease and for monitoring Marek's disease vaccination by real time-PCR. Avian Dis 55:302–310.
Dai, S. F., L. K. Wang, A. Y. Wen, L. X. Wang, and G. M. Jin. 2009. Dietary glutamine supplementation improves growth performance, meat quality and colour stability of broilers under heat stress. Br. Poult. Sci. 50:333–340.
Daykin, C. A., P. J. D. Foxall, S. C. Connor, J. C. Lindon, and J. K. Nicholson. 2002. The comparison of plasma deproteinization methods for the detection of low-molecular-weight metabolites by ^{1}H nuclear magnetic resonance spectroscopy. Anal. Biochem. 304:220–230.
de Graaf, R. A., and K. L. Behar. 2003. Quantitative ^{1}H NMR spectroscopy of blood plasma metabolites. Anal. Chem 75:2100–2104.
Dehnhard, M., A. Schreer, O. Krone, K. Jewgenow, M. Krause, and R. Grossmann. 2003. Measurement of plasma corticosterone and fecal glucocorticoid metabolites in the chicken (*Gallus domesticus*), the great cormorant (*Phalacrocorax carbo*), and the goshawk (*Accipiter gentilis*). Gen. Comp. Endocrinol. 131:345–352.
De Jong, I. C., A. S. Van Voorst, J. H. F. Erkens, D. A. Ehlhardt, and H. J. Blokhuis. 2001. Determination of the circadian rhythm in plasma corticosterone and catecholamine concentrations in growing broiler breeders using intravenous cannulation. Physiol. Behav. 74:299–304.
Desrochers, D. W., J. M. Reed, J. Awerman, J. A. Kluge, J. Wilkinson, L. I. Van Griethuijsen, J. Aman, and L. M. Romero. 2009. Exogenous and endogenous corticosterone alter feather quality. Comp. Biochem. Physiol. Part A Mol. Integr. 152:46–52.
Docherty, D. E. 2004. *Corvidae* feather pulp and West Nile virus detection. Emerg. Infect. Dis. 10:907–909.
Dryer, S. E. 2015. Glutamate receptors in the kidney. Nephrol. Dial. Transplant. 30:1630–1638.
Erf, G. F., and I. R. Ramachandran. 2016. The growing feather as a dermal test site: comparison of leukocyte profiles during the response to *Mycobacterium butyricum* in growing feathers, wattles, and wing webs. Poult. Sci. 95:2011–2022.
Etches, R. J. 1979. Plasma concentrations of progesterone and corticosterone during the ovulation cycle of the hen (*Gallus domesticus*). Poult. Sci. 58:211–216.
Fairhurst, G. D., T. A. Marchant, C. Soos, K. L. Machin, and R. G. Clark. 2013. Experimental relationships between levels of corticosterone in plasma and feathers in a free-living bird. J. Exp. Biol. 216:4071–4081.
Ferreira, N. G. C., L. P. Da Silva, C. Fonseca, A. M. V. M. Soares, and S. Loureiro. 2010. Habitats: can they stress birds? An example of the use of biomarkers as an evaluation tool. Integr. Environ. Assess. Manag. 6:779–780.
Giannuzzi, D., B. Biolatti, E. Longato, S. Divari, L. Starvaggi Cucuzza, P. Pregel, F. E. Scaglione, A. Rinaldi, L. M. Chiesa, and F. T. Cannizzo. 2021. Application of RNA-sequencing to identify biomarkers in broiler chickens prophylactic administered with antimicrobial agents. Animal 15:1751–7311.
Gou, Z., K. F. M. Abouelezz, Q. Fan, L. Li, X. Lin, Y. Wang, X. Cui, J. Ye, M. A. Masoud, S. Jiang, and X. Ma. 2021. Physiological effects of transport duration on stress biomarkers and meat quality of medium-growing Yellow broiler chickens. Animal 15:1751–7311.
Greene, E. S., H. Rajaei-Sharifabadi, and S. Dridi. 2019. Feather HSP70: a novel non-invasive molecular marker for monitoring stress induced by heat exposure in broilers. Poult. Sci. 98:3400–3404.
Guabiraba-Brito, R., and C. Schouler. 2015. Avian colibacillosis: still many black holes. FEMS Microbiol. Lett. 362:1–8.
Hangalapura, B. N., M. G. B. Nieuwland, J. Buyse, B. Kemp, and H. K. Parmentier. 2004. Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. Poult. Sci. Assoc. 83:1644–1649.
Harris, C. M., C. L. Madliger, and O. P. Love. 2016. Temporal overlap and repeatability of feather corticosterone levels: practical considerations for use as a biomarker. Conserv. Physiol. 4:1–11.
Harris, C. M., C. L. Madliger, and O. P. Love. 2017. An evaluation of feather corticosterone as a biomarker of fitness and an ecologically relevant stressor during breeding in the wild. Oecologia 183:987–996.
Hõrak, P., M. Männiste, R. Meitern, E. Sild, L. Saks, and T. Sepp. 2013. Dexamethasone inhibits corticosterone deposition in feathers of greenfinches. Gen. Comp. Endocrinol. 191:210–214.
Inglis, G. D., B. D. Wright, S. A. Sheppard, D. W. Abbott, M. A. Oryschak, and T. Montina. 2021. Expeller-pressed canola (*Brassica napus*) meal modulates the structure and function of the cecal microbiota, and alters the metabolome of the pancreas, liver, and breast muscle of broiler chickens. Animals 11:1–22.
Isaksson, C., J. Örnborg, E. Stephensen, and S. Andersson. 2005. Plasma glutathione and carotenoid coloration as potential biomarkers of environmental stress in great tits. EcoHealth 2:138–146.
James, C., L. Asher, K. Herborn, and J. Wiseman. 2018. The effect of supplementary ultraviolet wavelengths on broiler chicken welfare indicators. Appl. Anim. Behav. Sci. 209:55–64.
Jastrebski, S. F., S. J. Lamont, and C. J. Schmidt. 2017. Chicken hepatic response to chronic heat stress using integrated transcriptome and metabolome analysis. PLoS One 12:e0181900.
Jenni-Eiermann, S., F. Helfenstein, A. Vallat, G. Glauser, and L. Jenni. 2015. Corticosterone: effects on feather quality and deposition into feathers. Methods Ecol. Evol. 6:237–246.
Karu, N., L. Deng, M. Slae, A. C. Guo, T. Sajed, H. Huynh, E. Wine, and D. S. Wishart. 2018. A review on human fecal metabolomics: methods, applications and the human fecal metabolome database. Anal. Chim. Acta 1030:1–24.
Kiss, D., M. Ambeskovic, T. Montina, and G. A. S. Metz. 2016. Stress transgenerationally programs metabolic pathways linked to altered mental health. Cell. Mol. Life Sci. 73:4547–4557.
Koren, L., S. Nakagawa, T. Burke, K. K. Soma, K. E. Wynne-Edwards, and E. Geffen. 2012. Non-breeding feather

concentrations of testosterone, corticosterone and cortisol are associated with subsequent survival in wild house sparrows. Proc. R. Soc. B: Biol. Sci. 279:1560–1566.
Kriat, M., S. Confortgouny, J. Viondury, M. Sciaky, P. Viout, and P. J. Cozzone. 1992. Quantitation of metabolites in human blood serum by proton magnetic resonance spectroscopy. A comparative study of the use of formate and TSP as concentration standards. NMR Biomed 5:179–184.
Lattin, C. R., J. M. Reed, D. W. Desrochers, and L. M. Romero. 2011. Elevated corticosterone in feathers correlates with corticosterone-induced decreased feather quality: a validation study. J. Avian Biol. 42:247–252.
Le Roy, C. I., L. J. Mappley, R. M. La Ragione, M. J. Woodward, and S. P. Claus. 2016. NMR-based metabolic characterization of chicken tissues and biofluids: a model for avian research. Metabolomics 12:157.
Lin, C. Y., H. Wu, R. S. Tjeerdema, and M. R. Viant. 2007. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. Metabolomics 3:55–67.
Lin, H., E. Deculypere, and J. Buyse. 2004. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*) - 2. Short-term effect. Comp. Biochem. Physiol. B 139:745–751.
Lu, S. C. 2013. Glutathione synthesis. Biochim. Biophys. Acta 1830:3143–3153.
Lytou, A. E., G.-J. E. Nychas, and E. Z. Panagou. 2018. Effect of pomegranate based marinades on the microbiological, chemical and sensory quality of chicken meat: a metabolomics approach. Int. J. Food Microbiol. 267:42–53.
Markley, J. L., R. Bruschweiler, A. S. Edison, H. R. Eghbalnia, R. Powers, D. Raftery, and D. S. Wishart. 2017. The future of NMR-based metabolomics. Curr. Opin. Biotechnol. 43:34–40.
Maryvonne Dho-Moulin, J. M. F. 1999. Avian pathogenic *Escherichia coli* (APEC). Vet. Res. 30:299–316.
Matysik, S., C. I. Le Roy, G. Liebisch, and S. P. Claus. 2016. Metabolomics of fecal samples: a practical consideration. Trends Food Sci. Tech. 57:244–255.
McHugh, C. E., T. L. Flott, C. R. Schooff, Z. Smiley, M. A. Puskarich, D. D. Myers, J. G. Younger, A. E. Jones, and K. A. Stringer. 2018. Rapid, reproducible, quantifiable NMR metabolomics: methanol and methanol: chloroform precipitation for removal of macromolecules in serum and whole blood. Metabolites 8:93–108.
Mormède, P., S. Andanson, B. Aupérin, B. Beerda, D. Guémené, J. Malmkvist, X. Manteca, G. Manteuffel, P. Prunet, C. G. Van Reenen, S. Richard, and I. Veissier. 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92:317–339.
Nicholson, J. K., J. C. Lindon, and E. Holmes. 1999. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29:1181–1189.
Patterson, A. G. L., A. S. Kitaysky, D. E. Lyons, and D. D. Roby. 2015. Nutritional stress affects corticosterone deposition in feathers of caspian tern chicks. J. Avian Biol. 46:18–24.
Paxman, E. J., N. S. Boora, D. Kiss, D. P. Laplante, S. King, T. Montina, and G. A. S. Metz. 2018. Prenatal maternal stress from a natural disaster alters urinary metabolomic profiles in project ice storm participants. Sci. Rep. 8:12932.
Phang, J. M., W. E. I. Liu, and O. Zabirnyk. 2010. Proline metabolism and microenvironmental stress. Annu. Rev. Nutr. 30:441–463.
Post, J., J. M. Rebel, and A. A. ter Huurne. 2003. Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress. Poult. Sci. 82:1313–1318.
Poulsen, L. L., I. Thofner, M. Bisgaard, J. P. Christensen, R. H. Olsen, and H. Christensen. 2017. Longitudinal study of transmission of *Escherichia coli* from broiler breeders to broilers. Vet. Microbiol. 207:13–18.
Psychogios, N., D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman, T. Goodfriend, and D. S. Wishart. 2011. The human serum metabolome. PLoS One 6:1–23.
Rettenbacher, S., E. Möstl, R. Hackl, K. Ghareeb, and R. Palme. 2004. Measurement of corticosterone metabolites in chicken droppings. Br. Poult. Sci. 45:704–711.
Rettenbacher, S., R. Palme, R. Hackl, and E. Bamberg. 2006. Distribution and elimination of radiolabelled corticosterone in chicken broilers. Wien. Tierarztl. Monatsschr. 93:283–287.
Rodriguez-Siek, K. E., C. W. Giddings, C. Doetkott, T. J. Johnson, and L. K. Nolan. 2005. Characterizing the APEC pathotype. Vet. Res. 36:241–256.
Romero, L. M., N. E. Cyr, and R. C. Romero. 2006. Corticosterone responses change seasonally in free-living house sparrows (*Passer domesticus*). Gen. Comp. Endocrinol. 149:58–65.
Romero, L. M., and J. M. Reed. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comp. Biochem. Physiol. Part A Mol. Integr. 140:73–79.
Romero, L. M., and R. C. Romero. 2002. Corticosterone responses in wild birds: the importance of rapid initial sampling. The Condor 104:129–135.
Romero, L. M., D. Strochlic, and J. C. Wingfield. 2005. Corticosterone inhibits feather growth: potential mechanism explaining seasonal down regulation of corticosterone during molt. Comp. Biochem. Physiol. Part A Mol. Integr. 142:65–73.
Rosner, W. 1990. The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. Endocr. Rev. 11:80–91.
Scanes, C. G. 2016. Biology of stress in poultry with emphasis on glucocorticoids and the heterophil to lymphocyte ratio. Poult. Sci. 95:2208–2215.
Sharma, S., A. Gupta, S. M. S. T. Chik, C. G. Kee, B. M. Mistry, D. H. Kim, and G. Sharma. 2017. Characterization of keratin microparticles from feather biomass with potent antioxidant and anticancer activities. Int. J. Biol. Macromol. 104:189–196.
Shen, J.-c., L.-y. Lian, Y. Zhang, Q.-d. He, J.-l. Chen, L.-b. Zhang, M.-s. Huang, M. Liu, L.-c. Qian, C.-c. Liu, and Z.-b. Yang. 2019. Dynamic analysis of metabolic response in gastric ulcer (gu) rats with electroacupuncture treatment using ^{1}H NMR-based metabolomics. Evid. Based Complement. Alternat. Med. 2019:1–12.
Shini, S., A. Shini, and G. R. Huff. 2009. Effects of chronic and repeated corticosterone administration in rearing chickens on physiology, the onset of lay and egg production of hens. Physiol. Behav. 98:73–77.
Singh, R. 2015. Membrane technology and engineering for water purification. Pages 1−80 in Introduction to Membrane Technology. Butterworth-Heinemann , Waltham, MA.
Snytnikova, O. A., A. A. Khlichkina, R. Z. Sagdeev, and Y. P. Tsentalovich. 2019. Evaluation of sample preparation protocols for quantitative NMR-based metabolomics. Metabolomics 15:84–93.
Stettenheim. 2000. The integumentary morphology of modern birds: an overview. Am. Zool. 40:461–477.
Szymanska, E., E. Saccenti, A. K. Smilde, and J. A. Westerhuis. 2012. Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. Metabolomics 8:3–16.
Tesfaye, T., B. Sithole, and D. Ramjugernath. 2017a. Valorisation of chicken feathers: a review on recycling and recovery route − current status and future prospects. Clean Technol. Environ. Policy 19:2363–2378.
Tesfaye, T., B. Sithole, D. Ramjugernath, and V. Chunilall. 2017b. Valorisation of chicken feathers: characterisation of chemical properties. Waste Manag. Res. 68:626–635.
Tiziani, S., A.-H. Emwas, A. Lodi, C. Ludwig, C. M. Bunce, M. R. Viant, and U. L. Günther. 2008. Optimized metabolite extraction from blood serum for ^{1}H nuclear magnetic resonance spectroscopy. Anal. Biochem. 377:16–23.
Virden, W. S., J. P. Thaxton, A. Corzo, W. A. Dozier, and M. T. Kidd. 2007. Evaluation of models using corticosterone and adrenocorticotropin to induce conditions mimicking physiological stress in commercial broilers. Poult. Sci. 86:2485–2491.
Weimer, S. L., R. F. Wideman, C. G. Scanes, A. Mauromoustakos, K. D. Christensen, and Y. Vizzier-Thaxton. 2018. An evaluation of methods for measuring stress in broiler chickens. Poult. Sci. 97:3381–3389.
Whiley, H., and K. Ross. 2015. *Salmonella* and eggs: from production to plate. Int. J. Environ. Res. Public Health 12:2543–2556.

Will, A., K. Wynne-Edwards, R. Zhou, and A. Kitaysky. 2019. Of 11 candidate steroids, corticosterone concentration standardized for mass is the most reliable steroid biomarker of nutritional stress across different feather types. Ecol. Evol. 9:11930–11943.

Wishart, D. S. 2008. Quantitative metabolomics using NMR. Trends Analyt. Chem. 27:228–237.

Wu, H., A. D. Southam, A. Hines, and M. R. Viant. 2008. High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. Anal. Biochem. 372:204–212.

Wu, J., Y. An, J. Yao, Y. Wang, and H. Tang. 2010. An optimised sample preparation method for NMR-based faecal metabonomic analysis. Analyst 135:1023–1030.

Xiao, Z., Y. Luo, G. Wang, C. Ge, G. Zhou, W. Zhang, and G. Liao. 2019. ^{1}H-NMR-based water-soluble low molecular weight compound characterization and fatty acid composition of boiled Wuding chicken during processing. J. Sci. Food Agric. 99:429–435.

Zavala, G., M. W. Jackwood, and D. A. Hilt. 2002. Polymerase chain reaction for detection of avian leukosis virus subgroup j in feather pulp. Avian Dis 46:971–978.

Zaytsoff, S. J. M., V. F. Boras, R. R. E. Uwiera, and G. D. Inglis. 2022. A stress-induced model of acute necrotic enteritis in broiler chickens using dietary corticosterone administration. Poult. Sci. 101:101726.

Zaytsoff, S. J. M., C. L. J. Brown, T. Montina, G. A. S. Metz, D. W. Abbott, R. R. E. Uwiera, and G. D. Inglis. 2019. Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens. Sci. Rep. 9:19225–19238.

Zaytsoff, S. J. M., S. M. Lyons, A. M. Garner, R. R. E. Uwiera, W. F. Zandberg, D. W. Abbott, and G. D. Inglis. 2020. Host responses to *Clostridium perfringens* challenge in a chicken model of chronic stress. Gut Pathog 12:12–24.

Zhang, L., H. Y. Yue, H. J. Zhang, L. Xu, S. G. Wu, H. J. Yan, Y. S. Gong, and G. H. Qi. 2009. Transport stress in broilers: I. Blood metabolism, glycolytic potential, and meat quality. Poult. Sci. 88:2033–2041.

Zhao, F. Q., Z. W. Zhang, J. P. Qu, H. D. Yao, M. Li, S. Li, and S. W. Xu. 2014. Cold stress induces antioxidants and Hsps in chicken immune organs. Cell Stress Chaperones 19:635–648.