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Corticosterone in feathers: Inter- and intraindividual variation in pullets and the importance of the feather type

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ABSTRACT

Measuring corticosterone concentrations in feathers of poultry may be suitable to determine birds' exposure to stress. It is thinkable, that in laying hens such information could be helpful as an animal welfare indicator to evaluate adverse husbandry conditions and to predict the risk of developing behavioral disorders, such as feather pecking and cannibalism. Yet, there are some fundamental issues which remain unclear. Therefore, the objective of the current pilot study was to examine the inter- and intraindividual variation of pullets at the end of the rearing period, when most of the feathers are fully grown and animals are reaching sexual maturity. Flight feathers from both wings ($n = 4$), the tail ($n = 2 – 3$), and body feathers ($n = 1$ pool of 3 – 5 feathers) were taken from pullets ($n = 10$), genetics Lohmann Brown, at an age of 19 weeks who were reared in the same flock ($N = 728$). Corticosterone analysis was performed applying a validated protocol for laying hens. Results indicate not only high intraindividual, but also high interindividual variation. Mean over all samples was 75.2 pg/mg (± 38.58 pg/mg, $n = 76$), showing higher intraindividual variation (between feather types; SD: 23.75 pg/mg – 49.38 pg/mg; $n = 10$ pullets) than interindividual variation (within feather types; SD: 11.91 pg/mg – 49.55 pg/mg; $n = 6$ feather types). The variation between different feather types within one bird was higher than the variation within one feather type between different birds, indicating that birds a) may respond differently when exposed to stressors and b) corticosterone measurements should be done with the same feather type.

1. Introduction

Animal welfare and its assessment has become an important research field over the last years. The awareness of animals being creatures capable of suffering led to the question how to evaluate their welfare objectively. In pullets and laying hens plumage and skin condition is used to assess feather pecking and cannibalism as an indicator for poor animal welfare (Bestmann, Koene & Wagenaar, 2009; Welfare Quality, 2009). However, one major shortcoming of visual scoring systems is the lack of objectivity and repeatability, especially when undertaken by different persons (inter-observer repeatability). Additionally, the manual scoring requires labor, is time-consuming and birds need to be handled in order to carry out an exact assessment. The measurement and evaluation of corticosterone concentrations in feathers (*CORTf*[1]) was shown to be a suitable, non-invasive tool to detect and monitor birds that had to cope with adverse environmental situations over a long-term period (Bortolotti, Marchant, Blas, & German, 2008; Häffelin et al., 2020). Therefore, detecting *CORTf*[1] may be a promising approach to assess animal welfare in single birds and flocks, respectively. The stress-related hormone is deposited into feathers during the duration of feather growth when they are supplied with blood (Jenni-Eiermann, Helfenstein, Vallat, Glauser & Jenni, 2015; Romero & Fairhurst, 2016).

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[1] Corticosterone in feathers.

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Contrary, *CORT*[2] levels in the blood or *GCM*[3] in feces are subjected to circadian rhythm (Möstl, Rettenbacher & Palme, 2005; Touma & Palme, 2005) and short-term liabilities, such as capturing (Bortolotti, Marchant, Blas, & German, 2008; Dehnhard et al., 2003; Romero & Reed, 2005), immobilization by hand and heat stress (Beuving & Vonder, 1978). In older pullets or young laying hens, *CORTf*[1] may be a useful indicator to assess birds' ability to cope with their rearing conditions, and thus, to evaluate their susceptibility to develop behavioral disorders, such as feather pecking and cannibalism before their appearance (Häffelin et al., 2020), because they often emerge with a time lag to the period that is responsible for them. Thus, *CORTf*[1] would be beneficial regarding the evaluation of pullets before rehousing for production and the implementation of an adapted and preventive flock management (Weimer et al., 2018). Furthermore, *CORTf*[1] measurements may also be suitable to be implemented in breeding schemes in order to provide stress-resilient animals (Häffelin et al., 2020). However, prior of any interpretation of *CORTf*[1] values, there is a need to establish species-specific reference values (Fairhurst, Marchant, Soos, Machin & Clark, 2013, 2012; Kennedy, Lattin, Romero & Dearborn, 2013; Kouwenberg, Hipfner, McKay & Storey, 2016). To the best of our knowledge there is no such data available for pullets and laying hens. Therefore, the objective of the current study was to determine the variation of *CORTf*[1] within a pullet (feather types) and between the pullets of one flock.

2. Materials and methods

2.1. Animals and feather sampling

Animals were kept on a commercial farm in Germany, in accordance with local legislation (Council of Europe, 1995; Minimum Requirements for Pullets, 2000; Recommendations for preventing feather pecking & cannibalism, 2018; TierSchNutztV, 2017). Feathers were pulled *post-mortem* (Häffelin et al., 2020; Monclús et al., 2017) from pullets ($n = 10$) with untrimmed beaks, genetics Lohmann Brown, reared in the same flock ($N = 728$) in floor husbandry with a modified aviary system and without free range. Pullets were slaughtered at an abattoir as part of another project at the end of the rearing period with 19 weeks of age. Addressing the inter- and intraindividual variation, contour and flight feathers were taken from each animal. All selected feathers had to be full-grown which was verified through the absence of blood and feather pulp in the *calamus* (Jenni, Ganz, Milanesi & Winkler, 2020), and the completeness of the vane. The contour feathers ($n = 3 – 5$ / pullet) were taken from the region on the back between the scapulae (Carbajal, Tallo-Parra, Sabes-Alsina, Mular & Lopez-Bejar, 2014; Häffelin et al., 2020; Monclús et al., 2017) and are subsequently named as interscapular feathers (Häffelin et al., 2020; Monclús et al., 2017). Flight feathers taken from the tail (*Rectrices,* $n = 2 – 3$ / pullet) are subsequently named as tail feathers (Aharon-Rotman, Buchanan, Klaasen, & Buttemer, 2017; Häffelin et al., 2020; Harms et al., 2015; Jenni-Eiermann et al., 2015; Lendvai, Giraudeau, Németh, Bakó & McGraw, 2013; López-Jiménez et al., 2016; Robertson, Muir, Hurd, Hing & Quinn, 2017). Flight feathers from the wings (*Remiges;* $n = 4$ / pullet; based on Aharon-Rotman, Buchanan, Klaasen, & Buttemer, 2017; Bortolotti, Marchant, Blas, & German, 2008; Bortolotti, Marchant, Blas & Cabezas, 2009; Bosholn et al., 2020; Bourgeon et al., 2014; Fairhurst et al., 2011; Lattin, Reed, DesRochers & Romero, 2011; Monclús et al., 2017; Strong, Pereira, Shore, Henrys & Pottinger, 2015; Weimer et al., 2018; Will et al., 2014), most recently developed, were taken from their corresponding positions on the left and right wing – the third or fourth alula feathers (bastard wing) and the fifth primaries. Feathers recently developed were defined as feathers with absent blood and feather pulp in the *calamus* who are located next to a feather of the same type with a calamus containing blood and feather pulp and under consideration of the molting order of primary feathers. Interscapular feathers of each pullet were processed and analyzed as a pool (Freeman & Newman, 2018; Häffelin et al., 2020; Lattin et al., 2011), because unlike flight feathers from wings and tail, their individual growth cannot be determined for a specific period of time. Also, they are much lighter than flight feathers and one body feather does not provide enough mass to prepare one sample for analysis (Freeman & Newman, 2018). Thus, for each animal ($n = 10$) one sample consisted of three to five feathers ($n = 10$ interscapular feather samples). Every tail, alula and primary feather was processed and analyzed separately, providing the possibility to collect data for each feather ($n = 26$ tail feather samples, $n = 20$ alula feather samples, $n = 20$ primary feather samples). Consequently, a total of 76 samples existed. Feathers were stored dark and dry at room temperature in labeled paper envelopes before analysis (Bortolotti et al., 2009; Häffelin et al., 2020; Monclús et al., 2017).

2.2. Corticosterone extraction and analysis

In accordance with Bortolotti, Marchant, Blas, & German, 2008, the *calamus* of each feather was removed, and vane and rachis of the same feather were examined together. Corticosterone extraction and analysis was undertaken using the protocol of Häffelin et al. (2020). Samples were standardized by weighing 10 mg (9.5 mg – 10.5 mg; precision balance Mettler, Spoehrhase A.G. Giessen, Germany) of each pulverized feather sample for extraction, avoiding the small sample artifact (Berk, McGettrick, Hansen, & Breuner, 2016; Lattin et al., 2011). Samples were analyzed as triplets (Harris, Madliger & Love, 2016) in an ELISA (Carbajal et al., 2014; Jenni-Eiermann et al., 2015; Kouwenberg et al., 2016), using the Enzo Life Sciences Corticosterone ELISA Kit ADI-901–097 (Enzo Life Sciences Inc., New York, USA; also used by Bourgeon et al., 2014; Häffelin et al., 2020; Harris et al., 2016; Harris, Madliger & Love, 2017).

2.3. Calculations and statistical analysis

CORTf[1] concentrations were calculated in accordance with the product manual of Enzo Life Sciences Corticosterone ELISA Kit ADI-901–097 (Enzo Life Sciences Inc., 2019), using the Magellan™ data analysis software 7.2 (Tecan Group Ltd., Männedorf, Switzerland). In order to evaluate the precision and repeatability of the assay the intra-assay-variability for each triplet was expressed with the CV[4]. Only values amounting less than 20% were included in any further calculation (Häffelin et al., 2020; Kinn Rød, Harkestad, Jellestad & Murison, 2017). Values were expressed in pg/mg (Freeman & Newman, 2018; Häffelin et al., 2020; Monclús et al., 2017; Robertson et al., 2017). Due to the structure of the data set no statistical analyses were performed, but the descriptive arithmetic means for each triplet were given. Data management, calculations and visual representations were performed using the software package Minitab® 16.2.3 (Minitab LLC., State College, USA).

3. Results

Intra-assay CV[4] over all samples was 6.6% (median, 0.5% - 17.2%, $n = 76$ samples). *CORTf*[1] values of all 76 analyzed samples differed between 23.0 pg/mg and 189.5 pg/mg, with a mean of 75.2 pg/mg (± 38.58 pg/mg) and a median of 69.8 pg/mg.

3.1. Interindividual variation

The average of *CORTf*[1] values of the samples (feathers) originating from one bird ($n = 10$) ranged from 50.6 pg/mg to 104.7 pg/mg,

[2] Corticosterone.
[3] Glucocorticoid metabolites.
[4] Coefficient of variation.

showing a maximum difference ($\Delta CORTf^1$) between birds of 54.1 pg/mg (Fig. 1). $CORTf^1$ concentrations of the different feather types over all birds are depicted in Table 1 and Fig. 2, whereby interscapular feather pools ($n = 10$) and tail feathers ($n = 26$) showed $CORTf^1$ concentrations of 61.3 pg/mg (mean ± 17.58 pg/mg, median 61.2 pg/mg) and 91.9 pg/mg (mean ± 49.55 pg/mg, median 91.7 pg/mg), respectively. Flight feathers from the left and the right wings showed $CORTf^1$ concentrations of 90.6 pg/mg (mean ± 17.80 pg/mg, median 99.1 pg/mg) and 95.3 pg/mg (mean ± 13.45 pg/mg, median 96.9 pg/mg), for the primaries ($n =$ 10 each side), and for the alula feathers ($n = 10$ each side) 45.3 pg/mg (mean ± 12.46, median 42.4 pg/mg) and 40.2 pg/mg (mean ± 11.91 pg/mg, median 34.6 pg/mg) respectively.

3.2. Intraindividual variation

Standard deviations of $CORTf^1$ values for the feathers of one pullet (n = 7 or 8 feathers) ranged between 23.75 pg/mg and 49.38 pg/mg, maximum $CORTf^1$ difference ($\Delta CORTf^1$) between samples (feathers) within one bird was 146.2 pg/mg and minimum difference ($\Delta CORTf^1$) 61.7 pg/mg, respectively (Fig. 1). CV^4 of the tail feather values taken from one pullet ($n = 2 – 3$ feathers) was between 4.3% and 24.4% for nine pullets (no. 1 – 9) and 65.7% for pullet no. 10. Mean of the flight feathers from the left and right wings differed with a CV^4 of 2.5% for the primaries and 5.9% for the alulae ($n = 10$ flight feathers each side and type).

4. Discussion

In order to interpret $CORTf^1$ measurements correctly and, more importantly, draw reasonable conclusions one must know how different birds respond to the same stressor (interindividual variation) evoked in the very same environment. Furthermore, and from an intraindividual perspective, knowledge about potential variations regarding the deposition of $CORT^2$ from blood into different feather types is mandatory in order to establish a meaningful and standardized methodology. The current paper made an attempt to address both of these issues, initially, however, as a pilot study, no distinction was made between different stressors.

The examined feather types were chosen based on previous studies (inter alia, studies of Aharon-Rotman, Buchanan, Klaasen, & Buttemer, 2017; Bortolotti, Marchant, Blas, & German, 2008; Carbajal et al., 2014; Häffelin et al., 2020; Jenni-Eiermann et al., 2015). The flight feathers, in this case feathers from the tail (*Rectrices*) and wings (*Remiges*), have the advantages that their growth and growth cycle can be monitored closely and determined precisely (Bortolotti, Marchant, Blas, & German, 2008). Moreover, *Remiges* are available twice. Thus, they are expected to develop and grow at the same time and thus, while leaving fluctuating asymmetry aspects (Swaddle & Witter, 1994) aside, are exposed to $CORT^2$ from blood during the same period of time and consequently show the same $CORTf^1$ concentrations as shown in the current study and by Strong et al. (2015). Body feathers, however, are available numerously, but their respective time of growth according to the body region can hardly be narrowed down to a specific period of time. Also, due to their minor weight a larger amount of body feathers needs to be collected in order to prepare a pooled sample as done in the current study.

Based on Freeman and Newman (2018) and the validated protocol of Häffelin et al. (2020), 10 mg of each feather or feather pool was analyzed, avoiding the small sample artifact (Berk, McGettrick, Hansen, & Breuner, 2016; Lattin et al., 2011). The results indicate not only high interindividual variation, but also high intraindividual variation. It could be proposed that the interindividual variation is a result of different responses of the animals to certain liabilities (Cockrem, 2007;

Table 1
Feather corticosterone concentrations (pg/mg) in different feather types collected from 10 Lohmann Brown pullets.

Feather type	n (samples)	Mean ± SD	Median	Range	CV
Interscapular feathers	10	61.3 ± 17.58	61.2	38.0 – 93.7	28.7%
Tail feathers	26	91.9 ± 49.55	91.7	23.0 – 189.5	53.9%
Primaries left	10	90.6 ± 17.80	99.1	55.6 – 116.1	19.6%
Primaries right	10	95.3 ± 13.45	96.9	65.3 – 112.6	14.1%
Alulae left	10	45.3 ± 12.46	42.4	33.4 – 72.5	27.5%
Alulae right	10	40.2 ± 11.91	34.6	29.5 – 70.7	29.6%

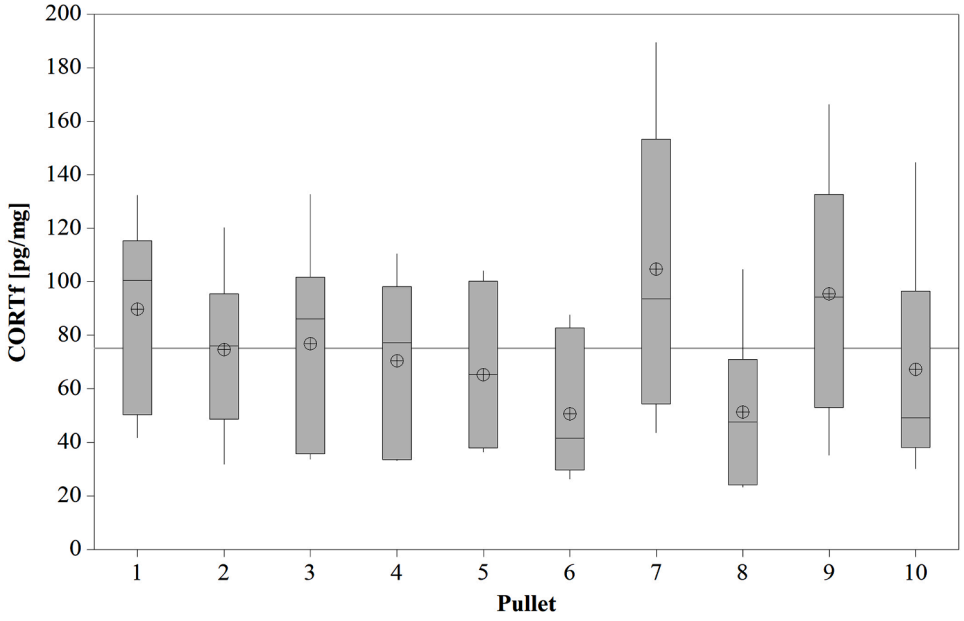

Fig. 1. Feather corticosterone ($CORTf$) levels of 19 weeks old Lohmann Brown pullets ($n = 10$). Mean over all samples = 75.2 pg/mg (± 38.58 pg/mg, $n = 76$; gray line). Box = interquartile range.

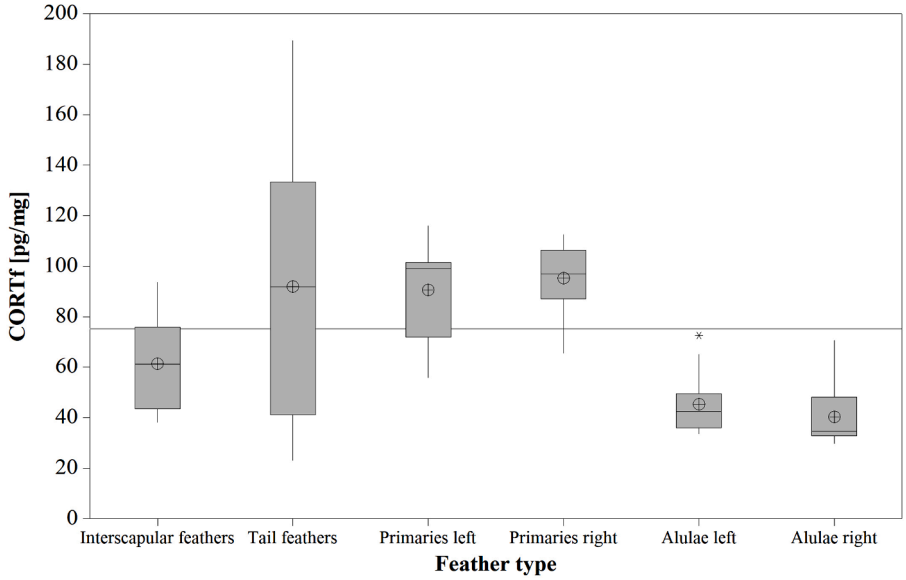

Fig. 2. Comparison of feather corticosterone (*CORTf*) concentrations of different feather types. Mean over all samples = 75.2 pg/mg (± 38.58 pg/mg, $n = 76$; gray line). Box = interquartile range.

Koolhaas et al., 1999) during rearing and thus, feather growth. Regarding liabilities, this could be for example inadequate lighting conditions (Kämmerling, Döhring, Arndt & Andersson, 2017) feeding (Rodenburg et al., 2013) or climate conditions, especially adverse ammonia concentration which is shown to provoke stress in poultry (Drake, Donnelly & Stamp Dawkins, 2010). Also, the social hierarchy in the flock might have an influence on the $CORT^2$ level of a single bird. This must be taken into account when interpreting the results for interindividual variation (Aharon-Rotman et al., 2017). However, the standard deviation over the different feather types within one pullet (SD: 23.75 pg/mg – 49.38 pg/mg, $n = 10$ pullets; Fig. 1) was higher than the values coming from calculations for the standard deviation over the feather types of all pullets (SD: 11.91 pg/mg – 49.55 pg/mg, $n = 6$ feather types; Table 1 and Fig. 2), indicating a higher variation between different feather types of one animal than within feather types of different animals. This suggests that there is an effect of the feather type and thus makes it necessary to sample the very same feather type when performing $CORTf^1$ analysis (Häffelin et al., 2020; Monclús et al., 2017) in poultry.

Interscapular feathers were analyzed as a pool sample which resulted in rather less variation within and between animals. The results indicate, that it is beneficial to pool the same feather type of a representative number of different individuals to get an impression about the flock. In any case, all samples have to be standardized to the same weight in order to be comparable and meaningful.

The CV^4 of the tail feathers was different from the other feather types with 53.9% ($n = 26$; Table 1). Furthermore, they had the widest range of all feather types (23.0 pg/mg – 189.5 pg/mg; $n = 26$ feathers), showing the lowest and highest $CORTf^1$ concentration of all samples measured (Fig. 2). This interindividual variation is interpreted as an indication, that the different $CORTf^1$ concentrations reflect individually experienced liabilities (Fairhurst et al., 2011), and animals reacting with different coping strategies (Cockrem, 2007; Koolhaas et al., 1999). In regard to this and with a closer look on the intraindividual variation, CV^4 between the tail feathers of one pullet ($n = 2 – 3$ feathers) indicate that there is an individual scope for each animal. However, in one of the animals ($n = 10$ pullets) CV^4 between the tail feathers was high with 65.7% ($n = 2$ feathers). Apparently, those feathers did not deposit comparable amounts of $CORT^2$ coming from the blood. An explanation would be, that the feathers had different periods of growth during which the bird was exposed to stressors, erratically. For future investigations this should be observed more closely and tail feathers (*Rectrices*) should be taken from the same position or feathers should get analyzed as pools. Another explanation for the high variation could be that the vanes deposit significantly more $CORT^2$ than the rachis and may have been diluted by them, as the weight and mass of a feather is dominated by the rachis (Freeman & Newman, 2018; Häffelin et al., 2020). Separating these two parts of a feather before processing and analyzing is, however, not practicable. High variations may also result from "fault bar allocation hypothesis" of Jovani and Blas (2004), stating that feathers which are less important for flying are more susceptible to develop fault bars during their feather growth when having physiological stress (Aharon-Rotman et al., 2017; Sarasola & Jovani, 2006) and thus, deposit higher $CORTf^1$ levels than sections without fault bars (Bortolotti et al., 2009; Fairhurst, Dawson, van Oort & Bortolotti, 2014; Robertson et al., 2017).

Although flight feathers from the wings (*Remiges*) offer the advantage of providing precise information about their growth period, they appear less meaningful than tail and interscapular feathers due to their $CORTf^1$ concentrations not being normally distributed. However, this should be verified in further studies with a higher sample size. Nevertheless, their CV^4 between the left and right wings (primaries = 2.5%, alulae = 5.9%; $n = 10$ each side and each type; Fig. 2) speaks in favor of the reliability of the protocol and the assay. Strong et al. (2015) also found no significant differences ($p = 0.4 – 0.8$) between the fifth primaries from both wings of sparrowhawks (*Accipiter nisus,* $n = 10$), barn owls (*Tyto alba,* $n = 5$), and tawny owls (*Strix aluco,* $n = 5$). Values twice as high in the primaries than in the alulae also support the thesis of respecting the different growth rate (Häffelin et al., 2020; Jenni-Eiermann et al., 2015; Monclús et al., 2017). Thus, Bortolotti, Marchant, Blas, & German, 2008 recommend the unit pg/mm, which requires an analysis of the whole feather, getting along with the small sample artifact (Berk, McGettrick, Hansen, & Breuner, 2016; Lattin et al., 2011) by the use of light feathers, such as interscapular feathers.

For further investigations on $CORTf^1$ variations within and between animals, the same position of a feather type should be chosen or in case this is not practicable, pools should get analyzed which would also be beneficial when comparing single feathers in order reduce the effect of different growth periods and/or different pigmentations (Fairhurst et al., 2014; Jenni-Eiermann et al., 2015). Regarding feather growth one must be aware to acknowledge that only for periods with feather growth in the life time of a bird a possible statement about welfare can be made.

Until today the method to extract and measure *CORTf*[1] is not standardized for each bird species. Despite the fact, that feather collection can be done by the farmer rather easily, the required laboratory work is very time consuming and not ready to be applied commercially just yet. Before establishing own methods and protocols, researchers could apply the already validated ones. The latter seems desirable, as it enables comparisons between studies.

5. Conclusion

It can be concluded, that decisions on which feather type should get analyzed depend on the research question and consequently on the time period which is wished to be covered. For this, the different molts during the rearing period of pullets need to be observed carefully. For now, we know different feather types of pullets deposit different amounts of corticosterone and there are also differences between animals. The latter may be due to the fact that the birds may have different coping strategies to get along with stressful situations. Respecting the differences between the feather types it can be concluded, that a reference value should be established for each feather type. In consideration of reference values related to traceable periods of feather growth, determination of periods which the animals perceive as particularly stressful could be done. Moreover, investigations are needed regarding the identification of environmental factors perceived as remarkably stressful by the animals and thus, influences *CORT*[2] deposition from blood into feathers. Therefore, investigations should be done in which certain stressors are implemented into birds' environment. To make a statement whether *CORTf*[1] could be used as an indicator for the risk of developing behavioral disorders in pullets or layers, a correlation between the actual appearance of behavioral disorders and altered *CORTf*[1] concentrations needs to be clarified. Further research should also cover possible effects on *CORTf*[1], such as genetics, pigments, and fault bars.

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Ethical permits

The current study was undertaken in accordance with the German legislation (TierSchG, 2019; TierSchNutztV, 2017).

Ethical statement

All animals in this study were kept on a commercial farm in Germany in accordance with the German Animal Welfare Act (2019) and the German Legal Standard on the Protection of Animals and Animal husbandry conditions (2017).

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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