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Mean measurable corticosterone in House Sparrow (*Passer domesticus*) primary feathers varies little across life-history stages

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ABSTRACT—The relatively new technique of measuring corticosterone (cort) levels extracted from feathers provides a less invasive, more integrated method of assessing a bird's stress physiology. Current understanding is that cort is deposited into the feather via blood when the feather is replaced during molt. The blood supply in the feather is cut off when the new feather completes growth, thereby ending the influx of cort. Previous studies assumed that cort deposited during feather growth remained constant throughout the feather's attachment to the bird, allowing an accurate retrospective index of circulating cort at the time of feather growth. We tested this assumption by measuring cort levels in feathers collected from different wild House Sparrows (*Passer domesticus*) across 6 important life history stages spanning a single year, thus representing feathers of different ages but grown during similar environmental conditions. If the common assumption is correct, we predicted that feather cort would not vary across the year. We further simulated substantial feather wear by removing the top 20% length of duplicate samples from the same birds across the same time span. We found significant differences in cort levels across life history stages, but no effect of sex or 20% feather removal. After excluding feather wear and sex and re-running the analysis, the effect of life history stage was no longer significant. Furthermore, despite uncontrolled individual and environmental variation, there was not a sustained decrease in feather cort over time. These data support the assumption that feather cort levels are stable while feathers are on the bird, regardless of feather age or typical wear. *Received 12 May 2020. Accepted 1 October 2021.*

Key words: feather age, feather corticosterone, feather growth, plasma corticosterone, stress mediator.

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La corticosterona media medible en las plumas primarias del gorrión *Passer dosmesticus* varía poco a lo largo de las etapas de su historia de vida

RESUMEN (Spanish)—La relativamente nueva técnica para medir niveles de corticosterona (cort) extraídos de plumas proporciona un método menos invasivo y más integrado para determinar la fisiología del estrés de un ave. El entendimiento actual es que la cort se deposita en la pluma por medio de la sangre cuando la pluma es reemplazada durante la muda. El suministro de sangre se suspende cuando la nueva pluma completa su crecimiento y concluye el influjo de cort. Los estudios previos asumían que la cort depositada durante el crecimiento de las plumas era constante a lo largo del periodo en el que la pluma permanecía sujeta al ave, permitiendo un índice retrospectivo preciso de cort circulante durante el tiempo de crecimiento de la pluma. Sometimos a prueba este supuesto por medio de mediciones de niveles de cort en plumas colectadas de diferentes gorriones *Passer domesticus* silvestres a lo largo de 6 etapas importantes de su historia de vida en un solo ciclo anual, representando con ello plumas de diferentes edades que sin embargo crecieron bajo condiciones ambientales similares. Si este supuesto común es correcto, predecimos que la cort en plumas no variaría a lo largo del año. Simulamos incluso un desgaste sustancial de plumas a través de la remoción del 20% de la longitud superior de la pluma de muestras duplicadas de los mismos individuos a lo largo del mismo periodo de tiempo. Encontramos diferencias significativas en los niveles de cort a lo largo de etapas de su historia de vida, mas no un efecto de sexo o remoción del 20% de remoción de pluma. Después de excluir el desgaste de pluma y el sexo, re-hicimos los análisis y el efecto de la historia de vida dejó de ser significativo. Además, aún sin controlar las variaciones por individuo y ambiental, no encontramos un decremento sostenido en el cort en plumas a lo largo del tiempo. Estos datos dan soporte al supuesto de que los

niveles de cort en plumas son estables mientras las plumas se encuentran en el ave, independientemente de la edad de la pluma o el desgaste típico.

Palabras clave: corticosterona en plasma, corticosterona en plumas, crecimiento de las plumas, edad de la pluma, mediador de estrés.

Measurements of stress mediators, like corticosterone (cort), can be useful in assessing an animal's probability of survival (Romero 2012). For many decades, avian researchers have focused on measuring birds' corticosterone levels in plasma (Nagra et al. 1963, Wingfield et al. 1992). As useful as this type of measurement is, it has the drawback of requiring handling of the bird in order to obtain a sample. The development of a technique to measure cort deposited in feathers (Bortolotti et al. 2008), and subsequent explorations of the practical and theoretical underpinnings of feather cort and its measurement (Bortolotti et al. 2009, Bortolotti 2010, Lattin et al. 2011, Fairhurst et al. 2013, Jenni-Eiermann et al. 2015), have provided a less invasive method of measuring cort levels in birds. This technique appears to yield an integrated profile of cort circulating in the bird's blood throughout the time of molt (genesis) of the feather during which time the feather is vascularized (Proctor and Lynch 1993, Maderson et al. 2009). Once it completes its growth, the blood supply then dries up, leaving behind detectable levels of cort deposited in the feather (Bortolotti et al. 2008, Jenni-Eiermann et al. 2015). The fully grown feather can then be harvested either by plucking the feather or by picking up feathers naturally dropped by the bird. The feather cort assay is proving to be a powerful tool providing the basis for a wide range of studies of birds both in the field and in captivity, and either alive or dead (Romero and Fairhurst 2016).

One assumption of this technique is that the concentration of measurable cort remains stable for the life of the feather. In other words, the amount of measurable cort would be equivalent, and reflect the amount originally deposited in the feather, regardless of what time of year the feather was plucked. This is a reasonable assumption considering a number of species only undergo one molt per year, and limited work has generally supported this assumption (Romero and Fairhurst 2016). However, because feathers are exposed to year-long conditions that can potentially deteriorate feather structure, the goal of this study was to test this assumption.

We analyzed primary feathers from House Sparrows (*Passer domesticus*) collected across 6 life history stages, with each individual sampled only once. However, all birds were captured from the same suburban site in the same year, and because House Sparrows have a limited adult range (Lowther and Cink 1992), we assumed that all feathers were molted under similar environmental conditions. We determined whether feather cort levels changed over the period of a feather's functional lifespan, i.e., from genesis to shed, regardless of the bird's life history stage at sampling. We then compared the feather cort levels to the significant differences in plasma cort that were previously reported from House Sparrows in general (Romero et al. 2005), and these birds in particular (Lattin et al. 2012). Because the feathers were grown under roughly equivalent natural conditions (during fall molt 2010), we did not expect to find differences in feather cort across life history stages. Furthermore, the ends of primary feathers often abrade over time; specifically, the distal 20% of the length can often wear away the longer the primary feather stays on the bird (Willoughby et al. 2002). Therefore, we also tested whether feather cort could change as a result of abrasion by removing the distal 20% of feathers prior to assay, thereby mimicking the effect of abrasion, and comparing cort concentrations to adjacent primaries that did not have the distal portion removed. In previous studies using European Starling (*Sturnus vulgaris*; Lattin et al. 2011) and Rock Pigeon (*Columba livia*; Jenni-Eiermann et al. 2015) feathers, higher cort concentrations were found in the distal portion of the feather. Consequently, we predicted lower levels of cort in the cut feathers (Fig. 1).

Methods

Study subjects

Note that this was not a repeated measures design—all birds were sampled only once during the study. However, all birds were captured at the same field site in Medford, Massachusetts, USA. During each life history stage, approximately equal numbers of males and females were

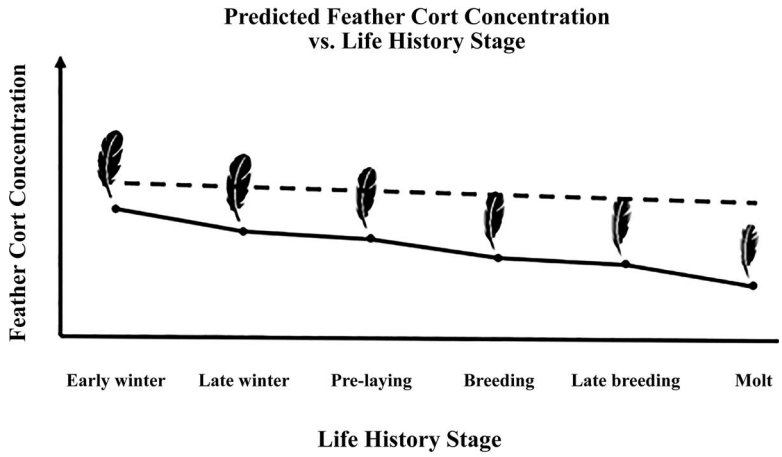

Figure 1. Predicted relationship of corticosterone concentrations of whole feathers (dotted line) and naturally or artificially eroded feathers (solid line and shrinking, fading feather symbols) across 1 year of life history stages in the House Sparrow. Previous studies measuring corticosterone levels in whole feathers assume that, once grown, feather corticosterone levels will not change. In naturally worn feathers, studies suggest that any decrease in feather corticosterone concentrations may result from loss of distal feather mass.

sampled. Feathers used in this study were collected as part of a different study (Lattin et al. 2012) and stored whole in a dark cabinet for ~8 years.

Briefly, wild House Sparrows were caught at 6 times of one year corresponding to important life history stages in New England and sampled for plasma cort (Lattin et al. 2012). These stages were defined as early winter (11–15 Dec 2010, $n_2 = 20$, $n_3 = 20$), late winter (1–14 Feb 2011, $n_1 = 8$, $n_2 = 18$, $n_3 = 20$), pre-laying, (31 Mar–7 Apr 2011, $n_1 = 8$, $n_2 = 12$, $n_3 = 12$), breeding (23–24 May 2011, $n_1 = 8$, $n_2 = 11$, $n_3 = 12$), late breeding (12–22 Jul 2011, $n_1 = 8$, $n_2 = 20$, $n_3 = 20$), and molt (9–12 Sep 2011, $n_1 = 9$, $n_2 = 9$, $n_3 = 8$), with $n_1 =$ plasma cort, $n_2 =$ whole feather cort, and $n_3 =$ cut feather cort.

Two whole feather samples in late winter, one whole feather sample in breeding, and one cut feather sample in molt were lost due to pipetting errors during the assay. We only measured old feathers from molting birds, so that they were grown during the previous year's molt (late Aug–Sep 2010), and all feathers used were primary feathers (P3–P9).

Feather preparation

Two groups of feathers per bird per life history stage were assayed, with each group consisting of 3–5 primary feathers. Combining multiple feathers in each group was necessary to ensure that all samples weighed at least 30 mg to control for effects of small sample mass (Lattin et al. 2011). In addition, storage containers did not label individual primaries, so we could not control for specific feather identity. For group 1, referred to as whole feathers, the calamus of each feather was removed and the rachis length measured from the proximal end of the feather to the distal tip (mm). For group 2, referred to as 80% feathers, after removal of the calamus, the rachis was measured to ascertain 100% length and then the top 20% of the feather was removed with a cut perpendicular to the rachis. The sum total length of all rachises per sample was recorded and the final cort concentration was expressed as cort/mm of feather as per recommendation of Bortolotti et al. (2008). Following this, all feathers were cut into pieces for the assay. Sample order (cut and intact feathers from 6 different life history stages) was randomized within each radioimmunoassay.

Feather cort assay

Prepared feathers were assayed following Bortolotti et al. (2008) with some modification. Feathers were mixed with 7 mL of methanol (HPLC grade, Fisher Scientific, Pittsburgh, Pennsylvania, USA), placed in a sonicating water bath at room temperature for 30 min, then incubated

overnight in a shaking 50 °C water bath. Methanol was separated from feather pieces using vacuum filtration with #4 Whatman filter paper in a filtration funnel. Feather pieces, the sample vial, and the filter paper were rinsed twice with ~2.5 mL of additional methanol, with the rinses added to the total methanol extract. Methanol extracts were dried under nitrogen gas in a 50 °C water bath. Extracts were reconstituted in Tris-HCl buffer and run through a standard radioimmunoassay as originally described by Wingfield et al. (1992). The anti-corticosterone antibody was produced in rabbit (Sigma-Aldrich C 8784-100TST, St. Louis, Missouri, USA; lot 047M4870V). This antibody has been used in many avian species (Musgrove et al. 2017, Studholme et al. 2018). Interassay variation from 3 assays was 16.3%, and intraassay variation was 3.4%.

Plasma cort concentrations

All baseline plasma cort concentrations were collected <3 min after capture from the same birds as those used for the feather assays. These concentrations were reported in an earlier study (Lattin et al. 2012) and reproduced here for qualitative comparison purposes. Plasma cort values were not included in statistical models of feather cort.

Statistical tests

Statistical tests were done using R 3.6.0 (R Core Team 2020) and SigmaPlot 14.0 (Systat Software Inc., San Jose, California, USA). After using a log transformation to normalize data distributions, a 3-way ANOVA was used to analyze differences in cort with life history stages, feather treatment (whole or 80% feathers), and sex as main factors, including all interactions. Because there were no effects of sex or feather treatment on cort (see results below), these factors were removed, and data reanalyzed solely on life history stage. However, after removing these factors, life history stage data were no longer normally distributed despite the log transformation, so we analyzed data using Kruskal-Wallis one-way analysis of variance on ranks.

Results

A 3-way ANOVA showed no effects of whole and 80% feather treatments ($F_{1,158} = 1.30$, $P = 0.26$) or bird sex ($F_{1,158} = 0.59$, $P = 0.45$) on feather cort levels. However, feather cort levels across life history stages were significantly different ($F_{5,158} = 3.44$, $P = 0.01$). We found no significant 2- or 3-way interactions ($P > 0.10$) for any tests. Therefore, the data were re-run excluding sex and treatment (in order to determine pairwise post hoc differences in life history stages), which failed normalcy. A Kruskal-Wallis one-way ANOVA on ranks showed no significant differences across life history stages ($H_5 = 10.88$, $P = 0.054$). Although approaching significance, the data do not fit the predicted pattern in Figure 1 with the highest cort being in the youngest feathers and the lowest being in the oldest (i.e., no systematic decrease over time). When the pattern of feather cort was compared to baseline plasma cort, the patterns did not align (Fig. 2).

Discussion

In this study, we examined the assumption that corticosterone deposited into feathers at the time of their growth remains stable across 1 year of life history stages as they remain attached to the bird. By necessity, this study had a factorial, not repeated measures, design. Cort measurements thus reflect the age of the specific feathers as they are maintained naturally by the bird, without confounding effects of repeated sampling required for a repeated measures design. It would be difficult, if not impossible, to repeatedly capture wild House Sparrows in order to test our hypothesis using repeated measures.

However, this design does present some limitations. We could not control for individual or environmental variation within each life-history stage, meaning that we only compared broad patterns. On the other hand, all birds were caught at the same site and within 12 months of the previous molt, so that all feathers in this study were grown during the same molting season (Aug–Sep 2010). Because House Sparrows have a limited adult range (Lowther and Cink 1992), it is reasonable to assume that all the feathers in this study were grown during similar environmental conditions, and thus should have started with approximately equivalent cort, notwithstanding individual variation and variation inherent in using multiple primaries from each bird. We consequent-

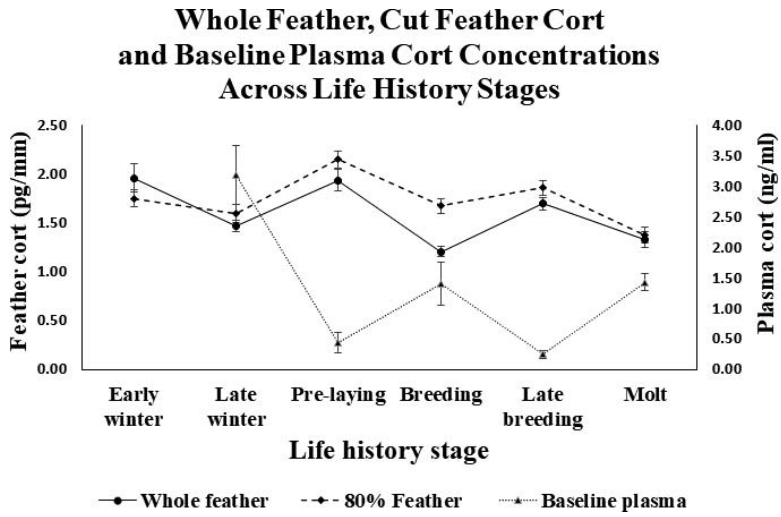

Figure 2. House Sparrow whole and 80% (cut) feather corticosterone concentrations and baseline plasma corticosterone concentrations across life history stages. Plasma corticosterone measurements were taken at the same time the feathers used for this study were collected (previously reported in Lattin et al. 2012). Bars indicate SE.

ly expected the differences in feather wear across life-history stages to be substantial enough to demonstrate a trend, especially when comparing winter (relatively new feathers) to molt (oldest feathers).

Our inability to demonstrate a declining trend as feathers aged, despite the substantial variation discussed above, suggests that perhaps feather cort concentrations remain constant across life history stages. The initial analysis showed the highest feather cort during pre-laying, a middle time point during the year, which did not fit the a priori prediction of a gradual decrease in feather cort and may reflect a methodological artifact. Furthermore, feather cort was equivalent in whole feathers and feathers cut to 80% of initial length. The lack of a difference between whole and cut feathers suggests that normal feather wear has no significant effect on measured feather cort levels. Consequently, despite studies showing that there can be a disproportionate amount of cort in the distal end of feathers (Lattin et al. 2011, Jenni-Eiermann et al. 2015), cutting distal ends off feathers in our study did not significantly affect total feather cort.

In contrast, baseline plasma cort varies significantly in these same birds over the year (Fig. 2; Lattin et al. 2012) and the feather cort pattern does not match the plasma cort pattern. This was expected since feather cort levels are believed to be an integrated measure of plasma cort during the 2–3 week span when the feather was grown, whereas plasma cort levels reflect cort in the blood at the moment of capture. This study provides further evidence that feather cort is isolated from the annual variability in plasma cort outside of molt, suggesting feather and plasma cort reflect distinct aspects of a bird's physiology.

These results support the assumption of a wide range of research that cort levels are stable across the functional life span of feathers. If feather cort levels remain stable, it allows collection of feathers outside of molt to infer physiological condition of the bird during the prior molt. This provides further support for using feather cort concentrations to assess physiological carryover effects from the prior molt to subsequent life-history stages such as reproduction (e.g., Bortolotti et al. 2002, Crossin et al. 2013, Grunst et al. 2015, Harms et al. 2015, Monclús et al. 2017), or to relate this to variation in feather pigmentation and mate choice (e.g., Grunst et al. 2015, Lendvai et al. 2013). It is important to point out, however, that this study only addressed whether feather cort changed during the year it was on the bird's body. Unaddressed is whether feather cort degrades over time once collected, such as in museum specimens.

In addition, the factorial experimental design used in this study did not allow us to conclude that feather cort remains stable despite feather wear in an individual bird, only that it does not appear that feather cort decreases on a population level as feathers age. Further studies could correlate feather cort with actual feather wear, either macroscopically (e.g., light penetration through the feather vanes) or microscopically (e.g., tip abrasion or barbule damage).

In conclusion, these data provide moderate support to the assumption that cort levels deposited in growing feathers remain stable at least through the time when that feather is replaced during the following molt. This should improve confidence that measurements of feather cort allow inferences of physiological condition at the time of feather growth.

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