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Long term stability of corticosterone in feathers

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ABSTRACT

Measuring corticosterone in feathers allows researchers to make long-term, retrospective assessments of physiology with non-invasive sampling. To date, there is little evidence that steroids degrade within the feather matrix, however this has yet to be determined from the same sample over many years. In 2009, we made a pool of European starling (*Sturnus vulgaris*) feathers that had been ground to a homogenous powder using a ball mill and stored on a laboratory bench. Over the past 14 years, a subset of this pooled sample has been assayed via radioimmunoassay (RIA) 19 times to quantify corticosterone. Despite high variability across time (though low variability within assays), there was no effect of time on measured feather corticosterone concentration. In contrast, two enzyme immunoassays (EIA) produced higher concentrations than the samples assayed with RIA, though this difference is likely due to different binding affinities of the antibodies used. The present study provides further support for researchers to use specimens stored long-term and from museums for feather corticosterone quantification, and likely applies to corticosteroid measurements in other keratinized tissues.

1. Introduction

The recent advancement of measuring corticosterone from feathers provides an exciting avenue for non-invasive, retrospective sampling in birds (Bortolotti et al., 2008; Romero and Fairhurst, 2016). It is thought that in the developing feather, corticosterone is deposited through the vascularized feather pulp, between the area of cell proliferation and keratinization/deployment (Bortolotti et al., 2008; Harris et al., 2016; Jenni-Eiermann et al., 2015). Once the feather is fully grown, the pulp cap is formed and the feather is no longer vascularized. At baseline, low levels of circulating corticosterone play an important role in regulating metabolism, while upon the onset of a stressor, an increase in corticosterone aids in survival and/or recovery from that stressor (Sapolsky et al., 2000). Thus, corticosterone measured from feathers represents an average value of circulating corticosterone during the period of feather growth (a timescale of weeks; Bortolotti et al., 2008; Gormally and Romero, 2020; Lattin et al., 2011; Romero and Beattie, 2021).

Measuring corticosterone from feathers is an attractive technique because feathers are easily and non-invasively collected, and can even be collected when the bird is dead or absent (Blas, 2015; Romero and Fairhurst, 2016). Furthermore, unlike plasma samples, feathers do not require specialized storage – storage at room temperature in an envelope or bag will suffice (Blas, 2015; Bortolotti et al., 2009; Romero and Fairhurst, 2016). Because of these advantages, there have been many attempts to correlate physiology and behavior with feather corticosterone since the technique was first described (Bortolotti et al., 2008). For example, feather corticosterone has been shown to correlate (either positively or negatively) with age (Adámková et al., 2019; López-Jiménez et al., 2017), recent previous clutch size (Bortolotti et al., 2008), food restriction (Will et al., 2014), reproductive performance (Will et al., 2014), feather coloration (Bortolotti et al., 2008; Kennedy et al., 2013), carryover effects (Crossin et al., 2017; Harms et al., 2014), temperature (Legagneux et al., 2013; Lynn et al., 2022), condition (Strong et al., 2015), hepatic metal concentration (Strong et al., 2015), and urbanization (Beaugeard et al., 2019).

Some researchers have quantified corticosterone in museum specimens to study birds of the past (Bortolotti et al., 2009; Fairhurst et al., 2015; Kennedy et al., 2013; Strong et al., 2015) and while there is no indication that corticosterone degrades with time, studies thus far have been of an independent measures design. To our knowledge, the present study is the first to quantify corticosterone from the same feather sample pool over time (repeated measures design). Determining the stability of corticosterone in keratinized tissues is critical to their use as an effective tool in both basic and applied biology (Harris et al., 2016).

2. Methods

Feathers were obtained from starlings caught using mist nets in

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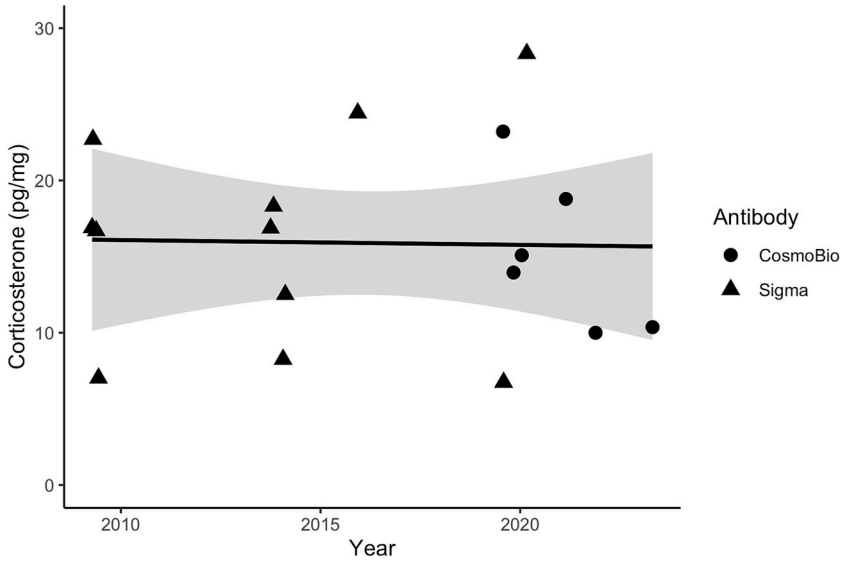

Fig. 1. Corticosterone quantification from starling feathers does not change over 14 years. A subset of the same pooled starling feather samples was assayed multiple times from 2009 to 2023. These assays were radioimmunoassays conducted with either a CosmoBio or Sigma antibody. Each point is the average of multiple replicates in a single assay.

Eastern Massachusetts. All animals were handled with the approval of the Tufts University Institutional Animal Care and Use Committee as well as in accordance for the guidelines to the use of wild birds in research (Fair et al., 2010). In 2009, a pool of starling feathers (138 feathers from four individuals) was ground into a homogenous powder using a ball mill (Lattin et al., 2011) for use as a cross-assay control in radioimmuno- and enzyme immuno-assays in the lab. The pool was stored in a 50 mL conical tube on the bench top, at room temperature and with varying light exposure, to mimic how feathers might be stored in museums, collections, or labs. We have compiled data from the past 14 years of assays using this pool to analyze changes in feather corticosterone over time.

2.1. Corticosterone quantification

All assays started with a steroid extraction protocol from Bortolotti et al. (2008) and Lattin et al. (2011) and described briefly here. First, 7 mL of methanol was added to at least 20 mg of the pooled feathers (Kintz, 2004; Thieme et al., 2003) and the mixture was left in a sonicating water bath for 30 min at room temperature and then in a shaking water bath overnight at 50 °C. Feather remnants were vacuum filtered out of the methanol/extract solution using #4 Whatman filters in a Buchner funnel. The sample tubes, feather remnants, and funnels were rinsed twice with 2.5 mL of methanol, for a total sample volume of 12 mL. Between samples, the filters were changed and the funnels were washed with methanol that was discarded. The methanol/extract solutions were dried under gaseous nitrogen and then extracts were reconstituted with 500 μL of buffer (see below).

The majority of the assays for corticosterone quantification were standard radioimmunoassays (Wingfield et al., 1992) using either a Sigma-Aldrich antibody (Cat. No. C8784, St. Louis, MO) or a CosmoBio antibody (Cat. No. FKA420, Carlsbad, CA). The antibody differences were due to a discontinuation of the Sigma-Aldrich antibody. These two antibodies have been shown to produce similar corticosterone values in European starlings (Fischer et al., 2021). Samples that were assayed with the Sigma-Aldrich antibody were reconstituted with phosphate buffered saline with 1 g/L of gelatin. Samples that were assayed with the CosmoBio antibody were reconstituted with Tris-HCl buffer. Quantified corticosterone values were normalized to sample weight and corrected according to a corticosterone standard run with each assay.

The remaining assays were an enzyme immunoassay from Arbor Assays (Cat. No. K014-H5, Ann Arbor, MI). Extracted samples were reconstituted with the kit-provided Assay Buffer and the assay was then run according to the manufacturer's protocol (with 50 μL of reconstituted extracts being added directly into the plate).

Many assays required multiple runs on the centrifuge, and a separate feather pool sample was included in each of these runs. Feather pools were analyzed both within single assays (replicates from multiple runs in the same assay) and between assays over time.

2.2. Statistics

All statistical analyses were run in R version 4.2.2. Multiple replicate pool samples from the same assay were averaged and statistics were run on average data (average coefficient of variation for replicates within the same assay was 10.8%). A linear model was used to test for an effect of corticosterone concentration over time. The model was run once with data from only the radioimmunoassays (CosmoBio and Sigma antibodies) and once with the addition of the enzyme immunoassay data (Arbor Assays antibody).

3. Results

Among radioimmunoassay-only data, neither time ($t = 0.20$, $p = 0.84$) nor antibody ($t = 0.34$, $p = 0.73$) affected corticosterone concentration. Similarly, when antibody is removed from the model, corticosterone quantification did not change over time ($t = -0.09$, $p = 0.93$; Fig. 1). The trendline has a slope of -8.65×10^{-5}. The average of all radioimmunoassay data is 15.89 pg/mg, the standard deviation is 6.36, and the coefficient of variation is 40%.

We did not have long-term data for corticosterone quantified with an enzyme immunoassay as the two datapoints we had were from March of 2023. The values were 36.99 pg/mg and 38.90 pg/mg but were not included in the figure. When the enzyme immunoassay data is included in the statistical model, corticosterone quantification still does not change over time ($t = 1.36$, $p = 0.19$). The trendline has a slope of 0.002. The average of the whole dataset is 18.21, the standard deviation is 9.18, and the coefficient of variation is 50%.

4. Discussion

The goal of this long-term study was to determine if corticosterone

was stable in the same pooled sample of feathers measured over a 14-year period. Feathers are an attractive matrix for assessing corticosterone as they can be sampled non-invasively and stored easily (Blas, 2015; Bortolotti et al., 2009; Romero and Fairhurst, 2016), but evidence on the stability of this steroid is limited (Harris et al., 2016). In 2009, we created a pool of starling feathers (ground to a powder using a ball mill and homogenized) to assess interassay variability (i.e. variability of replicates in a single assay with multiple centrifuge runs). Across radioimmunoassays (using two different antibodies), there was no change in corticosterone concentration, though the variability was high (Fig. 1). It is unclear why the variability was so high as the feathers were pulverized and highly mixed, but it is consistent across time. It should be noted however, that variability of separate extracts within the same assay was not high (10.8%).

Results from the enzyme immunoassay were higher than all radioimmunoassay results, however this is likely due to the difference in antibodies (Fischer et al., 2021). The current hypothesis is that different antibodies produce different concentrations of corticosterone because of different binding affinities, specificity, and cross-reactivity of the antibodies (Fischer et al., 2021; Lattin et al., 2011; Romero and Fairhurst, 2016). The feather pool in the present study has been consumed, but future experiments should investigate the long-term stability of corticosterone in feathers as measured by an enzyme immunoassay. Regardless of the elevated corticosterone according to the enzyme immunoassay, the results according to the radioimmunoassay are promising evidence that corticosterone is stable in feathers for at least 14 years. Furthermore, these results are also likely to apply to retrospective studies in other keratinized tissues that do not require specialized storage (reviewed in Gormally and Romero, 2020) such as hair (Macbeth et al., 2010; Mastromonaco et al., 2014), nails/claws (Baxter-Gilbert et al., 2014; Matas et al., 2016), snakeskin sheds (Berkvens et al., 2013), baleen (Hunt et al., 2014), scutes (Hamilton et al., 2018), and scales (Aerts et al., 2015), but stability should be independently validated.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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