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ORIGINAL ARTICLE

Can antibody-based assays consistently detect differences in feather corticosterone?

Clare Parker Fischer[1] · Maren N. Vitousek[2] · L. Michael Romero[1]

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Abstract

Measuring corticosterone (Cort) in bird feathers has become increasingly popular as a non-invasive method of obtaining an integrated profile of Cort exposure during the period of feather replacement. Most studies use antibody-based assays to assess Cort levels in feathers [radioimmunoassays (RIA) or enzyme immunoassays (EIA)]. However, it is still unclear whether differences in Cort can be reliably and consistently detected in feathers using antibody-based assays, in part because it is not known how much Cort is present in feathers and antibodies can differ in their ability to detect their antigens. In this study, we tested six commercially available polyclonal Cort antibodies in a feather Cort RIA in nine species. We found that different antisera detected very different levels of Cort in feathers. Additionally, we found that the broad patterns of Cort across species were not the same when measured with different antibodies. Further analysis by mass spectrometry indicated the presence of very little Cort in the feathers of any of the five species tested, suggesting that antibodies were instead binding with Cort metabolites or other substances. These data indicate a potential hidden source of variability when measuring feather Cort with antibody-based tests. The data further suggest caution in cross-species comparisons because patterns seen in feather Cort may reflect artifacts of the specific antibody used in the assay.

Keywords Feather corticosterone · Hypothalamic–pituitary–adrenal axis · Glucocorticoids · Non-invasive technique

Zusammenfassung

Können antikörperbasierte Untersuchungen konsistent Unterschiede im Federkortikosteron nachweisen?

Die Messung von Kortikosteron (Cort) in Vogelfedern erfreut sich als nicht-invasive Methode zunehmender Beliebtheit, um ein Gesamtprofil der Cort-Ausschüttung während des Federwechsels zu erstellen. Die meisten Studien verwenden antikörperbasierte Untersuchungen, um den Cort-Spiegel in Federn zu messen (Radioimmunoassays, RIA, oder Enzymimmunoassays, EIA). Es ist jedoch immer noch unklar, ob Unterschiede im Feder-Cort mit antikörperbasierten Untersuchungen zuverlässig und konsistent nachgewiesen werden können, zum Teil deshalb, weil unbekannt ist, wieviel Cort in Federn enthalten ist und inwiefern Antikörper sich in ihrer Fähigkeit, ihre Antigene zu erkennen, unterscheiden. In dieser Studie untersuchten wir sechs kommerziell erhältliche polyklonale Cort-Antikörper im Feder-Cort-RIA bei neun Vogelarten. Wir fanden heraus, dass die verschiedenen Antiseren sehr unterschiedliche Cort-Mengen in den Federn nachweisen. Darüber hinaus stellten wir fest, dass die allgemeinen Cort-Muster bei den verschiedenen Arten nicht gleich waren, wenn sie mit unterschiedlichen Antikörpern gemessen wurden. Weitere Analysen durch Massenspektrometrie zeigten eine sehr geringe Cort-Menge in den Federn fünf untersuchter Arten, was darauf hindeutet, dass die Antikörper sich stattdessen an Cort-Metaboliten oder anderen Substanzen banden. Diese Daten lassen möglicherweise auf eine versteckte Quelle der Variabilität

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✉ L. Michael Romero
Michael.romero@tufts.edu

[1] Department of Biology, Tufts University, Medford, MA 02155, USA

[2] Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA

bei der Messung von Feder-Cort mit antikörperbasierten Tests schließen. Weiterhin rufen die Daten bei artenübergreifenden Vergleichen zur Vorsicht auf, da die in dem Feder-Cort beobachteten Muster Artefakte durch die in den Untersuchungen verwendeten spezifischen Antikörper widerspiegeln können.

Introduction

Corticosterone (Cort) is the main circulating glucocorticoid hormone in birds (Holmes and Phillips 1976). It is secreted in high concentrations as part of the physiological stress response during a crisis that threatens an animal's homeostasis (Sapolsky et al. 2000). When stressors are repeated or ongoing, an animal can become chronically stressed (Romero and Wingfield 2016). While the acute release of Cort is considered an adaptive response, long-term chronic exposure to elevated levels of Cort is deleterious to health and fecundity (Wingfield and Romero 2001).

Detection of chronic stress in wild animals is an important tool to assess population health (Wikelski and Cooke 2006). Currently, plasma concentrations of Cort are most commonly used to assess the condition of an animal's hypothalamic–pituitary–adrenal axis. Plasma Cort gives an instantaneous snapshot of an animal's current Cort concentrations and changes rapidly in response to events in the animal's immediate past, including the stress of capture (Romero and Reed 2005). Cort is also frequently assayed in fecal samples, where it gives a short-term profile of hormone levels over several hours (e.g. Millspaugh and Washburn 2004; Goymann 2005; Sheriff et al. 2011). However, to assess an animal's long-term Cort exposure over days or weeks, hair (Lavergne et al. 2020; Heimburge et al. 2019a) and feather (e.g. Bortolotti et al. 2008; Aharon-Rotman et al. 2016) analyses are becoming more prevalent. By examining Cort in feathers, it is thought that a more long-term profile of a bird's Cort history can be obtained (Romero and Fairhurst 2016), although this assumption has lately been questioned (Kalliokoski et al. 2019).

Bortolotti et al. (2008) originally developed a method to extract Cort from feathers and assay it by radioimmunoassay (RIA). Cort is deposited in the feather as it grows (Jenni-Eiermann et al. 2015). This creates two features of feather Cort. First, because mature feathers are not connected to the blood once growth is completed, there is no known mechanism for plasma Cort to be deposited in feathers once feather growth is complete, although several mechanisms have been proposed (Gormally and Romero 2020; Heimburge et al. 2019b). Second, because Cort is slowly deposited in growing feathers throughout the several-weeks growing period, feather Cort reflects the overall integrated history of an animal's Cort experience during feather growth and is thus minimally impacted by events in the immediate past, such as capturing the animal to obtain a sample. Furthermore, feathers are shed naturally during molt, so non-invasive collection of samples is possible. Feather Cort also appears to be heat stable and may remain unchanged in feathers for decades (Bortolotti et al. 2009). This could allow very old specimens to be compared with more recently collected birds (Kennedy et al. 2013).

The technique for assaying feather Cort is still under development, and a number of recent studies have helped define important parameters for its use (e.g. Freeman and Newman 2018; Berk et al. 2016; Alba et al. 2019; Harris et al. 2016; Fischer et al. 2017). One key issue, however, is the use of different antibodies in enzyme immunoassays (EIA) or radio immunoassays (RIA) used in different commercial kits and in different laboratories. The antibodies are used to bind to Cort to allow quantification of Cort in the substrate, but different antibodies naturally have different affinities to their intended substrates. Lattin et al. (2011) found variability in the effectiveness of two different antisera in detecting feather Cort via RIA. The antiserum used by Bortolotti's group (from Sigma), detected Cort in feathers, but an antiserum often used for plasma Cort RIAs (produced by Endocrine Sciences) failed to detect any feather Cort. The reason for this difference is not presently clear but may reflect divergent binding affinities and cross-reactivities of the different antisera. This problem is similar to work in fecal samples, where different antibodies have proven to have different effectiveness in detecting Cort metabolites (Palme 2019; Bienboire-Frosini et al. 2018; Young and Hallford 2013; Stowe et al. 2008). However, because the antibodies supplied in commercial kits are often unique to each kit, and because EIA and/or RIA kits are currently the predominant way in which feather Cort is assessed, the variability associated with different antibodies is potentially a major hidden confounding variable in studies of feather Cort. In this study, we tested six polyclonal Cort antibodies produced by five companies in the feather Cort RIA. We tested feathers from nine species across a range of taxonomic groups, including waterfowl, raptors, and passerines. We further performed liquid chromatography-Mass Spectrometry (LC–MS/MS) in five of those species to determine how much Cort was actually in the feathers.

Materials and methods

Sources of feathers

Feathers from nine species of birds were analyzed. The Tufts Veterinary School (Medford, Massachusetts) donated

feathers from wild birds treated at their clinic. We used flight feathers from one each of: Mute Swan (*Cygnus olor*), American Black Duck (*Anas rubripes*), Common Loon (*Gavia immer*), Northern Gannet (*Morus bassanus*), Great Blue Heron (*Ardea herdias*), Cooper's Hawk (*Accipiter cooperii*), and Red-Tailed Hawk (*Buteo jamaicensis*). Additionally, one European Starling (*Sturnus vulgaris*) and five House Sparrows (*Passer domesticus*) were wild-caught and kept in the lab for several months. They had been involved in other experiments in the lab but their feathers had been grown in the wild the previous fall. Body and flight feathers from the five House Sparrows were pooled together to provide sufficient sample mass. Flight and body feathers from the European Starling were pooled. These animals were captured and housed according to AALAC guidelines and approved by the Tufts University Institutional Animal Care and Use Committee.

Bortolotti et al. (2008), further supported by Jenni-Eiermann et al. (2015), suggested that feather Cort is more accurately reported in pg Cort mm^{-1} feather to account for the way that feathers grow and the mechanism by which Cort is thought to integrate into the keratin structure of the feather. However, Cort may vary by feather mass and may not be uniformly distributed throughout the feather (Lattin et al. 2011; Harris et al. 2016). Therefore, to have samples that were consistent for each test, the calamus was removed from all feathers and the vanes and rachis were minced with scissors and ground into a homogenized powder using a ball mill. This means that every sample used in the following assays should have exactly the same concentration of Cort that reflects the average concentration in each feather. Cort concentrations were standardized by mass of feather powder and reported in pg Cort mg^{-1} feather.

Antisera

Six Cort antisera from five companies were tested in the RIA. The antiserum from Sigma (C8487, St. Louis, Missouri, USA Lot # 081M4788) was used by Bortolotti's group (2008, 2009) and has been used for the feather Cort RIA in our lab (Lattin et al. 2011). Antisera from EMD Millipore (AB1297, Billerica, Massachusetts, USA Lot # NG1749017) and Cosmobio USA (FKA 420, Carlsbad, California, USA) were specified for use in ^{3}H RIAs. We used two polyclonal antibodies from MP Biomedical (Solon, Ohio, USA), which we will refer to as MP #1 (07-120,016, Lot # 3R3PB-19E) and MP #2 (07120115, Lot # 3R6-5-9BO-3E). The MP #1 antiserum was also specified for use in ^{3}H RIAs. However, the MP #2 antiserum was specified for use in ^{125}I RIAs—we optimized the concentration for the tritium RIA. The antiserum from Pantex (CORTICO-3-#227, Amarillo, Texas, USA, Lot #227) was specified for use in EIAs—we adapted it for use in the ^{3}H RIA.

Details on the cross-reactivities for each antibody, as reported by the manufacturers, is in Table 1. The Cort RIA was tested for each antibody using 200 pg of purified Cort. They were also tested using 30 µl of a common plasma pool from a Chukar partridge (*Perdrix choukar*), which was

Table 1 Steroid crossreactivity values for the six antisera used in feather Cort RIAs

	Sigma	Cosmobio	MP #1	Pantex*	EMP Millipore	MP#2†
Corticosterone	100	100	100	100	100	100
Aldosterone	4	<0.01	0.08	<0.001	0.2	<0.01
4-Androstenedione	2.6	1.2	0.01	<0.001	n.r	0.02
Cortisol	4.5	2.2	0.19	0.06	<0.01	0.03
Cortisone	3.2	0.23	n.r	n.r	<0.01	n.r
11-Dehydrocorticosterone	n.r	4.7	n.r	n.r	0.67	n.r
Dehydroepiandrosterone	<10	<0.01	<0.01	<0.001	n.r	<0.01
11-Deoxycorticosterone	20	4.8	6.1	1.97	1.5	0.39
5α-Dihydrotestosterone	1.4	n.r	<0.01	<0.001	n.r	0.04
Estradiol	<30	<0.01	<0.01	<0.001	n.r	<0.01
17-Hydroxyprogesterone	1.8	0.3	<0.01	<0.001	n.r	<0.01
20α-Hydroxyprogesterone	8.8	n.r	n.r	n.r	n.r	n.r
20β-Hydroxyprogesterone	5.2	n.r	n.r	n.r	n.r	n.r
Progesterone	15.7	5.4	0.29	<0.001	n.r	0.04
Testosterone	7.9	0.35	0.08	<0.001	n.r	0.28

Crossreactivity information was supplied by the manufacturer. Reactivities were measured by ^{3}H RIA unless otherwise indicated

n.r. not reported

*Crossreactivities measured by EIA

†Crossreactivities measured by ^{125}I RIA

extracted in dichloromethane, dried under nitrogen, and run in a standard RIA protocol (Dickens et al. 2009; Wingfield et al. 1992) (the plasma pool was not run for the Sigma antiserum due to an oversight and subsequent exhaustion of the pool).

Extraction

100 mg of homogenized feather powder per species was used for each of three antisera (Sigma, MP #1, and EMP Millipore); 150 mg was used for each of the other three antisera (MP #2, Pantex, and Cosmobio). Extracts from feather masses greater than 50 mg have been shown to have repeatably measurable quantities of Cort (Lattin et al. 2011). Feather powder was mixed with 7 mL HPLC-grade methanol (Bortolotti et al. 2008), placed in a sonicating water bath for 30 min at room temperature, and incubated overnight in a 50 °C shaking water bath. Feather particles were removed with vacuum filtration using #4 Whatman filter paper and a Buchner funnel. The tubes, feather particles, and funnel were rinsed twice with 2.5 mL methanol, which was added to the methanol extracts. Methanol extracts were dried under nitrogen and kept at 4 °C until use. 1 mL RIA buffer (phosphate-buffered saline (PBS) containing 1 g L^{-1} gelatin) was added to samples the night before running the assay. Some feather powder was not removed by filtration—after samples were resuspended in RIA buffer, all particulate material was removed by centrifugation. The resuspended samples were diluted further in RIA buffer and run at 5 concentrations: undiluted, 1:2, 1:4, 1:8, and 1:16. For the MP #2 antiserum, only the first three dilutions were assayed. The feather extracts were then run through a standard RIA protocol (Lattin et al. 2011; Wingfield et al. 1992) using the six antisera. The amount of Cort per sample was determined from standard curves using JMP (Version 5. SAS Institute Inc., Cary, North Carolina, USA).

LC–MS/MS analysis

Feather extracts (10 µL of a 150 µL sample obtained from extracting 150 mg of homogenized feather—the same feather pool as described above) were analyzed for Cort in a triple-quadrupole LC–MS system (Pump, autosampler, and PDA detector: Accela; MS: TSQ Quantum Access; Thermo Scientific) equipped with an atmospheric pressure chemical ionization (APCI) interface in positive ion mode. Tune parameters were first tested with an electrospray (ESI) interface and then optimized with APCI using a 10 ng/µL solution of Cort in the mobile phase (50:50% acetonitrile:MilliQ water, both with 0.1% formic acid) in auto-loop injection mode at a mobile phase flow rate of 200 µL/min. Compounds were separated on a Kinetex 2.6 µm EVO C18 100 Å LC column (150 × 2.1 mm; Phenomenex) at a mobile phase flow rate of 200 µL/min with the following gradient: 0–2 min 30:70% B:C (acetonitrile:MilliQ water, both with 0.1% formic acid), 2–10 min 30% to 70% B, 10–12 min 70% B. Ultraviolet spectral data were collected at 200–600 nm and the MS was operated at selected ion monitoring for the transitions: *m/z* 347 to 121 (collision energy: 26 V) and *m/z* 347 to 329.1 (collision energy: 14 V) at a 1.5 mTorr collision pressure. The detection limit was 2 pg/µL or 0.3 ng/sample. Five species were chosen for analysis: mute swan, northern gannet, red-tailed hawk, house sparrow, and European starling.

Results

We obtained good standard curves for all six antibodies ($R^2 > 0.98$ for all). Pantex had a narrower range of detection than the other antibodies and was not able to detect low levels of Cort (Pantex could detect ≥32 pg per sample; all other antisera could detect ≥8 pg per sample). All antibodies were able to detect comparable amounts of the purified Cort standard (Fig. 1a, coefficient of variation = 9.7% as

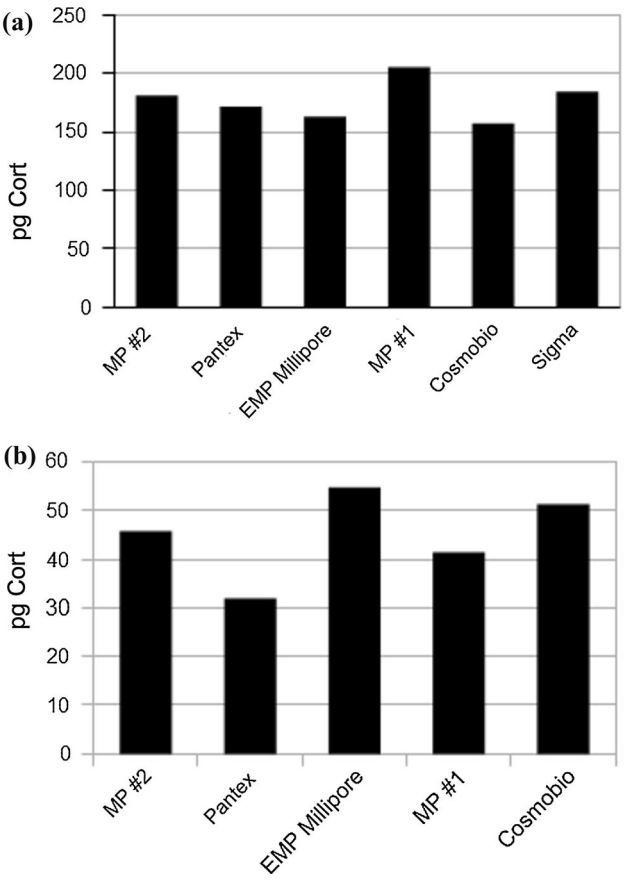

Fig. 1 **a** Detection of a standard quantity of purified Cort by the six antibodies. A known quantity of Cort (200 pg) was included per sample tube. **b** Detection of Cort in a plasma pool (chukar partridge). The plasma pool was run for all antibodies except Sigma

computed from the variation around the mean value from the 6 antibodies). The antibodies were more variable in their detection of Cort in the plasma pool, with Millipore detecting 1.7 times as much Cort as Pantex (Fig. 1b, coefficient of variation = 19.8%).

The six different antibodies yielded very different results in the feather Cort RIA (Fig. 2). Sigma, Cosmobio, and MP #1 detected higher levels of Cort for all species than the other antisera. In comparison, the Millipore, Pantex, and MP #2 antisera detected very low levels of Cort, frequently near or below the limit of detection. Pantex was not able to detect any Cort for five of the nine species. Across antibodies, the northern gannet consistently had the highest concentration of Cort. The house sparrow, European starling, and Cooper's hawk all had higher Cort concentrations relative to other species with all antibodies. However, there are some subtle differences in the broad patterns of detected Cort between the different antibodies that could lead to different interpretations of results, depending on which antibody was used in an assay (see "Discussion" section).

To ensure that nothing in the feather extracts was non-specifically interfering with the RIA, we performed the assay on serial dilutions of the feather extracts. We checked for parallelism with the standard curve by plotting the total Cort detected (on a log scale) against the percent of radioactivity bound to the antibody (Fig. 3). All antibodies generally had good parallelism, though more diluted samples were sometimes too low to detect. Note that because several antibodies did not detect any Cort from some species (e.g. the Pantex antibody failed to detect Cort in five species), there could be no dilution curves for those antibodies and those species (e.g. Fig. 3e reports dilution curves for the Pantex antibody and thus is "missing" five curves).

The analysis of purified corticosterone using LC–MS/MS produced a strong peak that eluted at 5.63 min (Fig. 4a). In only northern gannet, however, was a similar peak identified (Fig. 4c), but that peak was too small for quantitative analysis. No other species showed an elution peak that corresponded to corticosterone (Fig. 4b, d–f).

Discussion

Evidence is building that increased levels of immunoreactive Cort in the feather generally reflect heightened concentrations of Cort in the plasma. Indirect evidence comes from artificially elevating (e.g. Fischer et al. 2017; Lattin et al. 2011; Aharon-Rotman et al. 2017; Fairhurst et al. 2013) or inhibiting (e.g. Horak et al. 2013; Aharon-Rotman et al. 2017) plasma Cort and finding a corresponding signal in the growing feathers, but the strongest evidence is that radiation from radiolabeled Cort injected into the plasma is detected in the feather (Jenni-Eiermann et al. 2015). For the birds used in this study, plasma Cort at the time of feather growth was unknown. All individuals were free-living at the time of their previous molt when the tested feathers were grown. The nine species showed a wide range of feather immunoreactive Cort. Note, however, that the goal of this study was not to explore that variation, but rather to test the efficacy of various antibodies in detecting Cort in feathers.

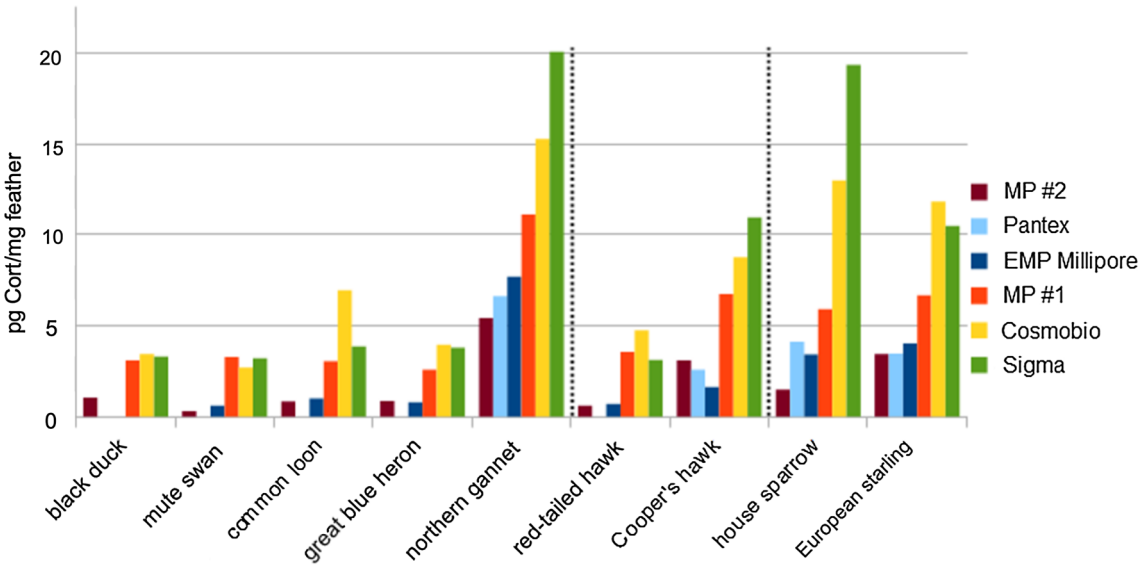

Fig. 2 Different antisera in the RIA detect different levels of Cort in feather extracts from nine species. The *dotted vertical lines* separate rough taxonomic groups—waterbirds, raptors, and passerines. The great blue heron, northern gannet, and Cooper's hawk were immature birds. Pooled flight feathers were used for all birds except the European starling (flight and body feathers pooled from one individual) and the house sparrow (flight and body feathers pooled from five individuals)

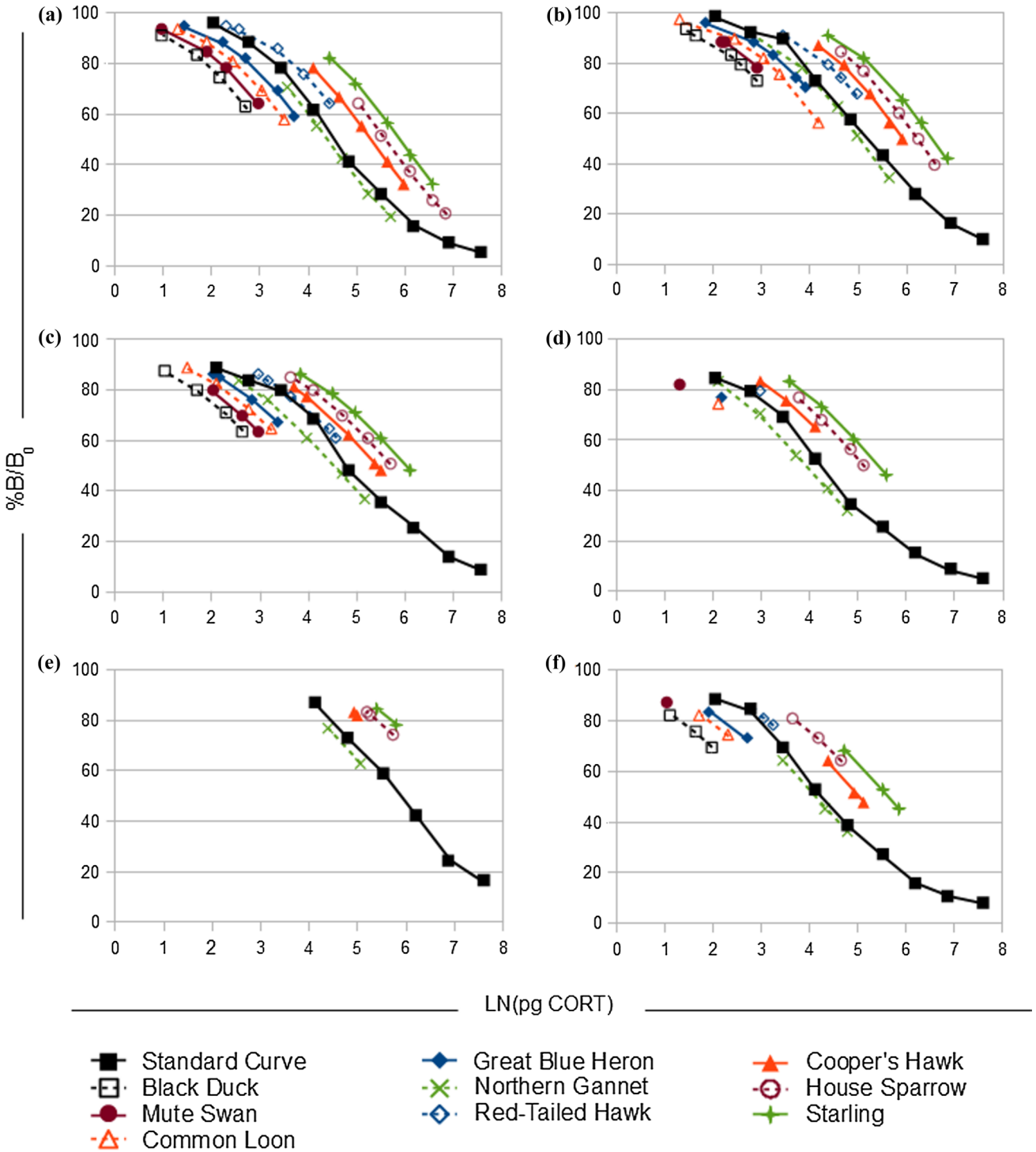

Fig. 3 Parallelism of serial dilutions of feather extracts from nine species with a standard curve. The natural log of the total Cort detected is graphed against the percent radioactivity bound to antibody over total radioactivity for each of the antibodies: **a** Sigma, **b** Cosmobio, **c** MP 1, **d** Millipore, **e** Pantex**,** and **f** MP 2. The points for the nine species were moved laterally for clarity

As a consequence, the use of a homogenized and standard concentration of Cort for each species was more important for this goal than the evaluation of either interindividual or intrafeather variation. Exploration of both of these topics would be an interesting target for future studies but beyond the scope of this study.

The six antibodies tested measured very different levels of feather immunoreactive Cort. The Sigma, Cosmobio, and

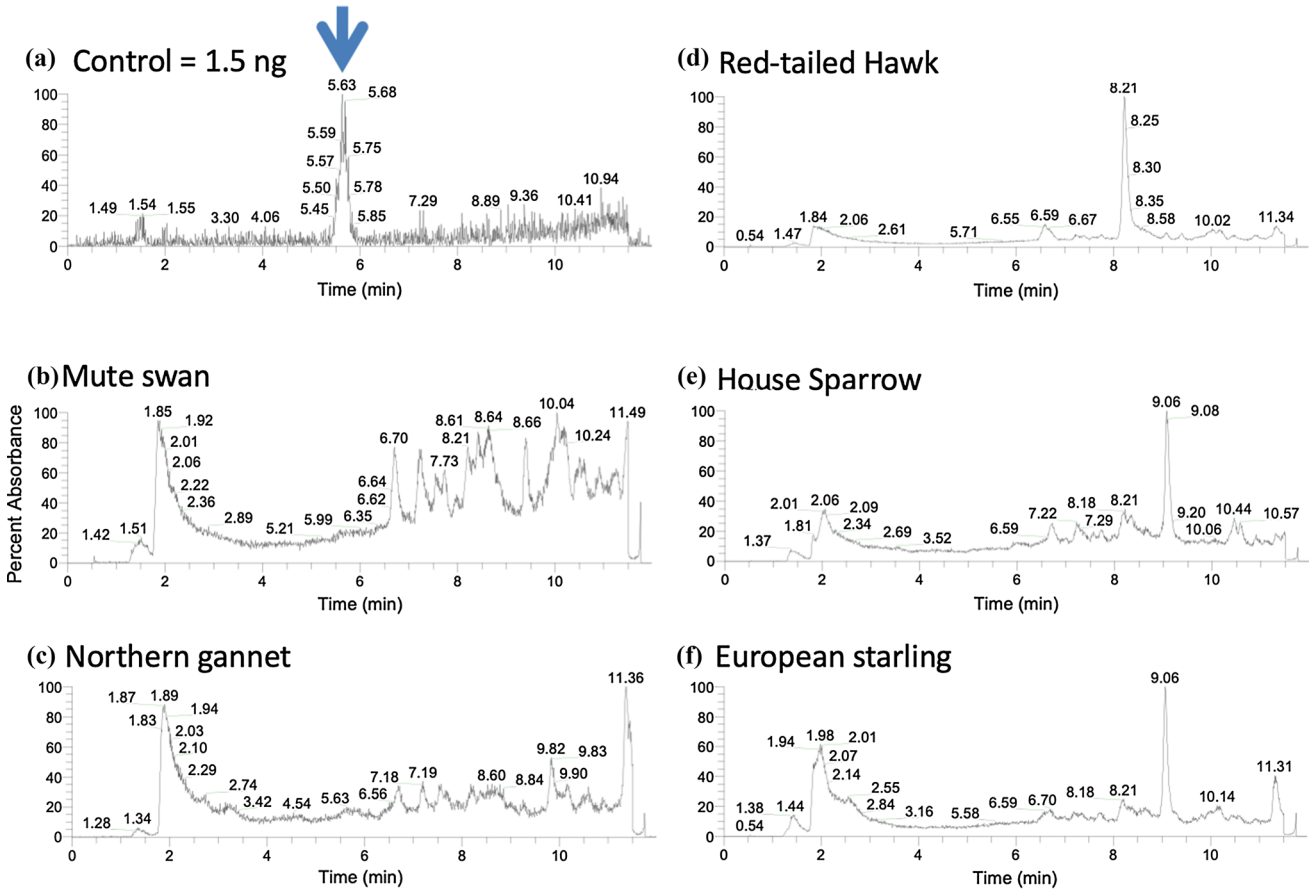

Fig. 4 Elution profiles of purified corticosterone (**a**) and steroid extractions from five avian species (**b**-**f**). *Blue arrow* indicates the elution peak (5.63 min) for the purified corticosterone. Elution peaks were automated and indicated by a time stamp

MP#1 antisera consistently measured higher concentrations of feather Cort in the nine species than the Millipore, Pantex, and MP#2 antisera. Two of the antisera that worked less well (MP #2 and Pantex) had been developed for use in other assays (^{125}I RIA and EIA, respectively) and concentrations for these antibodies in the ^{3}H RIA had to be determined experimentally. Although they still produced good standard curves, the total binding of radioactive Cort to these two antibodies was low relative to the other antisera (data not shown). All antibodies were able to detect comparable amounts of a purified Cort standard (Fig. 1a), suggesting that interactions with other molecules present in biological samples is driving the differences between the antibodies' performance. This is similar to reports from feces and hair (Jewgenow et al. 2020; Palme 2019). On the other hand, serial dilutions of feather samples from all species produced lines parallel to the standard curve (Fig. 3), suggesting that all antibodies performed adequately in a quasiquantitative manner (Lee et al. 2006; Valentin et al. 2011). Freeman and Newman (2018) also reported parallelism in dilution in feather extracts from other species. Furthermore, the antibodies that detected very low levels of immunoreactive Cort in the feathers did not consistently detect less Cort in the plasma sample (compare Figs. 1b and 2). For example, in feathers, MP #1 detected more immunoreactive Cort in all samples than MP #2. However, in plasma, the two antisera performed very similarly. This suggests that the factors that cause differences in the performance of antibodies in the RIA are unique to the sample substrate being analyzed. Note, however, that no currently commercially available kit has been optimized for measuring Cort in feathers and so we are reliant upon antibodies that were optimized for other biological substrates (e.g. plasma).

The pattern of relative feather immunoreactive Cort amount detected by the different antibodies was broadly similar—the antibodies always measured the most Cort in the Northern Gannet, and much less in the Black Duck, Mute Swan, Great Blue Heron, and Red-Tailed Hawk. However, there were some striking differences in relative Cort amounts detected across species for some antibodies. The Cosmobio antiserum detected relatively more Cort in the Common Loon than the other antisera. More Cort was detected in the House Sparrow than the European Starling when the Sigma antiserum was used, but the other antisera found those two

species to be similar. Therefore, results from an RIA with one antibody may differ from results from an RIA using a different antibody, so that results that appear to show differences between species may or may not be biologically real.

The differences between the antibodies (both in terms of overall amounts of steroid detected and in the relative differences between species) may be due to the different cross-reactivities of the antisera (Table 1). The low specificity of the Sigma and Cosmobio antibodies may mean that another steroid was interfering with Cort detection. Different Cort metabolite profiles might also explain the differences between antibodies. Bortolotti et al. (2008 supplementary material) found high levels of gluconidated and sulfonated Cort in feathers, indicating that Cort may be metabolized in the feather follicle or skin. The Sigma antibody was not reactive to these Cort metabolites but the other antisera may be. Berk et al. (2016) also found both Cort and other metabolites in the feathers using high-performance liquid chromatography. In contrast, Koren et al. (2012), using liquid chromatography and tandem mass spectrometry, found Cort in only 16 of 61 house sparrow feathers. These results are similar to the data reported here (Fig. 4) where the house sparrow did not have detectable levels of Cort. Other unidentified substances are also present in all five species analyzed here, which may be differentially detected by the different antibodies. Consequently, despite Cort injection studies indicating that Cort is being deposited in feathers, the data from this study and others indicate that the evidence is still mixed whether the antibodies used in RIA or EIA studies are detecting Cort. We were unable to separate and isolate the different substances indicated in Fig. 4, which may or may not be non-Cort steroids or steroid metabolites, and analyze them in our assays. This would have helped ascertain what the antibodies were actually detecting and would be important for a follow-up study.

Regardless of whether Cort metabolites are present and detected by the feather RIA, the concentration of immunoreactive Cort detected in the feathers may still be reflective of plasma Cort levels, but would be influenced by individual or species differences in Cort metabolism. Fecal Cort assays are known to measure metabolites of the steroid rather than the form of Cort present in the plasma—Cort is metabolized by the liver before it is secreted into the GI tract (e.g. Möstl et al. 2005). When analyzing fecal Cort, it is, therefore, necessary to consider what factors might change Cort metabolism (Goymann 2012; Millspaugh and Washburn 2004; Sheriff et al. 2010) and similar considerations may apply to measuring feather Cort.

Measuring feather Cort is an intriguing new tool that may reflect the history of Cort exposure experienced by birds. Our results indicate that feather Cort is detectable in a wide range of species. However, despite a growing number of studies, it is still not known precisely what molecules we are currently detecting when using antibody-based techniques. This is a major concern when relying upon antibody-based assays. Of further concern is that the binding characteristics of the antibodies used in various EIA and RIA kits that are often used for feather Cort analyses are generally unavailable, making it difficult to compare results across studies. The specific kit that is purchased for the study could have a profound impact on the ultimate results. This suggests caution: patterns seen in feather Cort, particularly in cross-species comparisons, as in this study, may be a mere artifact of the antibody used in the assay. Similar results were recently reported for different EIAs in measuring cortisol in the hair (Jewgenow et al. 2020). Further studies of both within-individual, within-species, and cross-species variation, especially in those species that do not have Cort in feathers, would be especially valuable.

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Code availability Not applicable.

Availability of data and material The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest None.

Ethics approval All animal experiments were approved by the Tufts University Institutional Animal Care and Use Committee.

Consent to participate Not applicable.

Consent for publication Not applicable.

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