Measuring Corticosterone in Feathers:
Strengths, Limitations, and Suggestions for the Future

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Abstract:

The recently introduced technique of measuring corticosterone in feathers currently provides the longest-term measure of corticosterone in birds. This review examines the strengths, weaknesses, and unresolved technical issues of the feather corticosterone technique. Feather corticosterone's major strengths are that it provides: a retrospective assessment of corticosterone physiology, including information from absent (unseen) or dead (e.g. museum specimens) individuals; a long-term measure of corticosterone exposure over the period of feather growth (days-weeks), integrating both baseline and responses to stressors; and flexible, minimally-invasive, sampling. However, researchers considering this technique should be aware of its limitations. Feather corticosterone only reflects hormone exposure during feather growth and, when sampling during molt, corticosterone titers and ecological conditions may not be representative of the majority of the annual cycle. Synchronization of molt is often unknown for a population, requiring assumptions when making inter-individual comparisons. Additionally, unresolved technical issues include: assessing whether corticosterone is the only hormone measured by assays; determining deposition dynamics to fully understand connections between feather and plasma corticosterone titers; studying the longevity and stability of corticosterone in the feather; establishing the impact of feather size and color on corticosterone deposition; and understanding the causes and implications of corticosterone variation along the length of the feather. Notwithstanding the above limitations and technical challenges, determining corticosterone titers in feathers is proving to be a useful technique for exploring some ecological and physiological correlates in individual birds. Given the unique perspective that feather corticosterone offers, we suggest that this measure complement, not replace, plasma measurements.

Introduction:

For over 80 years measuring plasma glucocorticoid titers has been the preeminent technique to determine whether an individual is experiencing stress (e.g. Romero and Wingfield 2016). Glucocorticoids are released from the adrenal or interrenal gland in response to either perceived or actual noxious stimuli (stressors) and can have powerful impacts on physiology (Sapolsky et al. 2000). These physiological changes are believed to help the animal counteract the stressor and return to normal life-history behaviors and functions (Wingfield 2013). However, substantial evidence indicates that extended, or chronic, secretion of glucocorticoids can have deleterious effects and can lead to stress-induced disease (Sapolsky et al. 2000). Attempts to define the balance between beneficial and deleterious effects of glucocorticoids has led to a number of theoretical models for glucocorticoid function. These include allostasis (McEwen and Wingfield 2003) and reactive scope (Romero et al. 2009), with a recent comparison of the two models (Blas 2015). Consequently, there has been considerable interest in using glucocorticoid responses to understand the impacts of natural and anthropogenic stressors on the health, survival, and coping mechanisms of free-living animals.

Glucocorticoids have been measured in free-living individuals from a large number of species (Romero and Wingfield 2016). Initially, field studies adapted laboratory techniques and focused on measuring glucocorticoid titers in plasma (e.g. Sapolsky 1982; Wingfield et al. 1982). These studies recognized that capture of wild animals would induce a stress response, but they used that fact to create a standardized stressor that allowed comparisons across individuals experiencing different conditions prior to capture (Wingfield and Romero 2001). Many species, however, are not amenable to capture and sampling of the plasma, either because they were difficult to catch, difficult to bleed quickly, or are endangered. Consequently, substantial effort went into establishing noninvasive methods for assessing or inferring glucocorticoid titers. The first such technique was to measure glucocorticoids in feces (e.g. Millspaugh and Washburn 2004; Möstl et al. 2005) and this was followed more recently with measurements in hair (e.g. Davenport et al. 2006), baleen (Hunt et al. 2014), and turtle claws (Baxter-Gilbert et al. 2014). An important feature of the last three examples is that they are keratinized tissues. Birds also have a major keratinized tissue – feathers.

Measurements of corticosterone, the major glucocorticoid in birds (Holmes and Phillips 1976), in feathers was pioneered by Bortolotti and colleagues (Bortolotti et al. 2008; Bortolotti et al. 2009). They established that corticosterone is deposited and measurable in feathers, and that the amount of corticosterone deposited is correlated with stressors experienced by the bird during feather growth. In the years since their inaugural paper, measurement of corticosterone in feathers has generated considerable interest as a noninvasive technique for monitoring stress and energy management in birds. Immunoassays allow a variety of researchers to measure corticosterone in feathers, creating new and exciting research avenues. This paper will explore the strengths and weaknesses of this relatively new technique and identify technical questions that still need to be addressed (Fig. 1). We hope that this review will be useful for helping researchers better understand feather corticosterone analysis, guide future research, and put feather corticosterone on even stronger theoretical and practical foundations.

Brief Description of the Method:

Most studies are currently measuring feather corticosterone using the general method originally described by Bortolotti et al. (2008), some with small modifications. Feathers are processed by removing and discarding the calamus, then mincing the rachis and feather vanes into pieces <5 mm^2. A device to remove static can be helpful during this step. Corticosterone is then extracted from feathers using methanol, with samples placed in a sonicating water bath at room temperature for 30 min and then incubated overnight in a shaking 50°C water bath. After separating methanol from feather using vacuum filtration, methanol extracts are dried under nitrogen gas and reconstituted in a saline buffer.

Samples are assayed using either a radioimmunoassay (Lattin et al. 2011; RIA, e.g. Fairhurst et al. 2012a), enzyme immunoassay (EIA, e.g. Jenni-Eiermann et al. 2015; Kouwenberg et al. 2015), or liquid chromatography coupled with mass spectrometry (e.g. Koren et al. 2012). An important step with any of these analytical techniques is estimating the efficiency of the extraction of hormone from feather. In extractions of plasma corticosterone, addition of radiolabeled corticosterone to plasma closely mimics the natural occurrence of corticosterone in solution. However, mimicking hormone

embedded within the feather matrix presents a challenge when estimating extraction efficiency with feathers. Some RIA studies have approached this by adding a small amount of radioactive corticosterone to feathers during the addition of methanol and tracing this radioactivity through the extraction process (e.g. Bortolotti et al. 2008; Lattin et al. 2011; Fairhurst et al. 2012b; Lendvai et al. 2013; Harms et al. 2015). However, the spiked steroid does not arise from the feather matrix and, thus, this procedure estimates the *recovery* of hormone through filtering, not the true *extraction* efficiency. An alternative control is to create a homogenous pulverized dust mixture of pooled feathers (e.g., using a Ball mill) and aliquots of this feather "dust" can then be used as standards in each assay to assess inter-assay variation and methanol extraction efficiency (e.g. Lattin et al. 2011). However, this process may increase retention of methanol (and thus corticosterone; see below).

Because of our current understanding of how feathers grow, elongating along their radial circumference, corticosterone values are generally normalized to feather length rather than mass (Bortolotti et al. 2008; Bortolotti et al. 2009; Bortolotti 2010). It also appears that corticosterone is deposited in the feathers during the cell differentiation phase of feather growth, when cells become keratinized and pigment are incorporated, rather than the cell proliferation stage (Jenni-Eiermann et al. 2015). This means that the cells incorporating corticosterone are in the quill that is directly exposed to the blood rather than in the follicle.

Categories of Feathers:

In theory, corticosterone can be measured in any feather because all feathers are connected to a blood supply while growing and, thus, contain hormone that is deposited from general circulation. However, this does not necessarily make any feather corticosterone measurement useful for physiological, biological, or ecological studies. As is the case with measurements of corticosterone from blood, the utility of feather corticosterone data is greatly improved if the researcher can characterize the basic ecophysiological state of the bird as close as possible to the period of hormone

deposition. For feather corticosterone, important ancillary data include the date and geographic location of feather growth, and the age, sex, biometrics, and body condition of the bird, some of which may themselves be endpoints of research. All of these variables are important for understanding (and standardizing) variation among individuals. Because feathers can be sampled months after they have grown, the range and reliability of ancillary data that can be collected vary with the source of feathers. Thus, researchers should be aware when using feather corticosterone of the trade-offs between quality of ancillary data (and thus the ability to interpret feather corticosterone), improved access to feathers, reduced cost of feather collection, and sample sizes. Below we introduce the main sources of feathers, discuss their benefits and limitations, and summarize this information in Table 1.

Known live birds

Researchers frequently have access to wild-caught or captive live birds, making known live birds (i.e., feather identity can be reliably assigned to a living individual) the most common source of feathers for feather corticosterone data. Although fieldwork and animal husbandry can be resource-intensive, feathers collected from live birds have several unique benefits. Perhaps most importantly, live birds provide opportunities for performing experiments during the feather growth period, either by plucking feathers and inducing feather regrowth (e.g. Fairhurst et al. 2011; Lattin et al. 2011; Sild et al. 2014), or by sampling individuals during natural molt (e.g. nestlings; Fairhurst et al. 2012a; Fairhurst et al. 2012b; Fairhurst et al. 2013a; Harriman et al. 2014). This is particularly valuable because the exact period of feather growth is known, the timing of feather regrowth and environmental conditions during that period can be better standardized among individuals, and a variety of ancillary data can be collected during feather growth and thus synchronized and/or correlated with feather corticosterone.

In many cases, however, feathers are already fully grown when collected from live birds. Although ancillary data can be collected at the time of capture, information from the feather growth period, including where and when the feather grew, must be inferred or assumed. Despite this, other metrics from the feathers themselves (e.g., stable

isotope ratios, growth bar width) can help reconstruct some of this information and provide ancillary data (e.g. Fairhurst et al. 2013b; Fairhurst et al. 2014; Fairhurst et al. 2015b). For example, species-specific molt phenology can be useful for estimating the timing of feather growth, and sex can be determined genetically using feathers (Griffiths et al. 1998; Horvath et al. 2005).

Thus, feathers from known live birds provide the greatest scope for research, best access to ancillary data, and potentially the best quality feather corticosterone data. These benefits come at a cost of having to capture/recapture the birds to collect feathers and, in the case of captive populations, house and maintain the birds.

Known dead birds

Carcasses provide a second source of feathers. Collection location and date, age, sex, and biometrics are often known for museum specimens, and carcasses from veterinary clinics and rehabilitation centers may come with additional clinical ancillary data collected while the bird was alive. However, the ancillary data from feathers from found carcasses are limited to what can be collected from the dead bird (e.g., age, sex, some biometrics) or the feathers themselves (e.g., stable isotopes, growth bar width). The timing and location of feather growth, and thus the corresponding ecological and physiological conditions, often must be assumed, though species-specific molt phenology can be useful for estimating the timing of feather growth. The benefits of using feathers from carcasses are that no trapping is needed, inexpensive opportunistic sampling is possible (e.g., road and window kills) and, in the case of museum specimens, a variety of unique questions can be addressed (see below). Note, however, that whereas collection of feathers from carcasses is often inexpensive, ascertaining the important ancillary data (e.g. stable isotope and sex analyses) may not be.

Unknown sources

Feathers found on the ground, lining old nests, or incorporated into cultural or archaeological artifacts (e.g., Native American war bonnets) comprise a third source of

feathers. This type of feather can be abundant in some places (e.g., communal roosts, breeding colonies) and can be collected relatively inexpensively. However, in many cases virtually nothing can be known of the individual(s) that grew the feather(s) other than ancillary data from the feather itself, which greatly limits the inferences that can be made about the feather corticosterone data. Like museum specimens, cultural artifacts provide a means of understanding historical levels of feather corticosterone (see below), though the opportunity to sample feathers from such valuable pieces is likely to be limited.

Overall Biological Strengths:

The strength of using feathers to measure corticosterone is that they provide a retrospective, long-term, integrated measure of corticosterone physiology. This provides a fundamentally different perspective than what is possible from blood sampling and should be viewed as complementing, not replacing, the latter. Using feathers allows corticosterone measurements to be made from species and time periods that are otherwise difficult or impossible to sample, and sampling feathers has practical advantages in the field. These unique strengths change the types of biological and ecological questions that can be asked using hormone data. Below we discuss these attributes in more detail, focusing on the benefits that feather corticosterone can bring to biological studies.

A retrospective measure

One of the greatest strengths of using feathers to assess corticosterone levels is that measurements can be made after the hormone has been secreted and deposited into feathers. This has advantages for storing feathers (see below), but also makes it possible to measure corticosterone from a range of species, in locations, and from time periods that are effectively inaccessible for blood sampling. For example, many species of migratory birds are logistically difficult or impossible to access or capture after the breeding period, but they molt during non-breeding and will return with those feathers to

breeding grounds the subsequent year, where the feathers can be collected more easily. Studies on pelagic seabirds (Bourgeon et al. 2014; Will et al. 2014; Fairhurst et al. 2015b; Gilmour et al. 2015; Kouwenberg et al. 2015), vultures (Carrete et al. 2013), warblers (Grunst et al. 2015), waterfowl (Legagneux et al. 2013; Harms et al. 2015), and aerial foragers (Fairhurst et al. 2015a) highlight this benefit. Similarly, post-breeding molt of many migratory species takes place on or near the breeding grounds, but breeding areas can be remote and span wide ranges (e.g., boreal forest). Because some such species concentrate during migration, banding stations provide access to feathers from individuals originating across broad geographic areas. To our knowledge, no published study has yet to take advantage of migration banding stations as a source of sampling. Regardless, studies commonly sample individuals returning to breeding grounds with feathers grown previously.

Feathers from hatch-year birds are particularly interesting because they are grown in the nest or post-natal area, providing opportunities to gain insight into how corticosterone correlates with natal conditions (Fairhurst et al. 2012a; Fairhurst et al. 2012b; Yosef et al. 2013; Harriman et al. 2014; López-Jiménez et al. 2015) or predicts subsequent fledging success (Fairhurst et al. 2013a; Lodjak et al. 2015), phenotype (Grava et al. 2013; Fairhurst et al. 2015c) or dispersal (Grava et al. 2013). The ability to correlate physiology from an earlier time period with subsequent condition and performance traits (e.g., reproduction) provides a novel means of investigating mechanisms underlying carry-over effects in birds (e.g. Crossin et al. 2013; Fairhurst et al. 2015b; Harms et al. 2015; Kouwenberg et al. 2015), which is likely to be a fruitful area of research.

A unique and powerful benefit of using a retrospective measure of corticosterone from feathers is that museum specimens can be exploited, which presents considerable scope for research. To date, only two published studies have taken advantage of this, showing that corticosterone in museum feathers is apparently detectable after many decades. One study analyzed feathers collected in the mid-1960s (Kennedy et al. 2013), and another assessed long-term trends in corticosterone from a 153-year time-series of feathers beginning in 1859 (Fairhurst et al. 2015b). This latter study additionally discussed how important ecological events could be detected from a historical time-

series. Thus, the retrospective measure of corticosterone that feathers provide opens up new research avenues because it is now possible to sample corticosterone physiology from a variety of novel spatiotemporal contexts, including extinct and extirpated species.

A long-term, integrated, measure of CORT

Corticosterone levels fluctuate dynamically with energetic need and in response to stressors (Dallman et al. 1993; Romero 2004; Romero et al. 2009). Feather corticosterone measurements are believed to reflect this dynamic range because corticosterone is deposited continually into feathers as they grow. Thus, by continuously incorporating the hormone at baseline levels, as well as registering the amplitude and frequency of responses to stressors, feather corticosterone is an integrated or total measure of corticosterone deposited during feather growth. In line with plasma corticosterone's role as an energy-regulating hormone, it is likely that feather corticosterone reflects changes in energy consumption or management, as has been suggested by several authors (Fairhurst et al. 2012a; Crossin et al. 2013; Fairhurst et al. 2015a; Fairhurst et al. 2015b; Harms et al. 2015). Direct tests of this hypothesis, particularly ones that manipulate energetic demand and measure physiological and/or metabolic correlates, would be particularly revealing.

Feathers take days to weeks to grow (depending on the species, life-history stage, and feather), so feather corticosterone provides a measure of physiology over a similar period. However, the contribution of any single short-term stressor (e.g., predator attack) to feather corticosterone values is likely quite small, reducing the ability of the technique to detect short-lived events or instantaneous corticosterone physiology (Fairhurst et al. 2013a, and see below). Thus, feather corticosterone should be seen as complementary to, not a replacement for, plasma measurements of corticosterone. Both the time period that feather corticosterone reflects, and the fact that it integrates all corticosterone secretion during that period, makes it a unique measure of glucocorticoid physiology. This measure may be more relevant for studying responses to environmental variation and stressors that operate over longer time periods (e.g., weather and climate, food availability, habitat quality).

Minimally-invasive

Generally, biologists seek to minimize harm to the species they study so it is desirable to use techniques that are as minimally invasive as practically possible. Given discussions about the potential effects of blood sampling on avian species (Sheldon et al. 2008; Brown and Brown 2009; Voss et al. 2010) using feathers for corticosterone analysis provides an alternative to bleeding. All impacts to the bird of feather collection are eliminated when using carcasses or collecting naturally-molted feathers, but when collecting feathers from live birds handling is obviously required. Even so, collecting feathers takes a few seconds and, assuming the feather is fully mature, greatly reduces the possibility of unexpected negative impacts (e.g., hematoma). The choice of feather to collect will depend on study questions, but researchers should be aware of the potential impacts. For example, pulling partially grown feathers can result in bleeding, removing feathers from plumage ornaments may alter their information content, removing body feathers could impact thermoregulatory properties, and collecting flight feathers may handicap the bird (McDonald and Griffith 2011). However, these caveats aside, the impact of collecting a few mature contour feathers is likely negligible for most birds.

Requires minimal special training or storage

Procedures for collecting and storing feathers are relatively simple and easy. Other than keeping them dry and clean, feathers do not need special storage and can be kept indefinitely at room temperate in paper or plastic envelopes or bags. This eliminates the need for special equipment (e.g., centrifuge) or conditions (e.g., refrigeration) during collection and transport, which adds flexibility in the field and laboratory, saves time and money, and produces no hazardous waste (e.g., bloody sharps). Moreover, assuming mature feathers are collected, there is apparently no possibility for investigator handling to affect feather corticosterone values, which eliminates the need to sample birds within

three minutes of capture (Romero and Reed 2005), and researchers do not need to make assumptions about the state of the bird prior to capture.

Overall Biological Limitations:

Notwithstanding the strengths and benefits of measuring feather corticosterone, several features intrinsic to feathers limit the usefulness of the technique. The following are biological attributes that restrict the kinds of questions that can be asked and limit the ultimate interpretations and conclusions that can be made using feather corticosterone.

Limited usefulness for short-term stressors

As with all hormones, the biological impact of corticosterone is mediated by its receptors and it is normally the plasma fraction of corticosterone that is interacting with receptors. Feathers grow slowly compared to the time blood circulates through the body. Thus, if short-term plasma titers of corticosterone are ultimately of interest, the resolution of corticosterone measured in feathers will only be as good as the smallest amount of feather that can be measured reliably. Studies have sectioned whole feathers and measured corticosterone from shorter time periods (Fairhurst et al. 2011; Lattin et al. 2011; Will et al. 2014; Jenni-Eiermann et al. 2015). However, substantial mixing of corticosterone along the growing calamus within the blood quill (Jenni-Eiermann et al. 2015) makes resolving very small differences along the length of the feather difficult for many species, particularly those with very small feathers.

Measuring the growth rate of feathers of the species of interest, although often not practical, can help determine how short a time period can be resolved. Important variables that may limit time resolution include the amount of feather mass (see below) and the size of the area of the calamus where mixing takes place. For example, adult European starling feathers grow at the rate of 3-5 mm/day, a whole feather takes approximately 16 days to grow after the pin forms (Strochlic and Romero 2008), and approximately an entire feather is required to reliably extract corticosterone, so a single measurement of corticosterone represents approximately a 2 week period. Feather

corticosterone levels are not likely to correlate with changes in plasma that are relatively modest and of short duration (Fairhurst et al. 2013a). A corticosterone response to a single acute stressor, even something as strong as a predator attack, generally lasts only a few hours before returning to baseline (Sapolsky et al. 2000) and is therefore unlikely to be reflected in whole-feather corticosterone levels.

Restricted to times of the annual cycle when molt takes place

Mature feathers are dead tissue (Gill 2007). Once the feather completes its growth, the blood supply retreats to lower in the skin and isolates the feather. This means that corticosterone circulating in the blood only has access to the feather while the feather is growing. In essence, the corticosterone in the feather is akin to an insect trapped in amber – it is frozen in time and only represents the period when the feather was actively growing. This limits when during the annual cycle corticosterone concentrations can be assessed from feathers. Adventitious molt (or researcher-induced regrowth) notwithstanding, in species that undergo a single post-breeding molt, feather corticosterone is not useful for comparing corticosterone across different life history stages or seasons within the same annual cycle. Comparatively few species molt during breeding, migration, or winter, making those life history stages inaccessible to this method in many species. As a consequence, the range of ecological correlates that can clearly be addressed is limited to those that have a bearing on the physiological state of the individual during molt.

On the other hand, those species with different molt strategies present some exciting opportunities. As examples, some duck species regrow feathers during incubation, providing an index of corticosterone levels during breeding. Other species perform a partial molt in one habitat and then migrate to a different habitat to complete the molt, allowing comparisons of different habitats by sampling those feathers molted in those habitats. In still other species, sympatric breeding populations molt in different locations, which can be distinguished by stable isotope analysis, thereby allowing comparisons of different environmental effects where molt took place (Bourgeon et al. 2014; Gilmour et al. 2015). In addition, when the molt of different feather types do not

overlap (e.g., flight feathers vs. body feathers), measuring corticosterone in these different feather types will reflect different life-history stages (e.g. Lendvai et al. 2013). These types of studies will allow evaluation of inter-individual variation associated with those different life history periods where molt occurs. Regardless of the molt strategy of the individual species under study, care must be taken to interpret feather corticosterone in light of those periods when the feather was actually grown.

Because corticosterone levels in feathers are determined during molt, the range of corticosterone concentrations that can be assessed with feathers may be limited. Although there are exceptions (e.g. Cornelius et al. 2011), in many species corticosterone concentrations are comparatively low during molt (Romero 2002), and some species exhibit virtually no further response to a stressor during this life-history stage (Romero et al. 1997). As a consequence, feather corticosterone in such species reflects a period when blood corticosterone concentrations and release dynamics are damped, suggesting that the range of possible feather corticosterone values also would be damped. Despite this, numerous studies report considerable variation in feather corticosterone values (although the source of that variation is still not entirely clear – see below), so the utility of feather corticosterone is not necessarily limited. But this does highlight the importance of considering biological context (e.g., corticosterone levels can be constrained during molt) when interpreting feather corticosterone in ecological relationships.

Synchronization of molt within populations is generally unknown

There can be substantial variation in molt onset within a population of the same species and between different species occupying the same habitat (e.g. Butler et al. 2008). This can create difficulties in correlating ecological conditions with feather corticosterone. For example, a bird that has a failed breeding attempt will often initiate molt earlier than a conspecific that successfully fledged chicks (Nilsson and Svensson 1996). Even comparing two individuals captured on the same day requires assumptions that the feathers were grown at the same time and in the same place, and that the conditions were equivalent when the feathers were growing. In addition, molt can be protracted, complex, or both in many large species. Birds cannot easily alter feather growth rates (Rohwer and

Rohwer 2013) and there is often limited time during the annual cycle to accommodate molt, leading most large birds to perform a complex molt that is not completed in a single year (Rohwer et al. 2009). This again means that the same feather from two different birds are not necessarily grown at the same time.

That being said, such assumptions are unavoidable when the date and location of feather growth is unknown, which is often the case in field studies. Several studies have analyzed corticosterone from the same feathers on opposite sides of the same bird (Lattin et al. 2011; Lendvai et al. 2013; Strong et al. 2015; Aharon-Rotman et al. 2016) and adjacent feathers (Strong et al. 2015) and found consistent corticosterone levels. This suggests that, at least over short time periods, slight between-feather asynchrony within an individual may not be problematic. However, we know far less about how much among-individual variation in feather corticosterone is due to intrinsic (physiological) factors vs. extrinsic (environmental) ones, so future research addressing this could be especially useful. Moreover, it is essential that researchers carefully consider molt and, when publishing, clearly articulate any assumptions made regarding the synchrony of feather growth.

Unresolved Technical Issues:

Similar to the preceding section on limitations of measuring feather corticosterone, there are several technical issues that have been raised for this relatively new technique. The following sections address and provide the state of our understanding of the major technical issues. A fuller understanding each of these issues will serve to increase confidence in interpretations arising from the measurement of corticosterone in feathers.

What are the antibodies actually measuring?

Mass spectrometry indicates that native corticosterone was found in all feathers of one study (Jenni-Eiermann et al. 2015), but only 16 of 61 samples (although cortisol was found in other samples) in another study (Koren et al. 2012). The consensus thus seems

to be that native corticosterone is present in feathers and that this corticosterone is what is being measured in the RIAs and EIAs. The inaugural paper by Bortolotti et al. (2008) used an RIA to measure feather hormone levels, and most subsequent studies have followed this analytical approach, using the same commercial antibody (Sigma-Aldrich product C 8784, Saint Louis, Missouri, USA). However, another antibody widely used for measuring plasma corticosterone (Endocrine Sciences/Esoterix B3-163 - no longer available, e.g. Romero et al. 2006) failed to measure any corticosterone in feathers (Lattin et al. 2011), and other commercial antibodies produce widely different concentrations from the same feather samples (LMR, unpublished data). Currently, the variability in the efficacy of different antibodies is puzzling considering that both the Sigma-Aldrich and Endocrine Sciences antibodies are excellent at measuring plasma corticosterone. An early explanation was that a conjugate of corticosterone, rather than native corticosterone, is deposited in the feather, and that antibodies differ in their abilities to bind to this conjugate (Lattin et al. 2011). Bortolotti et al. (2008) found substantial levels of sulfonated and glucuronidated conjugates of corticosterone in feathers, although the antibody used in their study did not appear to recognize those conjugates. Hydrolysis of samples also did not alter feather corticosterone measurements (Patterson et al. 2015). Discovering the exact reasons why different antibodies report different values should be a focus of future research.

It is important to recognize that antibodies differ substantially in their binding affinities, specificity, and cross-reactivities, all of which can produce differences in apparent hormone levels (Tate and Ward 2004). Sample preparation is likewise important because sample matrix can interfere with immunoassay performance, both on its own and by interacting with the antibody (Tate and Ward 2004). Feather extracts may contain a variety of substances, and variation in feathers within and among species and individuals, as well as variation in procedures used for cutting/pulverizing, extracting, and filtering feather samples, can all be expected to affect assay results on some level. One potential option for decreasing assay interference (at least from triglycerides) is to purify the feather extract after filtering. Kouwenberg et al. (2015) showed that treating Atlantic puffin (*Fratercula artica*) feather extracts with an acetonitrile/hexane purification resulted in significantly lower corticosterone values compared to untreated samples.

Some researchers prefer to use commercially available RIA and EIA kits, each with its own unique antibody, and different labs have departed from Bortolotti et al.'s (2008) originally published technique for extracting corticosterone from feathers (c.f. Lattin et al. 2011; Lendvai et al. 2013). Given these differences, the lack of comparability of absolute feather corticosterone concentrations across laboratories is expected. Although this does not affect comparisons within an individual study, it does limit the ability to perform meta-analyses across studies performed with different antibodies. However, these issues are not unique to feather corticosterone and are issues with immunoassays in general (Tate and Ward 2004). To move towards overcoming these issues, researchers must begin to standardize practices across labs (particularly extraction techniques), maintain high standards and repeatability within labs, and report antibody product names and assay quality parameters (e.g., variability) in published studies (Buchanan and Goldsmith 2004).

Connection between feather and plasma corticosterone?

Evidence to date suggests that the corticosterone in feathers comes from the general circulation and is deposited into feathers as they grow. Early work with semi-captive red-legged partridges (*Alectoris rufa*) showed that corticosterone in the whole feather correlated with handling-induced (but not baseline) titers of plasma corticosterone (Bortolotti et al. 2008) and, in captive Clark's nutcrackers (*Nucifraga columbiana*), feather corticosterone from segments of a regrown feather corresponded to experimental enrichment experienced during feather growth (Fairhurst et al. 2011). This initial evidence suggested that feather corticosterone reflects changes in circulating corticosterone at the time of feather growth, a hypothesis that was supported with a subsequent series of hormone manipulation studies.

Work with captive European starlings (*Sturnus vulgaris*) showed that corticosterone-filled silastic implants elevated plasma titers of the hormone, and feathers grown during this manipulation had correspondingly higher levels of feather corticosterone (Lattin et al. 2011). However, when separate segments of the feather were analyzed, feather corticosterone from each segment did not correspond to the plasma

corticosterone at the time of segment growth (Lattin et al. 2011, but see below). Additional work with time-release corticosterone pellets, which can provide a more consistent release of hormone than silastic implants (Fusani 2008), showed that experimentally-elevated levels of both baseline and handling-induced plasma corticosterone correlated with corticosterone in the whole feather on an individual level in free-living tree swallow (*Tachycineta bicolor*) nestlings, but only at the peak of hormone release when plasma titers were highest and most variable (Fairhurst et al. 2013a). Recent work has replicated the main finding of this study (Jenni-Eiermann et al. 2015; Aharon-Rotman et al. 2016). Horak et al. (2013) provided additional evidence of a plasma-feather connection by experimentally inhibiting deposition of corticosterone into the feather using injections of dexamethasone, a synthetic glucocorticoid that effectively suppresses corticosterone levels.

These hormone manipulation studies strongly suggest that the corticosterone in feathers arises from general circulation, and is consistent with the idea that feather corticosterone integrates plasma corticosterone at the time of feather growth. Limited additional evidence currently rules out an alternative explanation that external sources of corticosterone influence feather corticosterone. Although structures in hair follicles have been shown to secrete cortisol (Slominski et al. 2007), an analogous system has not been identified in birds (Taves et al. 2011), and preen oil apparently does not contain detectable levels of corticosterone (Lattin et al. 2011). In line with this, washing feathers with soap prior to extraction did not appreciably change levels of feather corticosterone (Bortolotti et al. 2008), though effects are mixed (see below).

Despite the evidence supporting a plasma-feather relationship, how corticosterone is deposited into the feather had only been hypothesized as coming from the blood supply connected to the feather as it grows. Work by Jenni-Eiermann et al. (2015) confirmed this by showing that radiolabeled corticosterone and corticosterone implants created similar patterns of deposition (and thus also demonstrated a positive relationship between plasma and feather corticosterone). They further demonstrated that deposition appears to occur in the blood quill where cell differentiation and keratinization occur, rather than at the base of the feather (i.e., follicle) where cell proliferation occurs (Jenni-Eiermann et al. 2015). There are important implications for this mechanism in the interpretation of

corticosterone deposition. Since circulating corticosterone is deposited in the entire blood quill (which is both inside and outside of the skin), increased corticosterone levels will be found both in the proximal and distal parts of the growing feather length measured at the time of corticosterone pellet implantation. Consequently, the findings of Jenni-Eiermann et al. (2015) explain why feather segment analysis as performed by Lattin et al., (2011) produced anomalous results.

Studies further elucidating this deposition mechanism will be especially important to understanding and interpreting feather corticosterone values. For example, feather corticosterone is likely determined by an interaction between the robustness of the activity of the HPA axis (either elevation or suppression) and the period over which activity is sustained (Fairhurst et al. 2013a), so it makes intuitive sense that feather corticosterone should reflect plasma titers of corticosterone. However, plasma corticosterone levels are typically in the nanogram range, whereas feather corticosterone levels are in the picogram range, suggesting that a bottleneck of some kind occurs during deposition.

The studies mentioned above that correlate plasma and feather corticosterone are almost entirely from experimental elevations of corticosterone. This should not be surprising since implants and injections produced a sustained increase in corticosterone that fits well with the integrated nature of corticosterone deposition in feathers (discussed above). Absent these manipulations, however, feather corticosterone levels should not necessarily be correlated with single plasma titers. It is unlikely that a "snap shot" of corticosterone titers from a plasma sample (representing minutes-to-hours) should correlate with an extended integration of corticosterone over the period of feather growth (representing days-to-weeks), so the relevancy of the presence (or lack thereof) of a correlation between a point sample of plasma corticosterone and feather corticosterone levels is likely limited. For this reason, we caution researchers against using feather corticosterone to directly infer plasma levels of the hormone over short time periods, and suggest that feather corticosterone be considered a separate, but complementary, measure of glucocorticoid physiology.

Stability of corticosterone in the feather

The majority of feather corticosterone measurements are made days, weeks, or months after the growth and collection of feathers, so there is an implicit assumption that the corticosterone in feathers is stable over time. Physical degradation of feather material has the potential to reduce the amount of corticosterone in the feather by simple mass loss. Chemical degradation of the hormone molecule could (theoretically) occur without mass loss, and could occur over time with or without the influence of external factors (e.g., sunlight). Chemical degradation is more problematic because, unlike indicators of physical degradation of the feather (e.g., changes in thickness or color), the loss of corticosterone by chemical means may not be detectable visually. Despite these possibilities, experimental evidence to date has failed to demonstrate such effects on feather corticosterone measurements. Below we introduce five common and pervasive factors: feather wear, sunlight, water, heat, and time.

The effect of feather wear (e.g., by abrasion or chewing by lice) on feather corticosterone has not been investigated, but it seems obvious that direct loss of feather material would reduce total hormone levels. Such physical degradation is problematic because it would likely be inconsistent among individuals. We strongly recommend that feathers analyzed for corticosterone be as comparable as possible (preferably whole) and any feathers with obvious signs of wear be avoided, especially if the amount of wear is clearly variable among individuals. Exposure of feathers to sunlight (and UV radiation), water, and heat could both physically and chemically affect the corticosterone in feathers. Although the effects of sunlight have not been investigated, this could be a confounding variable for some studies because feathers are exposed to sunlight in many free-living birds, and accumulated exposure could degrade corticosterone in older feathers vis-à-vis newly grown feathers. In contrast, heating feathers to 75 °C does not have any detectable effect on feather corticosterone measurements (Bortolotti et al. 2009). Studies washing feathers with a dilute soap-and-water solution show mixed effects. Bortolotti et al. (2008) found that feather corticosterone values were similar between washed and unwashed feathers. In contrast, Jenni-Eiermann et al. (2015) found decreases in feather corticosterone after washing, but only in feathers of birds with corticosterone implants and not in controls, suggesting that a portion of the elevated corticosterone was bound to

the feather surface. Although both these studies used soap and water, it is unclear whether water alone would have any appreciable effect on corticosterone in feathers.

The effect of time has been addressed comparatively. Across a wide variety of species, and contexts ranging from live birds to carcasses and museum specimens, feathers dating to 1859 contained detectable corticosterone (Bortolotti et al. 2008; Bortolotti et al. 2009; Kennedy et al. 2013; Fairhurst et al. 2015b). Moreover, no decrease in feather corticosterone was detected in a long-term time series of feathers, as would be expected if corticosterone in feathers is stable (see Fairhurst et al. 2015b for a more detailed explanation).

Thus, evidence to date suggests that the corticosterone in feathers is probably robust to the elements over short time periods and likely longer. Due to the importance of the stability of the hormone to measures of feather corticosterone, we recommend that future studies experimentally assess the ability of external factors (particularly UV light) to affect the stability of corticosterone in feathers. Additionally, researchers should be aware that physical degradation of feathers may have important consequences for feather corticosterone values, so it is important to ensure that feathers analyzed are as similar in wear and general condition as possible.

Do other attributes of a feather influence feather corticosterone?

Feathers, of course, are not uniform. There has been some work comparing structural differences. For example, although inner and outer vanes of primary feathers are asymmetrical and vary by mass, the amount of corticosterone does not vary when normalized to length (see above, Bortolotti et al. 2009). However, other structural properties have not been studied systematically. Body feathers and flight feathers differ substantially in structure, and the impact of these differences on incorporation of corticosterone is unknown. Controlled comparisons may be difficult since body and flight feathers are often molted at different times, and thus potentially exposed to different environmental conditions. A further factor potentially confounding these analyses is that corticosterone can alter the structural properties of feathers (DesRochers et al. 2009), so it will be difficult to establish the direction of causation if corticosterone content in feathers

does vary with different structural properties. Related to this, homogenizing can influence the surface area available to methanol for extraction (see below).

Feathers also differ in color, but exploration of the relationship between color and corticosterone incorporation is just beginning. The amount of corticosterone is positively correlated with the amount of carotenoids in the feathers (Kennedy et al. 2013; Lendvai et al. 2013; Fairhurst et al. 2014) and one study showed that carotenoids decrease in tandem with feather corticosterone when molting birds were exposed to internal parasites (Mougeot et al. 2010). Although these studies are exciting because of the growing work on corticosterone impacts on sexual signaling (Husak and Moore 2008), it is still unclear whether plasma corticosterone alters pigment deposition (important for a link between stress and sexual signaling) or pigment deposition alters corticosterone incorporation. At least one study indicates the latter – feather corticosterone increased with feather melanism, with darker segments incorporating more corticosterone (Jenni-Eiermann et al. 2015), but other work has failed to find relationships between feather corticosterone and melanin-based traits (Fairhurst et al. 2015c). Variation in carotenoid-based color may be an incidental bi-product of the metabolic effects of corticosterone on feather quality (Kennedy et al. 2013) or fat stores (Fairhurst et al. 2014). We recommend that studies experimentally test relationships among corticosterone and pigments in plasma and feathers in light of deposition into feathers.

Effect of unequal and small sample masses

Among the most commonly debated topics in the analysis of feather corticosterone is the significant effect of feather mass on feather corticosterone measurements. Two separate issues have arisen regarding mass, one due to deposition and another due to sample preparation. Why these effects exist, whether they pose an issue to interpretation of feather corticosterone measurements and, if so, how and why they should be controlled for, are sources of confusion for many researchers. Below we hope to define, clarify, and provide guidance on these points. We also suggest important experiments that will shed light on the relative importance of these issues.

The first mass-based issue was demonstrated by two early studies showing that the heavier parts and sections of the same feather contain less corticosterone per unit mass than the lighter parts and sections (Bortolotti et al. 2008; Bortolotti et al. 2009). This is essentially a mass-dilution effect. For any given point of growth along the length of the feather, the more massive rachis and the less massive vane would have been exposed to the same plasma titers of corticosterone. However, this same amount of corticosterone is relatively "diluted" when deposited into the rachis material because of its greater mass, resulting in a lower corticosterone/mg value in the heavier portion of the feather (see Fig. 4a in Supplemental Appendix of Bortolotti et al. 2008). Likewise, whole sections of feather (rachis plus vane) decrease in mass from the proximal calamus to distal tip of the feather. So, assuming that plasma corticosterone titers exhibit no trend during feather growth, the same amount of corticosterone is diluted when deposited into the more massive proximal sections, resulting in lower corticosterone/mg values compared to the less massive distal sections (see Fig. 1a and 2a of Bortolotti et al. 2009).

Bortolotti (2010) argued that this mass-dilution effect was an artifact that should be avoided. He reasoned that because corticosterone is not integrated into the keratin structure of feathers (as is, e.g., sulfur), its deposition is not mass-dependent, so corticosterone values should not be normalized by mass (i.e., expressed as a concentration, "corticosterone/mg"). Rather, he suggested that corticosterone deposition is incidental and therefore time-dependent, a hypothesis that has since been supported (Jenni-Eiermann et al. 2015). Because feather growth rate is fairly uniform within and across species (Rohwer et al. 2009), the best estimate of exposure time is a feather's length. This means that feather corticosterone values should be normalized by feather length (i.e., expressed as corticosterone/mm). Indeed, the significant mass-based relationships shown by Bortolotti et al. (2008; 2009) disappear when the corticosterone data is normalized by feather length, as would be expected (and desirable) if plasma hormone titers displayed no trend during feather growth. An important distinction to make here is that feathers in Bortolotti et al. (2008; 2009) were cut into small pieces for extraction.

When a homogenous mixture of pulverized feathers (i.e., "dust") is extracted, the above mass-dilution effect is largely eliminated because there is little or no variation in

feather structure in dust. However, when extracting feather dust a second mass-based issue arises, as demonstrated by Lattin et al. (2011). In theory, larger aliquots (i.e., greater masses) of homogenous feather dust should contain an equal amount of corticosterone per unit mass when compared to smaller aliquots. For example, 2 mg of feather dust should have twice the amount of corticosterone compared to 1 mg of dust but, when normalized by mass (i.e., expressed as corticosterone/mg), both samples should have similar values. However, when using feather dust, a non-linear inverse relationship arises between the mass of feather dust and corticosterone/mg (see Fig. 1 in Lattin et al. 2011).

Although superficially similar, the relationship reported by Lattin et al. (2011) is not the same as the one reported in Bortolotti et al. (2008; 2009) because the sample preparations in the two studies were different. Thus, it is important to draw a distinction between the mass-dilution effect reported by Bortolotti et al. (2008; 2009) and the extraction mass effect reported by Lattin et al. (2011) because these are separate issues.

Why measured corticosterone is higher in small-mass samples of feather dust is unclear, and it is currently unknown if extractions of (heterogeneous) feather pieces exhibit the same mass-based effect. One hypothesis is that the pulverization/extraction procedure could be influential and that the high surface area per gram of feather dust alters the efficiency of methanol extraction and/or the recovery of methanol during the filtration step. In the latter case, the larger masses of feather dust may retain proportionately greater volumes of methanol, allowing less methanol to be recovered. A study investigating the relative saturation with methanol of feather pieces and feather dust (e.g., by measuring recovered volume) would be an interesting test of this hypothesis. It is also unknown if extraction of very large masses of feather requires more than the standard 10 mL of methanol recommended by Bortolotti et al. (2008).

The extraction mass issue can be avoided by only measuring feather samples greater than 20 mg, but this is often difficult with smaller birds or when using body feathers. Body feathers are especially attractive, however, because sampling these feathers has less of an impact on flight dynamics in living birds and is often the only type of feathers permitted for sampling from museum specimens. For studies using relatively low feather masses (e.g., a single contour feather), potential solutions are to use feathers of only similar weights (e.g. Kouwenberg et al. 2015) or to make sure every feather

sample is of the same weight prior to analysis (e.g. Kennedy et al. 2013), even if they are of smaller mass than desired. A recent study (Will et al. 2014) controlled for feather mass statistically, though there can be a significant correlation between corticosterone normalized by mass and corticosterone normalized by length (e.g. Fairhurst et al. 2013a; GCF unpublished data).

More studies are needed to fully understand the influence of extraction mass on feather corticosterone values. It is clear that when different masses of homogenized feather dust are extracted an important mass effect occurs, but whether the effect is present in feather pieces is currently unknown, so studies comparing extraction procedures would be timely. As is the case generally, reducing variation by standardizing feathers as best as possible (including mass) will improve reliability of corticosterone measurements, and working towards standard practices for extraction will be important.

Influence of deposition dynamics

The deposition of corticosterone into the growing feather is believed to occur via diffusion of the hormone from the blood vessels of the pulp. Thus, factors that influence diffusion of the hormone can, in theory, affect feather corticosterone values. Diffusion of corticosterone into feathers is likely driven by three main factors. First is the concentration gradient among the bloodstream, dermal pulp, and keratinizing cells. It seems intuitive that circulating titers of corticosterone should be the major determinant of the concentration gradient and, thus, feather corticosterone values should be directly related to plasma titers of corticosterone (Baumgartner et al. 1989). Indeed, evidence from hormone manipulation studies supports this idea (see above). Plasma corticosterone is in nanogram concentrations, but feather corticosterone is at picogram levels, suggesting the presence of a strong concentration gradient or, alternatively, a limiting factor or bottleneck of some kind, which is currently not well understood.

Second, the surface area available for diffusion of the hormone into the growing feather may limit the amount of hormone that can be deposited. In feathers, the surface area of diffusion is determined by two interrelated factors. The volume of the pulp cavity is important because it provides the space for substances from the bloodstream to

accumulate near sites of feather growth, and the total number of growing feather cells is important because these are the sites for deposition. The complex geometry of the pulp cavity and its ramifications makes quantifying the surface area of diffusion prohibitively difficult for avian ecologists. However, both the volume of the pulp cavity and the number of growing cells are directly related to the mass of the feather, which can be measured easily. Thus, more massive feathers should have pulp cavities of greater volume and/or contain more growing cells and this increased surface area likely provides more opportunities for corticosterone to be deposited.

Third, the amount of time growing cells are in contact with the dermal pulp (and thus blood supply) should influence how much corticosterone diffuses to the sites of deposition. Because feathers elongate with time, in theory the faster the elongation of the feather (i.e., growth rate) the less time there is for corticosterone to diffuse into the growing feather and, hence, less corticosterone will be deposited. Even though feather growth rate is generally considered to be fairly consistent (Gill 2007), changes in feather growth rates can alter feather corticosterone measurements by altering exposure time to plasma corticosterone (Jenni-Eiermann et al. 2015). This is analogous to how changes in gut transport time help determine the levels of glucocorticoid metabolites measured in feces (Morrow et al. 2002).

Levels of plasma corticosterone and feather mass, length, and growth rate likely work in concert to determine how much corticosterone is deposited in a feather. However, stressors that can affect multiple aspects of diffusion simultaneously may be particularly influential to feather corticosterone values. For example, nutritional stress in captive Caspian tern (*Hydroprogne caspia*) chicks fed a 65% *ad libitum* diet resulted in lower growth rates and feather corticosterone levels, and feather corticosterone patterns that did not match plasma corticosterone patterns (Patterson et al. 2015). However, feeding captive rhinoceros auklets (*Cerorhinca monocerata*) a 50% *ad libitum* diet had opposite effects, resulting in higher feather corticosterone levels in food-restricted birds (Will et al. 2014). In both these studies, nutritional deficits resulted in high plasma corticosterone titers, but whether feather quality and growth rate were affected by nutrition, corticosterone, or both is not clear. Moreover, these effects were apparently species- and context-specific.

There remains the possibility that feather corticosterone is not derived solely from plasma corticosterone. Recent data from mammals indicate that corticosterone can be synthesized locally in the skin (reviewed in Taves et al. 2011), which would suggest the intriguing possibility that local, rather than (or in addition to) systemic, corticosterone is bathing cells in the feather follicles. Corticosterone implant studies would seem to contradict this hypothesis, but implants can create supraphysiological titers in the plasma that could overwhelm local production. However, to date no analogous structures capable of producing glucocorticoids have been identified in bird skin (Taves et al. 2011), so direct evidence for this hypothesized mechanism is currently lacking. Regardless, the unresolved possibility of local corticosterone production further underscores the need for continued research into the deposition mechanism underlying feather corticosterone.

Individual differences in physiology likely influence deposition such that a combination of genetics, condition, health and, importantly, exposure to environmental variation, collectively determine the amount of corticosterone that is ultimately deposited into the feather. Recent work suggests that feather corticosterone values may also be determined partly by previous levels of feather corticosterone (Aharon-Rotman et al. 2016). More fully understanding the factors that influence and limit corticosterone deposition should be a priority for future research. Collaborations among ecologists and cellular and molecular biologists will be especially valuable for identifying such factors, determining the circumstances under which they may be influential, and testing alternative hypotheses of deposition mechanisms.

What does variation in corticosterone along the feather represent?

Because distal portions of feathers lose their connection to the blood as they grow, it makes intuitive sense that corticosterone in the distal portion of the feather could reflect different environmental events than corticosterone in the proximal portion of the feather. This analysis of retrospective exposure has been used successfully with hair (Ashley et al. 2011; Meyer and Novak 2012). There appears to be no consistent pattern across studies in how variable corticosterone levels are along the length of feathers. Some studies have reported variable levels (e.g. Bortolotti et al. 2009; Fairhurst et al. 2011; Patterson et al.

2015) whereas other studies have not (e.g. Fairhurst et al. 2011; Lattin et al. 2011; Will et al. 2014).

The extent to which this variation represents either biologically relevant differences in plasma corticosterone titers (as discussed above) or underlying variability in deposition of corticosterone in the feather, or both, is still an open question. A lack of variation along the feather could result from deposition dynamics that serve to homogenize the distribution of corticosterone along the growing feather (see below) or from reflecting stable plasma corticosterone titers during the period of feather growth. Distinguishing between these possibilities may be very difficult because the relative contribution of each mechanism may be species- and context-dependent. However, one factor that has not yet been tested is the size of the bird (and thus the size of the feather).

Experimental evidence for the biological relevance of corticosterone variation along the feather length was provided by manipulations of environmental enrichment during experimental regrowth of feathers in Clark's nutcrackers, where changes in enrichment regimes were reflected in elevated corticosterone from sections of feathers grown during manipulations (Fairhurst et al. 2011). Correlative evidence includes a relationship between feather corticosterone and the presence of fault bars (Bortolotti et al. 2009). Since fault bars are thought to arise from periods of stress and can visually indicate those periods on the feather (Grubb 2008), data showing that small (~21mm) sections of feathers with fault bars are associated with higher feather corticosterone values in those sections (Bortolotti et al. 2009) reinforces the idea that corticosterone from small sections of feather may reflect punctuated stress events during that period of feather growth. However, relationships with fault bars alone should not be taken as strong evidence that CORT from small sections of feather can be interpreted easily. Fault bars frequently indicate nutritional stressors (Grubb 2008) which can have inconsistent effects on feather corticosterone (Will et al. 2014; Patterson et al. 2015), and the presence of fault bars may not be consistent across feather types (Jovani and Blas 2004).

The most comprehensive study to date indicates that corticosterone variation along sections of feather likely reflects differing environmental events only in the broad sense. Jenni-Eiermann et al. (2015) injected tritiated corticosterone into pigeons and found that radioactivity is incorporated into the feather. This indicates that feather

corticosterone does derive from plasma corticosterone. However, there is a broad rather than acute peak of radioactivity in the feather as a result of the corticosterone being deposited over the length of the blood quill. This indicates a diffuse incorporation into the feather that complicates fine-scale partitioning of environmental events along the length of the feather. More work in this area is clearly needed, but we caution researchers about interpreting corticosterone variation along the feather length from small sections of feathers without careful validation.

Conclusions and Future Directions:

In our opinion, the technical advancement of the measurement of corticosterone in feathers to date has been encouraging. New research findings are exciting and current data supports continued use of the technique. As with any new technique, there are still unresolved technical issues that have the potential to alter data interpretation, but other issues have been resolved to the point that carefully constructed and interpreted experiments can yield important data. However, we recommend that future studies continue to chip away at the remaining technical issues to put measuring feather corticosterone on even stronger theoretical and practical foundations. Studies addressing the stability of corticosterone in feathers, mechanism(s) regulating deposition of corticosterone into feathers, and factors that can simultaneously affect corticosterone, feather quality and their interactions will be especially valuable.

Feather corticosterone measurements have considerable promise to answer some longstanding questions in avian biology and facilitate a variety of new research avenues. The integrated nature of corticosterone incorporation allows for assessments of the impacts of corticosterone over periods of weeks – a time frame unavailable with any other technique. We encourage researchers to consider this measure in its own right, rather than expecting it to replace plasma measures of corticosterone. With careful avoidance of over interpretation, analysis of corticosterone and potentially other hormones (e.g. Koren et al. 2012) in feathers can be a powerful technique for the avian biologist.

Acknowledgements:

GDF thanks T. Marchant, G. Bortolotti, S. Cabezas, B. Clark, C. Soos, K. Machin, and R. Dawson and gratefully acknowledges the support of the University of Saskatchewan and Environment Canada. This work was partially funded by National Science Foundation (USA) IOS-1048529 to LMR.

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Table 1: Categories of feathers, including example sources, and their benefits and limitations.

Category of feather	**Potential sources**	**Potential quality of ancillary data**	**Pros**	**Cons**
known live birds	captive or wild-caught birds; naturally molted or plucked-and-regrown feathers	high	ancillary data can be chosen by researcher; manipulations possible during feather growth	can require significant resources to collect feathers
known dead birds	museums, veterinary clinics, rehabilitation centers, found carcasses	medium	inexpensive; samples can be abundant	ancillary data limited to information collected with specimen and available from feather (often expensive)
unknown sources	found feathers, cultural and archaeological artifacts	low	inexpensive; samples can be abundant	ancillary data limited to information available from feather (often expensive)

Fig. 1

Summary of advantages, limitations, and technical concerns when using feather corticosterone measurements to infer plasma corticosterone titers. CORT = corticosterone.

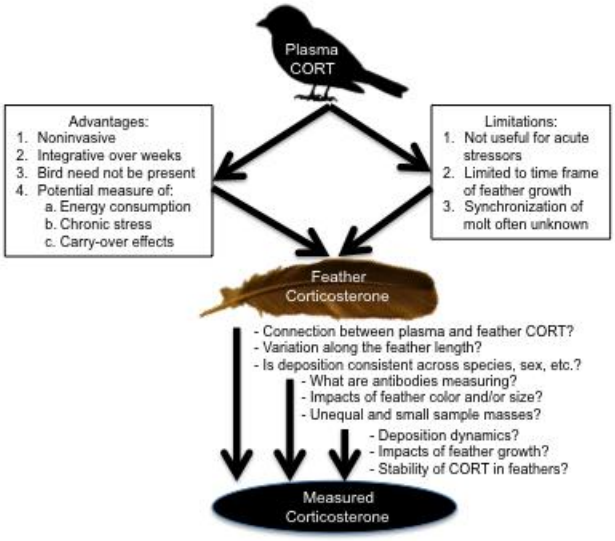
Plasma
CORT
Advantages:
1. Noninvasive
2. Integrative over weeks
3. Bird need not be present
4. Potential measure of:
a. Energy consumption
b. Chronic stress
c. Carry-over effects
Limitations:
1. Not useful for acute stressors
2. Limited to time frame of feather growth
3. Synchronization of molt often unknown
Feather
Corticosterone
- Connection between plasma and feather CORT?
- Variation along the feather length?
- Is deposition consistent across species, sex, etc.?
- What are antibodies measuring?
- Impacts of feather color and/or size?
- Unequal and small sample masses?
- Deposition dynamics?
- Impacts of feather growth?
- Stability of CORT in feathers?
Measured
Corticosterone

Figure 1