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Measuring stress in wildlife: techniques for quantifying glucocorticoids

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Abstract Stress responses play a key role in allowing animals to cope with change and challenge in the face of both environmental certainty and uncertainty. Measurement of glucocorticoid levels, key elements in the neuroendocrine stress axis, can give insight into an animal's well-being and can aid understanding ecological and evolutionary processes as well as conservation and management issues. We give an overview of the four main biological samples that have been utilized [blood, saliva, excreta (feces and urine), and integumentary structures (hair and feathers)], their advantages and disadvantages for use with wildlife, and some of the background and pitfalls that users must consider in interpreting their results. The matrix of choice will depend on the nature of the study and of the species, on whether one is examining the impact of acute versus chronic stressors, and on the degree of invasiveness that is possible or desirable. In some cases, more than one matrix can be measured to achieve the same ends. All require a significant degree of expertise, sometimes in obtaining the sample and always in extracting and analyzing the glucocorticoid or its metabolites. Glucocorticoid measurement is proving to be a powerful integrator of environmental stressors and of an animal's condition.

Keywords Glucocorticoids · Stress response · Salivary glucocorticoids · Fecal glucocorticoid metabolites · Hair and feather glucocorticoids

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Introduction

Environmental perturbations represent major selective forces in natural populations (Wingfield and Romero 2001; Reeder and Kramer 2005). Animals have evolved a suite of behavioral and physiological strategies to cope with them. The two most important physiological responses to stressors are the stimulation of the sympathetic nervous system (resulting in the release of catecholamines) and the activation of the hypothalamic–pituitary–adrenal (HPA) axis that helps restore homeostasis (Sapolsky et al. 2000; Reeder and Kramer 2005). The latter results in a secretion of glucocorticoids (GCs), lasting several minutes to hours (Sapolsky 1992; Wingfield and Romero 2001). The presence of short-term elevated GC concentrations facilitates the escape from life-threatening situations (Wingfield et al. 1998). However, chronic activation of the HPA axis and

elevated GC concentrations may have large deleterious effects on fitness (Boonstra et al. 1998; Blas et al. 2007; Sheriff et al. 2009a).

Since the amplitude and duration of this stress response often correlates with the overall health of an animal (Sapolsky 1993; Boonstra et al. 1998), GC concentrations are being used increasingly in ecological and conservation studies as indices of animal well-being (Busch and Hayward 2009). Measuring these hormones can help us understand how stressors affect the survival and reproductive success of free-living animals and thus how natural environmental challenges (e.g., conspecifics, predators, weather), climate change, relocation or reintroduction, and habitat disturbance impact populations (Boonstra and Singleton 1993; Wasser et al. 1997; Creel et al. 2002; Cyr and Romero 2007; Thiel et al. 2008; Monclús et al. 2009; Sheriff et al. 2009a). Although blood sampling has long been the most common method for measuring GC concentrations, it is not necessarily the most informative method and sometimes is not possible (Möstl and Palme 2002; Mormede et al. 2007). Therefore, a number of newer non-invasive procedures are gaining popularity for use with wildlife.

In this paper, we review the techniques for measuring GCs or their metabolites in four main biological matrices: blood, saliva, excreta (feces and urine) and integumentary structures (hair and feathers). For each matrix, we discuss the route of the GC entry, the collection and assay procedures, and the advantages and disadvantages of each in wildlife studies.

The neuroendocrine stress response

The neuroendocrine stress response is a cascade of events mediated by the HPA axis. The topic of stress physiology and the brain is enormous and complex, ranging from the cellular and molecular to the whole organism. For greater detail, a number of comprehensive sources can be consulted (e.g., Silverin 1986; Sapolsky et al. 2000; Wingfield and Romero 2001; Fink 2007). The HPA axis is comprised of the hypothalamic paraventricular nucleus (PVN), the anterior pituitary gland, and the adrenal cortex (Fig. 1). Under normal conditions, the hippocampus inhibits the HPA axis; as GC levels increase, the hormone itself acts as a negative feedback signal (De Kloet et al. 1998; Wingfield and Sapolsky 2003). In the brain, GCs bind to glucocorticoid and mineralocorticoid receptors, and in the body, to glucocorticoid receptors alone. This negative feedback loop regulates the normal circadian rhythm of GC release (De Kloet et al. 1998; Wingfield and Sapolsky 2003; Seckl 2004).

When an animal encounters a stressor (any physical or psychological event that disrupts homeostasis; Fig. 1) the

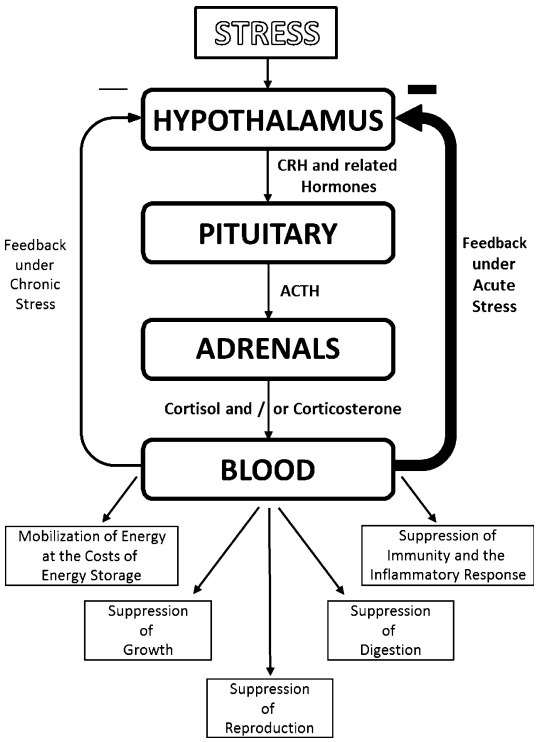

Fig. 1 The hypothalamic–pituitary–adrenal (HPA) axis, the negative feedback response of animals to an acute stressor compared with those under chronic stress, and the major impacts stressors have on bodily processes (*boxes*). Figure adapted from Boonstra et al. (1998)

PVN is stimulated, causing the parvocellular neurons to release corticotrophin-releasing hormone (CRH) and other secretagogues, such as arginine vasopressin (AVP) or its analogues, into the hypophyseal portal system that connects the hypothalamus and the anterior pituitary. CRH and AVP stimulate the anterior pituitary to synthesize and cleave the precursor molecule pro-opiomelanocortin into adrenocorticotropin hormone (ACTH). ACTH is released into the blood stream and stimulates the adrenal cortex to secrete GCs well above basal levels. It generally takes 3–5 min in vertebrates for this pathway to result in measurable increases in GC concentrations (Fig. 2; see review by De Kloet et al. 2005).

The cessation of the pathway is under the control of the GCs themselves through a negative feedback loop (Fig. 1). Stress-induced concentrations of GCs interact with glucocorticoid receptors in the hippocampus, hypothalamus and pituitary to suppress the initial steps of the HPA axis (De Kloet et al. 1998). GC concentrations usually reach peak levels 15–30 min after a stressor and return to basal levels within 60–90 min (De Kloet et al. 2005). However, the level at which GCs are elevated will depend on the severity of the stressor. Under conditions where the stressor is acute, the feedback mechanism operates efficiently and the system rapidly returns to normal. Under conditions where

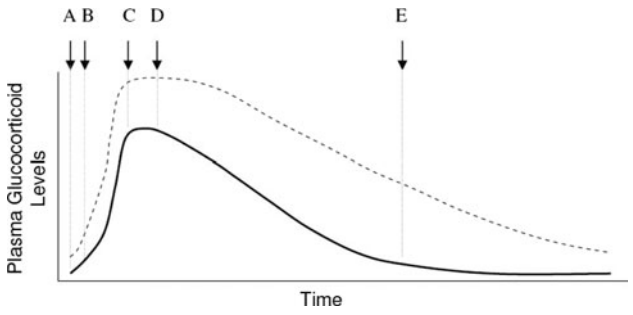

Fig. 2 Time course of glucocorticoid (GC) response (stress response) to an acute stressor. The stress-response is highly conserved across all vertebrate taxa (Sapolsky 1992; Guillette et al. 1995; Wendelaar Bonga 1997; de Kloet et al. 2005). Variations in the response (*solid line* vs. *hashed line*) can be due to many extrinsic and intrinsic factors (e.g., extrinsic: time of day, season, food availability, predation, disease, social status, habitat; intrinsic: species, body condition, reproductive status, age, sex); often a greater response (*hashed line*) is indicative of the animal being under greater stress (Sapolsky 1992; Wasser et al. 1997; Creel et al. 2002; Romero 2002; Wingfield 2003; Kitaysky et al. 2007; Sheriff et al. 2010b, 2011). *A* The onset of the acute stressor, *B* changes in GC levels are detectable after only 3 min of exposure to a stressor, *C* maximum GC levels are reached within 15–30 min, *D* in normally functioning animals GC levels begin to decline soon after peak levels have been reached; however, with animals in stressful situations GC levels may remain elevated for a prolonged period, *E* GC reach pre-stressor levels approximately 60–90 min later. Chronically stressed animals exhibit a heightened stress response in both the rate and magnitude of response. The decline in GCs in these animals is much slower compared to normally functioning animals

the stressor is chronic, feedback signals are weak and the system remains activated for longer periods (Fig. 2; Sapolsky 1992).

In any discussion of measuring GCs, an important distinction must be made between "free" and "bound" hormones (see Fig. 3 for GC pathway). In most species, a large proportion of total circulating GCs are tightly bound to a plasma protein, corticosteroid binding globulin (CBG). Because CBG is too large to leave the capillaries under normal circumstances, any GCs bound to CBG remain in circulation (GC also bind loosely to albumin, but it dissociates so rapidly that it is generally thought to be "free"; see Malisch and Breuner 2010). According to the "free hormone hypothesis", it is the concentration of free, unbound hormone that determines how much GC diffuses out of the capillaries and reaches the tissues (Rosner 1990). In some cases, trends in total and free GC concentrations will be identical, but this is not always the case. For example, house sparrows (*Passer domesticus*) showed seasonal changes in their baseline and stress-induced total GC concentrations. However, their CBG levels also varied seasonally, which resulted in similar free GC concentrations among seasons (Breuner and Orchinik 2001). Conversely, in a population of free-ranging snowshoe hares, food-supplemented hares (*Lepus americanus*) had similar total GC concentrations compared with controls, but because food-supplemented hares had more CBG they had significantly lower free GC concentrations (Boonstra and Singleton 1993). These studies, and numerous others, have illustrated the biological relevance of free GC concentrations. However, it is an oversimplification to claim that only free hormone is biologically active: there is good evidence that, in some specific cases, CBG-bound GCs can be released by enzymatic cleaving of the CBG molecule (Hammond 1995), and that, in some tissues, there are cell surface receptors for the CBG–GC complex (Singer et al. 1988). These cases are unlikely to be important to ecologists, which is why many comparative physiologists adopt the free hormone hypothesis. However, ecologists should be aware of this debate and its potential implications when interpreting results; see Malisch and Breuner (2010) for an excellent discussion of this issue.

Another often over-looked factor of GC physiology is that both the magnitude and duration of GC release are biologically important. Dallman and Bhatnagar (2001) found that the biological effects of the stress response result from the hormone–receptor interactions over the entire time course of the response, not just at the peak of GC release. The ability to measure this integrated response is extremely important when studying free-ranging animals, which may experience a variety of stressors at any given time. For example, in a population of free-living baboons, *Papio anubis*, subordinates had a lower maximal GC release to a stressor than did dominants. However, the subordinates had a much longer duration of GC response due to their weaker negative feedback, resulting in an overall greater total amount of GC release (Sapolsky 1993). This greater GC exposure was then linked to cardiovascular problems in subordinates (Sapolsky and Share 1994). Thus, the measurement of the total amount of GC released, a function of both the maximum and duration of release, is the important variable to measure, not simply the maximum alone.

GC analysis

Immunoassay is the common method for analyzing GC levels, and basically two versions are mostly used: radioimmunoassay (RIA) and enzyme immunoassay (EIA, sometimes erroneously called ELISA, enzyme-linked immunosorbent assay). There are less common methods that we will not discuss (e.g., gas or liquid chromatography coupled with mass spectrometry; Webb et al. 2007) that may be of use for identification and/or simultaneous measurement of complex mixtures of steroids. Both RIA and EIA are competitive binding assays and highly sensitive. Both necessitate an antibody directed against certain parts

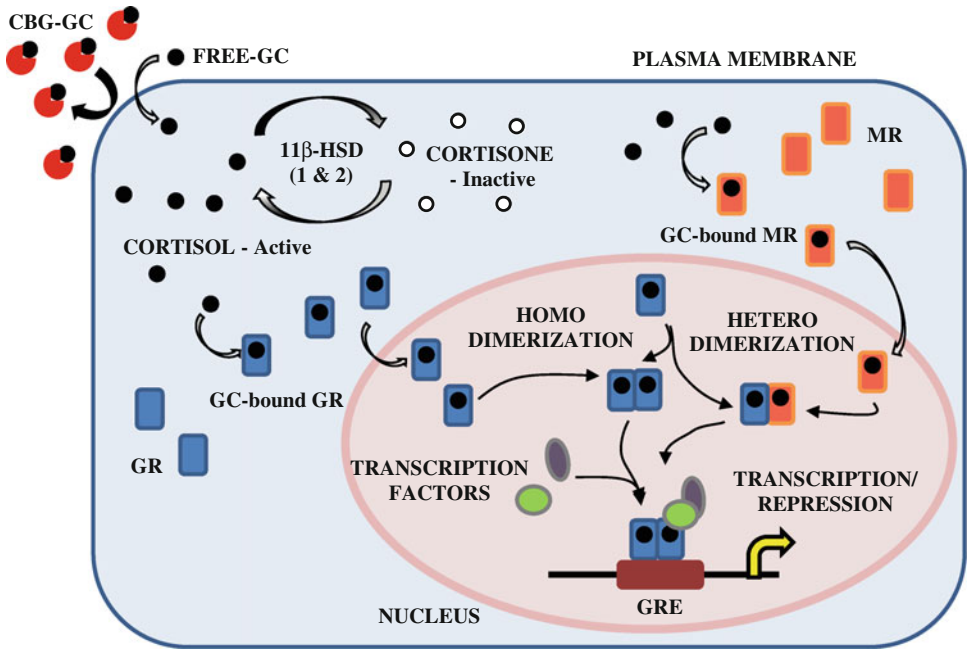

Fig. 3 Schematic of the glucocorticoid (*GC* cortisol or corticosterone, depending on the species) pathway into the cell and how it exerts genomic effects. Outside the cell, most GCs are bound by corticosteroid binding globulin (*CBG*), with only 5–10% being free and biologically active. Free GCs are transported across the cell membrane and are complexed to cytosolic receptors. Every cell in the body has glucocorticoid receptors (*GR*). In addition, in the higher stress axis regulatory center of the hippocampus and dentate gyrus, there are also mineralocorticoid receptors (*MR*). The now activated GR (or MR) complex translocates to the nucleus where it dimerizes with another ligand-bound steroid receptor. This dimer binds to glucocorticoid response elements (*GRE*) to modulate gene expression, often through its association with various co-activators, or co-repressors. Thus, it can both activate and repress. In the cytosol, free cortisol can also be converted to the inert form cortisone, via 11β-hydroxysteroid dehydrogenase (11β-HSD) (free corticosterone is converted to the inert 11-dehydrocorticosterone under the same action). This action can protect tissues and organs (e.g., kidney) from activation of GR and MR. (Rosner 1990; Smith et al. 1996; de Kloet et al. 1999; Matthews 2002; Owen et al. 2005; Malisch and Breuner 2010)

of the steroid molecule of interest. RIAs rely on a radioactive isotope (e.g., tritium or iodine) to generate a radioactive signal to quantify GC levels. EIAs use enzymes to generate a colorimetric signal to quantify GC levels. Both require reasonable technical skill and expensive instrumentation. The major upfront costs are greater for RIA, with a scintillation or a gamma counter being needed, whereas for EIA, a microplate reader is needed; the former are largely dedicated instruments whereas the latter can be used for multiple purposes. RIAs also have the disadvantage of using radioisotopes, and the disposal of radioactive material can be difficult.

There are basically three variants of these immunoassays and ongoing costs are a major consideration of which to choose. The common denominator is the antibody, which must be made or purchased. In-house, non-commercial assays have been developed by individual laboratories, and are still extensively used, especially where large numbers of samples are being processed (e.g., Wingfield; his students, and colleagues still use an RIA assay reported by Wingfield et al. 1992; see also Rich and Romero 2005; Fletcher and Boonstra 2006). Commercial RIA and EIA kits are the other two variants and have been developed primarily for human or laboratory animal use and have been adapted for wildlife studies. They usually come complete with all or most of the critical reagents and have been validated for their specific uses. However, most will not have been validated for use in wildlife species and will therefore need further validation.

Validation of GC measurements

For wildlife ecologists venturing into the measurement of hormone levels, it is critical to understand those variables that could compromise their validity and how to deal with and report them. Each assay must be validated for the species and matrix to ensure proper quantification of the GC or its metabolites. Though such validations are routinely done in the primary endocrinological literature and thus implicitly understood, the need for this rigor may not be appreciated by those new to hormone analysis. One cannot assume that, because the assay has been validated in a related species, it is unnecessary to carry it out again

(e.g., *Lepus timidus* vs. *L. americanus*; Rehnus et al. 2009; Sheriff et al. 2009b).

In blood (but also in all the other matrices), other components may interfere with the assay, and the validation procedure will identify if there is a problem. Thus, plasma components such as lipids (triglycerides and fatty acids; Rash et al. 1980; Newman et al. 2008b) may affect the assay in a concentration-dependent manner. To solve the problem, the extraction with a suitable solvent or pretreatment with suitable chemical (e.g., NH_4OH to saponify the triglycerides; Boonstra and Singleton 1993), or solid phase extraction to remove interfering compounds (Newman et al. 2008b), may result in high and consistent recoveries. Assays requiring extraction need solvents to be ultra-pure and free of peroxides (which can form when the solvent is simply sitting in the bottle). Thus, extracting solvents such as dichloromethane need to be redistilled immediately before use.

There are four general validation requirements for any immunoassay (whether a commercial kit or in-house preparation) applied to measure GCs (see the following for a comprehensive discussion of validation: Abraham 1975; Chard 1995; Buchanan and Goldsmith 2004; Möstl et al. 2005).

1. Specificity: other steroids known to be present in the matrix do not interfere significantly with the assay either because they do not cross-react with the antibody or they are not present in substantial amounts. The cross-reactivity of the antibody with other major steroids likely to be present in the matrix should also be reported.
2. Parallelism: demonstrate that serial dilutions of the sample result in a linear decrease in assay values that is parallel to the standard curve.
3. Accuracy: determine (in GC-free or previously assayed samples) that added GC over a range of concentrations correlates directly to the amount recovered by extraction.
4. Limitations of the assay: include nonspecific binding, absence of false positives or negatives (blanks), sensitivity (lowest GC concentration different from zero), and precision (the within assay and between assay variance by repeated measurements of the same sample and by measuring samples with a range of GC concentrations).

The actual methodology of these requirements is outside the scope of this review (but see Buchanan and Goldsmith 2004; Wada et al. 2007; Lynn and Porter 2008).

Besides the analytical validation of an immunoassay, its application in a given species and sample material should also be physiologically (and/or biologically) validated (Palme 2005; Touma and Palme 2005). The preferred technique for salivary assays and also fecal or urine metabolite assays is to use a hormonal challenge (ACTH stimulation and/or dexamethasone suppression tests). These challenges stimulate (ACTH) or suppress (dexamethasone) blood GC levels (Sapolsky 1983; Boonstra et al. 1998). By injecting ACTH or dexamethasone and measuring whether GC levels in the tested medium (saliva, excreta) reflect the predicted changes in the blood, the method can be physiologically validated (Touma and Palme 2005). We also recommend the use of a biological test by exposing an animal to a biological stressor (such as food deprivation, predation risk, or human disturbance) and measuring the GC (or their metabolites) in the samples. This test ensures that the method will appropriately measure GCs in the field when animals are exposed to genuine stressors. Radiometabolism studies have been used to gain information about the metabolism and excretion of GC (e.g., Palme et al. 2005), and can be especially useful when pioneering new assay techniques. Validating the relationship between plasma GC levels and those in hair and feathers is also a necessity; however, a much longer time scale is required (i.e., the exposure to a stressor must be long-lived over a period of days to months).

Blood (plasma or serum)

Blood has been the substance of choice from which to obtain glucocorticoid levels for many species of vertebrates. It gives an immediate snapshot of the state of the organism at that instant. This state is a composite of three components: endogenous cycles (circadian and seasonal cycles); immediate prior experience (e.g., actual or perceived acute stressors such as predator exposure or attack, or weather events); and longer term experience (chronic stressors induced by conspecifics, nutrition, predators, etc.).

The sole or primary glucocorticoid (GC) varies among and within taxa (Bentley 1998). If both corticosterone and cortisol are produced, cortisol predominates and it has a greater biological activity than corticosterone (Vinson et al. 2007). Sharks and rays are unusual in that the GC is 1α-hydroxycorticosterone. In the ray-finned fish (95% of the fish species: Actinopterygii), cortisol predominates but corticosterone is also present; in the rest, the dominant or sole GC varies. In amphibians, reptiles, and birds, the dominant GC is corticosterone. In mammals, most placentals and marsupials secrete primarily cortisol. However, some (e.g., echidna, rabbits, rats, house mice, and voles) secrete primarily or only corticosterone, whereas other closely related species (e.g., platypus, hares, spiny mice, and most other rodents) secrete primarily or only cortisol. Thus, the best advice in mammals as to which

glucocorticoid to analyze for, is to check the literature first and, if in doubt, analyze a heterogeneous sample (variation in age and sexual condition) for both. However, if one gets both, it is not clear what they mean or what to do with them; i.e., what is the functional significance biologically of the less abundant GC and how to treat levels of the two (leave them separate, e.g., Place and Kenagy 2000, or sum them, e.g., Reeder et al. 2004). An understanding of the relative importance of the two GCs will require studies to quantify the amount of both that is free (not bound to CBG), their binding affinities to CBG and GC receptors (mineralocorticoid receptors and glucocorticoid receptors), and their metabolic clearance rates. The need to ascertain the primary GC is not only important when measuring blood GCs but also when measuring GCs (or their metabolites) in all tissues discussed here.

Sample collection and storage

Prime considerations for blood collection are the well-being of the animal, the ease and rapidity of collection, the need for an anesthetic, and the safety of the investigator. Venous blood is usually collected, but arterial blood has also been obtained. The site of blood collection is usually specific for the species or family in question, and it is best to consult the enormous literature in this area. Sites include the caudal vein in fish and reptiles (e.g., Jessop et al. 2003; Webb et al. 2007), the brachial and jugular veins in birds, (e.g., Newman et al. 2008a), tail vein in mammals (kangaroos; e.g., Mckenzie et al. 2004), the jugular (large mammals) and the saphenous veins (Hem et al. 1998). In small mammals (mice, voles, lemmings, squirrels <1,000 g), the infra-orbital sinus has been used extensively (reviewed for efficacy and impact in Bradshaw 2003). In some species, the site is unique (e.g., in platypus, the vascular sinus on the anterior margin of the upper bill, Handasyde et al. 2003; in penguins, the medial metatarsal vein, Sergent et al. 2004; in bats, the interfemoral vein,, Wimsatt et al. 2005) and in others, where access to veins is difficult, blood-sucking bugs (Reduviidae, Heteroptera) have been used to obtain a blood sample (Voight et al. 2004; Arnold et al. 2008). The need for restraint is critical and may require an anesthetic (an inhalant such as isoflurane or an injectable such as telazol), but for the smaller species, a simple handling bag that covers the eyes of the animal and minimizes stress may be sufficient. The key is to have complete familiarity with both the veterinary and wildlife literature that may apply to the species in question, but to be prepared to try new approaches that may be more suitable and minimize stress. For example, although the standard protocol for obtaining blood samples from rabbits is holding them in a restraint device and bleeding from a marginal ear vein, snowshoe hares were less stressed by restraining them in a burlap bag with only the ear exposed and bleeding from an ear artery (Boonstra and Singleton 1993).

GC levels from either plasma or serum can be used as both give the same result. Foster and Dunn (1974) compared cortisol levels in both plasma and serum from the same blood sample using the same methodology and found they yielded the same GC concentration. Plasma is obtained from blood collected with an anti-coagulant such as heparin or EDTA and spun on a centrifuge to remove red blood cells; thus, it contains fibrinogen. Serum is obtained from blood with no anti-coagulant and samples simply sit and coagulate and then are spun; thus, the serum contains no fibrinogen. Upon collection of plasma samples, the blood should be kept on ice and spun in a centrifuge as quickly as possible to minimize steroid metabolism.

Plasma or serum GCs are extremely stable over time, with no deterioration occurring in plasma or serum held at −20°C for decades (Stroud et al. 2007). Reimers et al. (1983) found that GC levels were stable when kept at room temperature or 4°C for 72 h, but levels declined by 14% after 8 days at 4°C. Thus, we recommend that blood is collected, stored on ice until centrifuged as soon as possible (<24 h), processed either as plasma or serum, and stored at or below −20°C in a non-frost-free freezer (temperatures in frost-free freezers rise briefly to about 0°C every 24 h).

Analysis techniques

In analyzing GC from blood, the following need to be borne in mind. Volumes of plasma or serum from wildlife are often small and thus the extraction method and antibodies must be selected to account for this. Both, commercial or in-house immunoassays (RIAs or EIAs) may all have different detection limits. GC levels in wildlife species can range from a few nanograms/millilitre (e.g., many bird species) to thousands/millilitre (e.g., lemmings) (e.g., Romero et al. 2008). High concentrations can be diluted, but options for species with low GC concentrations may be more limited.

Unlike the other matrices that we discuss in this review, assays of plasma GCs measure total GC concentrations rather than reflecting free GC concentrations. However, because free hormone levels are the critical measurement (see "The neuroendocrine stress response", above), two further pieces of information are needed: the equilibrium dissociation constant (K_d) of CBG and the maximum corticosteroid binding capacity (MCBC or B_{max}) of the plasma/serum sample. These values are then used, along with the total GC concentration, to calculate the free hormone concentration. The equation most often used to generate a free hormone estimate is that of Barsano and

Baumann (1989). This equation generates an estimate that implicitly includes albumin-bound hormone as "free".

Whereas commercial kits can make the measurement of total GCs relatively easy, obtaining the K_d and B_{max} are significantly more involved. The K_d is typically calculated from a pooled plasma sample using saturation binding data analyzed by Scatchard plot (Bradley 1987) or non-linear regression (Orchinik et al. 1997; Delehanty and Boonstra 2009). The K_d is generally calculated for a species as a whole (although some researchers have looked for and sometimes found sex or seasonal changes; e.g., Silverin 1986; Romero and Wingfield 1998) and many species have published K_d values (see Westphal 1971; Sernia et al. 1979; Breuner and Orchinik 2002; Malisch and Breuner 2010). In contrast, the B_{max} is calculated for each individual plasma sample because it is a measure of CBG concentration which can vary between individuals, sexes, seasons, and with handling time (McDonald et al. 1981; Boonstra and Boag 1992; Breuner et al. 2006). It is beyond the scope of this review to describe these protocols in detail as they are quite varied and all highly technical (some starting points in the literature include Silverin 1986; Hammond and Lähteenmäki 1983; Charlier et al. 2009).

Use with free-ranging animals

Collecting blood samples from wild animals is invasive. It requires a sophisticated skill set that presupposes a detailed knowledge of the natural history of the animal (ecology, evolution, and behavior) together with a comprehensive knowledge of the physiology of the HPA axis and of stress to allow one to make sense of the sample. It includes being able to capture animals in their natural habitat, handle and restrain them, possibly anesthetize them, collect the sample with a minimum of stress, and finally process the sample in a rigorous, quantitative manner that recognizes the problems and limitations that could compromise the results. All in all, a daunting but holistic task.

The advantage of collecting blood samples are two. First, one is measuring the direct product of the adrenal cortex—the GCs, not a breakdown or excreted by-product—and thus there are numerous assay kits with the requisite antibodies developed by the biomedical community at one's disposal. Second, it permits a simultaneous collection of a whole suite of blood components that permit a comprehensive assessment of the state of the animal, including measures of CBG, of energy resources (glucose and free fatty acids), of indices of condition (hematocrit), of indices of immune function (leukocyte profiles), of other blood components (e.g., blood urea nitrogen, creatinine, ammonia), and of reproductive status (reproductive hormones). All these can change in concert with changes in an animal's response to environmental stressors (see the following for examples of use of many of these components in wildlife: Wingfield et al. 1983; Boonstra et al. 1998; Trumble and Castellini 2002; Wikelski and Romero 2003; Clinchy et al. 2004; Sheriff et al. 2010a, 2011). Ultimately, the significance of a given physiological signature can only become clear from comparative studies when one is contrasting animals from natural or experimental situations.

The disadvantage of collecting blood samples is that the very process we are interested in studying may be biased by the technique used to acquire the samples. A common problem for studies of stress in wild vertebrates is that the stress of capture and handling often prevents researchers from obtaining a "true baseline" stress profile. Thus the control—a benchmark of what constitutes the 'normal' unstressed condition—may not be readily available. There are now numerous wildlife studies showing that plasma GC levels increase significantly after 2–5 min from capture (e.g., Boonstra and Singleton 1993; Cash et al. 1997; Place and Kenagy 2000; Boonstra et al. 2001; Romero and Reed 2005; Mueller et al. 2006; Wada et al. 2007; Romero et al. 2008). It is often impractical or impossible to obtain blood samples from wildlife this quickly under field conditions. Instead, researchers are often limited to obtaining "nominal baseline" blood samples collected after an animal has been subject to capture protocol periods often measured in >5 min to hours (such as in cage traps; e.g., Lynn and Porter 2008).

There are three approaches to dealing with the problem of capture-induced stress. First, assess directly the impact of capture and handling on the stress response. Kenagy and Place (2000) and Place and Kenagy (2000) compared true baseline levels of GCs of yellow-pine chipmunks (*Tamias amoenus*) as soon as they entered the traps to those obtained on the same animals 30 min later. They found that the 30-min samples accurately depicted the seasonal changes in GCs. Wingfield and colleagues have used a similar capture-and-handling protocol to great effect in comparative studies on birds (e.g., Wingfield et al. 2004). Second, recognize that the bias occurs and to treat it as a constant by standardizing collection procedures and times and examining the differences that occur among animals independent of the bias. Such nominal base GC levels may still reveal the effects of seasonal changes, inter-annual changes, or experimental treatment effects (e.g., Boonstra et al. 1998). Third, use a hormonal-challenge protocol that overrides the immediate effects of capture and handling to get an integrated picture of stress axis responsiveness and what the animal has been experiencing in the recent physiological past (days to weeks). The protocol typically involves two steps sequentially: a dexamethasone suppression followed by an ACTH stimulation test. The first assesses whether the brain is registering GC levels

correctly, and making the necessary negative feedback adjustment by reducing ACTH and thus GC release; the second probes the responsiveness of adrenals directly. In most wild mammals studied, chronic stressors cause animals to be dexamethasone resistant (i.e., GC levels do not decline as far as they should) and, depending on the species, show a hypo- or hyper-GC response to ACTH stimulation (Sapolsky 1983; McDonald et al. 1986; Boonstra et al. 1998; Sheriff et al. 2010a; but see Harlow et al. 1992). This protocol or modifications of it has been successfully applied in comparative studies on stress in a wide variety of wildlife species (e.g., lizards, Gist and Kaplan 1976; sparrows, Astheimer et al. 1994; snowshoe hares, Boonstra et al. 1998, 2001; starlings, Rich and Romero 2005; rheas, Lèche et al. 2009).

The alternative problem of frequent capture and handling of animals in the field and obtaining blood samples from them is one of habituation. This could result in a reduction of the stress response with frequency of capture. Cyr and Romero (2009) discuss four approaches to assessing this situation.

Saliva

Salivary GC levels have been used as an alternative to serum GC levels in a number of clinical and captive studies (e.g., Lutz et al. 2000; Anisman et al. 2001; Yaneva et al. 2004; Pearson et al. 2008). The increasing popularity of this method is not surprising given it is a non-invasive procedure that reflects free GC concentration in the blood (Kirschbaum and Hellhammer 1994; Gröschl 2007; Wood 2009). The high correlation between salivary GC levels and free-unbound serum GC levels remains high during the circadian cycle and under the many tests used to validate this technique such as ACTH stimulation and dexamethasone suppression tests (Dorn et al. 2007; Hellhammer et al. 2009). However, there are a number of hurdles to the use of this technique in free-ranging animals.

Transfer of hormones

Lipid-soluble steroids like GCs pass freely from the blood into the saliva through the lipophilic layers and glandular epithelial cells of capillary walls and the salivary gland acinar cells and diffuse into the saliva (Riad-Fahmy et al. 1982). Because GCs in the blood are largely protein-bound, only the free hormone can pass into the saliva, and GC levels are unaffected by salivary flow (Kirschbaum and Hellhammer 1994). The concentrations of GCs in saliva are 10–60% of those found in plasma depending on the species (Katz and Shannon 1964; Umeda et al. 1981; Wood 2009), supporting the need to validate this technique for each species prior to use. However, there is a strong positive correlation ($r^2 = 0.94$) between salivary GC and free GC levels in the blood (Umeda et al. 1981; Vining et al. 1983; Wood 2009), and salivary GC levels peak approximately 20–30 min after the onset of a stressor (Kirschbaum and Hellhammer 1989).

It must be noted that the salivary glands contain the enzyme 11β-hydroxysteroid dehydrogenase type II (11β-HSD II; Smith et al. 1996). This enzyme irreversibly converts active GCs (cortisol or corticosterone) to their inactive forms (cortisone and11-dehydrocorticosterone, respectively).

Sample collection and storage

Most studies on salivary GCs involve the collection of saliva by either spitting through a tube, or by chewing on an absorbent material. In humans, these techniques are often very easy and participants can be asked to actively spit or passively drool into the collection vial. Several commercial kits have been developed to aid in the collection of saliva. The most common are the Salivette (Sarstedt), the Quantisal (Immunalysis), the Intercept (Orasure Technologies), and the Sorbette (Salimetrics). Certain materials such as polyethylene, parafilm and cotton are known to absorb target molecules from the saliva and need to be avoided or at least have their use validated (Chang and Chiou 1976; Banerjee et al. 1962; Höld et al. 1995). The application of citric acid has also been used to stimulate salivary flow, but it can interfere with immunoassay analysis by decreasing the pH of the sample (Gallagher et al. 2006). Blood contamination needs to be avoided when sampling. A pink coloration will appear at a blood concentration of 0.1–0.2% (by volume); thus, a visual inspection can detect contamination (Wood 2009).

In animals, the general collection principles are the same; however, the techniques need to be modified for each species. Lutz et al. (2000) developed two techniques for collecting saliva from awake, unrestrained adult rhesus monkeys. In the first, monkeys licked a piece of gauze sandwiched between two pieces of Plexiglas (one with a hole in it) and covered with a screen (to prevent monkey from being able to remove the gauze). Dry KoolAid crystals were sprinkled over the screen and, when a monkey licked the crystals from the screen, the gauze absorbed their saliva. The second method (adapted from Boyce et al. 1995 and subsequently used by Pearson et al. 2008) consisted of a polyvinyl chloride (PVC) tube housing a clamped, removable piece of braided cotton rope flavored with KoolAid and then dried. A monkey would chew on the rope, and thus deposit saliva. Gomez et al. (2004) modified previously learned behaviors in a female Indian rhinoceros held at the National Zoological Park,

Smithsonian Institute, and trained her for daily saliva collection. The female was trained to hold her mouth open and remain in position while a plastic spoon was used to collect about 5 ml of saliva from her lower lip. Other captive studies have successfully trained mammals as diverse as guinea pigs (with cotton swabs) to deer (with cotton swabs) to dolphins (with a syringe) for saliva collection (Fenske 1997; Millspaugh et al. 2002; Cross and Rogers 2004; Hogg et al. 2005). Although stress-free and standardized sample collection may be easy and advantageous in humans and trained captive animals, saliva collection is likely to be much more difficult in free-ranging animals.

After collection, centrifugation can be used to separate the saliva from the collection material. GCs are highly stable in saliva and samples can be stored at room temperature for a few days (Chen et al. 1992) or even up to 4 weeks prior to analysis (Kirschbaum and Hellhammer 1989; but see Garde and Hansen 2005 who found GC levels decreased 9.2% after 1 month at room temperature). Garde and Hansen (2005) recently showed that cortisol concentrations did not degrade at 5°C for 3 months or at −20 and −80°C for 1 year. Repeated thawing and freezing of samples (up to four times) did not affect cortisol concentration (Garde and Hansen 2005).

Analysis techniques

RIAs to measure cortisol levels in saliva have been developed in the past, some of which were modifications of commercially available serum kits (see Kirschbaum and Hellhammer 1989). More recently, salivary cortisol assay kits have become readily available from a variety of manufacturers for a reasonable cost. These kits are usually microtiter plate format assays using enzyme immunoassay or chemiluminescence immunoassay technology. An advantage to using the commercially available techniques is that samples usually can be very small, between 2–10 µl in some cases (Lutz et al. 2000; Millspaugh et al. 2002; Pearson et al. 2008).

Use with free-ranging animals

One of the major advantages to measuring GCs in saliva is that it has the ability to be a non-invasive measure of free serum GCs (Umeda et al. 1981; Vining et al. 1983). However, setting up an appropriate collection site, such as a lick or chewing site, could be extremely difficult in the field, and one would need to ensure that only a single, known animal deposited saliva. Collection by hand (with a swab or syringe), even for small animals, may be difficult, dangerous (to both the researcher and animal), time consuming, and possibly impose researcher-induced stress. Alternatively, animals could be anesthetized and a sample taken. However, if these methodological obstacles can be overcome, salivary GCs would be of great benefit in many mammalian studies where capture protocols frequently make it impossible or difficult to obtain blood samples that reflect unstressed GC levels (e.g., Place and Kenagy 2000; Delehanty and Boonstra 2009). Unlike blood in which GC levels increase within 3 min of a stressor (Romero and Reed 2005), GC responses in saliva are not detectable until 20–30 min have passed (Kirschbaum and Hellhammer 1989). Thus, saliva is a useful GC medium for researchers who are interested in measuring basal GC levels.

Another advantage to saliva GC measures is that, unlike other non-invasive tissues (feces or hair), it can be used to collect a controlled number of samples across relatively short time intervals (if the collection procedures allow this; Lutz et al. 2000). Furthermore, saliva GCs are relatively stable and can be kept at room temperature for a number of days (Chen et al. 1992). In remote field camps where access to freezers may be extremely limited, this can be very important. However, it should be noted that samples be spun soon after collection, if they dry up they cannot be analyzed.

Urine and feces

In the past 25 years, measuring glucocorticoid metabolite levels in excreta (urine and feces) has been increasingly used to measure stress levels in laboratory, domestic, zoo and free-ranging animals. Previous studies have reviewed the methods and guidelines for the use of these methods (Taylor 1971; Wasser et al. 2000; Buchanan and Goldsmith, 2004; Millspaugh and Washburn 2004; Möstl et al. 2005; Palme 2005; Palme et al. 2005; Touma and Palme 2005). Here, we review the use of assays to measure glucocorticoid metabolites (GCM) in urine and fecal samples from free-ranging animals and present practical guidelines for field biologists.

Transfer of hormones

GCs in the plasma are extensively metabolized by the liver and excreted into the urine via the kidneys or into the gut via the bile ducts (Taylor 1971; Palme et al. 2005). There are no native, unmetabolized GCs in feces (Touma and Palme 2005), while there can be a small amount of native GCs in the urine (Bahr et al. 2000). The metabolism of GCs is not straightforward. Some of the GCM produced in the first pass through the liver are reabsorbed into the blood stream from the gut and undergo further hepatic degradation (Taylor 1971; Klasing 2005). Intestinal microflora can also further alter the structure of GCM (Eriksson and Gustafsson 1970). Fecal glucocorticoid metabolites (FGM)

reflect the free (unbound) GC fraction of total GCs (Touma and Palme 2005; Sheriff et al. 2010b).

In mammals, the proportion and structure of metabolized GCs that are excreted in the urine and feces differs among species and can also differ between males and females (Touma et al. 2003; Palme et al. 2005). Radiometabolism studies (for review, see Palme et al. 2005) can be used to determine the proportion of GCM excreted in the urine and feces by injecting animals with radiolabeled GC and collecting all excreted material over a given time interval (usually a few days).

In avian and reptilian species, urine and feces are excreted together as droppings and, in some species, they are mixed together in the cloaca prior to excretion (Klasing 2005). As a result, the exact proportion of GCs excreted in each is frequently unknown. Some previous studies have collected and homogenized the entire droppings (e.g., Washburn et al. 2003) whereas others have only collected the fecal portion (e.g., Hirschenhauser et al. 2005).

Sample collection and storage

Although a large proportion of GCM can be excreted in the urine (Palme et al. 2005), few studies have measured GCM in urine of wild animals (but see Creel 2001) because of the logistical difficulties associated with collecting all of the urine voided under field conditions. The amount of urine may vary among bouts of excretion and can easily be lost during collection in the field, thus the concentration of GCM may be biased among samples (Millspaugh and Washburn 2004; Wasser and Hunt 2005). However, urine samples could be collected in a container underneath a wire-caged live-trap. Researchers should standardize the collection procedure and recognize the above caveats for urine collection particularly in avian/reptilian species (Millspaugh and Washburn 2004; Goymann 2005; Wasser and Hunt 2005).

FGM levels can be affected by environmental conditions (Rehnus et al. 2009; Washburn and Millspaugh 2002), age of the samples (Millspaugh and Washburn 2004), and bacterial or microbial degradation after defecation (Eriksson and Gustafsson 1970; Möstl et al. 1999, 2005; Lexen et al. 2008). Fecal samples collected under field conditions often cannot be preserved immediately, and the length of time between excretion and collection is often not known (depending upon interval of checking live-traps). Therefore, it is important to preserve fecal samples as soon as possible after defecation to minimize bacterial or microbial degradation of FGM. Freezing immediately after collection (followed by lyophilization if the samples cannot be kept frozen) is arguably the best choice (Lynch et al. 2002; Hunt and Wasser 2003; Palme et al. 2005). However, this can be difficult for field researchers in remote regions where freezers and lyophilizers may not be available. There are a variety of other methods that have been used to preserve fecal samples collected from wild animals. Fecal samples have been placed upon wet ice in the field until permanently frozen (Dantzer et al. 2010), preserved in alcohol at either ambient temperature or frozen (Wasser et al. 1988; Khan et al. 2002; Terio et al. 2002), dried in an oven (Terio et al. 2002), or immediately extracted and preserved on solid phase extraction cartridges (Beehner and Whitten 2004; Beehner and McCann 2008). The storage method and time of collection can also interact to introduce biases in FGM concentrations (Khan et al. 2002; Terio et al. 2002; Hunt and Wasser 2003; Millspaugh and Washburn 2004; Möstl et al. 2005; Palme et al. 2005). Therefore, preservation and storage methods (in combination with sampling and extraction procedures) must be chosen and validated carefully to determine how these may affect FGM concentrations (Hunt and Wasser 2003; Möstl et al. 2005; Palme 2005). It is not recommended to alter storage methods of fecal samples over the course of the study; however, if necessary, it is especially important to evaluate any consequent changes.

Analysis techniques

Wet or dry feces can be used for analysis, and both measures usually correlate quite well (Wasser et al. 2000). Dried feces are used when wet samples are not available (e.g., small animals' feces dry quickly), undigested materials need to be sorted out, or large differences in diet are present (Wasser et al. 1988, 1993; Millspaugh and Washburn 2004; Klasing 2005). Caution is advised in the case of longitudinal studies of herbivorous mammals or birds because seasonal changes in their diet may alter gut flora and therefore steroid metabolism. Complete homogenization of fecal samples helps distribute FGM uniformly in the fecal matrix and provides a more accurate estimate of FGM levels than subsampling from the defecate (Palme et al. 1996; Millspaugh and Washburn 2004; Beehner and Whitten 2004; Goymann 2005). To account for variation in water content in urine samples, creatinine levels can be measured (Palme et al. 1996; Bahr et al. 2000).

Metabolites can be extracted from the fecal matrix using a wide variety of techniques; however, the choice of the extraction procedure is very important because the metabolites may have different polarities (Palme 2005; Palme et al. 2005). The most common extraction method is through the use of a high percentage of methanol (or ethanol), where a proportional volume of methanol is added and then samples are vortexed, centrifuged, and an aliquot (in most cases after further dilution with assay buffer) of the supernatant measured in the respective immunoassay

(Palme and Möstl 1997; Wasser et al. 2000; Mateo and Cavigelli 2005; Möstl et al. 2005; Palme et al. 2005).

EIAs and RIAs have been used with equal frequency to measure FGM among various species. A major advantage of EIAs is that it is possible to develop group specific antibodies for the FGM (i.e., they react with several FGMs that share a common chemical structure) and they do not require the use of radioactive substances (see Goymann 2005; Möstl et al. 2005). In contrast, RIA kits use antibodies that were developed to react with a specific unmetabolized GC in plasma (i.e., cortisol or corticosterone) and depend upon unknown cross-reactivities with FGM (e.g., Wasser et al. 2000; Mateo and Cavigelli 2005). FGM can be characterized by high-performance liquid chromatography as this will aid in the selection of an appropriate antibody (Möstl et al. 2005). Alternatively, this step can be overcome by testing multiple antibodies (Goymann et al. 1999; Wasser et al. 2000; Millspaugh and Washburn 2004; Goymann 2005; Möstl et al. 2005). It has also been argued that, if the antibody accurately reflects known changes in adrenocortical activity (increase after ACTH stimulation), it is unnecessary to know the chemical structure of the FGM (e.g., Wasser et al. 2000). Fecal FGMs should also reflect known blood changes in GC levels as a function of reproductive status. Reproductive status in both males (testes active or not) and females (not breeding, pregnant, or lactating) cause major changes in plasma GC levels (see Handa et al. 1994), and these must be reflected in FGM.

Use with free-ranging animals

Collecting fecal samples from free-ranging animals is relatively non-invasive compared to the other methods and can be performed by untrained personnel. For example, fecal samples can be collected from individually marked ungulates by collecting feces in the field after an observer notes defecation (Ganswindt et al. 2010). Live-trapping is often required to collect fecal samples from small animal species. However, this is less invasive compared to the handling that is required to obtain plasma samples and even saliva or hair samples.

One disadvantage of fecal samples is that they cannot provide as many physiological indices of stress as blood samples (e.g., leukocyte profile, glucose, blood chemistry). However, the same fecal samples used to measure FGM can also be used to perform genetic studies (Huber et al. 2003) or measure other steroids (e.g., androgen levels; Möhle et al. 2002; Dloniak et al. 2004; Ganswindt et al. 2010; Dantzer et al. 2011), which would require similar validation procedures as described above (Palme 2005). In addition, assignment of collected samples to individuals is sometimes difficult (Huber et al. 2003).

Aside from the non-invasiveness of collecting fecal samples, there are two other major advantages to measuring FGM levels rather than plasma GC levels. The first is that FGM levels are not prone to researcher-induced biases introduced by restraint/handling or short-term fluctuations in GCs due to normal pulsatile changes in GC secretion. The time from the initial GC release due to a stressor until the signature appears in the feces (delay time) is much longer than the time the animal spends restrained or in a live-trap (FGM levels change over a period of 6–24 h depending upon the species; Palme et al. 2005). The second is that plasma GCs are point-of-time estimates and can be heavily influenced by time of day due to circadian patterns or other short-term fluctuations due to minor disturbances. FGM are thought to reflect an integrated average of plasma-free GCs that the animal has secreted, metabolized, and excreted over the course of a species-specific amount of time (depending on the frequency of defecation; Palme et al. 1996, 2005; Goymann 2005). Whether FCMs represent an accurate estimate of baseline GCs or an integrated average of total GCs experienced by the animal is unclear (Millspaugh and Washburn 2004; Goymann 2005; Sheriff et al. 2010b). However, a recent study clearly demonstrated that FGM levels are highly correlated with true baseline free GCs in plasma and that FGM levels predict an animal's plasma GC response to a standardized hormonal challenge (Sheriff et al. 2010b). As such, FGM levels reflect both true baseline GC levels and an animal's stress response, making this technique an invaluable non-invasive alternative to blood sampling in field studies.

Hair and feathers

Of the tissues presented in this review, hair potentially offers the longest record of an animal's GC exposure. The slow growth of hair means that the time scale over which one can measure the animal's GC levels is typically measured in weeks and months rather than hours and days. This could make hair a useful medium for studies examining the effects of chronic stressors, but unsuitable for investigating the impacts of more transient stressors (even if those transient stressors have significant biological effects). The exact time scale will depend on the rate of hair growth.

Feathers are also long-term records of GC levels, but the nature of their growth may offer more flexibility than hair. Some feathers have bands that correspond to daily growth cycles (Grubb 1989) which can then be used to provide a more detailed chronology of GC exposure. By clipping the feather at specific growth bands and measuring GC levels at several points along the length of the feather, a history of GC exposure with a resolution of a few days or a week can be achieved (Bortolotti et al. 2009a).

The study of GCs in hair and feathers is in its infancy; few studies (with mostly low numbers of samples) have used these matrices, and even fewer have made any attempts to properly establish that hair and feather GC levels accurately reflect long-term plasma GC levels. Any researchers wanting to use hair of feather GC levels should be prepared to first carry out a proper validation.

Transfer of hormones

We do not, as yet, have a comprehensive knowledge of how GCs are incorporated into hair and feathers. Hair grows from a root supplied by capillaries from which free GCs diffuse into matrix cells which ultimately become the dead hair shaft (Cone 1996). Blood supply is thought to be the major source of GCs, but the hair shaft can also be exposed to GC-containing secretions of various glands that may be absorbed (Cone 1996). A major shortfall to using this matrix is that currently we do not know the relative contribution of these various sources. In humans, the principal secretions are sweat and sebum, but numerous wild animals also secrete scents on certain areas of fur in ways that vary seasonally (e.g., Bradley and Stoddart 1993) or with dominance (e.g., Shimozuru et al. 2006). These secretions, as well as salivary GCs from grooming, could contribute to variation in GC levels. There is also a potential for localized GC synthesis in hair follicles in humans (Sharpley et al. 2009) which could affect the interpretation of hair GC levels as representative of systemic concentrations.

Feather growth is similar to hair growth in that feathers grow from a highly vascularized base that produces the dead, keratinized feather structures in which GC exposure is assumed to be stored. Like hair, feathers incorporate GCs through their vascular portion (Bortolotti et al. 2008), and it is likely that feathers can also absorb GCs that they are exposed to externally (as is the case with many heavy metals; Jaspers et al. 2004). But no research has been done to examine the contribution of glandular secretions to feather GC content.

Collection and storage of samples

One of the greatest advantages of sampling hair and feathers is the ease with which they can be collected and stored. Hair is simply clipped (plucking is to be avoided as the living follicle cells can skew results; Gow et al. 2010) and stored at ambient temperatures in an envelope (Raul et al. 2004; Accorsi et al. 2008; Sauvé et al. 2007). To obtain a real time record of GC levels, hair must be clipped immediately prior to the start of the period of interest and then the regrown hair can be clipped at the end. The regrown hair will provide an average GC exposure over that period; however, the minimum length of time will depend on the rate of regrowth which may, in turn, be affected by seasonal molting (e.g., Paul et al. 2007). Collection of hair samples without a "reclipping" protocol is unlikely to provide useful information, as the time period over which the hair grew would be unknown.

Feathers can be plucked or clipped. Analogous to the repeated clipping of fur, it is possible to stimulate feather regrowth by plucking a feather from a known location and then measuring the GC concentration in the regrown feather (e.g., Bortolotti et al. 2008). Again, the time frame over which GC exposure can be measured will be determined by the rate of feather regrowth. Although clipping of both hair and feathers may be acutely stressful and increase GC levels, this will not be relevant for the current sample but may be for future samples of re-grown hair or feathers.

Both hair and feather samples are routinely kept at room temperature prior to assay. Whether long-term storage affects GC levels has not been investigated (though see Bortolotti et al. 2009a, who reported much higher GC levels in specimens older than 25 years). In most studies that we reviewed, samples have been stored for <1 year and researchers have not stated any concerns about loss of GCs prior to analysis.

Analysis techniques

Much of the work on steroid detection in hair samples is focused on anti-doping in athletes or the detection of banned growth promoters in farm animals. For these purposes, liquid chromatography–mass spectrometry is frequently used for its ability to detect many different synthetic hormones that may have been given in small doses (e.g., Van den Hauwe et al. 2005). Due to the expense and high degree of expertise needed to use this technique, and because most ecologists will only need to measure natural GCs, we will limit our discussion of analysis techniques to immunoassays.

Some authors recommend washing hair to eliminate any GC contamination by secretions on the outside of the hair. Raul et al. (2004) and Van den Hauwe et al. (2005) both used brief methylene chloride washes to rid hair of external GCs. However, the wash procedure has the potential for penetrating the hair and removing sequestered GCs. Davenport et al. (2006) methodically tested organic, aqueous and alcohol washes and determined that an isopropanol wash was most appropriate for their macaque (*Macaca mulatta*) hair.

After hair or feathers are collected, they are typically weighed or measured. Feather GCs are best expressed as pg/mm due to the nature of their growth (see Bortolotti et al. 2009a). Hair GC levels have been expressed in pg/mg, but if hair growth rates vary seasonally, this may

generate misleading trends. The sample is either finely cut (Koren et al. 2002; Sauvé et al. 2007; Accorsi et al. 2008; Bortolotti et al. 2008; Sharpley et al. 2009) or pulverized with a ball mill (Raul et al. 2004; Davenport et al. 2006). The latter method presumably increases extraction efficiency by increasing the surface area exposed to solvent. Extractions for liquid chromatography-mass spectrometry are more involved (see Van den Hauwe et al. 2005), but most researchers using assay kits use a simple methanol extraction (or, rarely, ether; Martin and Réale 2008). Methanol is preferred for its ability to penetrate into the shaft of hair (Davenport et al. 2006). Exact procedures vary, but all typically involve a long incubation time (16–24 h) at various temperatures (20–52°C), with or without agitation (Davenport et al. 2006; Sauvé et al. 2007; Accorsi et al. 2008; Sharpley et al. 2009). The methanol is then pipetted and evaporated off, followed by resuspension of the hormone in buffer.

There are numerous commercial cortisol and corticosterone kits available, and most studies have simply adapted kits intended for saliva (Koren et al. 2002; Davenport et al. 2006; Sauvé et al. 2007) or used commercially available antibodies (Accorsi et al. 2008; Bortolotti et al. 2008) using standard assay procedures. Prior to commencing work on a new species, the mass of hair or length of feather needed for extraction and the appropriate reconstitution volume need to be empirically determined to ensure that the final concentration of hormone falls within the assay range.

As with all the techniques reviewed here, proper validation of the assay procedure is critical whenever techniques are applied to a new species (see Buchanan and Goldsmith 2004). We note, however, that only Bortolotti et al. (2009a) attempted a validation for the analysis of GCs in feathers.

Use with free-ranging animals

Measurement of GCs in hair and feathers is a technique that has a great deal to offer physiological ecologists, but there have been very few wildlife studies using this technique. Most studies have been correlational studies or have contrasted GC levels in individuals with different behaviours (Koren et al. 2002, 2008; Bortolotti et al. 2008; Martin and Réale 2008). Recently, a pair of manipulative field experiments in the red grouse (*Lagopus lagopus scoticus*) used feather GC levels (Bortolotti et al. 2009b; Mougeot et al. 2009). They found that higher corticosterone levels were related to increased susceptibility to parasites and reduced male ornamentation (Bortolotti et al. 2009b). As the grouse studies illustrate, the use of hair and feathers may have great potential for the study of chronic stress. Where long-term stressors vary naturally or can be manipulated (e.g., parasite infections, population density, predation pressure), hair and feather GCs can provide a record of GC levels throughout the entire period of stress and provide a more accurate measure of individual stress than before/after point measurements of GCs.

However, we currently do not have sufficient evidence to definitively conclude that hair and feather GCs accurately reflect long-term plasma free GC levels. We believe that researchers need to empirically establish: (1) that GCs are incorporated into hair and feathers proportionately to the concentration of free hormone in the blood; (2) whether saliva, scents, or other secretions (or excreta) contribute to measured GC levels in a way that compromises the interpretation of GC levels; (3) whether GCs "leach" out of hair and feathers while still on the animal or after being clipped; (4) whether the rate of hair regrowth varies and, if so, whether the faster growing hair has a lower GC concentration due to a dilution effect; and (5) if hair/feathers growing at different parts of the animal have similar GC levels. Currently, no researchers have made these basic, yet critical, examinations.

Miscellaneous techniques

The techniques presented so far have the broadest application for wildlife studies. However, there are some additional methods that have specialized applications. The first is measuring GC levels in water as a non-invasive measure of fish stress levels. Free GCs leak out of fish gills into the water where, after considerable processing to concentrate the hormones to a detectable level, they can be measured. This technique has recently been reviewed and evaluated (Scott et al. 2008), so we will not dwell on the methods and practical implications. Clearly, this technique requires fish to be held in tanks, which makes it difficult to apply to free-living fish.

In mammals, GCs can be transferred from mothers to nursing offspring through the milk (Zarrow et al. 1970). The transfer of GCs to offspring in this manner was reported to have profound and long-lasting effects on the number of glucocorticoid receptors in their hippocampus, on adrenocortical activity, and on behavioral stress response in the offspring (Casolini et al. 1997; Catalani et al. 2000). While quantifying levels of GCs in milk has been performed regularly in agricultural animals (Butler and Des Bordes 1980; Walker et al. 2008), few studies in natural populations have documented GCs in milk. Adequate milk samples for GC analysis can be obtained from both large and small species of mammals via manual ejection or by injecting oxytocin that facilitates milk letdown (Veloso et al. 2003; McDonald and Fuller 2005; Lang et al. 2005).

Another technique that has particular relevance to the study of maternal programming of the stress axis is GC analysis of eggs. In egg-laying animals, GCs are deposited in the egg by the mother and are thought to reflect the mother's GC status, although not necessarily in a straightforward manner. There can be among-female and within-clutch relationships, as reported in Love et al. (2008). It has to be noted that, in birds, egg yolk is produced in layers, which contain different amounts of steroids (Hackl et al. 2003), and that gestagens, which are present at very high concentrations, may interfere with the measurement of the low concentrations of GC (even if respective cross-reactions in the immunoassays are low; Rettenbacher et al. 2009). The ability to measure and manipulate egg GC levels has allowed researchers to investigate the effect of embryonic GC exposure on a number of measures of development, behavior, physiology and fitness (e.g., sex ratio in two species of lizard (Warner et al. 2009), stress sensitivity in fish (Auperin and Geslin 2008), survival and symmetry in fish (Gagliano and McCormick 2009), and flight performance in birds (Chin et al. 2009). However, caution is advised in birds as there is a growing body of evidence that maternal stress is not directly affecting the offspring via GCs in eggs (failure of an ACTH test to increase egg GCs and lack of increase of egg GCs in stressed birds; Rettenbacher et al. 2005; Janczak et al. 2007), but via other mechanisms (e.g., changes in content of gonadal steroid hormones; Henriksen et al. 2011).

Conclusion

This paper is directed largely toward the uninitiated who are considering measuring GC levels. We believe that stress physiology has a great deal to contribute to behavioral, population, and community ecology. Even a brief scanning of the recent literature will attest to the explanatory power and value of these measurements. However, like the blind use of statistics, GC levels cannot be measured or interpreted without sufficient background and knowledge and without meeting the assumptions. Measuring GC levels does not equate to measuring "stress", but they are a critical component of the stress response and, when taken together with other indices of stress (e.g., measures of immune function, metabolism, nitrogen balance), they offer considerable insight into how animals perceive and adapt to their environment. In this review, we have provided an introduction to the key papers in the literature, in the hope that more ecologists will make the somewhat daunting leap into exploring the role of GCs in animal ecology and evolution.

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