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Tracking stress: localisation, deposition and stability of corticosterone in feathers

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SUMMARY

How animals cope with stressors is an important determinant of their well being and fitness. Understanding what environmental perturbations are perceived as stressors, and quantifying how they are responded to, how often they occur and the negative consequences of exposure to glucocorticoids, has been problematic and limited to short-term physiological measures. By contrast, the quantification of corticosterone (CORT) in feathers represents a long-term, integrated measure of hypothalamic–pituitary–adrenal activity. In the present study, we show that by understanding how the hormone is deposited in feathers, in combination with specific sampling protocols, one can identify localised patterns of CORT deposition that reveal different temporal patterns of a bird's response to stressors. CORT in feathers appears to be stable over time, is resistant to heat exposure and is useful in determining both the overall exposure of the bird to the hormone over days or weeks, as well as identifying discrete, punctuated, stressful events. Variation in feather CORT can also be examined among individuals of a population at one point in time, as well as over years by using museum specimens. The ability to track stress over time allows for new questions to be asked about the health and ecology of birds and their environment.

Key words: feathers, stress physiology, glucocorticoids, fault bars, time frame.

INTRODUCTION

Few would deny the breadth and significance of the role played by stress in the biology of animals. Despite it being well documented as an important determinant of health and fitness, many components of stress physiology and their ecological and behavioural consequences are difficult to evaluate (Breuner et al., 1999; Romero, 2004). While some controversy exists regarding conceptual issues and terminology, it is clear that environmental perturbations, as threats to homeostasis, present fitness challenges and that animals are adapted to deal with them, albeit with considerable individual variation (Blas et al., 2007; Romero, 2004; Williams, 2008). A suite of behavioural and physiological changes known as the stress response is initiated in response to stressors, i.e. noxious stimuli (see Romero, 2004). The vertebrate hypothalamic–pituitary–adrenal (HPA) axis releases glucocorticoid hormones (Astheimer et al., 1992; Blas et al., 2007; Holberton, 1999; Koch et al., 2002; Sapolsky et al., 2000). This response redirects animals to a life-saving state or 'emergency life history stage' allowing them to overcome stress and re-establish homeostasis in the best possible physical condition (Wingfield and Ramenofsky, 1999; Wingfield and Silverin, 2002). However, chronically elevated levels of glucocorticoids have detrimental consequences to immune function, cognitive ability, growth, reproduction and survival (Kitaysky et al., 2003; Sapolsky et al., 2000; Wingfield and Ramenofsky, 1999; Wingfield and Silverin, 2002). Glucocorticoid levels are generally measured in the blood but the elevated levels of the stress response are found in circulation for a relatively short period of time (typically minutes). In addition, the protocol for measuring an individual's response generally requires handling and restraint, and it is not always clear how applicable this extreme protocol is to the plethora of natural stressors faced by an animal [see Bortolotti et al. (Bortolotti et al., 2008) for other methodological problems]. While blood sampling provides some idea of the magnitude of the response, what individuals perceive as stressors, how often they respond to them and therefore what is the total physiological exposure to corticosterone (CORT) over time is poorly known in most free-living animals.

Recently, Bortolotti et al. reported that CORT, the main avian glucocorticoid, can be measured reliably in feathers (Bortolotti et al., 2008). The levels of CORT in feathers represent an integration of HPA activity over a substantially longer period of time (days-to-weeks) than previously analysed using conventional blood sampling. Such results were found to be biologically meaningful for interpreting how individual birds respond to environmental perturbations and adjust to various life history stages (Bortolotti et al., 2008). In the present study, we ask a series of questions to further investigate the nature of CORT in feathers, with an emphasis on how one might monitor stress over time or evaluate it retrospectively, ultimately for a better understanding of its causes and consequences. We first explore in detail how the amount of CORT may vary along the length of individual feathers to give insight into how the hormone is incorporated into the feather, and to determine to what degree its localisation in the feather can be used to reveal when and how birds respond to stressors. We show that tracking stress over a number of time scales is possible at the level of the individual and population.

MATERIALS AND METHODS

Sample material and protocol

All feathers were prepared by first removing the calamus (i.e. the proximal, vaneless portion of the quill) and the length of the remaining portion, or segments of it, was measured and weighed. None of the samples were washed prior to hormone analysis as natural substances such as preen oils were found not to influence the results (Bortolotti et al., 2008). Feathers were stored in ordinary paper envelopes between collection (various years, see below) and analysis (2004–2008).

The feathers from wild birds used for this study were obtained from dead birds stored in a –20°C freezer for about a year, from museum specimens (University of Saskatchewan Biology Museum, Saskatoon, Canada), from freshly moulted feathers or, in the case of the great horned owl (*Bubo virginianus* Gmelin), a feather was pulled from live birds (see below). Details of the samples and a test for the potential effect of feather age on CORT are presented below.

How is CORT deposited in a feather?

It is imperative to understand how feathers grow and thus how CORT may be deposited for it is only during growth that this hormone may be incorporated into feathers (Bortolotti et al., 2008). The feather follicle is a tubular array of cells that is highly vascularised during growth. Growth rate for much of the length of the feather is at a relatively uniform rate (e.g. Bortolotti, 1984a; Bortolotti, 1984b) and at any point along the rachis, the vane on both sides, completely to the edge, is grown at the same time. Therefore, to capture the temporal nature of CORT deposition, one must cut feathers perpendicular to the rachis. Equal lengths of feathers from distal to proximal therefore represent approximately equal time periods.

To examine trends in CORT from proximal to distal along individual remiges (flight feathers of the wing), we used five freshly moulted feathers of bald eagles (*Haliaeetus leucocephalus* Linnaeus) collected from under nests at Besnard Lake, Canada (Gerrard and Bortolotti, 1988) between 1979 and 1982 and three primaries of a dead eagle in juvenal plumage (i.e. feathers were grown simultaneously as a nestling). The considerable length of eagle feathers, coupled with the availability of detailed knowledge of their growth rate (Bortolotti, 1984a; Bortolotti, 1984b), proved to be ideal for investigating patterns of CORT deposition. Segments 21 mm long were cut from these bald eagle remiges. Avoiding the worn tip, 10 segments from three feathers and 12 segments from two feathers were cut at right angles to the rachis.

To enhance the likelihood that within-feather patterns revealed in the analysis of bald eagle feathers could be generalised to other birds, we analysed remiges from five other species: prairie falcon (*Falco mexicanus* Schlegel), great horned owl, snow goose (*Chen caerulescens* Linnaeus), sandhill crane (*Grus canadensis* Linnaeus) and Swainson's hawk (*Buteo swainsoni* Bonaparte). These species were chosen in part because of availability and the ease of working with large feathers and also because they represented variation in body mass (approximately 0.6–4 kg), diet (carnivore, omnivore and herbivore) and habitat (terrestrial and wetland). A remige from the same position in the wing from one adult and one juvenile of the same sex were analysed for each species. Feathers were cut into three equal-length segments. We examined the proximal to distal variation in CORT in the rachis only to avoid the complication of varying degrees of vane asymmetry. The potential variation due to vane asymmetry was examined using the most proximal segment of each of these feathers.

Can punctuated stress events be identified?

For some time it has been known that stress is recorded in feathers in the form of fault bars – visible deformities (generally <1 mm in diameter) in the barbs running at approximately right angles to the rachis, which are believed to be caused by exposure to a variety of short-term stressors such as handling or bad weather (Bortolotti et al., 2002; Jovani and Blas, 2004). Only recently have they been found to be associated with a bird's fitness potential (Bortolotti et al., 2002). Using the bald eagle remiges, we examined whether such punctuated stress associated with fault bars could be detected using feather CORT.

Is CORT in feathers stable?

If CORT is to be compared among feathers it must be shown that it does not degrade appreciably over time or after exposure to the environment. To get some appreciation for whether CORT degraded, we analysed feathers from frozen carcasses of birds that had died relatively recently (<1 year before analysis in 2004) and those taken from the same age, sex and species as museum specimens collected between 1931 and 1972 (age=51±4.1 years, mean ± s.e.m.). We plucked a contour feather from the belly of 13 species representing a variety of birds: great horned owl, snow goose, sandhill crane, great blue heron (*Ardea herodeas* Linnaeus), mallard (*Anas platyrhynchos* Linnaeus), northern goshawk (*Accipiter gentilis* Linnaeus), gray partridge (*Perdix perdix* Linnaeus), American coot (*Fulica americana* Gmelin), peregrine falcon (*Falco peregrinus* Tunstall), snowy owl (*Bubo scandiacus* Linnaeus), ruffed grouse (*Bonasa umbellus* Linnaeus), Franklin's gull (*Larus pipixcan* Wagler) and western grebe (*Aechmophorus occidentalis* Lawrence).

In addition, we experimentally tested the stability of CORT by comparing segments of vane under ambient conditions with those exposed to 75°C for 30 min in a drying oven. We chose heat, in part, as it is one environmental variable that the feathers from most species would probably experience while still on the birds, it should increase the rate of degradation if indeed it was occurring and if proved unimportant it would be convenient for researchers to simply store dry feathers without regard to ambient conditions. Five remiges were pulled from one wing of each carcass of a sandhill crane, prairie falcon, great blue heron, Swainson's hawk, great horned owl, short-eared owl (*Asio flammeus* Pontoppidan), Canada goose (*Branta canadensis* Linnaeus), redhead (*Aythya americana* Eyton), Franklin's gull, Northern shoveler (*Anas clypeata* Linnaeus) and common raven (*Corvus corax* Linnaeus). The vanes of each feather were removed and kept separate from each other. The strands of vane were then separated from each other and mixed thoroughly within the individual feather sample. Each mixture was divided into 10 replicates, five of which were heated while the others remained as a control.

Variation within a population

To examine within-population variation and population variation over time, we collected back feathers from great horned owls from the vicinity of Saskatoon, Canada. In 2005 we collected one upper back feather from 45 carcasses of birds that died in 2004 and 2005 and whose carcasses were kept frozen. Although most specimens lacked information, the cause of death was typically suspected to be collision with a vehicle or simply found dead (likely to be starvation or, in two cases, birds tested positive for West Nile virus). Also in 2004 and 2005, a feather was collected from 10 live, wild owls obtained from various sources, e.g. trapped accidentally inside a building, captured by a bander. For comparison with these recent samples, we collected two back feathers (CORT averaged for analyses) from 21 museum specimens collected between 1931 and 1974 (1959.7±2.63, mean ± s.e.m.), also in the vicinity of Saskatoon. Of these latter birds, 15 date from the 1960s when they were pole-trapped as pests at a pheasant game farm. Sex was taken from museum labels (N=2 with no data) or determined by inspection of gonads or by DNA found in the feather (Doñana Biological Station, Seville, Spain) (Horvath et al., 2005).

Hormone analyses

A methanol-based extraction technique was used to extract CORT from feathers [complete details including validation of the methodology are presented in Bortolotti et al. (Bortolotti et al., 2008)]. The feather minus the calamus was first minced into pieces of <5×5 mm with scissors. Ten millilitres of methanol (HPLC grade, VWR International, Mississauga, Ontario, Canada) was added and the samples were placed in a sonicating water bath at room temperature for 30 min, followed by incubation at 50°C overnight in a shaking water bath. The methanol was then separated from feather material by vacuum filtration, using a plug of synthetic polyester fibre in the filtration funnel. The feather remnants, original sample vial and filtration material were washed twice with approximately 2.5 ml of additional methanol; the washes were added to the original methanol extract. The methanol extract was placed in a 50°C water bath and subsequently evaporated in a fume hood. Evaporation of the samples was completed within a few hours and the extract residues were reconstituted in a small volume of the phosphate buffer system (PBS; 0.05 mol l^{-1}, pH 7.6) used in the CORT radioimmunoassay (Blas et al., 2005). The filtration step was generally found to be sufficient to remove feather particulates but further particulate material could be removed, if needed, by centrifugation of the PBS-reconstituted samples. Reconstituted samples were frozen at –20°C until analysed for CORT. The efficiency of methanol extraction was assessed by including feather samples spiked with a small amount (approximately 4000 d.p.m.) of ^{3}H-corticosterone in each extraction. Greater than 90% of the radioactivity was recoverable in the reconstituted samples.

RESULTS AND DISCUSSION

Deposition of CORT

How steroids get deposited into feathers is central to our understanding of how to sample parts of a feather, as well as how to compare feathers and interpret biological significance of the variation. CORT could be bound in a specific manner so that for a given concentration in the blood, concentration in the feather would be consistent regardless of any variation in mass within or among feathers. Alternatively, Bortolotti et al. suggested that CORT may be deposited into the feather in a non-specific manner, e.g. without having to be bound to specific molecules (Bortolotti et al., 2008). Given that for most of their growth period, feathers elongate at an approximately even rate (e.g. Bortolotti, 1984a; Bortolotti, 1984b), the latter hypothesis predicts that the hormone would be deposited in a time-dependent [i.e. length (pg mm^{-1})] rather than mass-dependent [concentration (pg mg^{-1})] fashion and so portions of feathers with higher mass would be diluted. Differences in CORT among the rachis, vane and calamus of feathers suggest scaling CORT to length rather than mass is most appropriate (Bortolotti et al., 2008).

We tested the validity of concentration (pg mg^{-1}) *versus* a temporal expression of CORT (pg mm^{-1}) using natural sources of variation within five individual bald eagle feathers. For each of the five feathers cut into 10 or more segments of 21 mm, Spearman correlations showed that distal (segment number 1) to proximal position was always highly correlated with concentration (range r_s=–0.720 to –0.939, Ps<0.008) whereas we did not detect any significant correlations for length (range r_s=0.042–0.442, Ps>0.20). The amount of keratin per unit of length of a feather increases from distal to proximal as there is typically more mass of vane and because the rachis widens. CORT per mg clearly declined from distal to proximal (Fig. 1A) whereas no such pattern existed for CORT per mm (Fig. 1B). We cannot envision a biological explanation for the consistent distal to proximal variation in feather CORT (Fig. 1A). Instead, day-to-day variation in response to stressors should cause CORT to go up and down within the individual as shown in Fig. 1B. Note as well the large differences in inherent variation between concentration (Fig. 1A, a 5.9-fold range) and length (Fig. 1B, a 2.3-fold range).

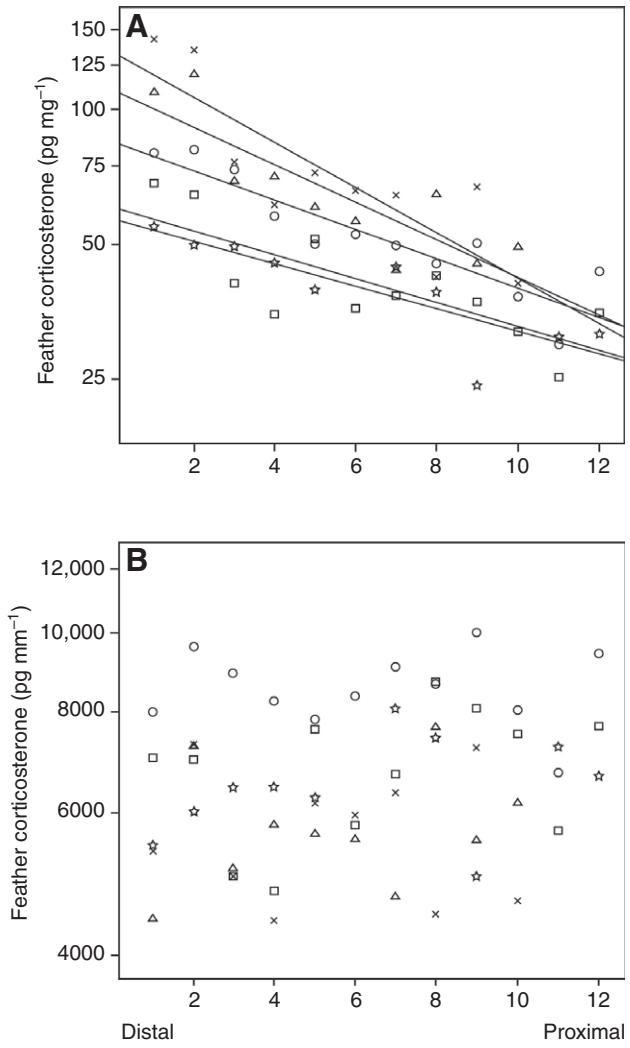

Fig. 1. Distribution of the amount of corticosterone in five primary feathers of bald eagles sampled in 21 mm segments from distal to proximal position along the shaft and expressed as (A) concentration (pg mg^{-1}) and by (B) a linear measurement (pg mm^{-1}). Symbols represent individual feathers, and lines show significant correlations (see text).

Bortolotti et al. found that feather CORT values correlated with maximal values of plasma CORT after an experimentally induced stress rather than baseline CORT (Bortolotti et al., 2008). Therefore, as one would expect, CORT values for individual feathers (Fig. 1B) reveal considerable within- and among-individual variation in exposure and/or response to stressors. The coefficients of variation (c.v.) averaged 15.7% (±1.6 s.e.m., range, 11–19%) for five feathers from breeding eagles and were 21%, 23% and 24% from three feathers grown concurrently on one individual while it was a nestling. The magnitude of the c.v. for this physiological trait is relatively high, considering that c.v. for among-individual variation for such a variable attribute as asymptotic body mass was 4% and

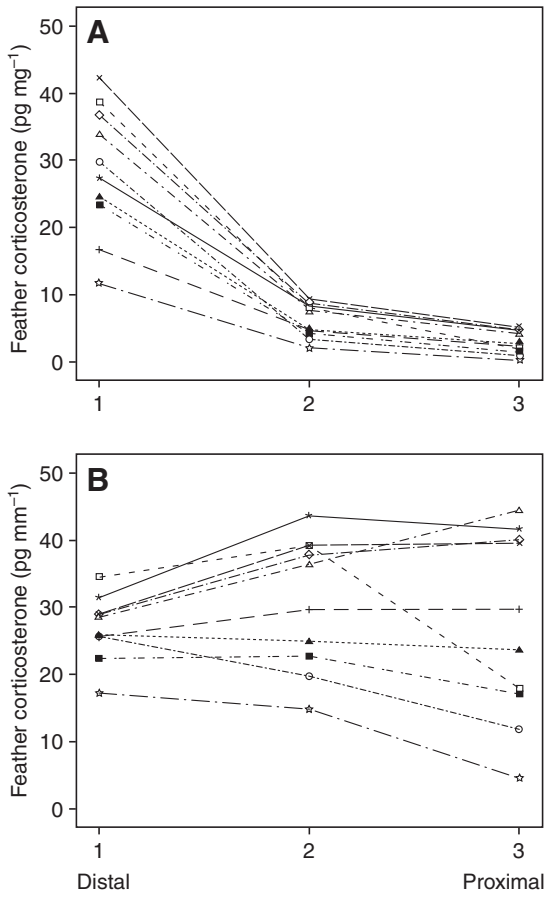

Fig. 2. Amount of corticosterone distal (1) to proximal (3) in feathers of five species (two feathers per species, see text) as measured by (A) concentration (pg mg^{-1}) and by (B) a linear measurement (pg mm^{-1}). Lines connect individual feathers.

for bill length it was 2% (calculated for data on N=47 eaglets) (Bortolotti, 1984b).

Distal to proximal variation was further explored for generality among five species with 10 flight feathers cut into three equal-length segments. For simplicity we show only results for the rachis (Fig. 2). Similar to the results for eagles, concentrations systematically decreased from tip to base (Fig. 2A) and were extremely variable among feathers (up to a 141-fold range). However, when examined in a linear measurement (Fig. 2B, only a 10-fold range), CORT went up, down or stayed the same along the feather as would be expected over time given the unpredictable nature of the appearance of, and response to, stressors.

Both Figs 1 and 2 suggest that mass drives the concentration by a dilution effect. To further examine this, avoiding the distal to proximal variation, we analysed the inner (trailing edge) *versus* the outer (leading edge) vane in the most proximal segment of the 10 remiges of the five species analysed above (i.e. only one comparison per feather). Feathers are naturally asymmetrical with the mass of the outer vane less than the inner vane. CORT in the outer vane was significantly different from the inner vane only for concentration (Wilcoxon paired test Z=–2.599, P=0.009 for concentration and Z=–0.866, P=0.386 for length). Collectively, the results presented here confirm that CORT is deposited in feathers in a time-dependent not mass-dependent fashion, and so the appropriate means of quantifying this hormone is per length of feather and not by concentration. Furthermore, these findings emphasise that the sampling of just part of a feather must include the rachis and entire vane, edge-to-edge perpendicular to the rachis to obtain all of the hormone deposited at any one point in time.

Identification of punctuated stress events

We used paired t-tests to compare two consecutive 21 mm sections of eagle feather. First using feathers that had fault bars, there was significantly more CORT in the length of a segment with one or more fault bars than in the adjacent section without bars (t=–2.192, P=0.049, N=13). Using these same feathers but comparing two sections lacking stress marks, there was no difference in CORT (t=1.053, P=0.317, N=11). Turning to other eagle feathers for which there were no fault bars at all along the entire feather, there was similarly no difference in CORT between two randomly selected consecutive sections (t=–0.466, P=0.658, N=7). These results should be considered as being very conservative in representing the technique's ability to resolve spikes in CORT. The sections we used spanned approximately three days of growth, even though the CORT pulse of induced stress associated with the event that caused a fault bar may have only lasted a matter of hours or minutes. Given that we do not know what stressor caused the fault bar, one must be cautious in interpreting or inferring causality. However, even if fault bars and CORT are not causally related, the bars should be good markers of stressful events and this is what seems to show up in the analysis of feather hormones.

Stability of CORT in feathers

While old feathers (>25 years) (Fig. 1) clearly contain measurable levels of CORT, the amount of hormone at the time of growth could not of course be known. From our sampling protocol, there was no evidence for museum specimens having less CORT as would be expected if degradation occurs (paired t-test, t=–0.1.41, P=0.184, N=13 pairs of fresh and old feathers per species). In fact, the mean for fresh carcasses (4.4±0.45 pg mm^{-1}, mean ± s.e.m.) was somewhat less than that for museum specimens (5.4±0.49 pg mm^{-1}, mean ± s.e.m.).

Feather CORT was also remarkably resistant to degradation by heat. We evaluated the effect of the heat treatment using generalised

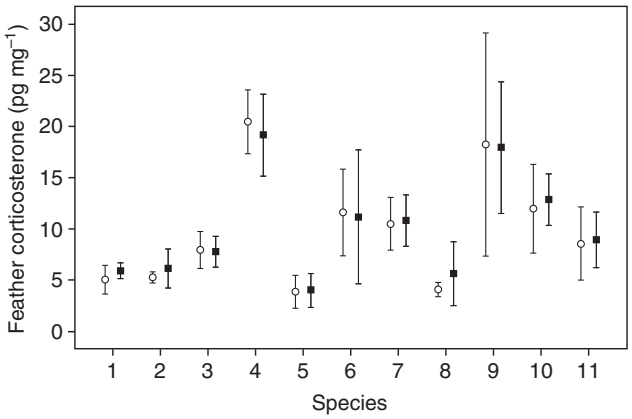

Fig. 3. Corticosterone levels (mean±95% C.I.) in remiges of 11 species comparing the heat treatment (closed squares) *versus* control (open circles). Species: 1, Canada goose; 2, great blue heron; 3, great horned owl; 4, prairie falcon; 5, common raven; 6, redhead; 7, Franklin's gull; 8, sandhill crane; 9, short-eared owl; 10, shoveler; 11, Swainson's hawk.

linear mixed models (GLIMMIX, SAS Institute, Cary, NC, USA). The dependent variable used was CORT concentration ($pg\,mg^{-1}$), which presented a gamma-type error distribution and a log-link function. The independent variable was treatment and we considered species as a random variable. There was no significant effect of the heat treatment on CORT ($F_{1,98}$=0.23, P=0.6359) (Fig. 3). An added benefit of this protocol is that it would sterilise zoonotic pathogens and thus increase security to investigators and local animals if the feathers were imported. Presently, heating feathers for 75°C for 20 min is required by Canadian regulations for the importation of feathers.

Population variation

We first tested for variation attributable to the state at sampling (alive or carcass) and sex of the recent samples (2004–2005) of great horned owls. There was no effect of sex ($F_{1,48}$=1.218, P=0.275) nor was there an interaction ($F_{1,48}$=0.524, P=0.473) but the difference between birds that were alive and dead approached significance ($F_{1,48}$=3.649, P=0.062) with the former having a higher level of CORT. There was no sex difference within the museum sample ($F_{1,18}$=0.066, P=0.800) so all historic specimens, i.e. including the two with no sex information, were compared with the recent samples ($F_{2,73}$=61.62, P<0.0001) (Fig. 4). Our intention here was merely to explore some potential sources of variation within a population (sex, alive *versus* dead and over time) and so a thorough investigation of causality is not appropriate. However, these results may suggest that either great horned owls are different today than they were 50 years ago or possibly a bias exists because of sampling. The museum specimens were either shot or pole-trapped and thus likely to be relatively healthy, free-flying birds. The more recent sample was probably more marginal members of the population. The fact that feather CORT from carcasses tended to be lower than that from living birds supports this interpretation. It is of interest that the only individual of the 2004–2005 sample that overlapped in CORT with the distribution of museum specimens was the only recent bird known to have been shot.

Conclusions

Unlike other tissues, feathers provide a historical record of past HPA activity. Even hair keratin, by virtue of its slow growth and other attributes, has limited application for detecting temporal variation in CORT (Accorsi et al., 2008). The analysis of CORT in feathers provides a temporal perspective on HPA activity that is unmatched in breadth, flexibility and ease of quantification. Of the many advantages of this non-invasive technique (Bortolotti et al., 2008), perhaps none is more significant than the ability to track stress over time. For example, at least four types of protocols for sampling provide very different time lines suitable for different types of questions. First, CORT can be evaluated for as short a time as a day or two to many weeks within a single feather. The hormonal response can be linked directly to behaviour, short episodes of environmental perturbations (e.g. inclement weather) or a physiological process as long as there is concurrence between the latter and feather growth. Because one can measure the length of a developing feather, natural or induced, it is a simple matter to observe or experimentally manipulate birds in a potentially stressful situation and subsequently measure the CORT at the exact point along the feather corresponding to the time of exposure (G.R.B., unpublished data).

Second, a considerably longer time line is possible by comparing among feathers grown at different times on an individual bird. Provided the growth rates of the type of feather are known or feathers of similar size and morphology are used (e.g. contours of the breast or back), then weeks or months of CORT record would be available. Repeated plucking of a feather, e.g. on the same position on the wing or tail, also allows one to get such an extended time line.

Third, many large species of birds such as Procellariiforms, Gruiforms, Falconiforms and Strigiforms do not undergo a complete annual moult of remiges. Individuals of such species can simultaneously possess feathers grown in three different calendar years, thus providing a remarkably long-term perspective into variation in CORT available from just one sampling point in time for the investigator.

Fourth, because exposure to CORT may have long-lasting physiological consequences, at least during development (Monaghan, 2008), there may also be causal links between CORT exposure at one point in time and a similar or different type of response at some point in the future. Therefore, researchers may have some insight on future or past events and how birds deal with them. When one considers extending sampling from within to among individuals, new ecological questions can be asked concerning population-level consequences of stress or CORT as a bioindicator of avian, or even ecosystem, health. Of particular value may be long-term monitoring of populations, or historical trends in CORT using museum specimens (Fig. 4), from the perspective of interpreting the significance of environmental change.

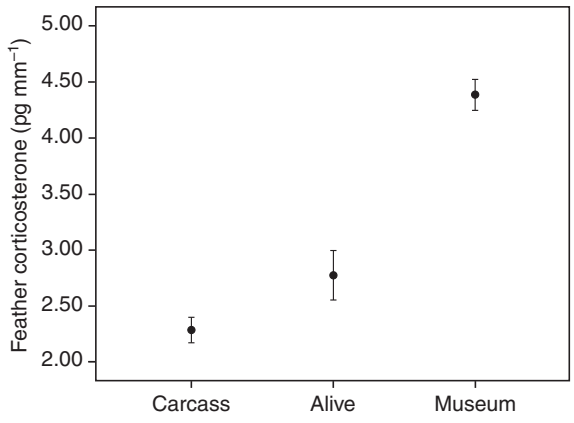

Fig. 4. Mean (±s.e.m.) level of corticosterone ($pg\,mm^{-1}$) in back feathers of great horned owls from a recent sample of alive and dead (i.e. carcass) birds, as well as an historical sample of museum specimens from Saskatoon, Canada.

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