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Research article

Utilising feather hormone measurements to study avian welfare

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Abstract

Feathers offer a non-invasive method for assessing the welfare of birds in human care. However, because few studies have utilized feather hormone measurements, little data exist for most taxa. Here, we measured concentrations of two hormones associated with welfare, corticosterone (CORT) and dehydroepiandrosterone-suphate (DHEA-S), and calculated CORT/DHEA-S ratios in feathers (n=979) from 46 individuals comprising 10 species of birds at the World Bird Sanctuary. Hormone concentrations based on feather mass and length were measured for primary and secondary wing feathers, and tail feathers. Concentrations were compared among feather types, and the variability in concentrations within feather types from individual birds was assessed. We also determined whether factors often associated with welfare, specifically sex, age, housing conditions, and participation in ambassador programs, were correlated with differences in hormone concentrations. We found that hormone concentrations differed significantly among feather types, with tail feathers having higher concentrations of CORT and DHEA-S compared to wing feathers. Concentrations of both hormones were less variable in tail feathers compared to wing feathers. Significant differences in hormone concentrations were also observed across taxa and in relation to sex and age. Ambassador birds had higher CORT/DHEA-S ratios than non-ambassador individuals, and birds participating in programs year-round had higher CORT/DHEA-S ratios than birds that participated in programs for 6 months of the year. Birds housed behind-the-scenes had lower concentrations of feather CORT and CORT/DHEA-S ratios than birds housed in view of guests year-round. These results provide comparative data and methodological recommendations for future studies of feather hormones.

Introduction

Maintaining high standards of animal welfare is a priority for the zoo community (Ward et al. 2018). While zoo-based welfare science has made substantial progress in recent years, most research has focused on mammals. Less than 10% of zoo-based welfare research has studied birds (Woods et al. 2022), despite the fact that birds make up 30% of the species in zoos, and zoos house twice as many bird species as mammals (Binding et al. 2020). Association of Zoos and Aquarium (AZA) institutions care for over 1,000 different avian species, and numerous authors have stressed the need for additional research to enhance the welfare of birds in human care (Binding et al. 2020; Kaplan 2021; Woods et al. 2022).

No single method can reliably quantify animal welfare, so researchers rely on a variety of measures to assess stress (Wielebnowski 2003). While a number of biological markers exist, glucocorticoid production is one of the most common measures used in zoo-based welfare studies (Hodges et al. 2010). Glucocorticoids are secreted by the adrenal cortex in response to excitatory and stressful stimuli and modulate responses to maintain homeostasis (Nelson 2011). While chronic increases in glucocorticoid production may be evidence of compromised welfare (Dembiec et al. 2004), acute changes are not always synonymous with stress, but can occur in response to beneficial behaviours that require activity, including mating behaviour, copulation (Lynch et al. 2002), and responses to enrichment (Cummings et al. 2007).

The ratio of glucocorticoids to dehydroepiandrosterone and its suphate ester, referred to as DHEA-S, has been proposed as a potentially more reliable indicator of stress than measuring glucocorticoids alone, with higher ratios associated with negative welfare. DHEA-S is also produced by the adrenal glands in response to stress, and it has been shown to have properties that mitigate some of the actions of glucocorticoids (Guilliams and Edwards 2010; Kamin and Kertes 2017; Whitham et al. 2020). Most research on DHEA-S has been conducted on mammals (e.g. Beer et al. 2023; Edes et al. 2022) and less is known about its role in avian species. However, DHEA has been shown to increase with changing social condition in northern cardinals *Cardinalis cardinalis* (Wright and Fokidis 2016), in response to territorial intrusions in song sparrows *Melospiza melodia* (Newman and Soma 2011), and was negatively correlated with dominance scores in Brant geese *Branta bernicla bernicla* (Poisbleau et al. 2010).

Endocrine analyses of avian species are often conducted with plasma samples (Cockrem and Silverin 2002; Wikelski et al. 1999). Blood sampling typically requires restraining an animal, and in a number of bird species, handling has been shown to affect concentrations of circulating hormones, including corticosterone (CORT) and DHEA-S (Fokidis 2016; Newman et al. 2008). Faecal hormone analyses, which are commonly performed for mammals (for a review, see Hodges et al. 2010), can provide a reliable and non-invasive method of assessing hormone production in birds (Brown et al. 2016; Kozlowski et al. 2018; 2023). However, collecting faecal samples from birds can be challenging. The collection process can be disruptive, particularly during the breeding and nesting periods. In addition, the small body sizes of many species make it difficult to collect enough faecal material for testing (Hayward et al. 2010), and birds are often group-housed, making it difficult to identify faecal samples from known individuals.

Feathers offer an alternative method for assessing hormone production in birds (Bortolotti et al. 2008; Meillère et al. 2016). Feather CORT measures were first conducted by Bortolotti et al. (2008), who demonstrated that CORT is deposited into growing feathers and that concentrations in feathers increase after stressful events. Additional studies have evaluated feather CORT concentrations in a variety of species and found significant relationships with reproductive success (Kouwenberg et al. 2013), survival (Koren et al. 2012), and immunocompetence (Sild et al. 2014). More recently, studies of feather DHEA concentrations have investigated the relationship between this hormone and body weight, body size, plumage coloration (Doyle et al. 2021), and health (Frongia et al. 2020).

Unlike blood and faecal samples, collecting feathers after moult is non-disruptive and relatively straightforward. Feathers also have the added advantage of being stable for years at room temperature (Beattie and Romero 2023; Bortolotti et al. 2009), unlike most biological samples that require cold storage. However, while blood samples provide real-time information on hormone production, and faecal samples have a time-lag of several hours, hormones are only deposited in feathers during the period of growth. Thus, knowledge of the time course of growth is necessary to interpret hormone data from feathers.

Because few studies have utilized feather hormone measurements, little reference data exists for most taxa, and whether certain feather types should be targeted for collection is unclear. Various authors have assessed hormone concentrations in primary (Will et al. 2014) and secondary wing feathers (Fairhurst et al. 2011), covert feathers (Frongia et al. 2020), tail feathers (Jenni-Eiermann et al. 2015), and contour feathers (Gormally et al. 2020), with few comparisons between types. Additionally, while the units of measure for hormones in blood and faeces are generally consistent, methods for measuring feather hormone concentrations vary. Bortolotti et al. (2009) demonstrated that CORT is deposited in feathers in a time-dependent manner and recommended that concentrations be quantified based on feather length. While many studies follow this recommendation (Fairhurst et al. 2011; Gormally et al. 2020; Haase et al. 2021; Reese et al. 2020; Legagneux et al. 2013; Will et al. 2015), other authors calculate concentrations based on feather mass (e.g. Frongia et al. 2020; Haffelin et al. 2021; Koren et al. 2012), and comparisons between concentrations based on length and mass are rarely performed.

In this study, we quantified concentrations of CORT, DHEA-S, and CORT/DHEA-S ratios in primary and secondary wing feathers, and tail feathers, from 43 individual birds comprising 10 species in human care. Our objectives were to 1) establish a reference database of feather hormone concentrations for a wide range of zoo-housed species, based on both length and mass; 2) assess variability in hormone concentrations between different feather types, as well as within feather types from individual birds; and 3) determine whether factors often associated with welfare, specifically sex, age, housing conditions, and participation in ambassador programs, were correlated with differences in feather hormone concentrations. The results from this study provide comparative data and methodological recommendations for future studies of avian welfare.

Materials and Methods

Study animals

Study animals consisted of 43 birds, comprising 10 species, housed at the World Bird Sanctuary (Valley Park, MO, USA). Feathers were collected from 21 males, 19 females, and three birds of unknown sex, which ranged in age from one to 38 years (mean=12.2±1.4 years of age). Birds were housed either singly (n=39 individuals), in opposite sex pairs (n=2 individuals), or in same-sex groups (n=2 individuals), in habitats that ranged from 3 × 3 × 3.5 (m) to 12 × 12 × 11 (m) (L × W × H). Birds differed in their exposure to visitors. Twenty-five birds were housed in habitats without visitor viewing. These habitats included both indoor and outdoor access. Eight birds were visible to visitors for part of the year (17–42% of the year). Ten birds were housed in outdoor habitats that were visible to visitors year-round.

Of our study birds, 27 individuals from nine species were classified as ambassador birds. (Table 1). Ambassador birds consisted of hand-reared owls, and wild-hatched diurnal birds that were deemed non-releasable following rehabilitation. Ambassador birds participated in routine training and interactive programs with the public, both at the World Bird Sanctuary, as well as nearby locations. Twenty birds participated in ambassador programming year-round, while five participated for six months out of the year, and two birds participated in ambassador programming for 8–9 months of the year.

Feather collection and extraction

Because of the non-invasive nature of data collection, ethical review by an IACUC committee was not necessary for this study. Feather samples were collected after moult and stored at room temperature until processing. In total, 979 feathers were collected (primary=417; secondary=329; tail=233), with an average of 22.8±1.7 feathers per bird (range=3–59 feathers) (Table 2). Feathers were collected over two seasons; 406 feathers were collected in 2022, and 573 feathers were collected in 2023. In 2022, an average of 3.7±0.6 primary, 3.3±0.4 secondary, and 2.7±0.4 tail feathers were collected from each bird in the study. In 2023, an average of 6.1±0.7 primary, 4.4±0.6 secondary, and 3.1±0.5 tail feathers were collected from each bird.

Prior to extraction, feathers were inspected for faecal material, and any feather with visible faeces was not included in the study.

Table 1. Study subjects at the World Bird Sanctuary.

Species	Males	Females	Unknown sex	Ambassador birds	Birds off-exhibit (all or part of year)	Birds visible to visitor year-round
American crow *Corvus brachyrhynchos*	0	0	3	1	2	1
American kestrel *Falco sparverius*	2	2	0	3	4	0
Augur buzzard *Buteo augur*	3	1	0	1	2	2
Barn owl *Tyto alba*	3	3	0	6	6	0
Cabot's tragopan *Tragopan caboti*	1	2	0	0	1	2
Eurasian eagle owl *Bubo bubo*	3	0	0	2	2	1
Great horned owl *Bubo virginianus*	2	1	0	2	3	0
Harris's hawk *Parabuteo unicinctus*	3	5	0	6	8	0
Peregrine falcon *Falco peregrinus*	0	3	0	2	2	1
Red-Tailed hawk *Buteo jamaicensis*	4	2	1	4	3	3

Next, the calamus was removed; feathers were then weighed on a digital scale to the nearest 0.01 g, and the length of the feather was measured to the nearest 0.1 mm with digital callipers. Vanes were then minced into pieces <5 mm in length with scissors and placed in a 50 ml polypropylene conical tube. Feather hormones were extracted following the protocol described by Bortolotti et al. (2008) with minor modifications. Ten ml of methanol (HPLC grade) was added to each feather sample. Samples were vortexed, then incubated at 50°C overnight. The following day, the methanol was separated from the feather material by filtration using filtered syringes (filter pore=0.2 µm). The feather remnants, conical tube, and filtration material were washed twice with 2.5 ml of additional methanol and the washes were added to the original methanol extract. The methanol extract was evaporated in an incubator at 50°C. The extract residues were reconstituted in one ml of assay buffer provided by the hormone assay manufacturer (Arbor Assays, Ann Arbor, MI, USA), incubated again overnight at 50°C, vortexed and pipetted into cryovials for storage at -80° C until assay.

Hormone assays

Feather extracts were assayed for CORT using Arbor Assays DetectX© Corticosterone K014 enzyme immunoassays (Ann Arbor, MI, USA) and DHEA-S using Arbor Assays DetectX© dehydroepiandrosterone-suphate EIA K054 enzyme immunoassays (Ann Arbor, MI, USA). The range of the standard curve for the CORT assay was 78.1 pg/ml to 10,000 pg/ml, and the range of the DHEA-S assay was 96 pg/ml to 60,000 pg/ml. Enzyme immunoassays were performed according to the kit instructions, and feather extracts were assayed neat or diluted 1:10 with assay buffer. For all assays, standards, samples, and quality control pools were assayed in duplicate. Mean intra-assay variation was 7.9% for CORT, and 9.3% for DHEA-S. Mean inter-assay coefficients of variation for two quality control pools were 9.2% for CORT, and 9.6% for DHEA-S.

Feather extracts were tested for linearity by diluting five samples that contained high concentrations of hormone by 1/2, 1/4, and 1/8 with extraction buffer. Serial dilutions measured an average of 107.4±9.0% of expected concentrations for CORT, and 97.5±4.4

Table 2. Number of primary, secondary, and tail feathers collected from each species at the World Bird Sanctuary.

Species	Primary	Secondary	Tail	Total
American crow *Corvus brachyrhynchos*	70	38	42	150
American kestrel *Falco sparverius*	50	24	5	79
Augur buzzard *Buteo augur*	15	14	16	45
Barn owl *Tyto alba*	46	50	12	108
Cabot's tragopan *Tragopan caboti*	25	22	23	70
Eurasian eagle owl *Bubo bubo*	25	38	9	72
Great horned owl *Bubo virginianus*	18	14	11	43
Harris's hawk *Parabuteo unicinctus*	70	58	53	181
Peregrine falcon *Falco peregrinus*	36	14	20	70
Red-Tailed hawk *Buteo jamaicensis*	62	57	42	161

% for DHEA-S, and all were parallel to the standard curve (test of equal slopes, P>0.10). The accuracy of the assays was assessed by adding a known amount of hormone to five feather extracts that contained low concentrations of hormone. Addition of known amounts of hormone at two dosage levels resulted in recovery of 104.7±3.2% of added CORT, and 100.2±3.5% for DHEA-S.

Statistical analyses

All statistical analyses were generated using the MIXED procedure within SAS/STAT software, Version 9.4 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA).

Hormone concentrations (CORT pg/mm, CORT pg/g, DHEA-S pg/mm, DHEA-S pg/g, and CORT/DHEA-S ratio) were compared between feather types (primary, secondary, and tail feathers) using a repeated measures ANOVA, where feather type, species, sex, collection year, ambassador status, and proportion of the year spent behind the scenes were fixed factors, individuals were the subject factor, and age at the start of study was a covariate. Hormone concentrations were compared within feather types using separate repeated measures ANOVAs, where species, sex, collection year, ambassador status, and proportion of the year spent behind the scenes were fixed factors, individuals were the subject factor, and age at the start of the study was a covariate. Data for the American crows *Corvus brachyrhynchos* were removed prior to analysis because the sex of these individuals was unknown. Additionally, due to a significant lack of homogeneity of variances between individuals, independent variances were calculated and used for all ANOVAs. Tukey's multiple mean comparison test was used to compare the pairwise means when the overall F-test was significant (α=0.05).

A subset of data (ambassador birds) were analysed to investigate differences in the proportion of the year a bird served as an ambassador (50% vs. 100%). For this analysis, birds that were not always housed behind the scenes were removed. Hormone concentrations (CORT pg/mm, CORT pg/g, DHEA-S pg/mm, DHEA-S pg/g, and CORT/DHEA-S ratio) were compared within feather types (primary, secondary, and tail feathers) using separate repeated measures ANOVAs, where sex, collection year, and proportion of the year spent in an ambassador role were fixed factors, individuals were the subject factor, and age at the start of the study was a covariate. Due to a significant lack of homogeneity of variances between species, independent variances were calculated and used. It should be noted that the proportion of the year spent in an ambassador role is confounded with species in this dataset, therefore species could not be included as a fixed factor in the model.

In order to evaluate the amount of variability in hormone concentrations (CORT pg/mm, CORT pg/g, DHEA-S pg/mm, DHEA-S pg/g, and CORT/DHEA-S ratio) within feather types, coefficients of variation (%CV) were calculated for each bird in each year by feather type for all five variables. %CV for hormone concentrations (CORT pg/mm, CORT pg/g, DHEA-S pg/mm, DHEA-S pg/g, and the CORT/DHEA-S ratio) were compared between feather types (primary, secondary, and tail feathers) using an ANOVA, where animal (i.e. individual) and feather type were fixed factors, and species was a random factor. Due to a significant lack of homogeneity of variances in %CV between species, independent variances were calculated and used. Tukey's multiple mean comparison test was used to compare the pairwise means for feather types when the overall F-test was significant (α=0.05).

Results

Hormone concentrations based on feather length and mass

Concentrations of CORT based on feather length averaged 4.9±0.17 pg/mm (range=0.4-75.2 pg/mm); when calculated based on feather mass, CORT concentrations averaged 3.30±0.14 pg/mg (range=0.43-40.6 pg/mg). DHEA-S concentrations were substantially higher, with mean concentrations of 311.4±46.1 pg/mm (range=7.6–33,208.9 pg/mm) and 188.5±22.3 pg/mg (range=5.7–13,196.9 pg/mg). Due to high concentrations of DHEA-S, CORT/DHEA-S ratios were low, averaging 0.04±0.001 (range=0.001-0.242). Concentrations of feather CORT and DHEA-S based on mass and length were significantly correlated for primary (CORT: r^2=0.35, $F_{1,417}$=224.7, p<0.0001; DHEA-S: r^2=0.96, $F_{1,417}$=8903.9, P<0.0001), secondary (CORT: r^2=0.40, $F_{1,329}$=221.7, P<0.0001; DHEA-S: r^2=0.94, $F_{1,329}$=5379.2, p<0.0001), and tail feathers (CORT: r^2=0.46, $F_{1,233}$=193.9, p<0.0001; DHEA-S: r^2=0.80, $F_{1,233}$=902.8, P<0.0001).

Differences among feather types

Hormone concentrations differed significantly among feather types (Figure 1), with tail feathers having higher concentrations of CORT, compared to primary and secondary feathers (length: $F_{2,64}$=82.05, P<0.0001; mass: $F_{2,64}$=13.34, P<0.0001). When calculated based on feather mass, DHEA-S concentrations in tail feathers were greater than primary or secondary feathers ($F_{2,64}$=4.94, P=0.0101). However, when calculated based on feather length, DHEA-S concentrations of primary, secondary and tail feathers were equivalent. Secondary feathers had the highest CORT/DHEA-S ratio ($F_{2,64}$=17.08, P<0.0001); no differences were observed between tail and primary feathers.

Intra-individual variation in concentrations of CORT and DHEA-S differed among feather types. Coefficients of variation (%CV) for CORT (pg/mm) averaged 33.2±1.9 % and 33.0±1.8% (pg/mg); %CV of DHEA-S concentrations averaged 46.9±2.9% (pg/mm) and 47.3±2.9% (pg/mg). Concentrations of CORT (length: $F_{2,139}$=14.42, P<0.0001; mass: $F_{2,139}$=9.89, P<0.0001) and DHEA-S (length: $F_{2,139}$=9.29, P=0.0002; mass: $F_{2,139}$=10.97, P<0.0001) were less variable in tail feathers, compared to primary and secondary feathers (Figure 2).

Species, sex, age-related and year differences

For all feather types, significant differences in both CORT (length: primary: $F_{8,23}$=5.11, P=0.001; secondary: $F_{8,21}$=4.62, P=0.0023; tail: $F_{8,21}$=11.05, P<0.0001; mass: primary: $F_{8,23}$=22.14, P<0.0001; secondary: $F_{8,21}$=4.85; P=0.0024; tail: $F_{8,21}$=11.05, P<0.0001) and DHEA-S concentrations (length: primary: $F_{8,23}$=52.89, P=0.0210; secondary: $F_{8,21}$=3.04, P=0.0194; tail: $F_{8,21}$=6.97, P=0.0002; mass: primary: $F_{8,23}$=6.87, P=0.0001; secondary: $F_{8,21}$=6.81; P=0.0002; tail: $F_{8,21}$=5.13, P=0.0012) were observed across species and varied based on the method of calculation (Table 3). When concentrations were based on length, Eurasian eagle owls *Bubo bubo* and Harris's hawks *Parabuteo unicinctus* had the highest concentrations of both hormones. When based on mass, American kestrels *Falco sparverius* had the highest concentrations of both hormones. This result was driven by large differences in feather mass among taxa (American kestrel feathers weighed on average 62±2.2 mg, while the range for other taxa averaged 201 to 666 mg). Mean feather lengths were less variable across taxa, with species averages ranging from 100 to 211 mm.

Sex- and age-related differences in hormone concentrations were also observed. Concentrations of CORT and DHEA-S were higher in secondary feathers of females when concentrations were based on feather mass (CORT: $F_{1,21}$=5.07, P=0.0352; DHEA-S: $F_{1,21}$=4.92, P=0.0377). DHEA-S concentrations were also higher in female feathers when concentrations were calculated based on feather length ($F_{1,21}$=6.03, P=0.0229). In addition, the ratio of CORT/DHEA-S was higher in primary ($F_{1,23}$=5.86, P=0.0238) and secondary feathers ($F_{1,21}$=24.7, P<0.0001) from female birds. There was also a trend for higher CORT/DHEA-S ratios in tail feathers from females ($F_{1,21}$=3.23, P=0.0869) (Figure 3). Concentrations of

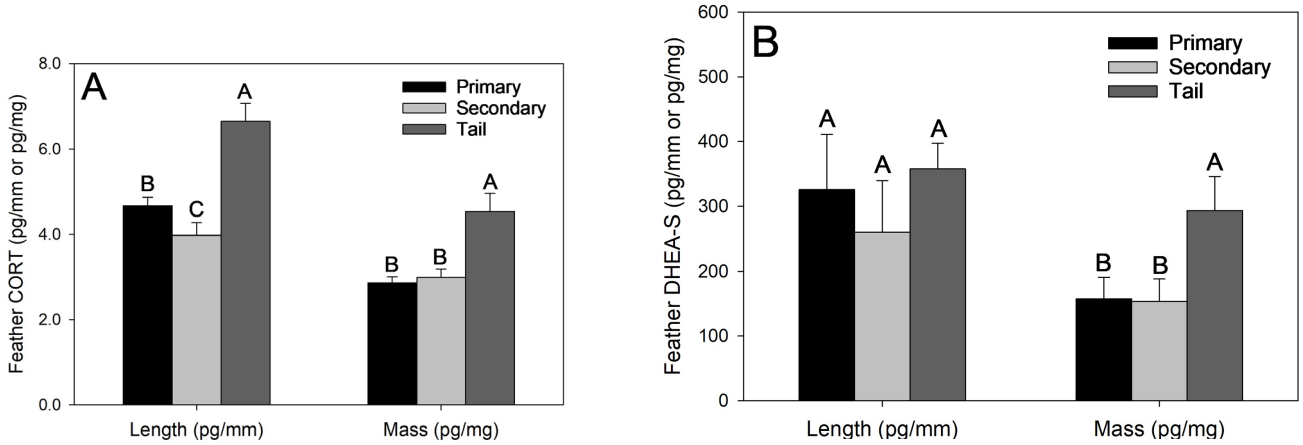

Figure 1. Mean (±S.E.) concentrations of (A) feather corticosterone (CORT) and (B) feather dehydroepiandrosterone-sulfate (DHEA-S) in primary, secondary, and tail feathers from birds at the World Bird Sanctuary. Feather types that do not share the same letter are significantly different (P<0.05).

CORT (mass: $F_{1,21}$=5.11, P=0.0346) and DHEA-S (mass: $F_{1,21}$=20.26, P=0.0002; length: $F_{1,21}$=29.62, P<0.0001), as well as the CORT/DHEA-S ratio ($F_{1,21}$=11.29; P=0.003) of secondary feathers were positively correlated with age. No age-related differences in concentrations of CORT and DHEA-S were observed for primary and tail feathers.

CORT and DHEA-S concentrations of primary (CORT length: $F_{1,17}$=4.87, P=0.0413) (DHEA-S length: $F_{1,17}$=5.22, P=0.0355; DHEA-S mass: $F_{1,17}$=6.41, P=0.0215) and tail feathers (CORT length: $F_{1,17}$=8.85, P=0.0085; mass: $F_{1,17}$=25.12, P=0.0001) (DHEA-S length: $F_{1,17}$=8.41, P=0.01) were higher in 2022, compared to feathers collected in 2023. CORT/DHEA-S ratios of tail feathers were also higher in 2022 ($F_{1,17}$=10.07, P=0.0056).

Housing and ambassador programming

The percentage of time birds were housed in view of visitors was associated with an overall increase in CORT concentrations for both primary (length: $F_{5,23}$=3.86, P=0.011; mass: $F_{5,23}$=3.29, P=0.0218) and tail feathers (length: $F_{5,21}$=7.54, P=0.0003), increased DHEA-S concentrations in primary (mass: $F_{5,23}$=2.8, P=0.0406), secondary (mass: $F_{5,21}$=3.12, P=0.0291), and tail feathers (length: $F_{5,21}$=6.46, P=0.0009; mass: $F_{5,21}$=3.10, P=0.0299), and higher CORT/DHEA-S ratios in all three feather types (primary: $F_{5,23}$=6.32, P=0.0008; secondary: $F_{5,21}$=14.61, P<0.0001; tail: $F_{5,21}$=5.26, P=0.0028). Birds housed behind-the-scenes had lower CORT/DHEA-S ratios than birds visible to visitors year-round. However, birds that were visible to visitors for 42% of the year had the lowest overall CORT/DHEA-S ratios (Figure 4).

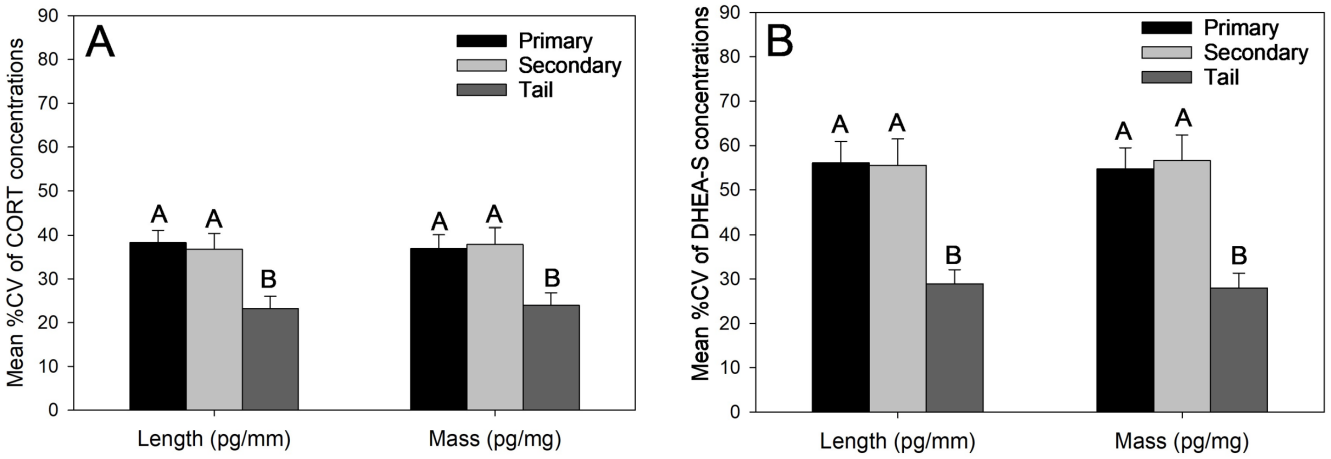

Figure 2. Mean (±S.E.) % coefficients of variation (%CV) for (A) feather corticosterone (CORT) and (B) feather dehydroepiandrosterone-sulfate (DHEA-S) in primary, secondary, and tail feathers from individual birds at the World Bird Sanctuary. Feather types that do not share the same letter are significantly different (P<0.05).

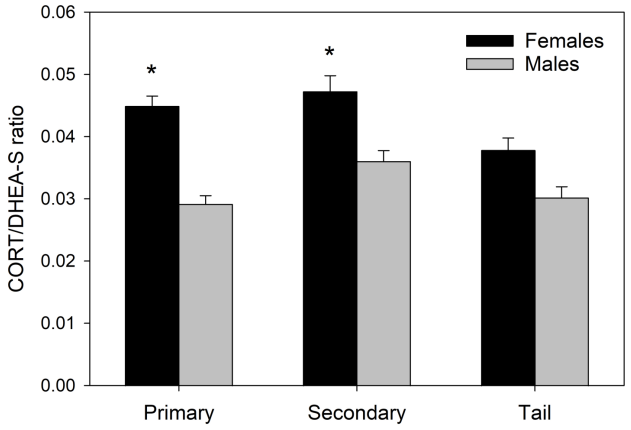

Figure 3. Mean (±S.E.) corticosterone/dehydroepiandrosterone-sulfate (CORT/DHEA-S) ratios of male and female birds at the World Bird Sanctuary. Asterisks indicate significant differences in CORT/DHEA-S ratios within feather type (P<0.05).

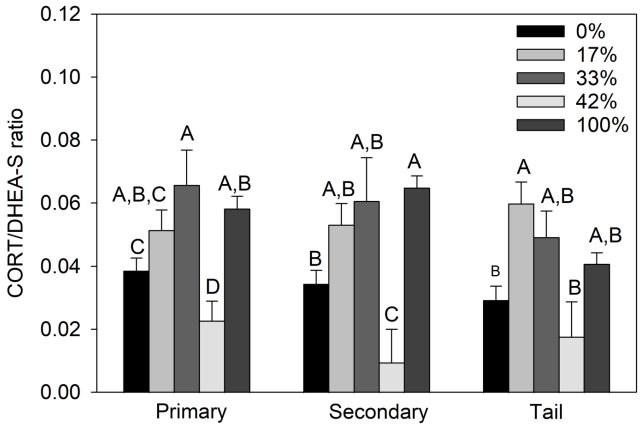

Figure 4. Mean (±S.E.) corticosterone/dehydroepiandrosterone-sulfate (CORT/DHEA-S) ratios of birds in relation the proportion of the year spent in view of visitors at the World Bird Sanctuary. Levels that do not share the same letter within feather types are significantly different (P<0.05).

Concentrations of CORT and DHEA-S did not differ between ambassador and non-ambassador birds. However, birds serving as ambassador animals had higher CORT/DHEA-S ratios in secondary wing ($F_{1,21}$=43.57, P=0.0003) and tail feathers ($F_{1,21}$=31.22, P<0.0001) (Figure 5). In addition, birds that worked as ambassadors for 6 months of the year had higher concentrations of CORT (primary: length: $F_{1,15}$=8.20, P=0.0119; tail: mass: $F_{1,13}$=6.24, P=0.0267) and DHEA-S (secondary: mass: $F_{1,13}$=6.57, P=0.0236; tail: length: $F_{1,13}$=5.99, P=0.0293; mass: $F_{1,13}$=14.04, P=0.0024), but lower CORT/DHEA-S ratios (secondary: $F_{1,13}$=14.82, P=0.0020; tail: $F_{1,13}$=5.55, P=0.0349) than birds that worked as ambassadors year-round (Figure 6).

Discussion

This study measured concentrations of two hormones associated with welfare, CORT and DHEA-S, in feathers from a variety of birds in human care. Our results demonstrated that concentrations differed among wing and tail feathers, as well as within feather types from individual birds. We also documented differences in hormone concentrations among species, as well as in relation to sex, age, participation in ambassador programming, and the proportion of time birds were visible to visitors.

Concentrations of both CORT and DHEA-S were highest in tail feathers, compared to primary and secondary feathers, emphasizing the importance of sampling the same feather type when performing hormone analyses. Previous work on pullets *Gallus domesticus* (Häffelin et al. 2021) and in Eurasian sparrowhawks *Accipiter nisus* (Monclús et al. 2017) also documented significant variation in CORT concentrations between feather types. Differences in hormone concentrations may be related to variation in growth rates (Jenni et al. 2020), the timing of moult between feather types (Edelstam 1984), or differences in the amount of rachis to vane (Freeman and Newman 2018). Pigmentation differences among feather types may also affect hormone concentrations, as the amount of CORT has been shown to positively correlate with the presence of carotenoids in feathers (Fairhurst et al. 2014; Kennedy et al. 2013; Lendvai et al. 2013).

High levels of variation in hormone concentrations were observed within feather types from individual birds, with greater variation amongst primary and secondary feathers than tail feathers. Steroids are thought to be incorporated into feathers primarily in the blood quill during the period of cell differentiation (Jenni-Eiermann et al. 2015), and feathers grown at different times are expected to vary in hormone concentration. While moulting patterns of many bird species are poorly understood, the pattern appears to differ between flight and tail feathers. Because the growth of flight feathers can take weeks to months, flight feathers cannot be shed in a single moult without compromising an individual's ability to fly. Instead, flight feathers are moulted serially, with each moult cycle lasting months, to a year or more in some larger birds (Clark 2004; Edelstam 1984; Newton 2009). In contrast, tail feathers in some species are shed in two rounds, with every second quill dropping in the first round, and the remaining ones dropping in the second round (Edelstam 1984). Thus, the lower level of variation in tail feathers may be due to the fact that many of these feathers are grown at the same time.

Concentrations of CORT and DHEA-S based on feather mass and length were highly correlated, suggesting that either metric may be appropriate for assessing hormone concentrations. However, while some relationships were consistent between concentrations based on mass and length, others varied significantly. Differences related to sex and age were detected when concentrations were based on feather mass, but not length. Hormones in feathers are believed to be derived from circulating levels in the blood during the period of growth. However, there is also evidence that CORT (and possibly other steroids) can bind to the surface of feathers (Aharon-Rotman et al. 2021; Jenni-Eiermann et al. 2015), and feathers with a larger surface area might be expected to have higher concentrations of hormones. Using mass to calculate hormone concentrations (which better reflects the surface area of a feather, particularly for asymmetrically shaped flight feathers) may more accurately reflect hormone concentrations than calculations based on length.

Significant differences in feather CORT and DHEA-S concentrations were observed across taxa. This is not surprising, given the range of feather CORT concentrations reported in various studies. The concentrations of CORT measured here fall

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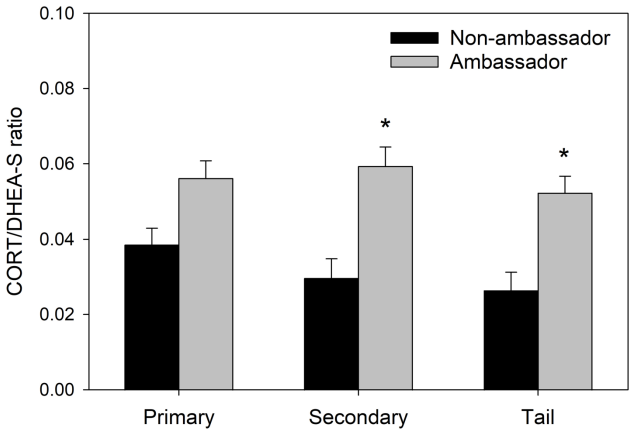

Figure 5. Mean (±S.E.) corticosterone/dehydroepiandrosterone-sulfate (CORT/DHEA-S) ratios of ambassador and non-ambassador birds at the World Bird Sanctuary. Asterisks indicate significant differences in CORT/DHEA-S ratios within feather type (P<0.05).

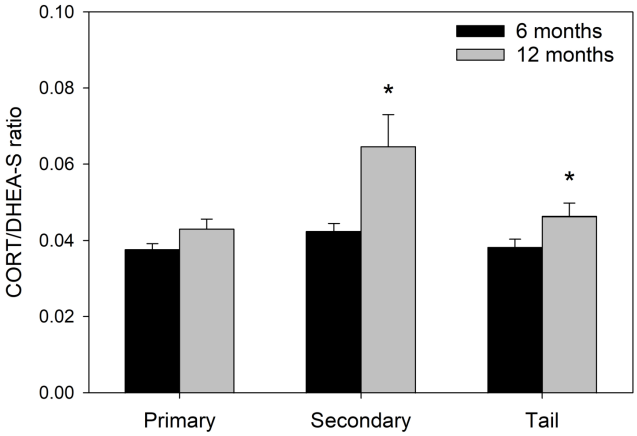

Figure 6. Mean (±S.E.) corticosterone/dehydroepiandrosterone-sulfate (CORT/DHEA-S) ratios of ambassador birds at the World Bird Sanctuary that participate in programs for 6 months or 12 months of the year. Asterisks indicate significant differences in CORT/DHEA-S ratios within feather type (P<0.05).

within the range published for other species (e.g. Berk et al. 2016; Bortolotti et al. 2008; Lendvai et al. 2013). In contrast, while little data exist for concentrations of DHEA-S in feathers, values for all of our species were substantially higher than DHEA concentrations measured in Griffon vulture *Gyps fulvus* feathers (Frongia et al. 2020).

We also found sex- and age-related differences in feather CORT and DHEA-S concentrations. Females had higher concentrations of CORT, DHEA-S and CORT/DHEA-S ratios than males, and older birds had higher levels of feather CORT, DHEA-S, and CORT/DHEA-S ratios than younger birds. Increased CORT production in females (Khan and Robert 2013; O'Reilly and Wingfield 2003; Tetel et al. 2002), and as well as in older birds (Beuving and Vonder 1978; Sims and Holberton 2000), has been described in a variety of avian taxa. Taken together, our results suggest that species differences, as well as the age and sex of individual birds, should be considered when interpreting feather hormone data.

No differences in concentrations of feather CORT and DHEA-S

Table 3. Mean (±S.E) concentrations of feather corticosterone (CORT) and dehydroepiandrosterone-sulfate (DHEA-S) based on feather length and mass for each species studied at the World Bird Sanctuary. Values in parentheses are the range of concentrations measured.

Species	Length (pg/mm)	Mass (pg/mg)	Length (pg/mm)	Mass (pg/mg)
American crow *Corvus brachyrhynchos*	3.6±0.3 (0.4–16.9)	2.1±0.2 (0.6–11.5)	137.7±18.8 (12.4–1126.3)	84.2±12.8 (10.6–766.5)
American kestrel *Falco sparverius*	5.6±0.4 (1.3–26.0)	9.2±0.6 (2.5–40.6)	320.6±54.7 (22.3–4504.2)	448.5±79.4 (51.1–6960.7)
Augur buzzard *Buteo augur*	4.1±0.3 (0.9–11.2)	1.7±0.2 (0.5–7.1)	119.6±15.6 (19.9–592.8)	50.5±7.2 (8.7–256.8)
Barn owl *Tyto alba*	3.9±0.3 (0.6–13.4)	3.0±0.2 (0.5–11.0)	143.6±27.0 (7.6–2845.1)	116.6±25.6 (5.7–2730.7)
Cabot's tragopan *Tragopan caboti*	2.9±0.2 (1.1– 11.1)	2.1±0.2 (0.7–11.3)	112.2±16.8 (17.5–1094.2)	76.9±10.9 (11.9–636.1)
Eurasian eagle owl *Bubo bubo*	7.6±0.7 (2.1–34.9)	2.5±0.2 (0.6–10.2)	739.6±153.3 (87.1–10442.7)	234.5±39.2 (34.3–2438.5)
Great horned owl *Bubo virginianus*	6.5±1.2 (1.7–52.9)	2.5±0.4 (0.6–19.3)	253.0±62.6 (32.9–2093.1)	99.9±24.7 (17.9–811.7)
Harris's hawk *Parabuteo unicinctus*	6.5±0.6 (0.8–75.2)	2.8±0.2 (0.6–29.6)	606.1±232.4 (18.7–33208.9)	258.5±94.7 (11.6–13196.9)
Peregrine falcon *Falco peregrinus*	4.0±0.4 (1.7–30.4)	1.8±0.3 (0.6–19.9)	147.0±20.8 (14.3–1066.7)	70.4±13.5 (8.2–697.8)
Red-Tailed hawk *Buteo jamaicensis*	3.7±0.4 (0.7–42.6)	1.4±0.1 (0.4–20.1)	204.8±32.8 (18.2–3236.2)	71.6±10.9 (8.0–1063.9)

were found between ambassador and non-ambassador birds. However, birds participating in ambassador programs had higher CORT/DHEA-S ratios than non-ambassador birds. To date, most research on ambassador animal welfare has focused on mammals, with results suggesting that impacts are both species- and situation-specific (e.g. Kozlowski et al. 2021; Normando et al. 2018; Powell et al. 2020). Research on ambassador bird welfare is limited, but participating in ambassador programs did not affect the behaviour of African penguins *Spheniscus demersus* (Saiyed et al. 2019), or domestic chickens *Gallus gallus* (Ramont et al. 2021), and did not increase glucocorticoid production of Magellanic penguins *Spheniscus magellanicus* (Hartell-DeNardo et al. 2023). However, handling for ambassador programs and husbandry was associated with increased glucocorticoid production by red-tailed hawks *Buteo jamaicensis* (Baird et al. 2016). We also found that birds participating in ambassador programming for only 6 months of the year had lower CORT/DHEA-S ratios than birds that participated in ambassador programming year-round. However, because species differences could not be taken into account in the analysis, these results should be viewed with caution, especially since we demonstrated that species differences in feather hormone concentrations exist.

Birds housed in view of visitors year-round had higher concentrations of feather CORT and higher CORT/DHEA-S ratios than birds housed behind-the-scenes. While most research on zoo visitor effects has focused on primates, an increasing number of studies have assessed the impact of visitors on other taxa (see review Williams et al. 2023). Birds remain understudied compared to mammals, but investigations on a variety of avian species have demonstrated mixed findings. For example, birds in a multi-species aviary changed their space use in the presence of zoo visitors, but changes in stress-behaviours were not observed (Blanchett et al. 2020). Similarly, the behaviour of flamingos *Phoenicopterus* spp. was not affected by the number of zoo visitors (Rose et al. 2018); however, little penguins *Eudyptula minor* increased preening, reduced vigilance, and spent more time near a viewing window when it was covered and not visible to guests (Chiew et al. 2020). In our study, birds housed behind the scenes had both indoor and outdoor access, while those visible to visitors were outdoors year-round. As differences in lighting (Bassler et al. 2013), environmental noise (Blanchett et al. 2020), and outdoor access (Rodenburg et al. 2005) have all been shown to impact indicators of avian welfare, it is unclear whether hormone differences are related to the presence of visitors or due to other factors associated with housing. Regardless, our results demonstrate that differences in hormone production by ambassador birds, as well as those associated with housing, are detectable in feathers.

Conclusion

This study provides comparative data, as well as methodological recommendations for future studies of feather hormones. Our results suggest that, when possible, multiple feathers should be collected for hormone analysis, and results based either on pooled samples or the population mean, not on single feather samples. For species of birds that moult flight feathers over long periods of time, hormone analysis of tail feathers may be preferred, as concentrations appear to vary less than flight feathers. Finally, for large feathers that are not affected by mass-dilution effects (Lattin et al. 2011), our results suggest that hormone concentrations based on mass may be a better metric than those based on length, particularly if concentrations are being compared across taxa. As collecting physiological data on avian species can be difficult, we propose that utilizing feather hormone measurements may help the zoo community better understand the factors that impact the welfare of birds in human care.

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