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Research article

Patterns of faecal hormone metabolites associated with reproduction in cinereous vultures *Aegypius monachus*

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Abstract

Cinereous vultures *Aegypius monachus* are listed as Near Threatened by the IUCN. Breeding efforts in zoos have had limited success and little is known about the species' reproductive biology. Here, patterns of faecal gonadal hormone metabolites associated with egg-laying for cinereous vultures are described. Glucocorticoid metabolites were also measured to assess the relationship between stress and egg production. Faecal samples (n=2012) were collected from 34 cinereous vultures at 14 institutions. Using enzyme and radioimmunoassays, concentrations of faecal oestrogen, progestogen, androgen and glucocorticoid metabolites were quantified. General linear models were used to assess differences in concentrations in relation to egg-laying, sex, age, month, housing conditions and rearing history (hand or parent). Metabolite concentrations did not vary in relation to housing, rearing or age. Of the 10 breeding pairs, 7 produced an egg and 1 pair hatched a chick. Oestrogen and progestogen metabolites were higher in laying females and concentrations of both metabolites increased prior to egg-laying. In contrast, androgen metabolites were lower in males whose partner laid an egg. Glucocorticoid metabolite concentrations did not vary in relation to egg production. Oestrogen and progestogen metabolites of laying females were highest in March, while glucocorticoid metabolites of both males and females were highest in January and declined during the following months. No monthly trend in androgen metabolite production was observed. This study provides new endocrine data for cinereous vultures and confirms that faecal hormone analysis is an effective way to monitor egg-laying and monthly changes in hormone levels.

Introduction

Cinereous vultures *Aegypius monachus* are a species of Old World vulture native to much of Europe, the Middle East and Asia. While populations in Europe are increasing, those in Asia, where the majority of birds are found, are declining as a result of consuming bait poisoned for predator extermination, as well as habitat destruction. Currently, the species is listed as Near Threatened by the International Union for the Conservation of Nature (IUCN; Garrido et al. 2021). While zoo populations have the potential to contribute to species conservation (Conde et al. 2013; McGowan et al. 2017), there are currently only 58 cinereous vultures in Association of Zoos and Aquariums (AZA) facilities (Willis 2019). Rates of reproduction are low in AZA facilities, despite the relatively large number of breeding pairs, and little is known about the reproductive biology of captive populations.

Hormone monitoring, which can be used to assess fertility, document seasonal reproductive patterns, detect the onset of puberty and evaluate stress, is an important tool to improve captive breeding (reviewed in Hodges et al. 2010). While blood samples are the most direct means for quantifying hormone

concentrations, they can be difficult to obtain as handling and physical restraint are often necessary and collection procedures can elicit an adrenal response due to the stress of capture (Kenagy and Place 2000). Instead, samples collected non-invasively (faeces, urine and saliva) are often preferred because collection allows for long-term data collection without the stress associated with repeated restraint procedures. While faecal hormone analyses are routinely performed for mammalian species, analyses of avian species are less common. Faecal glucocorticoid metabolite concentrations of passerines have received the most attention, with authors characterising glucocorticoid production in a variety of species, including Carolina chickadees *Poecile carolinensis* (Lucas et al. 2006), great tits *Parus major* (Carere et al. 2003), and dickcissels *Spiza americana* (Suedkamp Wells et al. 2003). Concentrations of faecal gonadal hormone metabolites have also been measured in a variety of passerine species (e.g. Goymann et al. 2002; Lee et al. 1995; Russ et al. 2015).

Information about reproductive endocrinology in non-passerine species is limited to a handful of taxa (Blas et al. 2010; Mays et al. 1991). Members of the Accipitridae family, which includes over 200 species of hawks, eagles, kites, harriers and Old World vultures, are particularly under-represented, specifically with regards to using a non-invasive approach. However, faecal hormone analyses have been used to document patterns of oestrogen and androgen production in bald eagles *Haliaeetus leucocephalus* (Bercovitz et al. 1982) and harpy eagles *Harpia harpyja* (Blank et al. 2020), and gonadal and adrenal function of golden eagles *Aquila chrysaetos* (Staley et al. 2007).

This study expands upon the work conducted by Long et al. (2001) and describes patterns of faecal hormone metabolite concentrations for cinereous vultures in North American zoos. Monthly patterns of hormone metabolite concentrations, changes related to egg-laying and differences associated with age, housing conditions and rearing history were documented. The faecal glucocorticoid metabolite assay used was validated by demonstrating a substantial rise in concentrations after a known stressful event (egg removal). In faeces, glucocorticoids are excreted as a mixture of different metabolites with unpredictable antibody affinities. Validation is necessary to demonstrate that an assay detects metabolites that correspond with glucocorticoid production. As cinereous vultures face increasing threats in the wild, the methods described here may support conservation efforts by increasing rates of reproduction in captive populations, as well as monitoring reproduction and stress of cinereous vultures in the wild.

Methods

Faecal sample collection and analysis

Thirty-four cinereous vultures, 14 males and 20 females, from 14 AZA institutions were included in this study (Table 1). Females laid eggs from February to April. The majority of eggs were laid in March (n=6). While not systematically recorded, breeding behaviour was observed in six pairs and occurred from January to March. In total, 11 eggs were laid by 7 pairs during the study.

Faecal samples were collected one to three times a week from January to July in 2018 to 2021. In total, 2012 faecal samples were collected, with an average of 59 per bird. All faecal samples were collected within 16 hours of defecation and stored at -80°C prior to extraction using previously published methods (Kozlowski et al. 2018). Briefly, samples were homogenised to evenly mix urates and faecal material, as the urates and faeces could not be separated prior to extraction. Approximately 0.25 g wet weight of faeces was then incubated at 37°C for 24 hours in 1.25 ml of modified phosphate-saline buffer and 25 µL of β-glucuronidase/arylsulfatase (Roche Diagnostics 10-127-698001). This enzyme hydrolysis step was added to deconjugate steroids present in the urates, as is commonly performed on avian faecal material (Crofoot et al. 2003; Hartup et al. 2005; Kozlowski et al. 2018). The following day, 1.25 ml of methanol was added to each sample and the samples were shaken overnight. Liquid extracts were decanted, and solids were removed through centrifugation at 4000 g. The remaining supernatants were frozen at -80°C until assay. Faecal material was placed in a drying oven overnight at 80°C. Hormone concentrations were determined in ng/ml, then divided by the dry weight of the extracted faeces to give the results in ng/g dry weight.

Samples from females were assayed for faecal progestogen metabolites using Arbor Assays DetectX © Progesterone EIA K025 enzyme immunoassays (Ann Arbor, MI, USA) and faecal oestrogen metabolites using Arbor Assays DetectX © Estradiol EIA K030 enzyme immunoassays (Ann Arbor, MI, USA). The standard curve range of the progesterone assay was 50–3200 pg/ml and the standard curve range of the oestradiol assay was 39–10 000 pg/ml. Samples from males were assayed for faecal androgen metabolites using Arbor Assays DetectX © Testosterone EIA K032 enzyme immunoassays (Ann Arbor, MI, USA). The standard curve range was 40–10 000 pg/ml. Enzyme immunoassays were performed according to the kit instructions and faecal extracts were diluted 1:10 or 1:100 with assay buffer, giving a final methanol concentration of 5% or 0.5%. Faecal glucocorticoid metabolites of both sexes were assayed using MP Biomedicals DA I-125 corticosterone radioimmunoassay (Solon, OH, USA). Assays were performed according to the kit directions with the exception that standard diluent was added to the faecal extracts and faecal extraction buffer (containing 50% methanol) was added to the kit standards. The standard curve range of the corticosterone assay was 26–20 000 pg/ml. In total, 34 progesterone assays, 32 oestradiol assays, 22 testosterone assays and 21 corticosterone assays were performed.

For all assays, standards, samples and quality control pools were assayed in duplicate. Mean intra-assay variation was 8.6% for progesterone, 8.1% for oestradiol, 7.9% for testosterone and 8.9% for corticosterone. Mean inter-assay coefficients of variation for two quality control pools were 9.1% for progesterone, 8.8% for oestradiol, 9.3% for testosterone and 8.7% for corticosterone.

Faecal 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 94.3±3.2% of expected concentrations for progesterone, 91.2±2.9% for oestradiol, 90.2±4.1% for testosterone and 96.1±3.4% for corticosterone, 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 faecal extracts that contained low concentrations of hormone. Addition of known amounts of hormone at three dosage levels resulted in recovery of 101.1±3.3% of added progesterone, 92.3±2.4% of added oestradiol, 102.3±4.1% of added testosterone and 94.5±3.8% of added corticosterone.

To further validate an assay for quantifying glucocorticoid metabolite concentrations in cinereous vulture faeces, faecal samples were collected before and after an egg removal at the Saint Louis Zoo. An egg removal involves removing an egg and replacing it with a dummy so that the egg can be artificially incubated. It is presumed to be a stressful event, as the vulture pair is very protective of their nest and multiple staff members must enter the enclosure to perform the removal. Faecal glucocorticoid metabolite concentrations for the female (#52) increased by almost five-fold the day after the egg removal, compared to before and after the removal (Figure 1). This provides evidence for the ability of the assay used to detect glucocorticoid production in response to stress.

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Table 1. Study population of cinereous vultures, including identification number, location, sex, age, housing conditions, rearing history (hand or parent), and the year(s) of sample collection. [1] indicates cinereous vultures that produced an egg during the study; [2] indicate the pair that hatched a chick.

ID #	Location	Sex	Age	Housing	Rearing	Year(s) of sample collection
44	Denver Zoo	Female	31	Single	Hand	2019
48[1]	Detroit Zoo	Female	29	Opposite sex pair	Hand	2019
52[1]	Saint Louis Zoo	Female	28	Opposite sex pair	Parent	2018-2021
56[1]	Living Desert Zoo and Garden	Female	28	Opposite sex pair	Hand	2019-2020
65	Toledo Zoo	Female	24	Opposite sex pair	Hand	2019-2020
67[1]	Denver Zoo	Female	24	Trio	Parent	2019
89	Detroit Zoo	Female	17	Opposite sex pair	Hand	2019
94[1]	David Traylor Zoo	Female	17	Opposite sex pair	Parent	2019
98	Blank Park Zoo	Female	14	Same sex pair	Hand	2019
100	Blank Park Zoo	Female	13	Same sex pair	Parent	2019
101	Virginia Zoo	Female	13	Same sex pair	Hand	2019-2020
105	Henson Robinson Zoo	Female	11	Single	Unknown	2019-2020
109	Lincoln Park Zoo	Female	10	Opposite sex pair	Parent	2019-2020
112[2]	Zoo Miami	Female	9	Opposite sex pair	Parent	2019-2020
117	Virginia Zoo	Female	8	Same sex pair	Parent	2019-2020
119	Ross Park Zoo	Female	8	Same sex pair	Parent	2019-2020
121[1]	Denver Zoo	Female	8	Opposite sex pair	Hand	2019
122	Pueblo Zoo	Female	6	Opposite sex pair	Parent	2019
125	Ross Park Zoo	Female	7	Same sex pair	Hand	2019-2020
126	Denver Zoo	Female	4	Trio	Parent	2019
40[1]	Detroit Zoo	Male	32	Opposite sex pair	Unknown	2019
41	Lincoln Park Zoo	Male	32	Opposite sex pair	Hand	2019-2020
42[1]	Saint Louis Zoo	Male	31	Opposite sex pair	Hand	2018-2021
46[1]	Living Desert Zoo and Garden	Male	31	Opposite sex pair	Hand	2019-2020
51[1]	Denver Zoo	Male	28	Trio	Hand	2019
63[1]	Toledo Zoo	Male	25	Opposite sex pair	Hand	2019-2020
82	Detroit Zoo	Male	20	Single	Hand	2019
86	Ross Park Zoo	Male	18	Opposite sex pair	Parent	2019-2020
90	Pueblo Zoo	Male	18	Opposite sex pair	Parent	2019
95[1]	David Traylor Zoo	Male	16	Opposite sex pair	Unknown	2019
107[2]	Zoo Miami	Male	10	Opposite sex pair	Parent	2019-2020
124[1]	Denver Zoo	Male	5	Opposite sex pair	Parent	2019
131	Living Desert Zoo and Garden	Male	3	Single	Parent	2019-2020
134	Toledo Zoo	Male	0.7	Single	Hand	2019-2020

Statistical analysis

Statistical analyses were generated using SAS/STAT software, Version 9.4 of the SAS System for Windows (SAS Institute Inc. Cary, NC, USA). Differences in faecal androgen, oestrogen, progestogen and glucocorticoid metabolite concentrations were evaluated within the MIXED procedure using a generalised linear mixed model, with sex (glucocorticoid metabolites only), month, egg production (produced egg: yes or no) and the month × egg production interaction term as fixed effects, the individual's ID as a random effect and age as a covariate, using the Satterthwaite adjustment for degrees of freedom. Where main effects were declared significant ($P<0.05$), Tukey's multiple comparison test was used to determine pairwise differences between months. Housing and rearing history (hand or parent) had no relationship with concentrations of any hormone metabolite and these factors were dropped from the final statistical model.

Changes in faecal androgen, oestrogen, progestogen and glucocorticoid metabolite concentrations over time leading up to egg laying were evaluated by sex within the MIXED procedure using a generalised linear mixed model using repeated measures within subjects, day relative to egg laying (only days prior to egg lay included) as a continuous fixed effect and the individual's ID as a random effect. Separate variances were calculated for each individual. Significant changes over time were declared when $P<0.05$.

Results

Concentrations of faecal oestrogen metabolites ($t_{20.8}$=-5.19, P<0.0001) and progestogen metabolites ($t_{72.3}$=-4.34, P=0.0012) were higher in females that produced an egg compared to non-laying females in March, but not in other months. Faecal androgen metabolite concentrations of males whose mate produced an egg were lower than males housed singly or those housed with females that did not produce an egg ($F_{1,47.6}$=7.97, P=0.0069). No differences in faecal glucocorticoid metabolite concentrations in relation to egg-laying were observed. Faecal glucocorticoid metabolite concentrations did not differ between male and female vultures.

Concentrations of faecal oestrogen and progestogen metabolites rose significantly in relation to egg-laying (oestrogen metabolites: B_1=4.2285, P<0.0001; progestogen metabolites: B_1=2.4449, P<0.0399) (Figure 2A). In contrast, faecal glucocorticoid metabolite concentrations of females declined in relation to egg-laying (B_1=-2.7738, P=0.0001). There was no relationship between faecal androgen or glucocorticoid metabolite concentrations of males and their partners' egg-laying date. Because not all females were sampled on the same days in relation to egg-laying, sample dates were converted to days relative to laying-date. Data from all females were then combined, and the average weekly faecal oestrogen and progestogen metabolite concentrations were calculated in relation to day of egg-laying. This allowed better visualisation of patterns of hormone production (Figure 3). Overall, faecal oestrogen metabolite concentrations increased starting approximately five weeks before egg-laying, and reached a maximum value three weeks before egg-laying. A second rise in faecal oestrogen metabolite concentrations was noted the week of egg-laying; concentrations then returned to pre-laying levels one week post-laying. Faecal progestogen metabolite concentrations reached a maximum concentration three weeks before egg-laying. Concentrations then declined, reaching pre-laying concentrations the week of egg-laying.

For egg-laying females, concentrations of faecal oestrogen metabolites averaged 223.6±23.2 ng/g and concentrations of faecal progestogen metabolites averaged 317.2±26.9 ng/g from 14 to 5 weeks preceding egg-laying. Maximum concentrations averaged 2128.6±632.6 ng/g for faecal oestrogen metabolites and 2207.1±931.5 ng/g for faecal progestogen metabolites (n=11 clutches from 7 females). Average concentrations for the 13 non-laying females were 138.2±8.1 ng/g for faecal oestrogen metabolites and 322.1±15.2 ng/g for faecal progestogen metabolites, and no apparent hormone increases were detected (Figure 2B).

Individual differences were observed for concentrations of faecal androgen (Z=1.9, P=0.0287), oestrogen (Z=1.78, P=0.0375), progestogen (Z=2.13, P=0.0164) and glucocorticoid (Z=3.28, P=0.0005) metabolites. Female #112, the only female to hatch a chick, had the lowest concentrations of faecal glucocorticoid metabolites of any bird in the study. Differences in faecal hormone

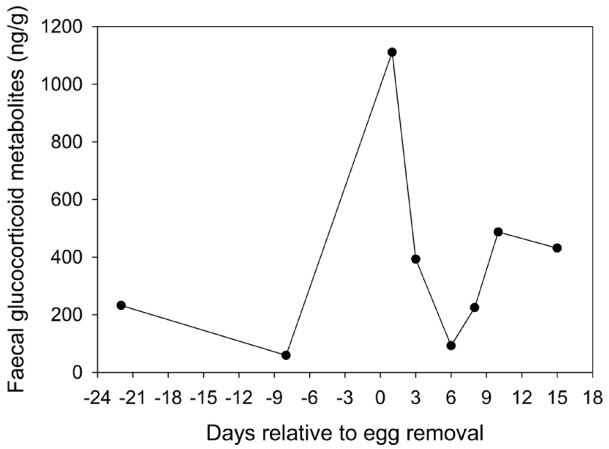

Figure 1. Faecal glucocorticoid metabolite concentrations (ng/g) before and after an egg removal (Day 0) for female cinereous vulture *Aegypius monachus* #52 at the Saint Louis Zoo (Saint Louis, MO, USA). An increase in faecal glucocorticoid metabolite concentrations was detected one day after the egg removal, providing evidence for the ability of the assay to detect glucocorticoid production in response to stress.

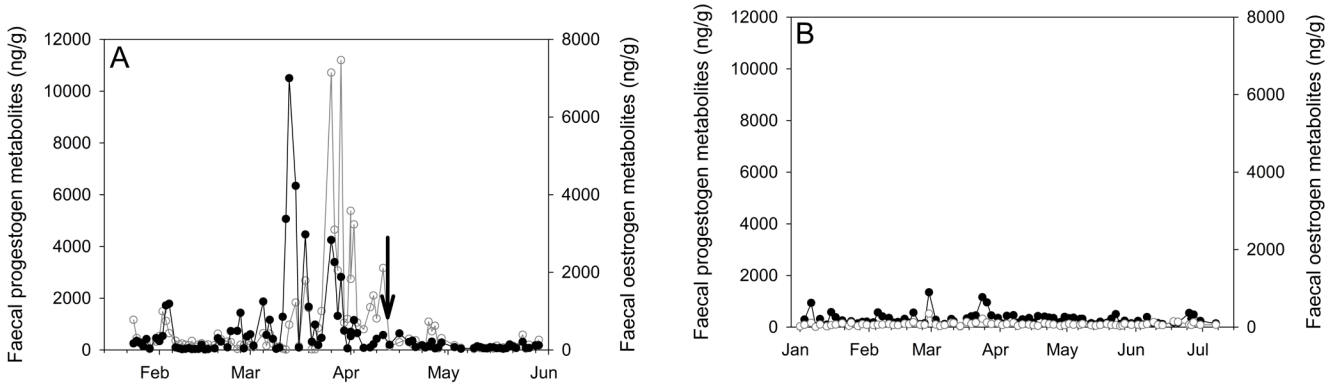

Figure 2. Faecal progestogen (○) and oestrogen (●) metabolite concentrations (ng/g) for (A) female cinereous vulture *Aegypius monachus* #52 that laid an egg on 13 April 2018 (indicated by arrow); and (B) female cinereous vulture #105 that failed to lay an egg.

metabolite concentrations were not related to rearing (parent or hand), age or social housing.

Seasonal changes in faecal hormone metabolite concentrations were observed. Faecal oestrogen metabolite concentrations in egg-laying females were highest in March ($P<0.05$), and faecal progestogen metabolite concentrations of egg-laying females were highest in March and April ($P<0.05$). No seasonal changes in oestrogen or progestogen metabolite concentrations were observed in non-laying females. Faecal glucocorticoid metabolite concentrations of both males and females were highest in January and decreased through April ($F_{6,1959}=7.06$, $P<0.0001$) (Figure 4). In contrast, concentrations of faecal androgen metabolites did not vary with month of sample collection.

Discussion

This study assessed concentrations of faecal hormone metabolites in captive cinereous vultures to better understand their reproductive biology. The results confirm that changes associated with egg-laying and seasonality can be monitored through non-invasive faecal hormone analysis. Gonadal hormone profiles of females differed according to their reproductive outcomes and seasonal changes in glucocorticoid production were observed.

Female vultures that produced an egg had higher concentrations of faecal oestrogen and progestogen metabolites during the breeding season than non-laying females, and concentrations of both hormones increased prior to egg-laying. Elevated concentrations of faecal gonadal hormones of laying, compared to non-laying, females have also been documented in piping guans *Pipile cumanensis* and horned guans *Oreophasis derbianus* (Kozlowski et al. 2018), great hornbills *Buceros bicornis* (Crofoot et al. 2003), whooping cranes *Grus americana* (Brown et al. 2016) and blue-fronted parrots *Amazona aestiva* (Pereira et al. 2018). In domestic chickens *Gallus gallus domesticus*, oestradiol is produced by follicular thecal cells. Production is highest in small, early stage follicles and declines in later-stage, yolky follicles. In contrast, progesterone is produced by large, yolky follicles (Etches 1996). Elevated concentrations of progesterone stimulate a surge in luteinising hormone and trigger ovulation (Sharp 1980). Oestradiol is correlated with accelerated growth of ovarian follicles. It also induces the synthesis and mobilisation of yolk components and increases development of the oviduct (Etches 1996). Behaviourally, oestradiol stimulates food intake (Johnson 1986) and initiates female reproductive and nest building behaviours (Hinde 1965; Searcy 1992). Progesterone is involved in the production of avidin, contraction of the myometrium, shell formation (Yoshimura and Bahr 1991) and regulates nesting and incubation behaviour (Sharp and Lea 1996).

Differences in timing between increases in faecal hormone metabolites were observed. Concentrations of oestrogen metabolites rose before progestogen metabolites, approximately five weeks before egg-laying. Both hormones peaked three weeks before egg-laying, with oestrogen metabolite concentrations showing a second peak the week of laying. Concentrations of both hormones then declined and were at baseline one week post-laying. These patterns are comparable to those described by Long et al. (2001), and similar patterns have been documented in a variety of avian taxa. However, the timing of hormone peaks varies, suggesting that there are species differences in the timing of hormone production by early and mature follicles. Harpy eagles show a pattern of hormone production similar to that of cinereous vultures, with concentrations rising three to four weeks before egg-laying (Blank et al. 2020). However, no secondary rise in oestrogen metabolite concentrations was noted. In contrast, progestogen metabolites in brown kiwi *Apteryx mantelli* only begin to rise one week prior to egg-laying (Jensen and Durrant 2005), and in piping and horned guans, increases in both hormones occur between one to two weeks prior to laying (Kozlowski et al. 2018).

No seasonal change in male androgen metabolite concentrations was detected. This is surprising, as breeding of cinereous vultures in North American zoos is highly seasonal. In the current AZA population, 91% of cinereous vultures hatched between April and June, with 53% of birds hatching in May (Willis 2019). It was expected that males would have elevated androgen metabolite concentrations from January to March, when breeding behaviour is observed, as androgen rises preceding breeding have been documented in a variety of avian taxa, including several raptors (Blank et al. 2020; Blas et al. 2010; Mays et al. 1991; Pereira et

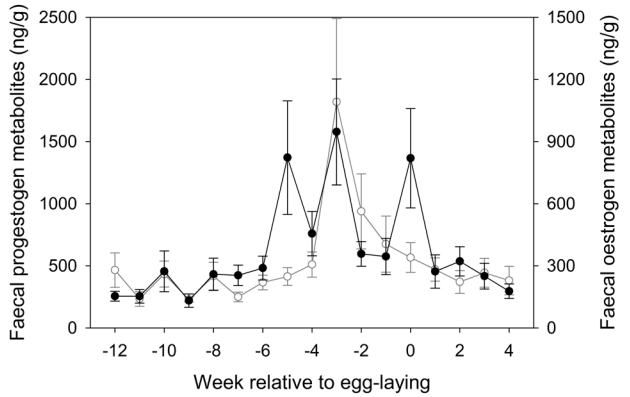

Figure 3. Weekly mean (±S.E.) faecal progestogen (○) and oestrogen (●) metabolite concentrations (ng/g) of female cinereous vultures *Aegypius monachus* (n = 11 clutches from 7 females) in relation to egg-laying. Week 0 is the week an egg was laid.

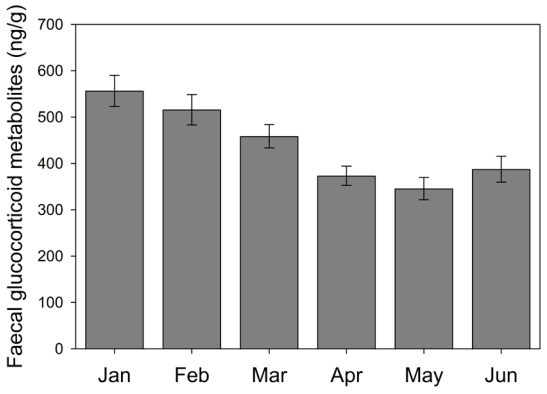

Figure 4. Mean (±S.E.) faecal glucocorticoid metabolite concentrations (ng/g) of cinereous vultures *Aegypius monachus* in relation to month of sample collection.

al. 2010). However, no change was detected, and males paired with laying females had lower androgen concentrations than other males in the population. While breeding behaviour was observed in a majority of the pairs, all but one failed to hatch a chick. Sperm production was not confirmed, and it is possible that low androgen concentrations may reflect that many of the males in this study were infertile. However, androgen concentrations for the single male whose egg hatched were similar to those of males whose mate produced non-hatching eggs. Interestingly, Long et al. (2001) also reported low concentrations of faecal androgens for male cinereous vultures, including for two males in which sperm production was confirmed. There is evidence that, in general, raptors may have lower concentrations of androgens compared to other avian species (Blas et al. 2010) as a result of reduced territoriality and strict monogamy (Mays et al. 1991). In addition, androgen concentrations may have been affected by the lack of intra-specific competition in captivity (Beletsky et al. 1992), as has been suggested for captive whooping crane males (Brown et al. 2016). More data are needed to understand the relationship between androgen metabolite concentrations and reproductive success in captive cinereous vultures.

Faecal glucocorticoid metabolite concentrations of both male and female vultures varied monthly. Concentrations were highest in January, during the known breeding season, and decreased throughout the spring. During the breeding season, birds generally become more active, and glucocorticoid production increases (Astheimer et al. 1994; Romero and Remage-Healey 2000; Romero et al. 1998) likely reflecting this increase in energy expenditure. There is also evidence that individuals become more sensitive to stress during the breeding season (Astheimer et al. 1994), and that glucocorticoids may regulate reproductive investment, as elevated concentrations were associated with mate-feeding, brooding and incubation behaviours in great tits (Ouyang et al. 2013).

No differences in faecal glucocorticoid metabolite concentrations were observed in relation to housing conditions or rearing history. There was also no evidence that stress was a contributing factor for the observed differences in egg-laying between pairs. However, the only pair to hatch a chick had lower faecal glucocorticoid metabolite concentrations than most other birds and the female had the lowest concentrations of any bird in the study. Similar findings have been documented in captive great hornbills (Crofoot et al. 2003) and suggest that adrenal activity of females may negatively affect egg viability. In birds, maternal stress has been shown to have carry-over effects in offspring. For example, elevated corticosterone production in female barn swallows *Hirundo rustica* increases corticosterone concentrations in egg yolk and reduces hatching rates (Saino et al. 2005). Further study is needed to determine whether glucocorticoid production in female cinereous vultures has a detrimental effect on egg viability.

Currently, there are fewer than 60 cinereous vultures in AZA zoos. Rates of reproduction are poor and maximising breeding success is a priority for the Raptor Taxon Advisory Group (TAG). The methods outlined here could contribute to ex-situ breeding efforts by helping to assess fertility and the impact of potential stressors on captive populations of cinereous vultures, as well as potentially other vulture species.

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