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Research article

Faecal glucocorticoid metabolite concentrations associated with illness, sex, age, and season in a kea *Nestor notabilis* population at the Cincinnati Zoo and Botanical Garden

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Abstract

The kea *Nestor notabilis* is an endangered New Zealand alpine parrot. After individuals became ill and died at the Cincinnati Zoo, it was suspected that prolonged hypothalamic-pituitary-adrenal axis activation may have precipitated illness onset. The main objective of this study was to determine if elevated concentrations of faecal glucocorticoid metabolites (fGCMs) preceded illness outbreaks. An effective protocol for extracting fGCMs from kea faeces was established, and a corticosterone enzyme immunoassay (EIA) was validated. The secondary objective was to ascertain if fGCM concentrations were impacted by sex, age, or seasonality. Samples collected over a year were analysed via EIA. fGCM concentrations in samples collected 30 days pre-symptom (PRE-SYMPT) and post-symptom (POST-SYMPT) onset were compared and found to trend higher in POST-SYMPT versus PRE-SYMPT samples (estimated marginal mean (EMM) and 95% confidence interval (CI)): 85.2, 62.7-133.1 ng/g and 58.1, 42.1-94.0 ng/g, respectively). Furthermore, POST-SYMPT values were significantly higher than those of healthy birds (NO-SYMPT) while PRE-SYMPT values did not differ (85.2, 62.7-133.1 ng/g; 53.9, 45.1-67.0 ng/g; and 58.1, 42.1-94.0 ng/g, respectively). Though not statistically significant, several trends were noted: 1) fGCM concentrations trended lower in males than females (48.5, 37.4-68.9 ng/g, and 51.0, 37.0-82.2 ng/g, respectively); 2) juvenile fGCM trended lower than that for adults (45.5, 34.1-68.2 ng/g and 54.8, 41.0-82.4 ng/g, respectively); and 3) fGCM concentrations in adult females trended higher during the breeding season (60.0, 39.5-124.3 ng/g), compared to non-breeding season (51.2, 36.2-87.2 ng/g). Contrary to the original hypothesis, fGCM concentrations in kea were not elevated prior to onset of illness.

Introduction

The kea *Nestor notabilis* is a species of alpine parrot found on the South Island of New Zealand. Unlike other parrots, kea are conditioned to the colder climates of their mountainous habitat (Diamond and Bond 1999). They are highly social, often congregating around food sources. Kea also are adept foragers which allows them to survive in the harsh alpine environment. However, their foraging habits, which include dining on dead sheep *Ovis aries* and pulling fat from live sheep, have also led to conflict with humans who have settled in New Zealand in relatively recent history. These proclivities relegated kea to nuisance animals targeted by farmers, and a bounty driven extermination of the species was carried out from approximately 1870 to 1970, during which an estimated 150,000 birds were killed (BirdLife International 2017; Elliot and Kemp 2004). More recent population threats to kea include consumption of toxic materials due to their wide ranging foraging, and introduced mammalian predators which target and destroy native avian nests, including those of kea (Diamond and Bond 1999; McLelland et al. 2010). As a result, kea were recently upgraded from vulnerable to endangered status on the International Union for Conservation of Nature (IUCN) Red List (BirdLife International 2017).

A population analysis of the North American ex situ population conducted by the Association of Zoos and Aquariums (AZA) Kea Species Survival Plan® (SSP) in 2012, found that the population was vulnerable and facing a decline. Factors contributing to this decrease and vulnerable status included a lack of females and disproportionate ratio of deaths to hatchings (Theis et al 2012). Nine institutions (eleven at the time of the study) in North America participate in the kea SSP, with variable breeding success. However, the Cincinnati Zoo and Botanical Garden (CZBG) successfully breeds, hatches and rears kea chicks consistently. From 2014-2015, out of 13 chicks hatched in North America, all but one originated from CZBG. Whereas the population appeared to be increasing from 2012 to 2015, the following year the population dropped by almost 40% leaving just 36 individuals in 2016. Currently, the population is still in decline with only 30 individuals remaining (Species360 2021). During this study, the CZBG flock represented a large portion of the animals in AZA care (n=18). Demographic variations within the flock coincided with those found within the total population, such as a subtle male sex bias and few young females, making this flock an ideal model for the kea population in AZA care (Theis et al. 2012).

The low numbers and vulnerable status of the kea SSP population make every individual important for the species' sustainability. In 2014 and 2015, multiple birds (adults and juveniles) in the CZBG population became ill with similar symptoms and some eventually died. Although involvement of avian bornavirus (ABV) was suspected due to animals in the flock previously testing positive for ABV it was difficult to prove perimortem due to the high occurrence of false positive and false negative results in ABV testing. (Dahlhausen and Orosz 2015; Rossi et al. 2018). This loss was a significant setback for the overall kea population within AZA institutions. Keepers at CZBG questioned if chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis, potentially caused by environmental conditions such as high heat and humidity, was leading to the illness outbreaks in the flock.

The influence of stress on immunity can be far reaching, however mechanisms involved are not always understood, nor well defined in wildlife (Martin 2009). The hypothalamic-pituitary-adrenal axis is one of the major stress response pathways; within it, glucocorticoids (GCs) are elevated, triggering an anti-inflammatory immune response. While initial bursts (minutes/hours) of HPA activation and GC secretion are able to increase immune function, it is thought that prolonged periods (days/weeks) of HPA activation and GC secretion decrease immune function (Dhabhar 2009; Webster Marketon and Glaser 2008). Assessing GCs as a measure of stress is common (Sapolsky et al. 2000). GCs can be measured directly in blood/plasma, saliva, hair, and feathers and indirectly in the form of glucocorticoid metabolites in faeces. (Goymann 2005; Harper and Austad 2000; Martin 2009). Due to the non-invasive nature of sample collection, faecal glucocorticoid metabolites (fGCMs) were considered the best biological matrix for determining if there was a connection between HPA activation and illnesses in kea.

Kea are notoriously intelligent and able to solve various puzzles and problems; therefore, most published research centres around this subject (Auersperg et al. 2011; Miyata et al. 2011; van Horik and Emery 2016) and publications regarding kea hormone analysis are lacking. However, fGCM analysis in other parrot species (blue-fronted parrots *Amazona aestiva*, macaws, budgerigars *Melopsittacus undulatus*, and red-tailed parrots *Amazona brasiliensis* has proven feasible (Ferreira et al. 2015; Watson et al. 2013; Young and Hallford 2013). Interpreting GCs relative to stress can be difficult (Millspaugh and Washburn 2004; Palme 2005). Various factors such as sample type, time of day collected, sex, age, and season can all influence GC concentrations (Schoenemann and Bonier 2018; Touma and Palme 2005). For example, basal GC concentrations in captive starlings *Sturnus vulgaris* exhibit distinct daily patterns (Romero and Remage-Healey 2000). Changes in GCs over time with relation to breeding season in northern spotted owls *Strix occidentalis caurina* and red-tailed parrots have been observed (Popp et al. 2008; Wasser and Hunt 2005). Additionally, differences between male and female northern spotted owl GCs have been reported (Wasser and Hunt 2005).

The main objective of this study was to establish an effective protocol for extracting fGCMs from kea faeces and confirm that they could be measured using a corticosterone enzyme immunoassay (EIA), thereby allowing for HPA axis activation assessment in this species. Once the protocol was established, the first goal of this study was to determine if sustained HPA activation, as indicated by fGCM concentration, was evident prior to outbreaks of illness in CZBG kea. A secondary goal was to investigate the impact of several other factors that might influence kea fGCM concentrations such as sex, age, and seasonality. It was anticipated that fGCM concentrations would vary between the

Table 1. Sex and age of individuals that showed symptoms or died during the study period. All animals that died during the study period had experienced symptoms associated with the illness.

	Total	Symptomatic	Died
Adult females	5	2	1
Juvenile females	2	2	2
Adult males	4	4	1
Juvenile males	7	3	2

sexes, as is seen in other bird species, and ages due to the age based hierarchy within a flock (Diamond and Bond 1999). It was also hypothesized that fGCM concentrations would be elevated in the summer in response to the higher temperatures that are uncommon in this alpine species' natural history (BirdLife International 2017; Diamond and Bond 1999; Ozella et al. 2015a).

Materials and methods

Animals

The birds in this study were all part of the CZBG breeding flock which consisted of adults (n=9) and juveniles (individuals under the age of three; n=9) of both sexes (Table 1). Six of the animals died during the study (two adults, four juveniles; Table 1). Throughout this study period the animals were housed in both in-door enclosures and an outdoor aviary. They were typically housed in social groups of males with a single female, and the remaining females were housed either with juveniles or solo. During breeding season, mated pairs were kept together.

Animals were used in accordance with CZBG IACUC protocol #14-123.

Sample collection and processing

Keepers collected faecal samples opportunistically from the birds from August 2014 until December 2015. This was a non-invasive and opportunistic study, so the samples were collected when known individuals were observed defecating or when animals were housed alone for other reasons, thus the frequency per animal varied. For the study, kea would generally be shifted into separate enclosures in the morning with faecal samples collected in the early afternoon. Individuals showing signs of illness (description below) were kept in smaller enclosures to prevent injury from illness symptoms which could include ataxia or aggression from the other birds. Faecal samples were collected off a clean concrete floor by scraping them into an aluminium tray and placing the sample in a plastic bag. Samples were immediately stored at -20˚C in a mini-freezer in the animal holding facility until transfer to the lab where they were stored at -20˚C until processing could occur. Once thawed, as much urate as possible was removed, and the samples were dried overnight in an oven at 37˚C. The samples were all cleaned and dried within a year of collection. The dried sample was then stored at room temperature (RT, 20-25˚C) until extraction which occurred within a year of cleaning and drying.

Sample extraction

Samples were extracted by slightly modifying a previously published method (Brown et al. 2016). Briefly, 0.98 g to 0.115 g of dried sample was weighed into 15 mL polypropylene conical tubes. Ethanol (70%; 5 mL) was added to each tube. Tubes were rotated for 1-3 hours, and then centrifuged at 1600 x g for 20 min. Supernatant was decanted into disposable borosilicate glass test tubes and another 5 mL of 70% ethanol was added to the sample. Samples were vortexed and centrifuged again. The supernatant was added to the previously decanted volume. Samples were dried in a fume hood with direct air, and then reconstituted using 1 mL of assay buffer (45.2 mM NaH_2PO_4, 61 mM Na_2HPO_4, 148.9 mM NaCl, 0.1% Tween 20, 0.1% BSA, 0.001% ProClin).

Corticosterone assay

Samples were analysed using a corticosterone EIA that relied on a corticosterone antibody and horse radish peroxidase-conjugated steroid (HRP) previously verified for use in several diverse taxa (CJM006, from Coralie Munro, University of California, Davis, USA (Watson et al. 2013)). Briefly, samples were diluted 1:5 in assay buffer, and 50 µl of samples, standards (0 pg/well to 1000 pg/well) and controls were added in duplicate to a 96-well plate (NUNC Maxisorp, ThermoScientific, New York , USA) pre-coated with goat-anti-rabbit IgG antibody (Arbor Assays, Ann Arbor, Michigan, USA). HRP (50 µl of a 1:85,000-100,000 dilution in assay buffer) was added to all wells, followed immediately by 50 µl of corticosterone antibody (diluted 1:85,000-1:100,000 in assay buffer). Each plate always included blank (no antibody), high control (100 pg/well), and low control (20 pg/well) wells to assess inter and intra-assay variability. Plates were shaken at RT for 2 hours, after which they were washed 3x with 300 µl of wash buffer (4.3 mM Na2HPO4·7H2O, 699.3 µM $NaH_2PO_4·H_2O$, 7.5 mM NaCl, 499.7 µM EDTA, 0.5% Tween 20, 0.0009% ProClin). Finally, 100 µl of chromogenic substrate ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) was added to all wells and the plates were covered and placed on a plate shaker to develop. The plate was read at a wavelength of 405 nm; when optical density of zero value wells reached approximately 1.0, data were recorded (Opsys MR plate reader and Revelation Quicklink software, Dynex Technologies, USA).

Assay validation

A pool of faecal extracts from five birds and extracts from three individual birds were all analysed to confirm parallelism with the standard curve. A spike/recovery was performed wherein pooled samples were spiked with standards prior to analysis. The concentrations from the pooled samples were compared to the concentration of the pooled samples spiked with the known standard amounts and the percent of recovered standard was calculated. Additionally, individual profiles were assessed to confirm that the assay would detect biologically relevant changes in fGCM concentrations. Baselines were calculated using an iterative process previously described using 2 standards deviations as the limit as opposed to 1.5 (Brown et al. 1996). The occurrence of two consecutive values above the baseline was considered a spike.

Assigning group classification

Symptoms of the illness in the CZBG flock were described by the veterinary staff as: lethargy, ataxia, nystagmus, anorexia, and peripheral neuropathy. Observations of these symptoms by keeper staff were quickly followed by an animal assessment by veterinary staff and treatment with an anti-inflammatory (celecoxib, Pfizer, New York, USA; 75mg orally BID) was initiated. Notes from the keepers were thoroughly investigated for information relating to onset of these symptoms of illness and treatment (Species360 2021). The combination of the physical symptoms followed by anti-inflammatory treatments prescribed by the veterinarian were used by the authors to identify birds that experienced the disease of concern in this study and made it possible to distinguish them from those exhibiting behavioural changes in response to other maladies such as heat stress or traumatic injuries.

Following discussions with the attending veterinarian, it was estimated that onset of symptoms may occur up to 10 days after the initiation of illness. Therefore, if elevated fGCM concentrations were associated with illness, an increase should be detected during the ~30 day period leading up to the day initial symptoms were noted. Values were first evaluated to assess if an animal was showing the described symptoms or not. Based on the above criteria and information the date symptoms were first initiated was determined for each individual. Samples were then classified into four different groups. NO-SYMPT indicates samples when no symptoms were being experienced by an individual and collection occurred more than 30 days before the start of symptoms. PRE-SYMPT samples denotes any collected -30 to -1 days prior to day of symptom on set as noted in ZIMS (day 0). POST-SYMPT was assigned to samples collected the first day of symptoms to 30 days post symptom onset. SYMPT classifies samples obtained when

Table 2. The symptom designation with the number of samples, the number of individuals, and the range of the number of samples per individual.

	n	# Individuals	# Samples
NO-SYMPT	170	17	1-41
PRE-SYMPT	9	5	1-3
POST-SYMPT	27	9	1-7
SYMPT	49	5	1-32

symptoms were still being experienced beyond in the initial 30 days post symptom onset. Due to the opportunistic nature of this study there were not matched samples for all groups for certain individuals (Table 2).

Sex, age and seasonal effects

The impact of sex, age and season (breeding/brooding or meteorological) on fGCM concentrations in kea were evaluated on a subset of the samples. Specifically, analyses were on those samples collected from when animals were not exhibiting illness symptoms listed above or being treated with the anti-inflammatory medication (NO SYMPT + PRE-SYMPT = WELL, n = 178). In the wild, kea breed in the southern hemisphere winter into spring/summer (July to January) (Diamond and Bond 1999). When housed ex situ, breeding shifts to the winter/spring months of the northern hemisphere. For this study exact dates of the breeding/brooding seasons were obtained from the keeper staff to accurately assess animal fGCM concentrations associated with breeding season activities of the CZBG flock. During the study, the breeding/brooding activities lasted from November 2014 to March 2015. Meteorological seasons were defined as: winter: December to February (n=24), spring: March to May (n=24), summer: June to August (n=38), and autumn: September to November (n=92).

Statistical analysis

Statistics were performed using the R statistical package (version 4.0.3). Several different models were built to analyse the different goals of the study. In the first model the effects of the illness symptoms on fGCM were analyzed using a generalized linear mixed effects model with Gamma distribution and the NO-SYMPT, PRE-SYMPT, POST-SYMPT, SYMPT variables included as fixed effects. In addition, animal ID was included as a random effect in this model to account for individual-based variation. In the second model the values were subset to the WELL values, and the effects of age, sex, and breeding season on fGCM were analyzed also using a generalized linear mixed effects model with Gamma distribution and the aforementioned variables included as fixed effects. Animal ID was included as a random effect in this model as well to account for individual-based variation. Because breeding season was correlated with meteorological season, the model was run a second time with meteorological season substituted for breeding season to assess the effects of meteorological season. For all models fit was assessed using ANOVA and quantile-quantile plots. Addtionally the POST-SYMPT fGCM values were compared between animals who ultimately lived and animals who ultimately died using a t-test with a Wilcoxon correction.

The effects of breeding season on adult female fGCM concentration was assessed by subsetting to only WELL adult females in a generalized linear model with Gamma distribution, with animal ID included as a random effect to account for individual-based variation, as well as a t-test with a Wilcoxon correction. Adult males were excluded from this analysis due to low sample size.

P-values were estimated for the models using Satterthwaite's degrees of freedom method and $P<0.1$ was considered significant. Estimated marginal means were calculated to isolate the estimated effects of each variable in the models.

Results

Samples and assay validation

Samples were collected opportunistically from the flock for over a year yielding 255 extracts. Pooled and individual faecal extracts diluted neat to 1:128 ran parallel to the standard curve ($r = 0.99$). Coefficients of variability (CVs) were less than 10% for both inter and intra assay variability and the assay sensitivity at 95% binding was 0.11 ng/g. Spiked samples demonstrated an average of 101% recovery. Data from several individual profiles demonstrated a spike in fGCM concentrations in the days preceding death. Furthermore, one profile contained low baseline concentrations of fGCMs until an injury (broken leg) was recorded, after which a spike in fGCM was clearly visible. These results provide evidence that elevated fGCM concentrations are detected in kea faeces following natural, stressful, physiological experiences.

fGCM concentrations relative to timing of illness

NO-SYMPT fGCM concentrations did not differ from PRE-SYMPT fGCM concentrations (NO-SYMPT EMM: 53.9 ng/g (95% CI: 45.1-67.0 ng/g), PRE-SYMPT EMM: 58.1 ng/g (95% CI: 42.1-94.0 ng/g), Figure 1). fGCM concentrations of POST-SYMPT were significantly higher than the NO-SYMPT values when they were not experiencing any symptoms and presumably healthy ($P<0.001$, POST-SYMPT EMM: 85.2 ng/g (95% CI: 62.7-133.1 ng/g); NO-SYMPT EMM: 53.9 ng/g (95% CI: 45.1-67.0 ng/g)). fGCM concentrations POST-SYMPT were not significantly higher than PRE-SYMPT, although they did trend higher (POST-SYMPT EMM: 85.2 ng/g (95% CI: 62.7-133.1 ng/g) PRE-SYMPT EMM: 58.1 ng/g (95% CI: 42.1-94.0 ng/g) Figure 1). Interestingly, fGCM concentrations trended higher during the first 30 days symptoms were exhibited than during the remainder of the symptomatic period (POST-SYMPT EMM: 85.2 ng/g (95% CI: 62.7-133.1 ng/g) SYMPT EMM: 65.9 ng/g (95% CI: 51.0-93.1 ng/g) Fig1). No differences were found between in the POST-SYMPT fGCM values animals who ultimately lived and animals who ultimately died ($P>0.4$)

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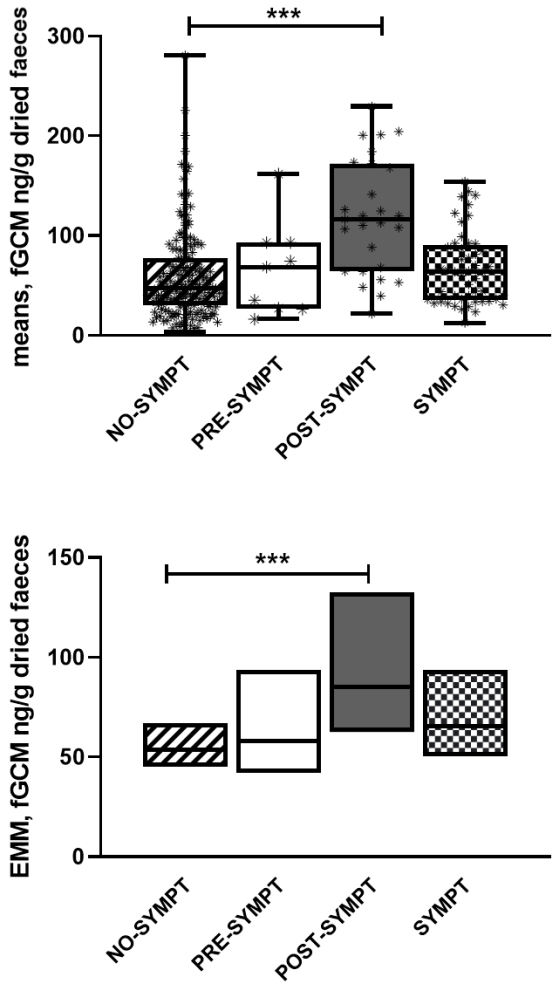

Figure 1. True means, interquartile range, maximum/minimum observations, and data points (upper); and Estimated Marginal Means (EMM) and EMM 95% confidence intervals (lower) of faecal glucocorticoid metabolite concentrations for sample values when no symptoms were being experienced by an individual and did not occur 30 days before the start of symptoms or 30 days after the start of symptoms (NO-SYMPT, diagonal marked bar, n=170), 30 days pre-symptom onset (PRE-SYMPT, open bar, n=9), 30 days post-symptom onset (POST-SYMPT, solid bar, n=49), and all other days with symptoms (SYMPT, checker bar, n=27). *** indicates P<0.001. The PRE-SYMPT and POST-SYMPT values are not included in the NO-SYMPT or the SYMPT.

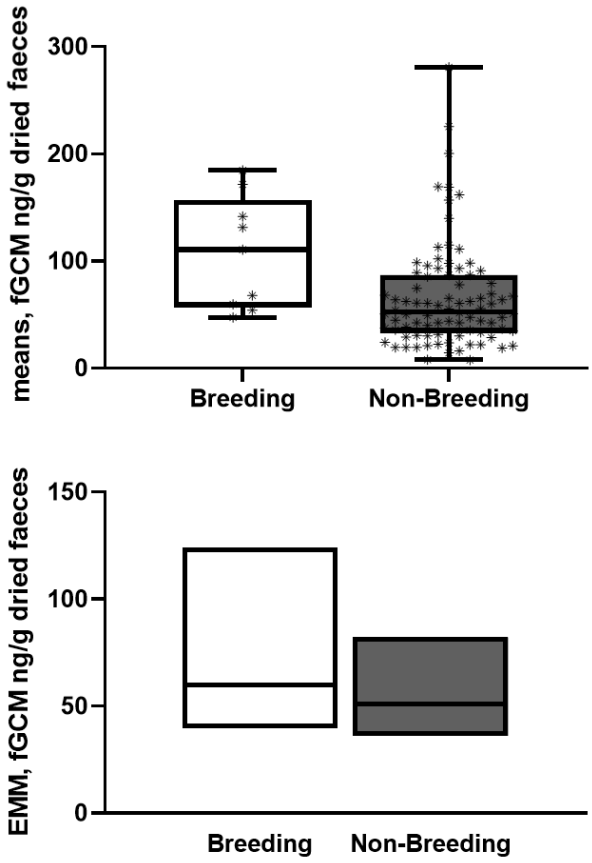

Figure 2. True means, interquartile range, maximum/minimum observations, and data points (upper); and Estimated Marginal Means (EMM) and EMM 95% confidence intervals (lower) for faecal glucocorticoid metabolite concentrations (fGCM) of adult female kea in breeding season (n=9), and non-breeding (n=86). fGCM concentration values only contain concentrations measured on adult female WELL dates when birds were not experiencing symptoms associated with illness.

Sex, age and seasonal effects

fGCM concentrations were not significantly different between females (n=95) and males (n=79), (female EMM: 51.0 ng/g (95% CI: 37.0–82.2 ng/g); male EMM: 48.5 ng/g (95% CI: 37.4: 68.9 ng/g)), nor did they significantly differ between juveniles (n=65) and adults (n=112), although juveniles trended lower than adults (juvenile EMM: 45.5 ng/g (95% CI: 34.1–68.2 ng/g); adult EMM: 54.8ng/g (95% CI: 41.0–82.4 ng/g)) .

fGCM concentrations in adult females appeared higher in the breeding season (n=9) than non-breeding season (n=86), (true mean: 107.65 ng/g vs 65.63 ng/g respectively, P<0.05). However, when the data were analysed using the generalized linear model with Gamma distribution, with animal ID included as a random effect to account for individual animal, significance was lost, though still trending towards breeding season values being higher than non-breeding season (adjusted mean 104.71 ng/g vs 68.75 ng/g, respectively (P=0.392); EMM: breeding: 60.0 ng/g (95% CI: 39.5–124.3 ng/g), non-breeding 51.2 ng/g (95% CI: 36.2–82.2 ng/g) Figure 2).

Whereas keepers did report physical signs of heat stress in the kea during the summer months, there were no differences in fGCM concentrations among meteorological seasons (autumn, EMM:

47.2 ng/g (95% CI: 38.3-61.6 ng/g), spring, EMM: 48.7 ng/g (95% CI: 38.3-66.8 ng/g), summer, EMM: 47.7 ng/g (95% CI: 38.1-63.8 ng/g), winter, EMM: 45.4 ng/g (95% CI: 35.4-63.3 ng/g) Figure 3).

Discussion

This is the first report of measuring fGCMs in the kea. After identifying an effective extraction protocol and validating the corticosterone EIA, it was possible to employ the assay to assess the relationship of fGCM concentrations and illness onset in this species. Previous studies have found associations between GC concentrations and severity of viral avian diseases. In zebra finches *Taeniopygia guttata* infected with West Nile virus, only those with elevated concentrations of corticosterone had infectious viral loads, and they were infected longer and had a higher mortality rate (Gervasi et al. 2017). Similarly, in chickens *Gallus gallus domesticus*, experimentally elevated concentrations of corticosterone increased the length of time of West Nile infection (Jankowski et al. 2010). In contrast, elevated fGCM concentrations were not present in kea prior to onset of illness in this study. Admittedly, because sample collections were opportunistic, some small sample subsets, as well as the variation of the individual animal could be influencing results. Regardless, the data do not appear to support the hypothesis that chronic activation of the HPA axis (which can be immunosuppressive) or even a more recent, short-term stressor event, are predisposing the birds to illness. Not surprisingly, kea did exhibit consistently elevated fGCMs after the onset of symptoms, most likely related to the physical stress and discomfort of the illness and possibly the associated examination/isolation. However, the lower fGCM concentrations after the initial 30 days in birds that survived suggest that the treatment was having the desired effect on animal wellbeing, and/or that the illness had resolved.

The breadth of the longitudinal data collected allowed exploration of other environmental and individual characteristics of the kea that could be associated with fGCM concentrations. Similar to data reported for European nuthatch *Sitta europaea* and greater rhea *Rhea americana*, fGCM concentrations did not differ between adult and juvenile kea (Landys et al. 2010; Ozella et al. 2015a). However, juveniles did trend lower than adults in this study, possibly because we measured fGCM in samples collected year-round, including the breeding season, whereas the other studies focused on a single season (winter or summer, respectively).

Study results indicated that adult females may excrete higher fGCM during the breeding season than the non-breeding season. Increased fGCMs in avian and mammalian species during the breeding season have been widely reported (Romero 2002; Touma and Palme 2005). Specifically, northern spotted owl females and males exhibit higher GCs during the nesting and fledging season (females) and breeding season (males) (Wasser and Hunt 2005). Higher fGCMs are also reported in male and female red-tailed parrots during the nesting and breeding season (Popp et al. 2008). Similarly, Landys et al. 2010 found that male and female European nuthatch corticosterone levels trended higher during the breeding season but were not significantly higher than those measured outside the breeding season (Landys et al. 2010). The higher concentrations of GCs/fGCMs around breeding season can be associated with defending a territory or nest, an activity typically taken on by males (Landys et al. 2010; Lèche et al. 2014) Kea, specifically females, are noted to be particularly aggressive during the breeding season (keeper communications). Additionally, it has been suggested that GCs will be highest during energetically taxing times (Romero 2002). The demands of defending a nest box, producing eggs, and caring for fledglings are all energetically taxing activities that could contribute to the elevated fGCMs that coincide with breeding season in kea. Therefore, it was not surprising that the trend toward higher fGCM concentrations in the females during the kea breeding season were noted. However, due to the low number of male samples collected during that season, a complete statistical analysis of males could not be done.

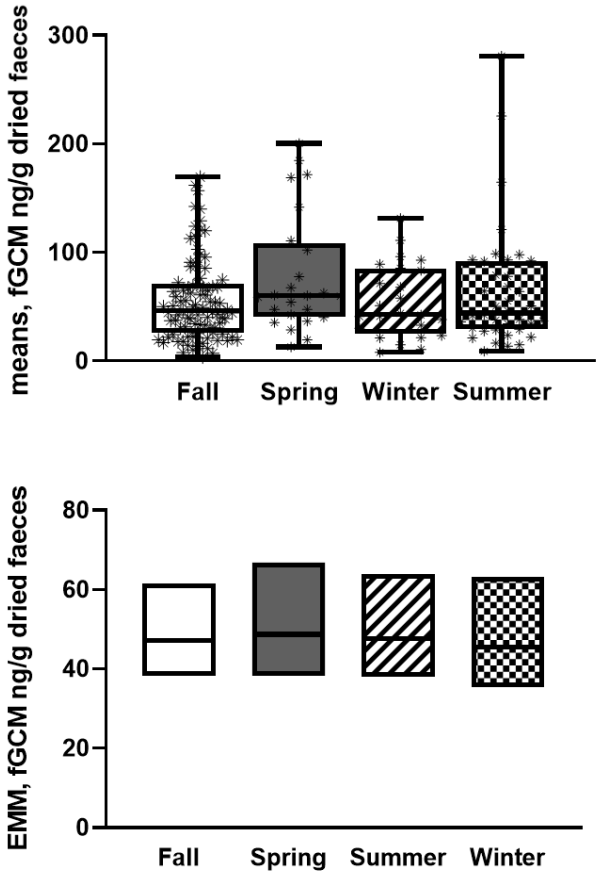

Figure 3. True means, interquartile range, maximum/minimum observations, and data points (upper); and Estimated Marginal Means (EMM) and EMM 95% confidence intervals (lower) for faecal glucocorticoid metabolite concentrations (fGCM) of kea during the different meteorological seasons. Meteorological seasons were defined as: winter: December to February (n=24), spring: March to May (n=24), summer: June to August (n=38), and autumn: September to November (n=92). fGCM concentration only contained samples measured on WELL dates when birds were not experiencing symptoms associated with illness.

While intriguing, this opportunistic finding is preliminary, and a more robust study of both males and females during the two seasons may yield different results.

Cincinnati experiences hot and humid summers; a climate that is markedly different from that of the species' natural history: thus the potential impact of season on fGCM concentrations was analysed. Keepers had reported that kea exhibit physical signs of heat stress: hunched posture, ruffled feathers, and heavy breathing, during the hot, humid conditions of the summer months. However, these obvious physical signs of discomfort did not translate to higher fGCM concentrations in our kea flock. There are mixed reports on corticosterone changing in response to heat in avian species. Heat was associated with elevated fGCM concentrations in African penguins *Spheniscus demersus* (Ozella et al. 2015b). However, no detectable difference was noted in individual rock pigeons *Columbia livia* in thermal stressed states (Angelier et al. 2016). Additionally, GC concentrations were not elevated during the summer months in Sonoran Desert birds, even during record high temperatures (Wingfield et al. 1992). These results are in agreement with what was found in the CZBG kea flock. However, it must be noted that GCs are part of the HPA axis which is only one of many stress pathways. The hypothalamic-pituitary-thyroid (HPT) axis is another route for regulating stress, and it has been suggested that the HPT axis is responsible for regulating thermogenesis in some birds (McNabb 2007). If this holds true for kea, it could explain why a change in fGCM concentrations was not detected in our animals despite reports from the keepers that the kea were displaying behaviours they associated with heat stress. However, even when the common stress hormones associated with the HPA and HPT axes (i.e., corticosterone, prolactin and thyroid hormones (triiodothyronine and thyroxine)) were assessed in rock pigeons, no differences associated with thermal stress were found (Angelier et al. 2016). These conflicting findings indicate more focused research is needed to accurately define biomarkers of heat stress in avian species, and conclusions regarding heat stress should not be made based on corticosterone alone. Because elevated fGCM concentrations where not detected during apparent heat stress in these birds, a physiological link between heat stress and onset of illness could not be identified in these animals.

Contrary to initial suspicions at the start of this study, chronic activation of the HPA axis did not appear to be linked to onset of the illness outbreak in the CZBG kea flock. The fGCM data do not support the hypothesis that either chronic or a recent acute HPA axis activation predisposed the birds to the illness. Instead, fGCMs only increased after individuals started to exhibit symptoms. Although this study does not resolve the cause of the illness in the flock, it does rule out environmental conditions or events that lead to HPA axis activation as primary targets for further investigation. Furthermore, it gives some basic insights into kea fGCM concentrations, while demonstrating the utility of fGCM analysis as a tool for assessing the management of these parrots.

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