



Feather corticosterone in Northern Bald Ibis *Geronticus eremita*: a stable matrix over time able to predict reproductive success

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Abstract

Measuring corticosterone concentrations in feathers (FCC) has been increasingly used as an integrated index of bird's response to stressors, offering valuable insights into subsequent carry-over effects. However, it is still unclear to what extent corticosterone levels deposited in feathers during the moulting period relate to individual physiology in high-energetic demanding situations. In addition, the stability and repeatability of FCC over the same feather generation and across successive generations are still unresolved questions. In this study, we assessed FCC in 24 captive Northern Bald Ibis, *Geronticus eremita*. We sampled body feathers on three occasions during two consecutive years (2015–2016) to explore within-individual stability of FCC in feathers grown during the same generation and test whether FCC was repeatable or varied between two consecutive generations. Then, we explored whether FCC (a) was associated with individual health status, (b) correlated with previous reproductive success and (c) was related to future reproductive success. Results showed high stability of FCC among feathers of the same generation, while FCC differed between successive generations. We also observed that FCC, age and health status were negatively associated with subsequent reproductive success the following season, while no association was found with previous reproductive success. Overall, our study supports the use of feathers as a stable measure of corticosterone over the same feather replacement generation and emphasizes the usefulness of FCC as a biomarker of subsequent reproductive success.

Keywords Birds · Chronic stress · Glucocorticoids · Non-invasive · Biomarker

Zusammenfassung

Feder-Corticosteron beim Waldraup *Geronticus eremita*: Eine zeitlich stabile Matrix ermöglicht Voraussagen über den Reproduktionserfolg

Das Messen der Corticosteronkonzentrationen in Federn (engl. *feather corticosterone concentration*, FCC) ist ein immer stärker genutzter, integrierter Index für die Stressreaktion eines Vogels. Dies ermöglicht wertvolle Einblicke in nachfolgende Carry-Over-Effekte. Jedoch ist immer noch unklar, in welchem Ausmaß der Corticosteronspiegel, der während der Mauserzeit in den Federn erreicht wird, mit der individuellen Physiologie in energetisch höchst anspruchsvollen Situationen zusammenhängt. Weiterhin ist die Stabilität und Messwiederholbarkeit der FCC innerhalb einer und zwischen aufeinanderfolgenden Federgenerationen noch ungeklärt. In dieser Studie haben wir die FCC von 24 in Gefangenschaft gehaltenen Waldraupen *Geronticus eremita* untersucht. Wir sammelten Körperfedern zu drei verschiedenen Zeitpunkten in zwei aufeinanderfolgenden Jahren (2015–2016), um die individuenspezifische FCC-Stabilität in Federn der gleichen Generation zu untersuchen und um zu testen, ob die FCC-Messungen zwischen zwei aufeinanderfolgenden Jahren

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wiederholbar waren oder Unterschiede aufwiesen. Daraufhin untersuchten wir, ob FCC (a) mit dem Gesundheitszustand des Individuums, (b) dem vorherigen und (c) dem zukünftigen Reproduktionserfolg korrelierte. Die Ergebnisse zeigten eine hohe Stabilität der FCC innerhalb der gleichen Federgeneration, während sich die FCC zwischen aufeinanderfolgenden Generationen unterschieden. Wir konnten ebenfalls feststellen, dass FCC, Alter und Gesundheitszustand negativ mit dem nachfolgenden Reproduktionserfolg der nächsten Saison korrelierten, während kein Zusammenhang zum vorherigen Reproduktionserfolg gefunden werden konnte. Zusammenfassend befürwortet unsere Studie die Nutzung von Federn als stabile Messung des Corticosterons innerhalb einer Federgeneration und bekräftigt die Nützlichkeit von FCC als Biomarker für den nachfolgenden Reproduktionserfolg.

Introduction

Under some stress stimulus, corticosterone (CORT; the main glucocorticoid in birds) is released in blood circulation by the hypothalamic–pituitary–adrenal (HPA) axis which enables the animal to direct its normal activity to an “emergency” state (Wingfield et al. 1998) and prioritize its resources towards self-maintenance (Jenni et al. 2000) (the so-called stress response). Despite this adaptive mechanism, the functioning of the HPA axis is altered when the stressor(s) is persistent or highly frequent resulting in elevated CORT levels in an extended period which can have detrimental consequences for the animal’s health, survival and reproduction (Romero et al. 2009; Wingfield and Sapolsky 2003).

Blood has been by far the most preferred matrix to quantify the stress response although it is considered an invasive matrix (Müller et al. 2007). As a consequence, minimally or non-invasive matrices, such as faeces (Möstl et al. 2005) and more recently feathers (Bortolotti et al. 2008; Romero and Fairhurst 2016), have emerged as alternatives. Unlike blood or faeces, feathers represent a unique matrix because they provide an integrative, long-term and retrospective picture of physiology over time. They incorporate CORT which was released during the whole period of feather growth, thus reflecting the HPA axis activity during this limited period of time which can extent from weeks to months (Bortolotti et al. 2009; Jenni-Eiermann et al. 2015; Aharon-Rotman et al. 2015). This matrix is easy to collect and does not require special conditions for transport or storage. Additionally, it has been shown that small daily variations in baseline CORT levels do not affect global feather CORT concentrations (FCC) (Bortolotti et al. 2008; Fairhurst et al. 2013), which is similar to what it occurs in mammalian hair (Tallo-Parra et al. 2016).

As a relatively new technique, feathers still present some methodological issues that need to be addressed for reliable interpretation of data (Romero and Fairhurst 2016). For instance, stability and repeatability of CORT in feathers is of special concern. It is vital that FCC are similar among feathers grown during the same moulting period, which represents the same feather generation, and that this

measure is stable in feathers over time. Researchers are often uncertain of the time gap between feather growth and feather sampling and analysis. Given that physical degradation of feather material occurs over time, this may affect FCC (Romero and Fairhurst 2016). Studying the stability of FCC across the same feather generation is, therefore, crucial for data interpretation. Another unresolved question is whether within-individual differences in FCC persist or vary across two different feather generations responding to internal or environmental conditions. This is a relatively understudied concept with mixed results reported. For example, great FCC variation was observed between successive years in greater snow geese (*Chen caerulescens atlantica*) and in common eiders (*Somateria millissima*) in relation to climate conditions (Legagneux et al. 2013), while high inter-annual repeatability was reported in house sparrows (*Passer domesticus*) highlighting the influence of individual quality in FCC (Aharon-Rotman et al. 2015).

Despite the potential methodological limitations, the use of FCC in literature has grown exponentially since its very beginning (Bortolotti et al. 2008). Several authors have applied this matrix in an ecological context, i.e., relating FCC to reproductive parameters, sexual selection, nutrition (Crossin et al. 2013) as well as studying the effects of stressful or challenging environments such as pollution [i.e., organic pollutants (Monclús et al. 2019)]. More recently, researchers have used FCC as a biomarker of carry-over effects relating FCC to subsequent migratory (Harms et al. 2015), survival (Koren et al. 2012; Monclús et al. 2017) and breeding performances (Harms et al. 2015; Will et al. 2014; Ramos et al. 2018), and as a biomarker of allostatic overload (Johns et al. 2018). However, the reported relationships are complex, and it remains unclear whether FCC can reflect and predict individual physiology in front of high energetically demanding situations such as reproduction (Fairhurst et al. 2017; Harris et al. 2017).

The present study aimed to evaluate the usefulness of FCC as a biomarker of fitness in an endangered bird species, the Northern Bald Ibis *Geronticus eremita* (hereafter called ibis). This long-lived species, classified as Critically Endangered (BirdLife International 2016), has suffered a big decline in numbers over the past decades due to

different anthropogenic disturbances (Boehm and Bowden 2010; Serra et al. 2015; BirdLife International 2016). So far, several captive breeding and reintroduction programs have been established around Europe (e.g., Waldrapp team in Austria, Germany, Italy and *Proyecto Eremita* in Southern Spain). However, high euthanasia rate is described in captive ibis due to chronic ulcerative dermatitis that appear to be related to feather picking and self-mutilation compulsive disorders (Quevedo 2009). Despite its critically conservation status, very few authors have investigated CORT levels in this species (Dorn et al. 2014; Puehringer-Sturmayer et al. 2018).

We divided the present study into two parts. The first part was designed as a methodological approach with two objectives. First, (i) we evaluated within-individual stability of FCC in feathers of the same generation by comparing FCC among feathers sampled on the same day and feathers sampled on different months. If FCC is indeed a robust and reliable measure, we should find equal hormonal levels among the different feather pools collected. Second, (ii) we investigated whether within-individual differences in FCC persist in the subsequent feather generation and thus the potential of this method to be used as an indicator of individual state. The second part of this study addressed two biological questions: (iii) whether FCC related to reproductive success and (iv) whether ibis with chronic diseases presented differences in FCC and reproductive parameters compared to “healthy” birds. Specifically, we evaluated whether FCC: (a) was associated with individual health status, (b) correlated with previous reproductive success, and (c) was related to future reproductive success.

Materials and methods

Animals, housing conditions, diet and management

We used a mixed-sex flock of 24 captive ibis (mean age 18.5 years; range 4–30 years; 14 males, 10 females). The group was held at Zoobotánico Jerez (Cádiz, Spain) and was part of the *Proyecto Eremita* set in Southern Spain. All animals were born in captivity and kept under the same conditions in an outdoor flight aviary (11 m width × 15 m length × 8 m height), with natural vegetation, water ad libitum, subjected to natural photoperiod and temperature, and fed with the same diet (mixture of minced heart of beef, chicken, carrots, pellets for insectivorous birds, multivitamins and carotenes).

Ibis are monomorphic and gregarious with a clear social hierarchy (Cramp 1998). They generally reach sexual maturity around 3 years (Cramp 1998; del Hoyo et al. 1992). Breeding activity starts in March (Sorato and Kotrschal 2006) and eggs are usually laid between March

and May, although some flexibility in the timing has been described due to climate conditions (del Hoyo et al. 1992). Both parents participate in building the nest, incubating the eggs and raising the chicks. Average clutch size is 3 eggs and hatching is asynchronous within intervals from 1 to 3 days (Sorato and Kotrschal 2006). Nestlings are fully fledged after 42–50 days (own observations). Extramarital copulatory behaviour is registered during the breeding season and breeding pairs can change in the following year (Cramp 1998). Ibis used in this study were free to pair and breed. In 2016, as part of the reintroduction programme *Proyecto Eremita*, eggs were removed from their parents, artificially incubated and hand reared by foster parents to increase hatching ratio and chick survival.

Study design

Three samplings were carried out encompassing two consecutive feather generations (Fig. 1). From the first feather generation, we sampled 11 ibis in October 2014 (Sampling A) and 22 in March 2015 (Sampling B). From the second feather generation, we sampled 22 ibis in November 2015 (Sampling C). In this latter case, we simultaneously collected a second pool of feathers from the same anatomical region in 18 individuals. Note that individuals sampled were different for each Sampling case (see Table ESM 1 in Electronic Supplementary Material). Each sampling procedure achieved different objectives. Samplings A and B were used to evaluate within-individual FCC stability between feathers sampled at different times during the same feather generation ($n = 11$). Samplings B and C were used to assess within-individual FCC variation between two consecutive feather generations and to explore the relationship with health and breeding success ($n = 22$). Finally, Sampling C was used to test within-individual FCC stability between pools of feathers sampled simultaneously ($n = 18$).

Feather and data collection

In total, we analysed 75 samples (ESM 1). In all cases, we pulled feathers during routine medical examinations and stored them in paper envelopes at room temperature. We sampled interscapular feathers as their collection was judged to have less impact on flight dynamics. These body feathers are replaced each year during the moulting period, from August to October (Gill 2007; Hardy et al. 2006). Medical data and reproductive success were recorded during all the study. Of the 24 ibis, 2 (9.1%) presented ulcerative chronic dermatitis and 5 (22.7%) presented chronic disabilities such as flight disabilities ($n = 2$), partial beak amputation ($n = 2$) and microphthalmia ($n = 1$).

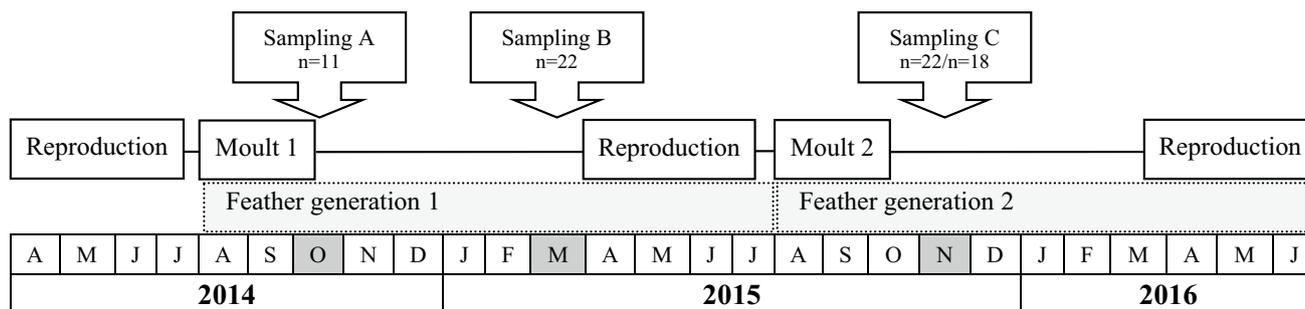


Fig. 1 Time frame of feather sampling and data recording. Northern Bald Ibis were sampled in three occasions: Sampling A (October 2014); Sampling B (March 2015); and Sampling C (November 2015)

encompassing two consecutive feather generations. Reproductive data was recorded for 2014, 2015 and 2016

Feather steroid extraction

In this study, CORT concentrations were normalized per length (mm) and feather mass (mg) was controlled to eliminate any potential confounding effects on FCC as recommended (Lattin et al. 2011; Crossin et al. 2013; Fairhurst et al. 2013; Patterson et al. 2015). Three to five interscapular feathers were analysed per individual to obtain the same longitude range ($21.9 \text{ cm} \pm 1.5 \text{ cm}$) and a minimum mass of 50 mg was required. In all cases, the two measures (pg mm^{-1} and pg mg^{-1}) were highly correlated (Pearson correlation test; $r=0.84$, $P<0.01$).

For FCC extraction, a modified protocol described by Bortolotti et al. (2008) was followed with modifications based on Monclús et al. (2017). Briefly, from each sample, the calamus was removed since it has a different growth rate than the rest of the feather. Then, the selected feathers were minced together with scissors into pieces of $<3 \text{ mm}^2$. Methanol (10 mL; Scharlab, S.L., Sentmenat, Spain) was added and samples were vortexed (Vortex Mixer S0200-230 V-EU; Labnet International Inc, NJ, USA) for 30 min at room temperature before they were shaken (G24 Environmental Incubator Shaker; New Brunswick Scientific Co. Inc., Edison, NJ, USA) for 18 h at $32 \text{ }^\circ\text{C}$ for steroid extraction. The methanol was then separated from the feather material using vacuum filtration. The obtained methanol extract was dried in an oven (Kendro Laboratory Products, Langenselbold, Germany) at $37 \text{ }^\circ\text{C}$. Once the methanol was completely evaporated, extract residues were reconstituted in $250 \text{ }\mu\text{L}$ buffer solution provided by the enzyme immunoassay kit (Corticosterone ELISA kit; Neogen[®] Corporation, Ayr, UK) and shaken for 30 s. Samples were then immediately stored at $-20 \text{ }^\circ\text{C}$ until analyses.

Steroid analysis and validation tests

We determined FCC using competitive CORT enzyme immunoassay kits (Neogen[®] Corporation, Ayr, UK) and

validated the immunoassay following the essential criteria of precision, accuracy, specificity and sensitivity (McCallister and Smith 1996; Sink et al. 2008). Extracts from 20 ibis were pooled and used for validation. A mean (\pm SD) intra-assay coefficient of variation (CV) of $5.74 (\pm 3.78) \%$ and an inter-assay CV of $12.53 (\pm 4.20) \%$ were obtained. All samples were above the detection limit (0.556 pg mm^{-1} feather). Pooled samples showed parallel displacement with the standard curve (standard curve: $y = 0.092x - 0.477$, $R^2 = 99\%$; pool feather samples: $y = 0.060x + 0.305$, $R^2 = 92\%$). The linearity of dilution showed a R^2 of 98% and a mean percentage error of $-11.09 \pm 11.29\%$. The average recovery percentage from spike-and-recovery test was $107.86 \pm 13.77\%$. In accordance to the manufacturers' instructions, the cross reactivity of the kit was as follows: 38% deoxycorticosterone; 19% 6-hydroxycorticosterone; 5.1% progesterone; 2.7% tetrahydrocorticosterone; 1.5% prednisolone; 1.1% cortisol. Steroids with cross reactivity $<0.9\%$ are not presented.

Statistical analysis

All analyses were performed using R version 3.2.2 (R-project, R Development Core Team, University of Auckland, New Zealand). For all models, the standardized residues were assessed to meet normality and homogeneity of variance and, when they were not normally distributed, log10 transformations were applied. The level of significance was set at $\alpha=0.05$ throughout the study.

To test within-individual variations on FCC, we used mixed-effects models. First, we tested whether the two pools of feathers sampled at the same time from the same individual showed similar FCC ($n=18$; Sampling C). Second, we tested whether individual FCC varied among months belonging to the same feather generation ($n=22$; Sampling A and B). In both cases, we conducted FCC (expressed as pg CORT mm^{-1} feather) as the dependent variable and individual identity as random effect to account for repeated samples on the same individuals. The intra-class correlation

coefficient (ICC), expressed as $d^2/(d^2 + \sigma^2)$, was used as a measure of repeatability. It was calculated based on: the variance explained by the model (between-individual variance) (d); and the variance explained by the residual (within-individual variance) (σ) (Nakagawa and Schielzeth 2010). The ICC ranges from 0 to 1 with values close to 1 meaning that most of the variance is explained by between-individual differences.

We then determined whether FCC were consistent between two consecutive feather generations ($n=22$; Samplings B and C). We used mixed-effects model with normal distribution and identity function. FCC was set as the dependent variable and period (Sampling B/Sampling C), sex and age were placed as fixed factors. Individual identity was used as random effect to account for repeated samples. A second mixed-effects model was performed to test for differences in FCC as a function of health and previous reproductive success. Given that the goal was to explore the relationship with physiological parameters, the different periods (Sampling B/Sampling C) as well as individual identity were placed as random effects. Sex and age were placed as fixed factors. For the previous reproductive success, recorded at 2014 and 2015 (Fig. 1), different variables were recorded: (1) breeding success (three levels: successful paired-successful breeding, successful paired-failed breeding, and failed paired-failed breeding); (2) reproductive success (two levels: successful paired-successful breeding, failed paired or failed breeding); (3) fecundity (number of eggs); and (4) fledging success (number of young that successfully fledged). We used different models to test each variable separately. Individuals with flight pathologies ($n=2$) were eliminated as these birds were not able to arrive at the upper nests. Regarding health, we tested separately in two models: (1) general health (two levels: healthy vs. unhealthy with the latter including chronic disabilities and skin disease) and (2) pathological alterations (three levels: healthy birds, birds with chronic disabilities, birds with skin diseases).

A generalised linear mixed model, with binomial distribution and logit link function, was performed to analyse the relation of FCC and subsequent reproductive success, recorded in 2015 and 2016 (Fig. 1). In the case of 2016, eggs were collected and incubated artificially. Therefore, fecundity and fledging success variables were excluded from the analyses and we only distinguished reproductive success (two levels: successfully paired vs. failed paired). Reproductive success (yes/no) was placed as the dependent variable and the explanatory variables included FCC, age, sex and health (different models for general health and for pathological alterations, see above). Individual identity and periods (Sampling B/Sampling C) were placed as random effects.

In all cases, the initial model contained all the main effects and all the possible interactions. The models were evaluated and ranked using the Akaike's Information

Criterion corrected for small sample size (AIC_c) (Burnham and Anderson 2002). The four most supported models and the null model are reported in AIC_c tables (ESM 2, 3 and 4). All models with AIC_c scores within 2 points of the lowest scoring model ($\Delta AIC_c < 2$) were considered within the candidate model selection. We used package 'nlme' and 'lme4' (R Core Team 2015).

Results

Within-individual stability of FCC

The two pools of feathers collected in Sampling C showed similar FCC (Table 1). The ICC of the model was 0.82 (with $d^2 = 2.29$ and $\sigma^2 = 1.08$) showing low within-individual variation. When comparing FCC between Samplings A and B, no statistical difference was found (Table 1) and ICC also indicated low variation (ICC = 0.77 with $d^2 = 2.12$ and $\sigma^2 = 1.16$).

Repeatability of FCC across consecutive feather generations

The best-supported model explaining differences in FCC between feather generations included sex and period (ESM 2). We found that FCC significantly decreased from Sampling B to Sampling C (Table 2, Fig. 2a). In addition, individual FCC greatly varied between the two feather generations (Fig. 2b). Sex did not influence FCC ($P > 0.05$).

Relationship with health status and reproduction

Health status and previous reproductive success

The best-supported model explaining FCC variation only included sex (ESM 3) and this was not significant (all $P > 0.05$). In fact, the null model produced $\Delta AIC_c < 2$ from the best-supported model. Thus, neither health, reproduction nor age were explanatory variables for FCC.

Subsequent reproductive success

The most parsimonious model explaining differences on subsequent reproductive success included health, FCC and age (ESM 4). The amount of CORT accumulated in feathers grown during the moulting period was negatively associated with the following reproductive success (Table 3), with successful birds displaying lower FCC than birds which failed to reproduce (Fig. 3a). Age and health also influenced subsequent reproductive success (Table 3), with unhealthy and older birds being associated with reproductive failure

Table 1 Comparison of corticosterone levels among feathers sampled during the same feather generation in Northern Bald Ibis (*Geronticus eremita*)

	<i>n</i>	pg CORT mm ⁻¹ feather		Statistic output		
		Mean ± SD	Range	Estimate ± SE	<i>t</i> value	<i>P</i> value
Sampling C—Pool 1	18	7.42 ± 2.05	[2.61–10.97]			
Sampling C—Pool 2	18	7.99 ± 2.90	[3.61–12.36]	-0.26 ± 0.36	-0.72	0.48
Sampling A	11	9.76 ± 1.97	[6.81–13.11]			
Sampling B	11	9.01 ± 2.78	[4.75–13.52]	-0.71 ± 0.94	-0.75	0.46

Two pools of samples were collected in Sampling C (pool 1 and pool 2) reflecting feather generation 2. Two pools of samples were collected at different times (Sampling A and B), both reflecting feather generation 1. Mean ± SD, range and statistic output are shown

Table 2 Comparison of corticosterone levels between two feather generations in Northern Bald Ibis (*Geronticus eremita*)

Sex	<i>n</i>	pg CORT mm ⁻¹ feather		Statistic output		
		Mean ± SD	Range	Estimate ± SE	<i>t</i> value	<i>P</i> value
Sampling B						
Males	12	8.90 ± 1.79	[5.63–10.88]			
Females	10	8.06 ± 3.12	[4.75–13.52]			
All	22	8.14 ± 2.23	[4.75–13.52]	-1.49 ± 0.62	-2.44	0.02
Sampling C						
Males	12	7.38 ± 2.18	[2.53–10.62]			
Females	10	6.35 ± 2.91	[2.61–11.08]			
All	22	6.89 ± 2.46	[2.53–11.08]	1.10 ± 0.84	1.32	0.21

Note that Sampling B refers to feathers sampled in March 2015 (Feather generation 1) and sampling C to feathers sampled in November 2015 (Feather generation 2). Mean ± SD, range and statistic output are shown

(Fig. 3b, c). The second-best model with reasonable support ($< 2 \Delta AIC_c$; ESM 4) included health, FCC, age and sex. In this case, the averaged parameter estimates also showed that FCC, age and health were significantly associated with subsequent reproductive success (all $P < 0.001$), while sex was non-significant ($P = 0.33$). When health status was classified in three groups (healthy, chronic disabilities and skin disease), candidate models were similar than healthy–unhealthy classification (ESM 4). In this case, health was non-significant (all $P > 0.05$).

Discussion

Within-individual stability of FCC

Consistent FCC were observed between the two pools of feathers sampled at the same time from the same individuals. Close agreement in FCC was previously reported in different raptor species between two opposite primary flight feathers (P5) (Strong et al. 2015) and between adjacent secondary flight feathers (S1 and S2) (Lattin et al. 2011), although these types of feathers may have an asynchronous moult which can last over 2 years (Rohwer et al. 2009). Instead, body feathers grow during a limited and more synchronized

period of time, after the breeding season and preceding winter stressors and migration (Gill 2007; Hardy et al. 2006); therefore, they appear to provide more time-specific and controlled information on the HPA axis activity (Monclús et al. 2017). The small mass of body feathers especially in small bird species may limit their use, since FCC are significantly overestimated when feather mass is below a critical threshold (Berk et al. 2016; Lattin et al. 2011). To counteract this effect, we pooled 3–5 interscapular feathers to increase sample mass. Our results confirm that pooling body feathers is a consistent method for the assessment of CORT levels. In addition, this method allows standardization of the amount of feather material across samples, which reduces the effects of factors such as asynchrony in moulting (Romero and Fairhurst 2016) and poor feather quality (Aharon-Rotman et al. 2015). Notwithstanding, it is worth mentioning that the use of feather pools may also dilute single feather variations related to stress factors and thus mask potential differences. This should be further studied in future experiments.

Our results also showed stability of FCC between feathers from the same feather generation collected at two different timepoints 6 months apart. These results suggest that CORT levels remain fixed in feathers for 6 months after the end of vascularization, complementing the limited amount of literature available on CORT stability over time (Romero

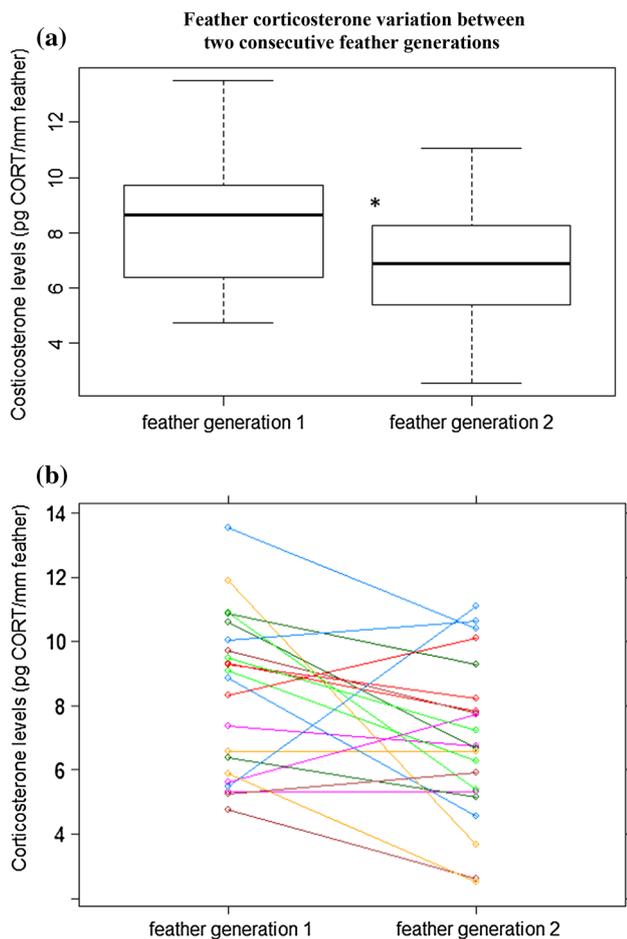


Fig. 2 **a** Global and **b** individual differences of feather corticosterone values (pg CORT/mm feather) between two consecutive feather generations in Northern Bald Ibis ($n=22$). Feather generation 1 refers to corticosterone levels obtained from feathers of Sampling B (reflecting moult 1) and feather generation 2 refers to corticosterone levels obtained from feathers of Sampling C (reflecting moult 2). Asterisk (*) indicates significant differences ($P<0.05$)

and Fairhurst 2016). Previous studies (Bortolotti et al. 2009; Fairhurst et al. 2015) have shown no evidence of CORT degradation among long-time series of feathers collected from

museum specimens. However, the fact that these studies sampled different individuals and used feathers from different years could allow external factors interfere with the results. By exploring CORT levels between feathers grown at the same time but collected at different times, we provide evidence that FCC is a stable metric of long-term CORT levels within individuals over the same feather generation.

Variation of FCC across periods and relationship with health and reproduction

FCC significantly varied between the two consecutive feather generations analysed and showed individual variation, suggesting that birds with high FCC the first year do not necessarily show high levels the following year (Fig. 2b). We could not relate this variation to previous reproductive success nor individual health status. We suggest that FCC could rather be related to external factors, as shown in the study of Legagneux et al. (2013). The ibis in this study were kept under captive conditions with available food resources. Albeit, other external factors could have affected their physiology, for instance cold temperature. This species is very sensitive to weather changes and they can adjust their migration and reproductive behaviour depending on temperatures (del Hoyo et al. 1992), which in turn could affect their stress status and CORT secretion. In addition, pair building at the start of the reproductive season can enhance stress in ibis, and pairs can change year to year (Puehringer-Sturmayer et al. 2018). In this study, we found a relationship between FCC and subsequent reproductive success measured as successful paired—failed paired (see below).

The relationship between increasing FCC and subsequent reproductive failure the following season (Fig. 3a) is consistent with previous studies in which females of house sparrow (Ouyang et al. 2011) and European starlings (*Sturnus vulgaris*) (Cyr and Romero 2007) with low plasma CORT levels showed high subsequent reproductive output. Experimentally, it has been shown that elevated CORT levels suppress reproductive behaviour (reviewed in Wingfield and Sapolsky 2003). However, recent work (Pérez et al. 2016)

Table 3 Model-averaged parameter estimates and associated standard deviation, z - and P value for the variables contained in the best-supported model explaining success of subsequent reproduction in Northern Bald Ibis (*Geronticus eremita*) ($n=22$)

Variable	Estimate \pm SE	z value	P
Intercept	$-44.54 \pm 9.56e-04$	$-434,451$	<0.001
FCC	$8.53 \pm 9.68e-04$	8811	<0.001
Age	$14.48 \pm 1.08e-03$	$13,788$	<0.001
Unhealthy	$24.67 \pm 9.52e-04$	$259,206$	<0.001
Random effects	Variance		SD
Period	6.73		2.59
ID	$1.51e+05$		$3.87e+02$

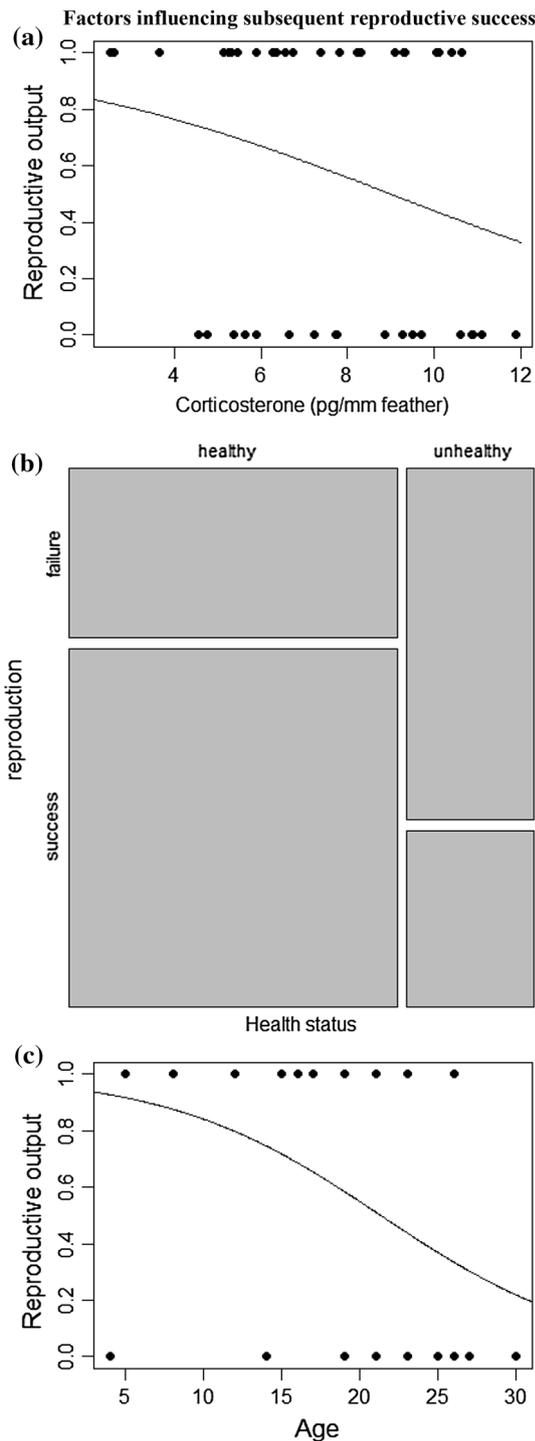


Fig. 3 Relationships between reproductive output in the subsequent breeding season and **a** levels of corticosterone deposited in feathers grown during the previous autumn season ($\mu\text{g CORT mm}^{-1}$ feather); **b** health status; and **c** age, in Northern Bald Ibis ($n=22$). In graphs **a** and **c** lines represent the significant logistic regression model with 1 meaning successful reproduction and 0 meaning failure reproduction

reported an inverse relationship and stated that high CORT levels prepare individuals for reproductive investment. In fact, the direction of the relationship between CORT and successful reproduction may depend on environmental conditions. While under predictable environment, birds with low CORT responses have greater fitness, birds with high CORT levels breed most successfully under unpredictable or changing environment (Cockrem 2013). In this line and given that the studied ibis were kept under stable conditions, we hypothesized a negative relationship between FCC and subsequent reproductive performance. As expected, birds with low FCC succeed in their breeding, while birds with a long-term increase of FCC failed to breed the next reproductive season. However, and in agreement with a previous study (Pérez et al. 2016), we did not find any relationship between FCC and past reproductive performance; birds that fail to breed do not necessarily show an increase of the stress response in the following months.

Additionally, no association was found between FCC and chronic diseases or disabilities. This could possibly be explained by habituation or acclimation of birds to the adverse situation (Bonier et al. 2009). In our study, unhealthy birds presented skin diseases or other type of physical disabilities (see “Feather and data collection” section). It is plausible that under captive conditions and after some time, ibis with physical disabilities were able to adapt to new circumstances without presenting any HPA alteration. Experimentally, this hypothesis was proven in captive great tits (*Parus major*) (Cockrem and Silverin 2002), captive American kestrels (*Falco sparverius*) (Love et al. 2003) and free-living eastern bluebirds (*Sialia sialis*) (Lynn et al. 2010), that under repeated stress stimulus they first showed an increase of CORT levels that progressively decreased over time. On the other hand, we found that unhealthy birds showed worse reproductive success the following breeding season (Fig. 3b). Thus, despite having no effect on the HPA axis activity, the presence of chronic disabilities or diseases appear to affect the reproduction in captive ibis. Given the strong social hierarchy of ibis and that the mating phase is particularly stressful for this species, low-ranked individuals may have more difficulties in pairing and breeding successfully (Sorato and Kotrschal 2006). In this situation, those birds that arrive at the reproductive season chronically stressed (high FCC) or those with chronic physical disabilities may have more difficulties to breed. However, this situation may change between consecutive years, at least for birds with high FCC given that their levels may vary yearly.

Lastly, no FCC differences were found among ages and sex. These findings corroborate previous research by Sorato and Kotrschal (2006) and may be explained by the sexual monomorphic and symmetrical social structure of ibis. Although some previous studies in ibis showed sex-differences (Dorn et al. 2014), they measured CORT metabolites and thus it is not

comparable to our results. In fact, studies performed on feathers of several other bird species also reported no sex-related differences (Carbajal et al. 2014; Koren et al. 2012; Monclús et al. 2017; Strong et al. 2015). Regarding age, similarities in FCC have been described in raptor species between immature, subadult and adult individuals (Monclús et al. 2017; Strong et al. 2015). Conversely, immature ibis were reported to show higher CORT levels compared to adults, probably reflecting a HPA response to the low-ranking position of these individuals in social hierarchy (Sorato and Kotrschal 2006). In the present study, the age of the individuals ranged from 4 to 30 years and therefore, we could not account for differences between immature and adult individuals. However, when studying parameters affecting future reproduction, we observed that the reproductive success declined in older birds (Fig. 3c), representing an important parameter for breeding performance as suggested in the literature (Blas et al. 2009; Sergio et al. 2011; López-Jiménez et al. 2016).

Conclusions

We showed that FCC exhibits high stability across a single feather generation regardless of the feather sampling timepoint, which evidences the usefulness of body feathers as a stable metric. In addition, within-individual variation in CORT levels was observed between two consecutive years. Birds showing high FCC one year do not necessarily show high levels the following year. Although we could not determinate the cause for high FCC, we provided evidence for the utility of FCC as a biomarker of future reproductive success, which is of special interest for the management of this species. Increases in energetic challenges or long-lasting stressful conditions may ultimately have consequences on the following reproductive season, particularly in pairing, and this can be predicted by FCC measured in body feathers. Due to the critically endangered status of ibis and the already great effort performed in developing reintroduction programs, we highly recommend further research focused on this issue and on elucidating the causes of high FCC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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