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Monitoring corticoid metabolites in urine of white-tailed sea eagles: Negative effects of road proximity on breeding pairs

Oliver Krone[a,*], Liam D. Bailey[a], Susanne Jähnig[a], Torsten Lauth[b], Martin Dehnhard[a]

[a] Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany
[b] Töpferstrasse 35, 17406 Morgenitz, Germany

A B S T R A C T

The white-tailed sea eagle (*Haliaeetus albicilla*) is known to be sensitive to disturbance. To better understand potential stressors, we measured corticosterone metabolite levels in *H. albicilla* excreta and recorded the nest success of breeding pairs. We tested the ability of four enzyme immunoassays (EIA) to measure urinary glucocorticoid metabolites (uGM) in the excreta of one adult female eagle subjected to a controlled physiological stress treatment. We identified corticosterone-21-HS to be the most sensitive EIA to changes in uGM concentration. To exclude a sex bias, we confirmed the assay's applicability with samples collected from similar stress treatments in two juvenile males. We used the identified EIA to measure uGM in wild breeding pairs and tested effects of disturbance. Breeding pairs nesting closer to roads and paths had higher uGM concentrations ($p = 0.02$), which is likely an effect of human recreational activity and disturbance. There was no difference in uGM concentrations between failed and successful nests. Our results highlight the potential impact of road and path proximity on white-tailed sea eagles, with potential importance for species management and conservation, particularly with respect to nest protection zone legislation.

1. Introduction

Intensive nature conservation efforts over the past century have saved the white-tailed sea eagle (*Haliaeetus albicilla*) from extinction in Germany. Eagles were legally protected in the 1920s, and the range around the nest was defined as a special protected area in the 1960s. The ban of DDT and other pesticides in the 1970́s enabled the sea eagle population to grow, with an estimated 800 breeding pairs currently active in Germany, and a predicted 1200 pairs expected by the year 2040 (Sulawa et al., 2010). The range of *H. albicilla* has slowly expanded to the north, west and south, with successful recolonization of Denmark in 1996 (Ehmsen et al., 2011) and The Netherlands in 2006 (van Rijn et al., 2010). However, in regions with the highest density of breeding pairs reproductive success has stagnated, a trend that may be caused by intraspecific competition (Hauff, 2009; Heuck et al., 2017).

One potential driver of reproductive success in white-tailed sea eagles may be human disturbance. The white-tailed sea eagle is known to be sensitive to human disturbance (Korsman et al., 2012), with a well-documented case of human disturbance resulting in the reproductive failure of a breeding pair in Berlin (Altenkamp et al. 2007). Breeding white-tailed sea eagles are territorial year-round (Krone et al. 2009, 2013) and start working on the nest as early as two to three month before oviposition, leaving a broad window during which birds may be affected by disturbances. There is a particular need for research to understand the effects of disturbance caused by recreational activities (Bathe, 2007). Tourism numbers have increased in some of the core breeding areas of *H. albicilla* (e.g., Mecklenburg Lake Plateau, Baltic Sea coast and islands; Statistisches Jahrbuch, 2017). These changes may lead to increased disturbance and negative effects on *H. albicilla* breeding pairs.

Birds and other vertebrates respond to unexpected physical or social changes in their environment with a rapid elevation of plasma corticosterone levels. Corticosterone mediates facultative behavioural and physiological responses of the organism to unpredictable events and allows the individual to cope with unexpected change (Wingfield et al., 1998). Within a narrow range, organisms can adjust to predictable environmental stimuli to maintain their homeostasis, but unpredictable or uncontrollable environmental stressors may disturb this status and cause stress (Hofer and East, 2012). Environmental stressors are often divided into natural (e.g. resource availability, predators, diseases) and anthropogenic effects (e.g. habitat change, human structures, recreational activities, etc.). The magnitude of a stress reaction in organisms can vary between (Wingfield et al., 1992), but also within species (e.g. Elliott et al., 2014; Kelm et al., 2016; Peel et al., 2005; Scheun et al., 2018).

Measuring glucocorticoids is a useful tool to evaluate effects of disturbance on wild birds, such as timber harvesting (Wasser et al., 1997), ecotourism (Müllner et al., 2004) or recreational activities like winter sports (Thiel et al., 2008). Glucocorticoid assays have been found to be effective indicators of stress, consistently showing elevation

* Corresponding author.
E-mail address: krone@izw-berlin.de (O. Krone).

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in adrenal activity after times of presumed physiological or psychosocial stress.

Typically, corticosteroids are measured in blood plasma (Müllner et al., 2004, Walker et al., 2006); however, this method requires animals to be caught and handled, followed by invasive blood collection, which can result in increases of glucocorticoid levels. Additionally, individual levels of blood glucocorticoid at a given time point are influenced by circadian variations and pulsative glucocorticoid secretion (Millspaugh and Washburn, 2004; Touma and Palme, 2005; Romero and Reed, 2005).

Non-invasive methods, such as measuring steroid metabolites in excreta (faeces and urine), feathers and hair have been developed for many species (Bortolotti et al., 2009; Sheriff et al. 2011; Will et al. 2014; Blas, 2015; Jenni-Eiermann et al., 2015; Romero and Wingfield, 2016; Monclús et al., 2017). Measuring glucocorticoid metabolites in excreta is particularly well suited for studying birds since they can easily be collected from nesting sites during breeding. Rather than the actual steroid concentration in blood, fecal and urine hormone metabolite analysis reflects the secretion, metabolism and excretion of glucocorticoids from the organism (Blas, 2015). Consequently, depending on the research question, steroid metabolite concentrations measured in feces and urine might represent the hormonal status of an animal more accurately over a longer time frame than a single plasma sample since it will represent an average concentration of glucocorticoids produced, metabolised and excreted during the hours since last defecation (Keay et al., 2006; Romero and Wingfield, 2016). The expected intestinal passage time, is 10–24 h in most mammal species, but might be shorter in birds (Palme et al., 2005).

In contrast to blood, glucocorticoid metabolites in excreta mainly consist of hormone metabolites (Palme, 2005), while cortisol and corticosterone are barely detectable. Steroid metabolism by the liver as well as microbial impact during the intestinal passage, and re-absorption into enterohepatic circulation generates a vast number of steroid metabolites in excreta. As a consequence, a multitude of different metabolites may be excreted in a given species (Möstl et al., 2005), although these metabolites may differ markedly between even closely related species (Heistermann et al., 2006; Palme et al., 1996; Young et al. 2004).

Metabolites in urine and particularly in feces represent metabolic products of the native hormones as a result of a series of chemical changes before their elimination from the body. The nature and extent of these metabolic processes can differ substantially between species, resulting in the presence of a vast number of different metabolites of the same parent hormone. This has been shown in primates (Bahr et al., 2000; Möhle et al. 2002). Based on these species variations, great care must be taken when applying endocrine measurements from excreta to a new species since extrapolation of findings from one species to another can be misleading (Schwarzenberger, 2007). However, it is advisable to perform a species-specific validation for excreted glucocorticoid metabolites before measurements can be utilized (Touma and Palme, 2005; Wasser et al., 2000). The aim of the comparison is to select the one that shows the largest expressed differences between basal and peak concentrations under controlled conditions, and therefore shows the best signal-to-noise ratio. This applies to fecal (Dehnhard et al., 2003; Goymann et al., 2002; P. Legagneux et al., 2011) as well as to urinary glucocorticoid metabolites.

1.1. Project objectives

The aim of the present study was to test the potential effect of anthropogenic structures (roads and forest paths) on stress levels in white-tailed sea eagles, using long-term monitoring in north-eastern Germany. We validated the suitability of different in-house enzyme immunoassays (EIA) that have been designed to measure corticosterone metabolites in the excreta of various species (see Dehnhard et al., 2003). We then applied the most effective EIA to investigate the impact of road proximity and nest success on urinary glucocorticoid metabolite (uGM) concentrations. Due to the documented sensitivity of white-tailed sea eagles, we predicted that breeding pairs with nests closer to roads and paths should show higher levels of uGM.

2. Material and methods

2.1. Study area and sample collection

H. albicilla nests were monitored in sites across Germany from 2003 to 2012. Monitored nests were classified as being at either high or low breeding density based on classification by Herrmann (2018). Nossentiner/Schwinzer Heide (NSH) and nature park island Usedom (IU) were classified as high density areas (> 2 pairs/121 km^2). All nests sampled outside these two areas were at low densities (1 pair/121 km^2). Low density areas were located in the federal states of Brandenburg, Schleswig-Holstein, Thuringia, and Lower Saxony. All these areas are hereafter simply termed 'low density area' (LDA). At 6–8 weeks post hatching, the number of nestlings in each nest was counted and all surviving nestlings were ringed.

At IU, we measured glucocorticoid metabolites concentrations in urine samples of *H. albicilla* collected under nesting trees and the distance of nesting trees to the nearest road/path. In birds, excreta typically consist of a fecal (brown) and a urinary (white) part that can be difficult to separate in some species, in birds of prey the fractions can easily be distinguished. Some researchers prefer to select the fecal part for analysis (Kotrschal et al., 1988; Dehnhard et al., 2003) whereas others take the whole (mixed) sample (Goymann et al., 2002; Albano et al., 2015; Fletcher et al., 2018; Jepsen et al., 2019) expecting that a proper separation is impossible because urine and fecal parts are already mixed inside the cloaca. In the wild, we were not able to detect fresh fecal samples and therefore collected the white (urinary) fraction only. Therefore, we focus specifically on the urinary fraction of excreta and measure urinary glucocorticoid metabolites (uGM). To the best of our knowledge, a study specifically analysing uGM in a bird species has not been conducted so far. Evidence from chicken excreta following a radiometabolism study demonstrated equal proportions of radiolabelled metabolites excreted in urine and feces (Rettenbacher et al., 2004), suggesting that concentrations of glucocorticoid metabolites in urine and feces are likely to be comparable.

Excreta were collected at the same time that nestlings were counted and ringed (i.e. 6 – 8 weeks post hatching). At 6–8 weeks, parents spend limited time sitting at the nest due to chick aggression (Willgohs, 1961). Therefore, we expected excreta collected from the nest to be almost exclusively from nestlings. In cases where we were able to identify the resting tree of the adult birds, supplementary excreta samples were also collected at this site. Where possible, samples of fresh excreta were still collected from nests when nestlings were no longer present.

Pooled samples, consisting of 5–10 single urine samples, were collected within a radius of three meters below each nest site in plastic bags. Sampling from soil was avoided and only dry samples on vegetation (mainly leaves) were used. All samples were temporarily kept at ambient temperature during transport after which they were stored at − 20 °C at the Leibniz Institute for Zoo and Wildlife Research (IZW). All urine samples were stored within 8–10 h of collection. As we could not determine the exact age of urine samples, we limited our collection to dry, but fresh, clear white samples. Liquid urine usually dries within a few hours, leaving it bright white with spots of dry matter. With aging, there is a gradual fading of the colour from bright white, to dull white and then grey-yellowish white. Old urine is usually washed away by periods of rain every one to two weeks; therefore, we assumed all urine samples were less than one to two weeks old. With this method we aimed to minimize the inclusion of older samples that might have been subject to microbial degradation or water loss and thus possible changes in uGM concentration. We also conducted a lab experiment to explicitly test effects of sample age on uGM concentration (see below).

We collected data on the number of nestlings in a total of 214 *H. albicilla* nests between 2003 and 2012 in NSH (42), IU (1 0 3), and LDA (69). In 52 of the nests located on IU we also measured uGM and distance to the nearest road/path to test potential effects of human disturbance. At 12 of the nests from IU, samples were also taken below adult perches to quantify uGM levels of breeding adults, giving a total of 64 samples where both uGM and road/path distance was known.

2.2. Sample processing

From the urinary portions collected in the field 0.05 or 0.1 g (depending on the available amount) were extracted for 30 min with 0.9 ml of 90% methanol. After centrifugation (15 min at 3000 × g) the supernatant was transferred into a new tube and diluted 1:1 with water, and aliquot portions of 20 µl were subjected to the EIAs for uGM in excreta. All hormone measurements were conducted in duplicates. As dry weight usually refers to lyophilized or oven dried excreta (Scheun,et al., 2018) our results are expressed as ng/g urinary wet weight (ww).

2.3. Enzyme immunoassay testing

Before analysing samples from the wild we tested four in-house EIAs (Ludwig et al., 2013) on samples from a five year old (adult) female sea eagle that underwent an adrenocorticotropin hormone (ACTH) challenge test (Touma and Palme, 2005). Using this method we could identify the EIA that was most sensitive to changes in glucocorticoid excretion. A single individual was used as we expected the most sensitive EIA to be applicable for all individuals of the species. The adult female was maintained in a rehabilitation centre, transferred to the IZW the day prior to the ACTH challenge, and returned to the rehabilitation centre the day following the trial. During the challenge, the sea eagle was maintained in a cage with an inner size of 174 × 94 × 70 cm (type 18.107T, manufacturer Schlievet, Holzkirchen, Germany). The interior of the cage was covered by plastic to facilitate the sampling of excreta. The bird was fed 300 g of fish at the beginning of the experiment and 12 h into the trial. Water was available *ad libitum*.

Synthetic ACTH (Synacthen depot, 100 I.E., Sigma-Tau Farmaceutliche Riunite SpA, Pomezia, Italy) was injected into the pectoral muscle of the bird (100 I.E. in 1 ml/bird). Dosage was calculated based on the body weight of the birds following concentrations given by Wilson and Holberton (2001). Urine samples were collected from 18 h before to 27 h after ACTH injection, with the cage checked every 30 min. Samples were stored in plastic bags at −20 °C until further analysis.

The methanol extracted samples from the ACTH challenge in the adult female were analysed with the four EIAs (Table 1). The antibodies of all EIAs were polyclonal and raised in rabbits against the corresponding 21-HS- and 3-CMO-steroids coupled with bovine serum albumin (BSA). The corresponding 21-HS- and 3-CMO-peroxidases were used as label for the EIAs. Microtitre plates were prepared as described earlier (Finkenwirth et al., 2009). Duplicates of 20 µl urine extract or glucocorticoid standard prepared in 40% methanol ranging from 0.2 to 100 pg/20 µl were then simultaneously pipetted into respective wells along with 100 µl of the respective label and 100 µl of the respective antibody and incubated overnight at 4 °C. After washing the microtitre plates 4-times with washing solution, 150 µl of substrate solution was added to each well. Following incubation for 40 min in the dark, the color reaction was stopped by adding 50 µl of 4 N sulfuric acid and then the optical density was determined with a microtitre plate reader (Infinite M200, Tecan) at 450 nm. Hormone concentrations were calculated using Magellan software V 7.0 (Tecan, 2008). Antibody cross-reactivities were calculated with different steroids (Table 1). The corticosterone assay based on the antibody directed against corticosterone-21-HS-BSA was most sensitive to changes in urine glucocorticoid concentrations after the ACTH challenge and was used for all subsequent analyses. Inter-assay and intra-assay coefficients of variation for two biological samples were 19.6% ($n = 18$) and 12.7% ($n = 26$), respectively.

To exclude a sex bias, physiological validations were carried out applying the corticosterone-21-HS-BSA assay to samples collected from ACTH challenges performed in two juvenile males in 2010 and 2012. The two juvenile males suffered from a pinching off syndrome characterised by feather abnormalities which rendered the birds unable to fly (Müller et al., 2007). The ACTH challenge and sample collection was applied in the same way as described above for the adult female. Following the ACTH challenge both young males were euthanized as their release into the wild would have been senseless particularly due to the pinching off syndrome of both birds.

All procedures were performed in accordance with the requirements of the Leibniz Institute for Zoo and Wildlife Research Ethics Committee on Animal Welfare.

The experiment was approved by the federal state of Berlin under LaGeSo G 0315/12.

2.4. Stability of urinary glucocorticoid metabolites at ambient temperature

The concentration of uGM might change over time when samples cannot be kept frozen at −20 °C immediately after defecation. Changes in uGM with sample aging may potentially occur through both bacterial degradation of glucocorticoid metabolites (which may decrease uGM concentration) and water loss (which would increase uGM concentration). To investigate this possibility, 5 fresh, dry urine samples from each of the two ACTH challenges were stored in the lab in open Petri dishes at ~ 20 °C allowing for both water loss and bacterial degradation. Aliquots were removed at 3, 6 and 9 days to simulate sample aging in the field. These samples were stored at −20 °C until assayed.

2.5. Statistical analyses

All statistical analyses of wild birds was conducted in R (v. 3.5.1; R Core Team, 2018), with the package lme4 (Douglas Bates, Martin Maechler, Ben Bolker, Steve Walker (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. doi: https://doi.org//10.18637/jss.v067.i01).

Firstly, we tested whether reproductive success differed between our low and high density areas to reconfirm observations by Hauff (2009) and Heuck et al. (2017) of reduced reproductive success at high densities. We used nest success (whether a nest produced nestlings) as a measure of reproductive success in *H. albicilla* breeding pairs. We followed Steenhof and Newton (2007) who recommended that nests of birds of prey can be considered successful if at least one nestling has reached 80% of the average age at first flight. Mortality after this age until first flight is usually minimal. Nests were classified as either failed (0 nestlings) or successful (≥1 nestling). We then fitted a generalised linear mixed effects model with a binomial distribution (logit link). We included a random intercept term for year to account for stochasticity in nest success between years, which was assumed to follow a Gaussian distribution. We included a categorical fixed effects term for study area to test for differences in nest success between areas. This analysis used all nests where nestling numbers were recorded (n = 214). Statistical significance of study area (alpha = 0.05) was determined using a likelihood ratio test. A post-hoc Tukey's test was used to identify significant differences between individual areas, using the Wald Z-statistic and p-value adjustments with the Holm-Bonferroni method.

We next tested for the effect of human disturbance on the uGM concentration of *H. albicilla* using data collected at IU. We fitted a general linear mixed effects model with a Gaussian error distribution. uGM was transformed using a Box-Cox power transformation ($\lambda = 0.18$) to ensure model residuals followed a Gaussian distribution and demonstrated homogeneity of variance. We included a random intercept term for year to account for stochasticity between years. We

Table 1
. Summary of cross-reactivities (%, previously determined in the lab of the authors) between the four polyclonal antibodies used in the EIAs (i.e. raised against 21-HS- and 3-CMO-corticosterone and cortisol) and other relevant steroids, including native (unconjugated) cortisol and corticosterone.

EIAsAntibody directed against	Cortisol-21-HS[1]	Cortisol-3-CMO[2]	Corticostero-ne-3-CMO[3]	Corticostero-ne-21-HS[4]
4-pregnen-11α,17,21-triol-3,20-dione (cortisol)	100	100	< 0.1	13.4
5α-pregnan-11β,17,21-triol-3,20-dione (5α-dihydrocortisol)		3.2	< 0.1	
5β-pregnan-11β,17,21-triol-3,20-dione (5β-dihydrocortisol)		< 0.1	< 0.1	
5α-pregnan-3α,11β,17,21-tetrol-20-one (5α-tetrahydrocortisol)		< 0.1	< 0.1	
5β-pregnan-3α,11β,17,21-tetrol-20-one (5β-tetrahydrocortisol)		< 0.1	< 0.1	
5α-pregnan-3β,11β,17,21-tetrol-20-one (3β,5α-tetrahydrocortisol)		8.4	< 0.1	
4-pregnen-11β,21-diol-3,20-dione (corticosterone)	13.2	6.3	100	100
4-pregnen-21-ol-3,20-dione (desoxycorticosterone)	< 0.1	< 0.1	9.0	24.4
4-pregnen-3,20-dione (progesterone)		< 0.1	2.0	21.8
5α-pregnane-3,20-dione		< 0.1		
5α-pregnane-3β-ol-20-one		< 0.1	< 0.1	
estradiol		< 0.1	< 0.1	
testosterone		< 0.1	< 0.1	
dihydrotestosterone			< 0.1	

[1] First described in Voigt et al. (2004).
[2] First described in Benhaiem et al. (2012).
[3] First described in Ludwig et al. (2013).
[4] First described in Dehnhard et al. (2003).

included a further random intercept term for territory to account for non-independence between urine samples taken from the same territory. Our model included a continuous measure of distance to the nearest road/path (meters), a categorical term for sample source (i.e. adult perch or nest), and a term for nest success (whether nestlings were present at 6 – 8 weeks). Data from one territory was classified as an outlier in this analysis as it had a substantial impact on model estimates. The inclusion of data from this territory caused a 400-fold change in the coefficient of road distance. This particular territory was considered biologically unusual compared to all other sampled territories because it was in an unusual nest location in a single tree within a cattle fence leaving it highly exposed (visible at a distance of 470 m). Therefore, data from this territory was removed from our analysis. We collected 64 uGM measurements from nests where the distance to the nearest road/path was known (52 from nestlings, 12 from adults). Of these, 3 records were removed due to their unusual nest location (described above) and 3 additional records were excluded in cases where nest success was unknown. Therefore, our final analysis used a sample size of 58 (46 from nestlings, 12 from adults). Statistical significance (alpha = 0.05) of all model terms in the linear mixed effects model was calculated using the Kenward-Roger method for estimation of the degrees of freedom and t-statistic.

3. Results

3.1. Comparison of enzyme immunoassays

The comparison of EIAs revealed substantial quantitative differences between assays. In general, higher quantities were measured with corticosterone than with cortisol EIAs. The lowest quantities of glucocorticoid metabolite concentrations were measured with the cortisol-3-CMO EIA in samples collected during the ACTH challenge in the adult female. Baseline concentrations of the cortisol-3-CMO assay before and after the ACTH challenge were close to the detection limit, which prevent additional dilution steps (Fig. 1). Therefore this assay was not considered for further analyses.

As an alternative to cortisol-3-CMO, an assay based on an antibody against cortisol-21-HS was applied. This assay revealed peak (maximum) levels of 1490 ng/g corresponding to a 5-fold increase after 7.5 h when compared to pretreatment levels (300 ng/g ww, Fig. 1). A more distinct increase was also obtained when applying an assay based on an antibody against corticosterone-3-CMO. Here corticosterone metabolite concentrations increased from pre-treatment levels of 48 ng/g ww to peak levels of 1970 ng/ng 5.5 h following ACTH application making

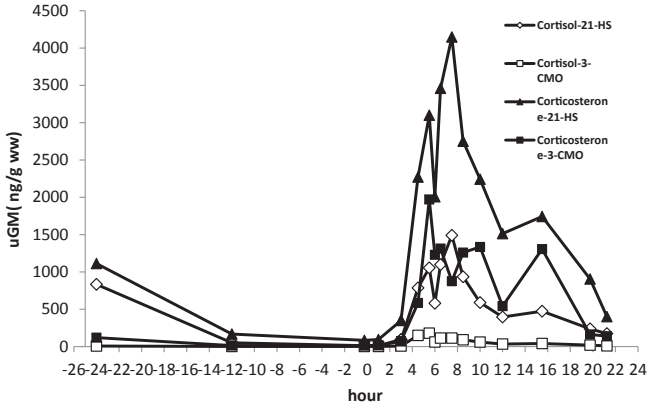

Fig. 1. Changes in urinary glucocorticoid metabolite (uGM) concentrations in wet weight (ww) during an ACTH challenge experiments in one adult female sea eagle as measured with four different antibodies against cortisol and corticosterone. Hour 0 indicates time of application of the adrenocorticotropic hormone (ACTH).

this assay suitable for adrenocortical monitoring in the sea eagle. However, the highest corticosteroid metabolite concentrations were obtained when an assay based on an antibody against corticosterone-21-HS was utilized. Peak levels of 4150 ng/g ww were reached 7.5 h after ACTH injection corresponding to a 9.2-fold increase from pre-treatment baseline (450 ng/g ww). Even if this assay showed a lower ratio of peak vs pre-treatment level compared to the corticosterone-3-CMO assay, we considered the corticosterone-21-HS assay to be more sensitive, which would allow more reliable conclusions in the case of subtle changes in adrenocortical activity. Therefore, corticosterone-21-HS was used for all subsequent analyses.

To exclude a possible sex bias in corticosterone metabolism and to prove the assay's applicability for both sexes two additional ACTH challenges were conducted with two juvenile males and their urine samples were analyzed applying the corticosterone-21-HS-BSA (Fig. 2). In all three individuals of both sexes (1 ad. female, 2 juv. males) we detected an increase in uGM after ACTH administration (i.e. hour 0 in Figs. 1 and 2) with a peak between 6 and 8 h followed by a decline to baseline levels, although there was variability between the individuals in baseline concentrations, peak values, and return to baseline. These results confirmed the reliability of the corticosterone-21-HS-BSA assay as an indicator of adrenocortical activity in both male and female white tailed sea eagles.

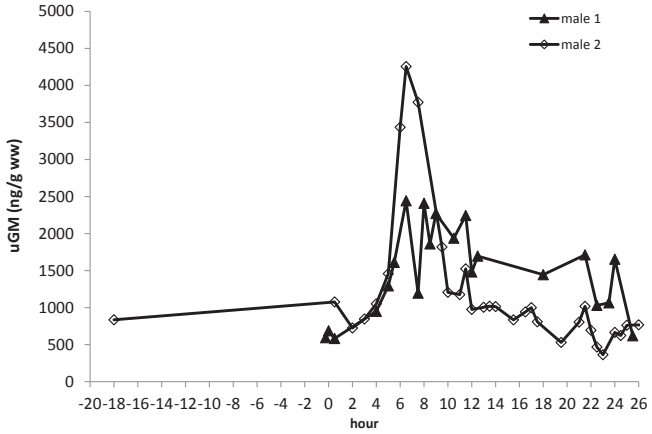

Fig. 2. Changes in uGM concentrations in wet weight (ww) during ACTH challenge experiments in two juvenile males measured with an antibody directed against corticosterone-21-HS. Hour 0 indicates time of application of the adrenocorticotropic hormone (ACTH).

3.2. Stability of glucocorticoid metabolites at ambient temperature

To investigate the stability of the corticosterone metabolites in excreta of white-tailed sea eagles, 10 urine subsamples were exposed to room temperature up to 9 d and subjected to corticosterone measurements. The results revealed no significant increase or decrease of corticosterone metabolites within a 9-d storage period (Kruskal-Wallis test, $n = 90$, $p = 0.328$), indicating that metabolite levels were stable even at an elevated temperature for at least 9 d (Table 2) (tab. 2). Compared with the initial concentrations (immediately frozen; 0 days), the recoveries were 127.9, 117.5, and 115.2% after 3, 6, and 9 days of storage, respectively. Recoveries were calculated in percentage with setting the mean concentration of day 0 as 100%. The overall recovery rate was 120.2%.

3.3. Variation in nest success between study areas

Nest location (NSH, IU or LDA) had a significant effect on nest success ($\chi^2 = 12.15$, df = 2, p = 0.002). A post-hoc Tukey's test showed that nest success of *H. albicilla* was significantly higher in the low density area (LDA) than either of the high density areas (LDA – NSH: Z = −3.33, p = 0.003; LDA - IU: Z = −2.31, p = 0.042; Table XX). Mean nest success was lowest in NSH (Fig. 3; Table 3).

3.4. Effect of road proximity on uGM concentration

Distance of *H. albicilla* nests to a road or path had a significant

Table 2
. Concentration of uGM in 10 samples at room temperature (20 °C) over the course of nine days, measured in three day intervals. Concentrations are given in µg/g ww.

	0 d	3 d	6 d	9 d
# 1	2.33	2.57	2.07	2.24
# 2	2.00	1.84	1.66	1.70
# 3	1.84	2.29	1.84	1.83
# 4	2.44	2.86	3.06	2.45
# 5	1.85	2.15	1.74	1.66
# 6	0.95	1.53	1.44	1.51
# 7	0.97	1.46	1.56	1.47
# 8	1.40	2.00	1.64	1.56
# 9	1.49	2.58	2.68	2.31
# 10	1.20	1.80	1.69	2.28
Mean (SD)	1.65 (0.53)	2.11 (0.47)	1.94 (0.53)	1.90 (0.38)
Recovery (%)	100.0	127.9	117.6	115.2

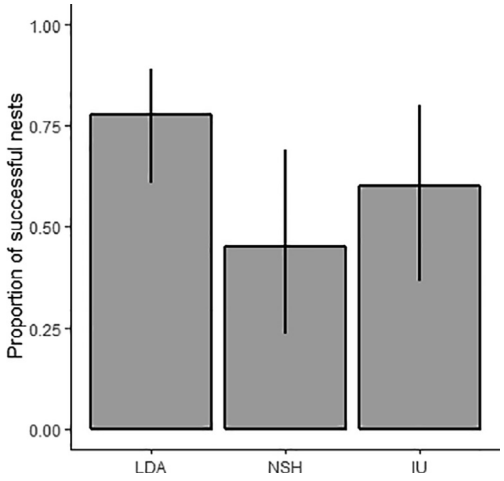

Fig. 3. Variation in *H. albicilla* nest success between three different study areas (High density areas at Island of Usedom (IU) and Nossentiner/Schwinzer Heide (NSH) and low-density areas in Brandenburg, Schleswig-Holstein, Thuringia, and Lower Saxony (LDA). Nest success was higher in the low-density area than either of the high-density areas. Figure shows mean nest success with bars representing 95% confidence intervals calculated using the Wilson method. Letters above bars show results of a post-hoc Tukey's test.

Table 3
. Comparison of white-tailed sea eagle nest success (whether nestlings were present at 6–8 weeks) between different study areas. (High density areas at Island of Usedom (IU) and Nossentiner/Schwinzer Heide (NSH) and low-density areas in Brandenburg, Schleswig-Holstein, Thuringia, and Lower Saxony (LDA)). Table shows coefficient estimates from a generalised linear mixed effects model (binomial with logit link) and 95% confidence intervals calculated using parametric bootstrapping with 1,000 iterations. p-values are the result of a post-hoc Tukey's test with Holm-Bonferonni p-value adjustments.

Areas compared	Estimate	[95% confidence interval]	Z value	*p*
LDA - NSH	−1.44	[0.69/2.08]	−3.33	0.003**
LDA - IU	−0.84	[-2.38/-0.65]	−2.31	0.042*
IU - NSH	−0.60	[0.15/1.66]	1.64	0.102

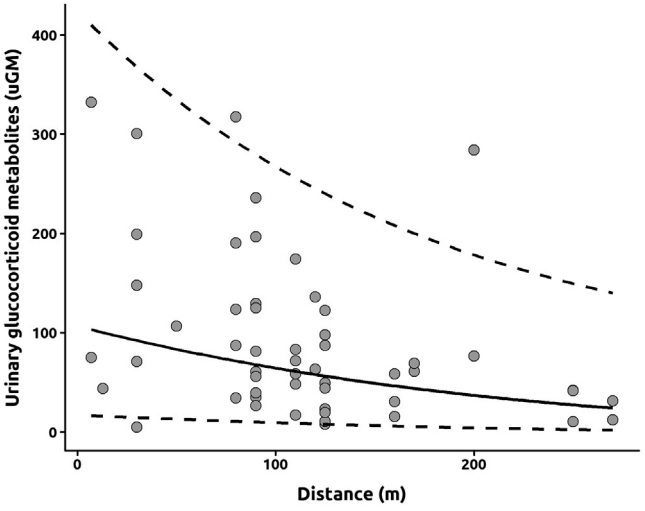

Fig. 4. Effect of road/path distance (m) on *H. albicilla* uGM concentration. Urine samples collected from territories further from roads showed significantly lower concentrations of uGM. Points show measured levels of uGM in samples. The solid line represents predicted uGM concentrations from a general linear mixed effects model with Box-Cox transformation. Dashed lines represent the 95% predication interval.

Table 4
. Effect of road/path distance (m), sample source (adult or nestling), and nest success (whether nestlings were present at 6–8 weeks) on white-tailed sea eagle urinary glucocorticoid metabolites (uGM). Table shows coefficient estimates from a general linear mixed effects model and 95% confidence intervals calculated using parametric bootstrapping with 1000 iterations. The response variable was transformed using a Box-Cox power transformation (lambda = 0.18). Parameter estimates are provided on the transformed scale. Urine samples from nestlings taken from a successful nest (with nestlings present at 6–8 weeks) are usedas the reference category.

Variable	Estimate	[95% confidence interval]	df	t	*p*
Intercept	7.36	[5.78/9.07]	30.88	9.16	< 0.001**
Distance	−0.01	[−0.02/−0.002]	24.89	−2.34	0.03*
Source	0.56	[−0.69/1.72]	45.81	0.90	0.37
Nest success	0.15	[−1.22/1.33]	49.54	0.23	0.82

negative effect on uGM concentration (t = 2.34, df = 24.88, p = 0.03; Fig. 4). uGM concentration was similar between samples taken from the adult perch or the nest (Table S2). There was no difference in uGM concentartion between failed and successful nests (Table 4).

4. Discussion

4.1. ACTH-Challenge and evaluation of enzyme immunoassays

We identified a corticosterone EIA (corticosterone-21-HS) as the most suitable for measuring immunoreactive corticosterone metabolites in urine samples of white-tailed sea eagles, following three ACTH challenges in two male and one female. This EIA has been previously used for sea eagles (Dehnhard et al., 2003). Our EIA is based on a polyclonal antiserum raised in rabbits against corticosterone-21-HS, and has been successfully used to analyze corticosterone metabolites in excreta of domestic chickens, great cormorants (*Phalacrocorax carbo*), and goshawks (*Accipiter gentilis*) (Dehnhard et al., 2003). Two other EIAs with antibodies directed against the 3-CMO conjugate of corticosterone and the 21-HS conjugate of cortisol had lower baseline (pre-ACTH challenge) and lower peak (post-ACTH challenge) concentrations, respectively, but comparable results regarding the time lag to reach peak levels. Thus, both assays previously applied to monitor adrenocortical activity in the cheetah (*Acinonyx jubatus*; Ludwig et al., 2013) and the domestic rabbit (*Oryctolagus cuniculus*; Voigt et al., 2004) may offer additional options to monitor stress in the sea eagle. In contrast, the cortisol-3-CMO assay was unsuitable for the sea eagle. The variation in results between different EIAs demonstrates the importance of evaluating a range of immunoassays for each species under investigation. Similar results were shown by Albano et al. (2015) who tested the suitability of six different antibodies regarding their suitability to monitor adrenocortical activity in gull-billed tern chicks (*Gelochelidon nilotica*). Only a cortisone EIA was useful to reveal the physiological response of chicks after a stressful event while all the others EIAs failed to show any significant difference after the ACTH injection.

The results appear to be remarkable, as it seems that our antibody is applicable for a wider range of bird species, at least for northern goshawks (*Accipiter gentilis*; Dehnhard et al., 2003) and white-tailed sea eagles but likely for a larger range within the family Accipitridae. This contradicts many experiences in mammal species where an assay successfully developed for one species is often inappropriate even for other closely related species. The species-specific suitability of an immunoassay depends on the ability of the antibody, often generated against the native steroid, to detect the relevant metabolites in excreta. Therefore each analytical method must be validated prior to its application to a particular species (Wasser et al., 2000; Young et al., 2004).

Although our corticosterone-21-HS antibody is specifically designed for corticosterone it also showed significant cross-reactivity towards desoxycorticosterone and progesterone (Table 1). This might explain the higher pre-treatment and peak levels following ACTH compared to the more specific corticosterone-3-CMO antibody. However, a radiometabolism study in chickens by Rettenbacher et al. (2004) did not demonstrate any amounts of cortisol and corticosterone. Instead glucocorticoids were metabolised into about 10 unknown mainly more polar compounds. Virtually congruent results were obtained in European stonechats (*Saxicola torquata rubicola*) (Goymann et al., 2002) and the Japanese quail (Hirschenhauser et al., 2012). Thus, the applicability of an assay is primarily dictated by the antibody's cross-reactivity towards commonly unknown metabolites. The polarity seen in the cited studies suggests that conjugated metabolites such as sulfates are part of the metabolite composition. However, an enzymatic hydrolysis did not yield higher amounts of extractable corticosterone metabolites (Rettenbacher et al., 2004). Moreover, Hirschenhauser et al. (2012) showed that in both the chicken and the quail neither corticosterone nor cortisol could be detected in urinary or fecal parts of the excreta. Thus we assume that corticosterone was not detected in *H. albicilla* urine using the corticosterone-21-HS assay.

Using the corticosterone-21-HS antibody, we demonstrated a considerable difference between the two male and one female sea eagle regarding baseline and peak levels following ACTH administration. This is not unusual as many authors who have performed ACTH stimulations in a larger number of animals described significant variations between individuals (Touma and Palme, 2005; Hadinger et al., 2015). However, our individuals did not deviate in the time before peak levels were reached. Testing the biological validity in both sexes might be important as results from mice revealed significant differences between the sexes (Touma et al., 2003). Sex differences were also observed in tigers, where mean levels of fecal glucocorticoid metabolites were significantly higher in females than in males (Narayan et al., 2013), and also in birds such as the black grouse (Baltic et al., 2005), Japanese quail (Hirschenhauser et al., 2012), European stonechats (Goymann et al., 2002), and the domestic chicken (Rettenbacher et al., 2004).

Non-invasive stress assessment techniques, including glucocorticoid analyses, have great potential for providing important information regarding the adrenocortical activity of free-ranging wildlife species. However, accurate stress assessment requires samples that contain biologically representative amounts of glucocorticoid metabolites. Non-fresh samples may not accurately reflect glucocorticoid concentrations due to bacterial metabolism of glucocorticoids or water loss. To simulate the effects of elevated environmental temperature conditions and delayed collection, samples were exposed to room temperature over a period of nine days. This treatment did not affect the accuracy of corticosterone metabolite measurements in sea eagle excreta. Corticosterone metabolite measures did not differ significantly from pre-treatment baseline levels during 9 days storage at room temperature, suggesting that elevated temperatures and the storage have little impact on uGM stability in our samples. The most likely explanation is that the samples were already concentrated (dry) and no relevant water loss occurred during the lab trial. This might also explain the lack of bacterial degradation. This is in contrast to other studies which detected both increases (African buffalo: Ganswindt et al., 2012; sheep: Lexen et al., 2008) and decreases of glucocorticoid metabolite levels in feces stored at ambient temperature. However, other ambient factors, especially prolonged rainfall, may also impact uGM measurements (Washburn and Millspaugh, 2002).

4.2. Stress factors affecting uGM concentrations

Non-invasive monitoring of stress hormones is of growing interest as a useful tool to assess disturbances that activate the hypothalamic-pituitaryadrenal (HPA) axis stress response. Blood as a matrix for stress levels provides only a short-term picture and may be complicated by increases in glucocorticoid levels induced by restraining and sampling

the animal. Measuring fGM/uGM concentration has an advantage over blood sampling as it does not require the animal to be disturbed. Glucocorticoid metabolites concentrations in feces can be closely related to blood glucocorticoid values (Sheriff et al., 2010) and are frequently used in wildlife (Dickens and Romero, 2013; Dantzer et al., 2014; Elliott et al., 2014; Heidinger et al., 2008; Kelm et al., 2016; Schmidt et al., 2012). Additionally, assessing glucocorticoid metabolite levels from excreta provides information over longer time periods than blood samples (Sheriff et al., 2011). The measurement of glucocorticoid metabolites in excreta has become a valuable tool in conservation biology and ecology to study stress load (Gladbach et al., 2011). Mammals and birds largely differ in their digestion and excretion physiology. While excreta from the former is largely digestive, that from the latter has a significant urinary fraction (Shoemaker, 1972). In the carnivorous birds of prey, which have a very effective digestive and urinary system, the excreta mainly consists of a white urinary part with a very small digestive part. Therefore, one could expect that the signal should be clearer and more homogeneous in birds than in mammals.

We showed that white-tailed sea eagles in low density areas had higher nest success than eagles in high density areas, which may be an example of density dependence in *H. albicilla* reproduction. Evidence for density dependence in bird populations has been shown for a wide range of species (Newton, 1998). For example, golden eagles (*Aquila chrysaetos*) showed reduced breeding effort at higher population densities (Fasce et al., 2011), while an analysis of a 25-year data set of a bearded vulture (*Gypaetus barbatus*) population revealed negative density-dependent effects on productivity in Spain (Carrete et al., 2006). For the congeneric bald eagle (*H. leucocephalus*) population density affected reproduction in Saskcatchewan, Canada (Mougeot et al., 2013) and British Columbia; Canada (Elliott et al., 2011). Density dependent effects on reproduction will mean that population growth of *H. albicilla* is expected to slow as populations near carrying capacity, a prediction which was also proposed by Krüger et al. (2010) for breeding birds in the German state of Schleswig-Holstein. Increasing frequency of territorial intrusion and territorial fighting are potentially the first signs of density-dependent effects (Krüger et al., 2010).

Several previous studies have documented negative impacts of anthropogenic disturbance on stress levels in wild birds, such as land use and habitat alteration (Wasser et al., 1997), capture and translocation (Dickens et al., 2009), unregulated wildlife tourism (Ellenberg et al., 2007), recreational winter activities (Thiel et al., 2008) and traffic intensity (Strasser and Heath, 2013). In our study we demonstrated that the distance of nests to a road or path had a significant effect on uGM levels in *H. albicilla* on Usedom. Tourists on Usedom mainly concentrate at the beach of the Baltic Sea, but during adverse weather conditions, recreational activities such as walking, hiking, and biking increases. With more than 1 million guests per year Usedom is a tourist stronghold in Germany (Reiseverkehrsstatistik, 2017). Roads, trails and paths along dikes and in the forest constitute the infrastructure for tourists to explore the area and are used for recreational activities. The use of these trails may act as a source of anthropogenic disturbance, with breeding birds becoming more stressed when tourists come close to the nest. This seems to be supported by the negative relationship we observed between uGM and road distance. Repeated disturbances might cause adult birds to flush from the nest, abandon the nest, reduce feeding and guarding of the chicks, and may even lead to the death of offspring (Altenkamp et al., 2007). Forest paths on Usedom have been constructed as a 300 × 300 m grid in many parts of the forest. This may limit the opportunities for breeding birds to build nests in undisturbed locations.

Along with the total protection of the species in the 1920 s and the ban of DDT and PCBs, the implementation of nest protection zones in legislation has helped to facilitate the recovery of the white-tailed sea eagle in Germany. Nest protection zones are legislated in two German states (Mecklenburg-Western Pomerania and Brandenburg), where the highest density and total number of white-tailed sea eagle breeding pairs is found. The Nature protection law of the federal state Mecklenburg-Western Pomerania defines protection zone I as a radius of 100 m around the nest, in which it is prohibited to cut tillering or to change the character of the zone. In both zone II, within a radius of 100 to 300 m from the nest, and zone I there is a ban on all agricultural, forestry and fishery activity, while hunting is prohibited from March 1st to August 31st (NatSchAG M-V, 2010). The Nature protection law of the federal state of Brandenburg differs slightly in the regulations regarding zone II, where it is illegal to build hunting constructions. In addition, in zone II it is generally not permitted to hunt and to perform machine based agricultural and forestry from February 1st to August 31st (BbgNatSchAG, 2016). Defining nest protection zones is an excellent example of using legislation to protect endangered species, and we would recommend that similar legislation should be considered as a tool for bird conservation. However, while nest protection legislation provides protection from large scale disturbance activity (e.g. forestry, hunting), our results suggest it may not be sufficient in areas with intense human recreational activity. We would recommend that nest protection legislation should be extended to recreational activity to exclude the use of forest trails/paths within a radius of at least 100 m around eagle's nests. If possible off-trail activities in breeding areas of eagles should be prohibited. Preserving undisturbed forests for breeding eagles will become more important with increasing recreational activities in the future.

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Appendix A. Supplementary data

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References

Albano, N., Santiago-Quesada, F., Masero, J.A., Sánchez-Guzmán, J.M., Möstl, E., 2015. Immunoreactive cortisone in droppings reflect stress levels, diet and growth rate of gull-billed tern chicks. Gen. Comp. Endocrinol. 213, 74–80.

Altenkamp, R., Stoewe, D., Krone, O., 2007. Verlauf und Scheitern einer Brut des Seeadlers (*Haliaeetus albicilla*) in Berlin und Konsequenzen für den Schutz der Brutplätze. Berlin. Ornithol. Ber. 17, 31–41.

Bahr, N.I., Palme, R., Möhle, U., Hodges, J.K., Heistermann, M., 2000. Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. Gen. Comp. Endocrinol. 117, 427–438.

Bathe, G., 2007. Political and social drivers for access to the countryside: the need for research on birds and recreational disturbance. Ibis 149, 3–8.

Baltic, M., Jenni-Eiermann, S., Arlettaz, R., Palme, R., 2005. A noninvasive technique to evaluate human-generated stress in the black grouse. Ann. N.Y. Acad. Sci. 1046, 81–95.

BbgNatSchAG [Brandenburgisches Naturschutzausführungsgesetz], 2016. Brandenburgisches Ausführungsgesetz zum Bundesnaturschutzgesetz. https://bravors.brandenburg.de/gesetze/bbgnatschag_2016#19 (accessed 18 July 2018).

Benhaiem, S., Dehnhard, M., Bonanni, R., Goymann, W., Eulenberger, K., East, M.L., 2012. Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in spotted hyenas (*Crocuta crocuta*). Gen. Comp. Endocrinol. 178, 265–271.

Blas, J., 2015. Stress in Birds. In: Scanes, C.G. (Ed.), Sturkiés Avian Physioloy, sixth ed. Elsevier, London, pp. 769–810.

Bortolotti, G.R., Marchant, T., Blas, J., Cabezas, S., 2009. Tracking stress: localisation, deposition and stability of corticosterone in feathers. J. Exp. Biol. 212 (10), 1477–1482.

Carrete, M., Donazar, J.A., Margalida, A., 2006. Density-dependent productivity depression in Pyrenean bearded vultures: implications for conservation. Ecol. Appl. 16,

1674–1682.
Dantzer, B., Fletcher, Q.E., Boonstra, R., Sheriff, M.J., 2014. Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species? Conserv. Physiol. 2 (1). https://doi.org/10.1093/conphys/cou023.
Dehnhard, M., Schreer, A., Krone, O., Jewgenow, K., Krause, M., Grossmann, R., 2003. Measurement of plasma corticosterone and fecal glucocorticoid metabolites in the Chicken (*Gallus domesticus*), the Great Cormorant (*Phalacrocorax carbo*), and the Goshawk (*Accipiter gentilis*). Gen. Comp. Endocrinol. 131, 345–352.
Dickens, M.J., Romero, M., 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. Gen Comp Endocrinol. 191, 177–189. https://doi.org/10.1016/j.ygcen.2013.06.014.
Dickens, M.J., Delehanty, D.J., Romero, L.M., 2009. Stress and translocation: alterations in the stress physiology of translocated birds. Proc. R. Soc. B. 276, 2051–2056.
Ehmsen, E., Pedersen, L., Meltofte, H., Clausen, T., Nyegaard, T., 2011. The occurrence and reestablishment of White-tailed Eagle and Golden Eagle as breeding birds in Denmark. Dansk Orn. Foren. Tidsskr. 105, 139–150.
Ellenberg, U., Setiawan, A.N., Cree, A., Houston, D.M., Seddon, P.J., 2007. Elevated hormonal stress response and reduced reproductive output in yellow-eyed penguins exposed to unregulated tourism. Gen. Comp. Endocrinol. 152, 54–63.
Elliott, K.H., Elliott, J.E., Wilson, L.K., Jones, I., Stenerson, K., 2011. J. Wildl. Man. 75, 1688–1699.
Elliott, K.H., O'Reilly, K.M., Hatch, S.A., Gaston, A.J., Hare, J.F., Anderson, W.G., 2014. The prudent parent meets old age: a high stress response in very old seabirds supports the terminal restraint hypothesis. Horm. Behav. 66 (5), 828–837.
Fasce, P., Fasce, L., Villers, A., Bergese, F., Bretagnolle, V., 2011. Long-term breeding demography and density dependence in an increasing population of Golden Eagles *Aquila chrysaetos*. IBIS 153, 581–591. https://doi.org/10.1111/j.1474-919X.2011.01125.x.
Finkenwirth, C., Jewgenow, K., Meyer, H.H.D., Vargas, A., Dehnhard, M., 2009. PGFM (13,14-dihydro-15-keto-PGF2a) in pregnant and pseudo-pregnant Iberian lynx: a new noninvasive pregnancy marker for felid species. Theriogenology 73, 530–540.
Fletcher, K., Xiong, Y., Fletcher, E., Gustafsson, L., 2018. Glucocorticoid response to both predictable and unpredictable challenges detected as corticosterone metabolites in collared flycatcher droppings. PLOS ONE. https://doi.org/10.1371/journal.pone.0209289.
Ganswindt, A., Tordiffe, A., Stam, E., Howitt, M., Jori, F., 2012. Determining adrenocortical endocrine activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis. Afr. Zool. 47, 261–269.
Gladbach, A., Gladbach, D.J., Koch, M., Kuchar, A., Möstl, E., Quillfeldt, P., 2011. Can faecal glucocorticoid metabolites be used to monitor body condition in wild Upland geese *Chloephaga picta leucoptera*? Behav. Ecol. Sociobiol. 65, 1491–1498.
Goymann, W., Möstl, E., Gwinner, E., 2002. Corticosterone Metabolites can be Measured Noninvasively in Excreta of European Stonechats (*Saxicola torquata rubicola*). Auk 119, 1167–1173.
Hadinger, U., Haymerle, A., Knauer, F., Schwarzenberger, F., Walzer, C., 2015. Faecal cortisol metabolites to assess stress in wildlife: evaluation of a field method in free-ranging chamois. Methods Ecol. Evo. 6, 1349–1357.
Hauff, P., 2009. Current population status of the white-tailed eagle (Haliaeetus albicilla) in mid-central Europe, in: Stubbe, M., Mammen, U. (Eds.), Populationsökologie von Greifvögel- und Eulenarten, Vol. 6, 27–46.
Heidinger, B.J., Nisbet, I.C., Ketterson, E.D., 2008. Changes in adrenal capacity contribute to a decline in the stress response with age in a long-lived seabird. Gen. Comp. Endocrinol. 156, 564–568.
Heistermann, M., Palme, R., Ganswindt, A., 2006. Comparison of different enzyme immunoassays for assessment of adrenocortical activity in primates based on fecal analysis. Am. J. Primatol. 68, 257–273.
Herrmann, C., 2018. Adlerland Mecklenburg-Vorpommern: See-, Fisch- und Schreiadler im Nordosten Deutschlands. Nationalpark-Jahrbuch Unteres Odertal 2017, 108–125.
Heuck, C., Herrmann, C., Schabo, D.G., Brandl, R., Albrecht, J., 2017. Density-dependent effects on reproductive performance in a recovering population of White-tailed Eagles *Haliaeetus albicilla*. Ibis 159, 297–310.
Hirschenhauser, K., Spreitzer, K., Lepschy, M., Kotrschal, K., Möstl, E., 2012. Excreted corticosterone metabolites differ between two galliform species, Japanese Quail and Chicken, between sexes and between urine and faecal parts of droppings. J. Ornithol. 153, 1179–1188.
Hofer, H., East, M.L., 2012. Stress and immunosuppression as factors in the decline and extinction of wildlife populations: concepts, evidence and challenges. In: Aguirre, A.A., Daszak, P., Ostfeld, R.S. (Eds.), Conservation medicine: applied cases of ecological health. Oxford University Press, New York, pp. 82–107.
Jenni-Eiermann, S., Helfenstein, F., Vallat, A., Glauser, G., Jenni, L., 2015. Corticosterone: effects on feather quality and deposition into feathers. Methods Ecol. Evol. 6, 237–246. https://doi.org/10.1111/2041-210X.12314.
Jepsen, E.M., Ganswindt, A., Ngcamphalala, C.A., Bourne, A.R., Ridley, A.R., McKechnie, A.E., 2019. Non-invasive monitoring of physiological stress in an afrotropical arid-zone passerine bird, the southern pied babbler. Gen Comp Endocrinol. 276, 60–68. https://doi.org/10.1016/j.ygcen.2019.03.002.
Keay, J.M., Singh, J., Gaunt, M.C., Kaur, T., 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. J. Zoo. Wildl. Med. 37, 234–244.
Kelm, D.H., Popa-Lisseanu, A.G., Dehnhard, M., Ibáñez, C., 2016. Non-invasive monitoring of stress hormones in the bat *Eptesicus isabellinus* - Do fecal glucocorticoid metabolite concentrations correlate with survival? Gen Comp Endocrinol. 226, 27–35. https://doi.org/10.1016/j.ygcen.2015.12.003.
Korsman, J.C., Schipper, A.M., Lenders, H.J.R., Foppen, R.P.B., Hendriks, A.J., 2012. Modelling the impact of toxic and disturbance stress on white-tailed eagle (*Haliaeetus albicilla*) populations. Ecotoxicology 21, 27–36.
Kotrschal, K., Hirschenhauser, K., Möstl, E., 1988. The relationship between social stress and dominance is seasonal in Graylag geese. Anim. Behav. 55, 171–176.
Krone, O., Berger, A., Schulte, R., 2009. Recording movement and activity pattern of a White-tailed Sea Eagles (*Haliaeetus albicilla*) by a GPS datalogger. J Ornithol. 150, 273–280.
Krone, O., Nadjafzadeh, M., Berger, A., 2013. White-tailed Sea Eagles (*Haliaeetus albicilla*) defend small home ranges in north-east Germany throughout the year. J Ornithol. 154, 827–835.
Krüger, O., Grünkorn, T., Struwe-Juhl, B., 2010. The return of the White-tailed Eagle (*Haliaeetus albicilla*) to northern Germany: modelling the past to predict the future. Biol Conserv. 143, 710–721.
NatSchAG M-V [Naturschutzausführungsgesetz], 2010. Gesetz des Landes Mecklenburg-Vorpommern zur Ausführung des Bundesnaturschutzgesetzes.
Legagneux, P., Gauthier, G., Chastel, O., Picard, G., Bêty, J., 2011. Do glucocorticoids in droppings reflect baseline level in birds captured in the wild? A case study in snow geese. Gen. Comp. Endocrinol. 172, 440–445.
Lexen, E., El-Bahr, S., Sommerferfeld-Stur, I., Palme, R., Möstl, E., 2008. Monitoring the adrenocortical response to disturbances in sheep by measuring glucocorticoid metabolites in the faeces. Wien Tierarztl Monatsschr. 95, 64–71.
Ludwig, C., Wachter, B., Silinski-Mehr, S., Ganswindt, A., Bertschinger, H., Hofer, H., Dehnhard, M., 2013. Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*). Gen. Comp. Endocrinol. 180, 15–23.
Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen. Comp. Endocrinol. 138, 189–199.
Möhle, U., Heistermann, M., Palme, R., Hodges, J.K., 2002. Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates. Gen. Comp. Endocrinol. 129, 135–145.
Monclús, L., Carbajal, A., Tallo-Parra, O., Sabés-Alsina, M., Darwich, L., Molina-López, R.A., Lopez-Bejar, M., 2017. Relationship between feather corticosterone and subsequent health status and survival in wild Eurasian Sparrowhawk. J. Ornithol. 158, 773–783. https://doi.org/10.1007/s10336-016-1424-5.
Möstl, E., Rettenbacher, S., Palme, R., 2005. Measurement of corticosterone metabolites in birds' droppings: an analytical approach. Ann. N.Y. Acad. Sci. 1046, 17–34. https://doi.org/10.1196/annals.1343.004.
Mougeot, F., Gerrard, J., Dzus, E., Arroyo, B., Gerrard, P.N., Dzus, C., Bortolotti, G., 2013. Population Trends and Reproduction of Bald Eagles at Besnard Lake, Saskatchewan, Canada 1968–2012. J. Raptor Res. 47, 96–107.
Müller, K., Schettler, E., Gerlach, H., Brunnberg, L., Hafez, M., Hattermann, K., Johne, R., Kollmann, R., Krone, O., Lierz, M., Linke, S., Lueschow, D., Mankertz, A., Müller, H., Prusas, C., Raue, R., Soike, D., Speck, S., Wolf, P., Frölich, K., 2007. Investigations on the aetiology of pinching off syndrome in four white-tailed sea eagles (*Haliaeetus albicilla*) from Germany. Avian Pathol. 36, 235–243.
Müllner, A., Linsenmair, K.E., Wikelski, M., 2004. Exposure to ecotourism reduces survival and affects stress response in hoatzin chicks (*Opisthocomus hoazin*). Biol. Conserv. 118, 549–558.
Narayan, E.J., Parnell, T., Clark, G., Martin-Vegue, P., Mucci, A., Hero, J.M., 2013. Faecal cortisol metabolites in Bengal (*Panthera tigris tigris*) and Sumatran tigers (*Panthera tigris sumatrae*). Gen. Comp. Endocrinol. 194, 318–325. https://doi.org/10.1016/j.ygcen.2013.10.002.
Newton, I., 1998. Population limitation in Birds. Academic Press, London.
Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused C-14-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 43, 43–63.
Palme, R., 2005. Measuring fecal steroids: guidelines for practical application. Ann. N.Y. Acad. Sci. 1046, 75–80.
Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Möstl, E., 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion and non-invasive measurement in fecal samples. Ann. N.Y. Acad. Sci. 1040, 162–171. https://doi.org/10.1196/annals.1327.021.
Peel, A.J., Vogelnest, L., Finnigan, M., Grossfeldt, L., O'Brien, J.K., 2005. Non-invasive fecal hormone analysis and behavioral observations for monitoring stress responses in captive western lowland gorillas (Gorilla gorilla gorilla). Zoo Biol. 24, 431–444.
Core Team, R., 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria URL: https://www.r-project.org/.
Reiseverkehrsstatistik, 2017. https://www.kaiserbaeder-auf-usedom.de/wp-content/uploads/2018/06/2018_06_04_KB_Reiseverkehrsstatistik2017.pdf (accessed 2112.18.).
Rettenbacher, S., Möstl, E., Hackl, R., Ghareeb, K., Palme, R., 2004. Measurement of corticosterone metabolites in chicken droppings. Br. Poult Sci. 45, 704–711.
Romero, L.M., Reed, J.M., 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comp. Biochem. Physiol. A Mol. Integr. Physiol. 140, 73–79.
Romero, L.M., Wingfield, J.C., 2016. Tempest, poxes, predators and people. Stress in wild animals and how they cope. Oxford University Press, Oxford.
Scheun, J., Greeff, D., Ganswindt, A., 2018. Non-invasive monitoring of glucocorticoid metabolite concentrations in urine and faeces of the Sungazer *(Smaug giganteus*). Peer J. https://doi.org/10.7717/peerj.6132.
Schmidt, K.L., Furlonger, A.A., Lapierre, J.M., MacDougall-Shackleton, E.A., MacDougall-Shackleton, S.A., 2012. Regulation of the HPA axis is related to song complexity and measures of phenotypic quality in song sparrows. Horm. Behav. 61, 652–659.
Schwarzenberger, F., 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. Int Zoo Ybk 41, 52–74.
Sheriff, M.J., Krebs, C.J., Boonstra, R., 2010. Assessing stress in animal populations: do

fecal and plasma glucocorticoids tell the same story? Gen. Comp. Endocrinol. 166, 614–619. https://doi.org/10.1016/j.ygcen.2009.12.017.
Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–887. https://doi.org/10.1007/s00442-011-1943-y.
Shoemaker, V.H., 1972. Osmogerulation and excretion in birds. In: In: Farner, D.S., King, J.R. (Eds.), Avian Biology Vol. 2. Academic Press, London, pp. 527–574.
Statistisches Jahrbuch, 2017. Kapitel 24: Gastgewerbe & Tourismus. Mecklenburg Vorpommern, Landesamt für innere Verwaltung, Statistisches Amt pp. 555–570.
Steenhof, K., Newton, I., 2007. Assessing nesting success and productivity. In: Bird, D.M., Bildstein, K.L. (Eds.), Raptor research and management techniques. Hancock House Publisher, Surrey, Canada, pp. 181–192.
Strasser, E.H., Heath, J.A., 2013. Reproductive failure of a human-tolerant species, the American kestrel, is associated with stress and human disturbance. J. Appl. Ecol. 50, 912–919.
Sulawa, J., Robert, A., Köppen, U., Hauff, P., Krone, O., 2010. Recovery dynamics and viability of the white-tailed eagle (*Haliaeetus albicilla)* in Germany. Biodivers Conserv. 19, 97–112.
Thiel, D., Jenni-Eiermann, S., Braunisch, V., Palme, R., Jenni, L., 2008. Ski tourism affects habitat use and evokes a physiological stress response in capercaillie *Tetrao urogallus*: a new methodological approach. J. Appl. Ecol. 45, 845–853.
Touma, C., Sachser, N., Möstl, E., Palme, R., 2003. Effects of sex and time of day on metabolism and excretion corticosterone in urine and feces of mice. Gen. Comp. Endocrinol. 130, 267–278.
Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann. N.Y. Acad. Sci. 1046, 54–74.
van Rijn, S., Zijlstra, M., Bijlsma, R.G., 2010. Wintering White-tailed Eagles *Haliaeetus albicilla* in The Netherlands: aspects of habitat scale and quality. Ardea 98, 1–10.
Voigt, C.C., Faßbender, M., Dehnhard, M., Wibbelt, G., Jewgenow, K., Hofer, H., Schaub, G.A., 2004. Validation of a minimally invasive blood-sampling technique for the analysis of hormones in domestic rabbits, *Oryctolagus cuniculus* (Lagomorpha). Gen. Comp. Endocrinol. 135, 100–107.
Walker, B.G., Dee Boersma, P., Wingfield, J.C., 2006. Habituation of adult Magellanic Penguins to human visitation as expressed through behavior and corticosterone secretion. Conserv. Biol. 20, 146–154.
Washburn, B.E., Millspaugh, J.J., 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. Gen. Comp. Endocrinol. 127, 217–222.
Wasser, S.K., Hunt, K.E., Brown, J.L., Cooper, K., Crockett, C.M., Bechert, U., Millspaugh, J.J., Larson, S., Monfort, S.L., 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen. Comp. Endocrinol. 120, 260–275.
Wasser, S.K., Bevis, K., King, G., Hanson, E., 1997. Noninvasive physiological measures of disturbance in the Northern Spotted Owl. Conserv. Biol. 11, 1019–1022.
Wilson, C.M., Holberton, R.L., 2001. An alternative method for delivering adrenocorticotropin hormone in birds. Gen. Comp. Endocrinol. 122, 349–353. https://doi.org/10.1006/gcen.2001.7648.
Will, A.P., Suzuki, Y., Elliott, K.H., Hatch, S.A., Watanuki, Y., Kitaysky, A.S., 2014. Feather corticosterone reveals developmental stress in seabirds. J. Exp. Biol. 217, 2371–2376. https://doi.org/10.1242/jeb.098533.
Willgohs, J.F., 1961. The white-tailed eagle in Norway. Acta Univ. Bergensis. Ser. Math. Rer. Nat, 1–212.
Wingfield, J.C., Vleck, C.M., Moore, M.C., 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. J. Exp. Zool. 264, 419–428.
Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M., Richardson, R.D., 1998. Ecological bases of hormone–behavior interactions: The emergency life-history stage. Am. Zool. 38, 191–206.
Young, K.M., Walker, S.L., Lanthier, C., Waddell, W.T., Monfort, S.L., Brown, J.L., 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. Gen. Comp. Endocrinol. 13, 148–165.