IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Non-invasive monitoring of adrenocortical activity in captive African Penguin (Spheniscus demersus) by measuring faecal glucocorticoid metabolites

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1545997 since 2023-01-07T17:04:20Z

Published version:

DOI:10.1016/j.ygcen.2015.06.014

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

11 July 2024

UNIVERSITÀ DEGLI STUDI DI TORINO

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in Gen.Comparat.Endocrinol., 2015, 224, 104-112 (10.1016/j.ygcen.2015.06.0140016-6480).

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

(1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.

(2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.

(3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en), (DOI: 10.1016/j.ygcen.2015.06.0140016-6480)

Non-invasive monitoring of adrenocortical activity in captive African Penguin (*Spheniscus demersus*) by measuring faecal glucocorticoid metabolites

Ozella L.[a*], Anfossi L.[b], Di Nardo F.[b], Pessani D.[a]

[a] Department of Life Sciences and Systems Biology, University of Turin

Via Accademia Albertina 13, 1023 Turin, Italy

[b] Department of Chemistry, University of Turin

Via Pietro Giuria 5, 10125 Turin, Italy

*to whom correspondence should be addressed. Tel: +390116704538, fax: +390116704508. E-mail: laura.ozella@unito.it

ABSTRACT

Measurement of faecal glucocorticoid metabolites (FGMs) has become a useful and widely-accepted method for the non-invasive evaluation of stress in vertebrates. In this study we assessed the adrenocortical activity of five captive African penguins (*Spheniscus demersus*) by means of FGM evaluation following a biological stressor, *i.e*. capture and immobilization. In addition, we detected individual differences in secretion of FGMs during a stage of the normal biological cycle of penguins, namely the breeding period, without any external or induced causes of stress. Our results showed that FGM concentrations peaked 5.5 to 8 h after the induced stress in all birds, and significantly decreased within 30 h. As predictable, the highest peak of FGMs (6591 ng/g) was reached by the youngest penguin, which was at its first experience with the stressor. This peak was 1.8-2.7 fold higher compared to those of the other animals habituated to the stimulus. For the breeding period, our results revealed that the increase in FGMs compared to ordinary levels, and the peaks of FGMs, varied widely depending on the age and mainly on the reproductive state of the animal. The bird which showed the lowest peak (2518 ng/g) was an old male that was not in a reproductive state at the time of the study. Higher FGM increases and peaks were reached by the two birds which were brooding (male: 5552%, 96631 ng/g; female: 1438%, 22846 ng/g) and by the youngest bird (1582%, 39700 ng/g). The impact of the reproductive state on FGM levels was unexpected compared to that produced by the induced stress. The EIA used in this study to measure FGM levels proved to be a reliable tool for assessing individual and biologically-relevant changes in FGM concentrations in African Penguin. Moreover, this method allowed detection of physiological stress during the breeding period, and identification of individual differences in relation to the reproductive status. The increase in FGM levels as a response to capture and immobilization suggests that the measured metabolites are appropriate indicators of adrenal activity in these birds.

Key Words: African Penguin (*Spheniscus demersus*), Stress, Corticosterone, Fecal Glucocorticoid Metabolites, Biological Validation, Breeding.

1. INTRODUCTION

Stress can be defined as a physiological response elicited when an individual perceives a threat to its homeostasis (Hulsman *et al*., 2011). Exposure to stressful stimuli usually results in an increased secretion of glucocorticoid hormones (GCs) subsequent to the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Möstl and Palme, 2002; Touma and Palme, 2005; Sheriff *et al.*, 2011; Benhaiem *et al*., 2012). The main GCs produced by the adrenal gland are cortisol and corticosterone, with the latter being predominant in birds (Möstl *et al*., 2005; Palme *et al.*, 2005; Cockrem, 2007).

The quantification of cortisol and corticosterone in blood sampling provides valuable information as an index of stress. However, blood sampling can be problematic if the collection procedure itself induces a stress response and therefore, in many species, non-invasive sampling techniques are preferred (Watson *et al*., 2013). A widely-employed alternative method is the measurement of GC metabolites from faecal samples (Palme *et al.*, 2005). This method offers several practical advantages, for example, easy collection of the samples, and repeated sampling of the same individual is possible without affecting its behaviour or its endocrine status (Touma and Palme, 2005). Moreover, while the blood sample values are an instantaneous indication of the hormone concentration at a specific time point, in faecal samples, hormone levels are integrated over a certain time period, and thus represent cumulative secretion (Goymann *et al.*, 1999; Keay *et al.*, 2006; Cockrem, 2007; Palme, 2012).

There are clear interspecies and gender differences regarding the metabolism and excretion of FGMs, and a careful validation of FGM excretion for each species and sex investigated is therefore obligatory (Goymann, 2005; Touma and Palme, 2005). A common practice is to carry out a physiological validation by administering exogenous adrenocorticotropic hormone (ACTH), which induces synthesis and release

of adrenocortical glucocorticoids, which is reflecting in the secretion pattern of FGMs after a specific time period (Wasser *et al.* 2000; Touma and Palme, 2005; Pribbenow *et al.*, 2013).

An alternative accepted method is biological validation by exposing an animal to a biological stressor and measuring FGMs in the samples (Palme, 2005; Touma and Palme, 2005; Sheriff *et al.* 2011). In previous studies, a variety of biological stressors have been used for validation, namely capture and restraint (*e.g.* Terio *et al.* 1999, Dehnhard *et al*., 2001; Wells and Washburn, 2003; Rettenbacher and Palme, 2009), institutional translocation (*e.g.* Goymann *et al*., 1999; Pribbenow *et al.*, 2013; Watson *et al*., 2013), transport (*e.g.* Dehnhard *et al*., 2001; Palme *et al*., 2002; Rettenbacher and Palme, 2009), social tension (Goymann *et al*., 1999; Young *et al*., 2004), and anaesthesia (Young *et al*., 2004; Benhaiem *et al.,* 2012). Validation using a biological stressor is recommended in endangered or intractable species when physiological validation is impracticable (Palme, 2005; Touma and Palme, 2005). Moreover, this test ensures that the method will appropriately measure GCs in the field when animals are exposed to genuine stressors (Sheriff *et al.*, 2011).

Despite the remarkable adaptability of certain species to live and reproduce in captivity, stress is one of the major issues currently facing zoological facilities around the world, and alleviating stress should be a key consideration for management programmes for captive animals (Narayan *et al*., 2013). In general, for zoo species, the effects of the animals' surroundings such as space, food availability, social conditions (Hogan *et al*., 2012), and presence of visitors and noise (*e.g.* Birke, 2002; Davey, 2007), have crucial implications for their welfare and health. Persistent stress causes prolonged secretion of glucocorticoids, which has deleterious consequences on individual fitness and reproductive success, and induces immunosuppression (Munck *et al.*, 1984; Liptrap, 1993; Munck and Naráy-Fejes-Tóth, 1994; Benhaiem *et al.*, 2012; Pribbenow *et al*., 2013). However, stressful circumstances facing animals in captive environments are not always easy to identify. Furthermore, stress responses can differ markedly between species and individuals (Palme, 2012) and many factors influence FGM levels, such as age, sex, diet, metabolic rate, social status, and early life experiences (Touma and Palme, 2005; Goymann, 2012).

Monitoring adrenal activity through non-invasive faecal hormone sampling is rapidly gaining popularity as a tool to assess zoo animal welfare (Clark *et al.*, 2012) in order to ascertain the causes of stress and to develop mitigating strategies.

The African Penguin (*Spheniscus demersus*) is a marine bird endemic to South Africa and Namibia. The current conservation status of this species is "Endangered", and it is indicated in the Red List of Threatened Species of the IUCN (International Union for Conservation of Nature) that the wild population has dramatically decreased in recent years to less than 75,000 – 80,000 mature individuals (BirdLife International, 2013). This decline is mainly due to loss of habitat, reduction of fish stocks, environmental pollution (including oil spills), and egg collection (Barham *et al*. 2006; Crawford *et al*. 2011). The African Penguin nests in large colonies, breeds throughout the year with peak breeding months varying locally, and usually lays two eggs (Crawford *et al*., 2006). This bird has a monogamous mating system with intensive biparental care during hatching and chick feeding (Crawford *et al*., 2013). As the African Penguin faces a great risk of extinction, *ex situ* conservation programs are becoming increasingly crucial. Due to the capability of this species to adapt to temperate environments (Frost *et al*., 1976), it is exhibited in zoos and aquaria all over the world.

Here, we investigate stress in a captive animal, using a non-invasive approach for measuring GCs in African penguins held in a zoological park (Zoom Torino, Italy). The aim of the study was to use a validated enzyme immunoassay (EIA) (Anfossi *et al*., 2014) to assess ordinary FGM concentrations, to assess the alteration of FGMS following a handling stress protocol, and during the breeding season without any extraneous or induced stress.

2. MATERIAL AND METHODS

2.1 Animals and Housing

Three male and two female African Penguins were involved in this study. The birds were housed in an exhibit (named “Bolder Beach”) of the biopark Zoom Torino (44° 56’ N, 7° 25’ E) in Italy. The exhibit reproduces the habitat of “Boulder Beach”, a natural nesting site in South Africa, near Cape Town, and covers an area of 1,500 m^2, including a pond of 120 m^2 (water depth maximum 3 m; temperature constantly maintained at 15°C). The exhibit substrate is made from sand and pebbles; trees and bushes are present to serve as hiding places or cover for penguins, and artificial nests are present in sufficient numbers to accommodate each pair. All penguins involved in the study were born and reared in captivity; sex, date of birth and zoological facility of birth are indicated in Table 1. All penguins had an adult plumage. The penguin younger than 2 years old, looking for a mate and/or a nest was classified as “prospector” (Warham, 1990), penguins aged 2-20 years as “adult”, and the penguins older than 20 years as “old”, as the average lifespan of *Spheniscus demersus* is 10 to 27 years (Pearce, 2011) (Table 1).

2.2 Sample collection

The birds were observed from a distance higher than 5 m, through binoculars to avoid disturbing the animals if necessary, by a researcher standing motionless outside the exhibit. Each penguin involved into the study was identified by means of a coloured flipper band on its wing. After a defecation event, the researcher entered the exhibit and gathered the expelled faeces. Sample collection took place immediately after defecation to avoid bacterial and microbial degradation (Millspaugh and Washburn, 2004). As urinal and faecal excretion are combined in birds, we only collected the faecal portion from droppings, which was distinguishable by colour (Ninnes *et al.*, 2010). Samples were gathered in cryovials and frozen immediately after collection at -20 °C (Millspaugh and Washburn, 2004; Palme *et al.*, 2005; Sheriff *et al.*, 2011). Throughout the study period the penguins’ diet was not modified in order to avoid alterations in gut flora and as a consequence steroid metabolism (Sheriff *et al.*, 2011).

The study was conducted between September 2013 and April 2014. To assess the ability of the EIA to detect physiologic changes in adrenocortical activity and to evaluate individual differences, faecal

samples from five penguins were collected during three sampling periods: during an ordinary day without known stress (named: “*Ordinary*”), after a stressful event for the penguins (named: “*Stress*”), and during the breeding period (named: ”*Breeding*”). Sample number and frequency depended on the individual.

2.2.1 Ordinary day *(Ordinary)*

A total of 16 samples were collected during an ordinary day, defined as a day lacking any known stress for the penguins. The colony was observed during the day prior to collection and during the early morning before starting sample collection in order to ensure that no stressful circumstances disturbed the animals. None of the penguins in the colony were subjected to veterinary checks. The keepers only entered into the exhibit for daily feeding and did not perform any maintenance work. Faeces collection continued over a period of 10 h, starting at 9:30 AM and ending at 7:30 PM of the same day.

2.2.2 Stressful event *(Stress)*

A total of 21 samples were collected from the five penguins after capture and immobilization for veterinary health investigation. It is commonly accepted that immobilization is a stressor for captive animals (Terio *et al.*, 1999; Morgan and Tromborg, 2007; Franceschini *et al*., 2008). We used this method to biologically validate the EIA as an alternative to conducting an adrenocorticotropic hormone (ACTH) challenge, as the Zoological facility did not approve any exogenous chemical treatment that was not necessary to the animals’ health. In Zoom park the capture of penguins for veterinary investigations takes place about twice yearly; besides that, animals are captured every time that they have healthy problems or for management procedures (*e.g*. flipper band or microchip applications). Nevertheless, the youngest penguin had never been captured before the study, either for veterinary procedures or microchip application.

Capture took place within the exhibit and the animals were immobilized for about 5 minutes each; after veterinary procedures the animals were immediately released. Sample collection started after the

stressful event, and continued over the subsequent 30 h, except at night.

2.2.3 Breeding period *(Breeding)*

A total of 28 samples were collected during the breeding period. However, the five penguins varied in sex, age and breeding stage; individual differences are listed in Table 2. In particular, we recorded whether the penguin hatched, if it fed its chicks and if it had a regular partner and nest. Sample collection started at 9:30 AM and continued over the subsequent 30 h until 3:30 PM of the following day, except at night.

2.3 Faecal Glucocorticoid Metabolite extraction

FGM extraction was carried out following the procedure described by Anfossi *et al*. (2014). Briefly, penguin faeces were transferred to a 15 ml tube and extracted with 5 ml of methanol: water (70: 30, v/v). After centrifugation, supernatants were transferred to a weighted tube, and the amount of the extracted sample was calculated by the difference between the total weight of the extract and the weight of the extraction solvent. Sample extracts were immediately stored at -20°C until required for analysis.

2.4 Enzyme Immunoassay (EIA)

Before FGM analysis the immunoassay was validated for African Penguins by testing specificity, precision, limit of detection, and accuracy (Anfossi *et al.*, 2014).

The assay is based on a polyclonal antibody raised against cortisol-3-(O-carboxymethyl)oxime-BSA (Bovine Serum Albumin). Antibody cross-reactivity to different steroids were as follows: 4-pregnen-11β,21-diol-3,20-dione (corticosterone), 100%; 4-pregnen-3β,17,21-triol-3,20-dione (cortisol), 100%; **1,4-Pregnadiene-11β,17α,21-triol-3,20-dione** (prednisolone), 38%; **11β,17α,21-Trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione** (methyl-prednisolone), 26%; 4-pregnen-3,20-dione (progesterone), 8%; 4-

androsten-17β-ol-3-one (testosterone), 7%; 4-pregnen-17,21-diol-3,11,20-trione (cortisone), 3%; 5b-dihydro-4-androsten-17β-ol-3-one (5b-dihydrotestosterone), 3%; **1,4-Pregnadiene-17α,21-diol-3,11,20-trione** (prednisone), 1%; 4-Androsten-3,11,17-trione, 1%; 5a-Androstan-3,17-dione, 0.2%; and <0.04% for 5β-pregnan-3α,11β,21-triol-20-one (tetrahydrocorticosterone), 5β-pregnan-3α,11β,17,21-tetrol-20-one (tetrahydrocortisol), 5-Androsten-3b,17b-diol, and 5a-Androstan-3b, 17b-diol. The accuracy of the optimized EIA method, the within-assay precision and between-assay precision were measured to be 83-116%; 7–8%, and 5–16%, respectively.

The experimental protocol has been previously reported (Anfossi et al., 2014). Briefly, calibration curves were obtained by dispensing 150 μl of the conjugate (cortisol-3-(O-carboxymethyl)oxime-horse radish peroxidase diluted at 1.5 mg L^{-1} in 20 mM TRIS buffer pH 8.0 supplemented with 0.3 M NaCl, 1% BSA, and 0.1% Tween 20) and 50 μl of corticosterone (diluted in 35% methanol at concentrations ranging from 0 to 50 μg l^{-1}) into immunoreactive wells obtained by coating with the polyclonal antibody. After 1-hour incubation, wells were washed, and colour development was obtained by 30 min incubation with TMB (200 μl per well). A volume of 50 μl of sulphuric acid (2 M) was used as a stop solution, and absorbance was recorded at 450 nm. Unknown sample concentrations were measured by replacing the corticosterone standard solution with sample extracts diluted 1 + 1 with water and then 1 + 4 or 1 + 9 with 35% methanol. All standards and samples were measured in duplicate.

2.5 Data analysis

For each penguin, ordinary levels of glucocorticoid metabolites in the faeces were defined as the mean of the concentrations of all samples collected during the ordinary day. For each penguin, the average FGM concentration (expressed as mean±SEM), for each sampling period was calculated. The increase of maximum values reached by each bird during the sampling periods *Stress* and *Breeding*, compared to the ordinary levels, was assessed.

Data were tested for normality and equality of variance. The average FGM concentrations in each sampling period were compared using the *Kruskal-Wallis test*; likewise, the maximum values of FGM concentrations reached by penguins in each sampling period were compared using the *Kruskal-Wallis test*.

All values were allocated into time frames (Dehnhard *et al*., 2001; Coradello *et al.*, 2012) of 5 h and the concentrations of FGMs were compared for each interval using the *Mann-Whitney-Wilcoxon test* for the sampling period *Ordinary*, and the *Kruskal-Wallis test* for *Stress* and *Breeding*, followed by the post-hoc *Nemenyi Damico Wolfe Dunn test*. The coefficient of variation (CV) was calculated for each penguin and for each sampling period. The CV is defined as the ratio of the standard deviation to the mean, and is expressed as a percentage.

According to the method outlined by Hogan *et al*. (2012), analyses of the profiles of FGM secretion after the stressful event (*Stress*) and during the breeding period (*Breeding*) were carried out in order to identify significant peaks. Values ≥ 1SD above the overall mean for a given animal were considered 'spikes'. Baseline FGM levels were derived from a re-calculation of the mean value after excluding the 'spike' concentrations. A FGM concentration >2SD above the baseline was accepted as a significant peak.

Statistical analyses were carried out using the R software, Version 3.1.2 (R Core Team, 2014), p<0.05 was considered as significant.

3. RESULTS

3.1 Comparison of FGM concentrations between sampling periods

There was inter-animal variability in the mean values of all samples obtained during *Ordinary* period, in the maximum values reached both in the *Stress* and *Breeding* periods and in the increase compared to the ordinary level (Table 3). The overall average FGM concentrations did not differ between the sampling periods (*Ordinary*, *Stress*, and *Breeding*) (*Kruskal-Wallis* X^2=1.486, *df*=2, *p*=0.475). The maximum values

reached during the sampling periods differed significantly (*Kruskal-Wallis* X^2=7.62, *df*=2, *p*=0.022). The *Nemenyi Damico Wolfe Dunn post-hoc test* showed that the maximum values of FGMs differed significantly between *Ordinary* and *Breeding* (*p*=0.006), and the maximum FGM concentrations were higher during the breeding period (Figure 1).

3.2 Handling Stress

Capture and immobilization resulted in an increase in maximum values of FGM concentration in all five penguins compared to the ordinary level (Table 3). Moreover, there was a significant difference between the FGMs evaluated in time frames of 5 h each (*Kruskal-Wallis* X^2=11.909, *df*=3, *p*=0.007). The *Nemenyi Damico Wolfe Dunn post-hoc test* showed that the concentration of the metabolites was significantly increased during the second time frame after the stressful event (5-10 h) compared to the first (0-5 h) (p=0.007) and the fourth time frame (25-30 h) (p=0.006) (Figure 2). In particular, the maximum values were reached for all five animals between 5.5 and 8 h after the stressful event, and these values corresponded to significant peaks (Table 3 and Figure 3).

3.3 Breeding period

During the breeding period, there was an increase in FGM concentration in all five penguins compared to the ordinary level (Table 3). The higher significant peaks were reached by penguins S, Z, and G (Table 3 and Figure 4). There was no significant difference between FGM values evaluated in the time frames of 5 h each (*Kruskal-Wallis* X^2=1.154, *df*=3, *p*=0.764).

3.4 Comparison between penguins according to sampling period

The individual ordinary level, calculated as the mean of FGMs in samples collected during the ordinary day, differed between penguins. In particular, the lowest values corresponded to the oldest birds (R: 804±927 ng/g; K: 1456±465 ng/g), while the highest ordinary level corresponded to the prospector (G:

2509±780 ng/g) (Table 3). An old male penguin (R) showed the highest CV in mean FGM values (99%), while the remaining birds showed similar levels of CV in their means (Figure 5).

Regarding the samples collected after the stressful event for the biological validation, the highest mean values corresponded to the prospector (G: 3294±2868 ng/g), as well as the highest peak value after the stressful event (G: 6591 ng/g) (Table 3): this peak was 1.8-2.7 fold higher compared to those of the other penguins. This animal was born at the Zoom Park, it was the youngest bird, and before the study had never been captured. An old male penguin (R) showed the highest CV in its mean FGM values (82%) (Figure 5).

The highest means of FGM levels in samples collected during the breeding season corresponded to the adult penguins (S: 17809±38632 ng/g; Z: 14301±9480 ng/g), followed by the mean of the prospector (G: 7577±14514 ng/g). The lowest means of FGM levels corresponded to the oldest penguins (R: 846±943 ng/g; K: 2389±1160 ng/g) (Table 3). Both adult penguins (S and Z) were hatching eggs at the time of sample collection, and penguin S was also feeding a chick. The prospector (G) was, instead, looking for a partner and a nest. One of old penguins (K) was feeding a chick but had no eggs and the other old penguin (R) had a nest but had neither eggs nor chicks. The highest peak FGM values were reached during the breeding period by penguins S (96631 ng/g) and G (39700 ng/g), followed by Z (22846 ng/g) (Table 3). Penguins S and G showed the highest CV in their mean FGMs (198% and 177%, respectively) (Figure 5).

4. DISCUSSION

We assessed the adrenocortical activity of captive African Penguins (*Spheniscus demersus*) using a non-invasive technique. We validated the measurement of faecal corticosterone metabolites using a biological stressor for this species. To be able to reliably assess glucocorticoid metabolites in faeces of African Penguins, we previously developed and optimized a dedicated enzyme immunoassay (EIA) (Anfossi *et al*., 2014). Samples belonging to the stress sampling set were employed at the purpose, as

they were expected to cover a wide range of FGM levels and were representative of variability associated to individuals and time of collection compared to biological functions (*i.e.*: feeding). For this study we have re-analysed the faecal samples from the stress-protocol used to validate the EIA. Notably, the means values and the maximum values of four individuals (R, K, Z and G) (Table 3) showed similar results to those reported in Anfossi *et al*. (2014), confirming the reliability of the employed method. In addition, we estimated the ordinary levels of FGM by measuring samples collected during an ordinary day, defined as a day lacking of any known stress for the penguins, and we studied individual differences in FGM secretion during a stage of the normal biological cycle of penguins, namely the breeding period, without any external or induced causes of stress. Two female and three male captive penguins were involved in the study, with different ages and different breeding statuses.

We demonstrated an increase in FGM levels after 5 h, and a decrease within 30 h, with respect to biological stress, *i.e.* the capture and immobilization of animals (Figure 2). Naturally, there is a time delay between stimulation of the HPA axis and increase in FGMs excreted in faeces following a biological challenge (Narayan *et al*., 2013). This delay corresponds to the time for food to be digested and passed from the duodenum to the rectum, it is species-dependent, and may be influenced by different factors such as feed intake (Palme *et al*., 2005). Determination of this time delay is important to identify the causes of elevation of FGM levels, and to be able to impute the increases to specific events (Narayan *et al*., 2013). In general, the time delay between the stress and the excretion of FGMs from the adrenal gland in birds is a few hours. Accordingly, our data showed that FGM concentrations peaked 5.5 to 8 h after the stressful event (Table 3 and Figure 3). This range is in good agreement with those previously obtained after ACTH administration in chickens (*Gallus domesticus*) (4-8 h) by Dehnhard *et al*. (2003), and in Adélie penguins (*Pygoscelis adeliae*) (6-18 h) by Nakagawa *et al*. (2003). Nevertheless, time delays may vary between bird species; for instance, in European Stonechats (*Saxicola torquata rubicola*) (Goymann *et al.*, 2002), Great tits (*Parus major*) (Carere *et al*., 2003), and Black grouse (*Tetrao tetrix*) (Baltic *et al*., 2005), the peak was reached within 3 h; while in Florida sandhill cranes (*Gru canadensis pratensis*)

(Ludders *et al*., 2001), in Capercaillies (*Tetrao urogallus*) (Thiel *et al*., 2005), and in Mourning doves (*Zenaida macrura*) (Washburn *et al.*, 2003) FGM levels increased after 2-3 h following ACTH stimulation. Wasser *et al.* (1997) showed an increase in FGMs within 2 h after ACTH injection, with a peak at 12 h in a single female Northern spotted owl (*Strix occidentals caurina*).

For the biological validation, we used capture and immobilization as a stressor, which is a commonly accepted method to stimulate the adrenocortical activity in captive animals (*e.g.* Terio *et al*., 1999). It is generally assumed that birds view capture and handling as a form of predation (Silverin, 1998). Activation of the HPA axis when an animal perceives a stimulus to be a threat, such as predation, is considered to occur simultaneously with the emotion of fear (LeDoux, 1996). Fear responses help the animal to avoid or reduce the possible consequences of exposure to danger (Cockrem, 2007). In addition, animals may adapt to repeated stimuli and, consequently, have less intense fear responses (Furini *et al*., 2014). Our results showed that the intensity of response to the biologically induced stress differed between the five animals (Table 3 and Figure 3). The highest mean value and peak of FGM levels reached after the stressful event corresponded to the youngest animal (Table 3 and Figure 3). There are two potential factors explaining the high level of FGMs observed in this individual: an age-effect, as it is known that the magnitude of the corticosterone response to a stressor decline with age, and the fact that this individual never experienced the stressor before. This female bird had never been transported from other zoological facilities and, before the study, had never been captured, not even for veterinary procedures; it was therefore its first experience with the stressor. The remaining four penguins had already been captured several times previously, and showed similar mean values and peaks after the stress compared with each other (Table 3 and Figure 3). We hypothesize that the difference in intensity of adrenocortical responses observed between the youngest female and the other penguins could be explained in terms of the different past experience with the stressful event rather than in terms of age-effect, which is, however, a possible justification of the findings. Individual variation between animals, in term of response to the same stressful event is not an unexpected outcome (Hogan *et al*., 2012), as

adrenocortical responses are known to reflect inter-animal variation in the perception of a stimulus, depending on factors such as previous experience, temperament, age, physical and physiological states (Owen *et al*., 2004).

The most common method to stimulate GC secretion is by ACTH challenge (Touma and Palme, 2005), in which there is a pharmacological stimulus, in addition to the biological stress (capture and handling for the injection). Therefore, ACTH challenge has the potential to provide useful information on the effect that an intense stimulus has on animals' stress levels (Narayan *et al.*, 2013). However, this particular method induces a potent stress response, which may not reflect the adrenocortical activity that occurs under natural stress conditions. The use of a biological stressor for the validation could be a more effective means to mimic natural stress causes and to detect the genuine levels of FGMs reached by animals as a response to those stressors. Furthermore, individual differences in corticosterone responses are more evident when birds are challenged with mild rather than strong stressors, as they are mainly caused by differences in adrenal sensitivity to ACTH and not by intensity of ACTH releases from the pituitary (Beuving and Vonder, 1986). We can define the biological stressor that we used as a "mild" stressor, because it lasted only 5 minutes, and the birds were immediately released back into their exhibit following handling, without changing their environment (*e.g*. use of cage). Notwithstanding, the analytical method employed allowed us to detect increasing and decreasing FGM levels associated with penguin status and to identify significant peaks connected to the stressful event for all penguins (Table 3 and Figure 3).

The reproductive status of animals influences the concentration of faecal GCs (Touma and Palme, 2005). In birds, corticosterone appears to facilitate reproductive activity (Edwards *et al*., 2013). During the breeding season, adrenal activity is up regulated (Romero and Wingfield, 1998) and breeding individuals tend to have a higher level of GCs compared with non-breeding individuals (Edwards *et al*., 2013). In addition, the magnitude of the adrenocortical response is positively related to the ability to defend the

nest from predators, as observed in White-rumped-sandpiper (*Calidris fuscicollis*) and in Red phalarope (*Phalaropus fulicarius*) by Edwards *et al*. (2013), and has been associated with the reproductive success in Hen harrier (*Cyrcus cinereus*) (García, 2003). Our results showed higher levels of FGMs during the breeding season relative to ordinary levels (Table 3 and Figure 1). Moreover, the increases and peaks are decidedly higher compared to those reached after the stressful event in three out of five penguins (Table 3 and Figure 4). The birds that showed the lowest increases and peaks were the two old males: one of these was not in a reproductive state, had no partner, eggs or chicks, and the other penguin had a partner, fed a chick but had no eggs.

High peaks were reached in two penguins (one male and one female) that were hatching eggs during sample collection (Table 3 and Figure 4). According to the hypothesis based on the vulnerability of offspring to predation (Skutch, 1949; Harvey and Greenwood, 1978; Brunton, 1990), the greater intensity of nest defence is correlated with a phase of major vulnerability of offspring (*i.e.* the egg phase), and this is associated with higher levels of FGMs. Furthermore, the highest peak of FGM levels was reached by the male penguin, which was not only hatching an egg, but was also feeding a chick, at the time of data sampling.

The prospector bird also showed a high peak of FGM levels during the breeding period (Table 3 and Figure 4). Although the prospector had not yet started to breed, we hypothesize that the continuing search for a partner and a nest could result in competition with other birds and consequently it could induce stress, particularly in captivity where these resources are limited. This result is in agreement with what was observed in Greylag Geese (*Anser anser*), which showed an elevated level of FGMs in birds lacking partners during the breeding season (Kotrshal *et al*., 1998).

We could not find any statistically significant sex-related differences in FGM levels due to the limited number of animals evaluated.

In all sampling periods we detected different variations (CV%) in individual FGM levels (Figure 5). A possible explanation for the observed variations in FGMs between animals and sampling periods could be

related to individual personalities of birds. The personality is defined as a consistent behavioural profile displayed in different situations (Garamszegi *et al*., 2008), and it determines how individuals generally deal with challenges in their physical and social environment (Quinn and Cresswell, 2005). The corticosterone stress response and behavioural responses to stimuli vary markedly between individual birds, depending on the individual bird's personality (Cockrem, 2007). Despite the captive conditions, with standard environment and management of animals, we detected differences in FGM concentrations between the penguins involved in the study. Further research could be useful to investigate the relation between FGM profiles and personalities in this species.

In conclusion, FGM analysis appears to be a useful method for monitoring adrenal activity, both after a known induced stress, and during a common stage of biological cycle in captive African Penguins. We have shown that the EIA used in this study is a reliable tool to biologically measure individual and relevant changes in FGM concentrations in African Penguin. The increase in FGM levels as a response to capture and immobilization suggests that the measured metabolites are appropriate indicators of adrenal activity in these birds. Moreover, this method allows detection of physiological stress during the breeding period, and highlights individual differences of the concentration of FGMs in relation to reproductive status. In combination with behavioural observations, the evaluation of FGMs provides a very useful method to assess the welfare of African Penguins. Therefore, this method is helpful to detect and outline potentially stressful situations, which may influence the welfare of animals held in zoological facilities. Furthermore, because the collection protocols are non-invasive, without causing any stress, faecal measurements can improve the ability to identify a genuine physiological response to external events such as human disturbance or mismanagement of animals.

Tables and Figures

Table 1. Details of African penguins involved in the study.

Table 2. Individual differences during the breeding period.

Table 3. Individual Ordinary levels (*Ordinary*), Mean values, Maximum values (ng/g faeces and %increase), Peak values (ng/g faeces) (*Stress* and *Breeding*), and time which were reached after the stressful event (*Stress*). Sample sizes are indicated in [ ]. "Maximum values" are the highest values of FGM concentration observed for each penguin, "Peaks" are intended as the values of FGM concentration >2SD above the baseline, calculated according to Hogan *et al*. (2012).

Figure 1. Maximum values of FGMs (ng/g faeces) measured for the five penguins for each sampling period (*Ordinary*=Ordinary day; *Stress*=Stressful event; *Breeding*=Breeding period). The box and whisker plots illustrate the interquartile range, and the black lines indicate the median. The error bars extend from the box to the highest and lowest values. The circles indicate the outliers data.

Figure 2. FGMs (ng/g faeces) measured for the five penguins pooled in time frames of 5 h each (except at night) after the stressful event. The box and whisker plots illustrate the interquartile range, and the black lines indicate the median. The error bars extend from the box to the highest and lowest values. The circles indicate the outliers data.

Figure 3. FGM (ng/g faeces) profile for each penguin after the stressful event during the subsequent 30 h. Data represent FGM concentrations of each sampling time, error bars indicate standard deviations, and significant peaks are labelled with *. The grey band indicates the time interval during which the peaks occurred (5.5-8 h after the stressful event).

Figure 4. FGM (ng/g faeces) profile for each penguin during the breeding period. Sample collection started at 9:30 AM and continued during the subsequent 30 h (3:30 PM of the following day). Data represent single FGM sampling times; error bars indicate standard deviations, and significant peaks are labelled with *.

Figure 5. Box and Whisker plots of FGM levels (ng/g faeces) of each penguin during the ordinary day (*Ordinary*), after the stressful event (*Stress*) and during the breeding period (*Breeding*). Coefficient of Variation (CV%) for each penguin is shown above each individual plot. The box and whisker plots illustrate the interquartile range, and the black lines indicate the median. The error bars extend from the

box to the highest and lowest values. The circles indicate the outliers data (note the different scale of FGMs in breeding period).

Acknowledgements

We would like to thank Zoom Torino S.p.A. (http:// www.zoomtorino.it) for free access to their animals and in particular Dr. Daniel Sanchez.

References

- Anfossi, L., Ozella, L., Di Nardo, F., Giovannoli, C, Passini, C., Favaro, L., Pessani, D., Möstl, E., Baggiani, C., 2014. A broad-selective enzyme immunoassay for non-invasive stress assessment in African penguins (*Spheniscus demersus*) held in captivity. Anal. Methods 6, 8222-8231.
- 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, 81-45.
- Barham, P.J., Crawford, R.J.M., Underhill, L.G., Wolfaardt, A.C., Barham, B.J., Dyer, B.M., Leshoro, T.M., Meÿer, M.A., Navarro, R.A., Oschadleus, D., Upfold, L., Whittington, P.A., Williams, A.J., 2006. Return to Robben Island of African Penguins that were rehabilitated, relocated or reared in captivity following the Treasure oil spill of 2000. Ostrich 77, 202–209.
- Benhaiem, S., Dehnhard, M., Bonanni, R., Hofer, H., Goymann, W., Eulenberger, K., East, M.L., 2012. Validation of an enzyme immunoassay for the measurement of fecal

glucorticoid metabolites in spotted hyenas (*Crocuta crocuta*). Gen. Comp. Endocrinol. 178, 265-271.

- Beuving, G., Vonder, G.M.A., 1986. Comparison of the adrenal sensitivity to ACTH of laying hens with immobilization and plasma baseline levels of corticosterone. Gen. Comp. Endocrinol. 62, 353-358.
- BirdLife International, 2013. *Spheniscus demersus*. The IUCN Red List of Threatened Species. Version 2014.3. <www.iucnredlist.org>. Downloaded on 22 February 2015.
- Birke, L., 2002. Effects of browse, human visitors and noise on the behaviour of captive orang-utans. Anim. Welf. 11(2), 189-202.
- Brunton, D.H., 1990. The Effects of Nesting Stage, Sex, and Type of Predator on Parental Defense by Killdeer (*Charadrius vociferous*): Testing Models of Avian Parental Defense. Behav. Ecol. Sociobiol. 26(3), 181-190.
- Carere, C., Groothius, T.G.G., Möstl, E., Daan, S., Koolhaas, J.M., 2003 Fecal corticosteroids in a territorial bird selected for different personalities: daily rhythm and the response to social stress. Horm. Behav. 43, 540-548.
- Clark, F.E., Fizpatrick, M., Hartley, A., King, A.J., Lee, T., Routh, A., Walker, S.L., George, K., 2012. Relationship between behaviour, adrenal activity, and environment in zoo-housed Western Lowland Gorillas (*Gorilla gorilla gorilla*). Zoo Biol. 31, 306-321.
- Cockrem, J.F., 2007, Stress, corticosterone responses and avian personalities. J. Ornithol. 148 (2), 169-178.
- Coradello, M.A., Morais, R.N., Roper, J., Spercoski, K.M., Massuda, T., Nogueira, S.S.C., Nogueira-Filho, S.L.G., 2012. Validation of a fecal glucocorticoid metabolite assay for collared peccaries (*Pecari tajacu*). J. Zoo Wild. Med. 43(2), 275-282.

- Crawford, R.J.M., Barham, P.J., Underhill, L.G., Shannon, L.J., Coetzee, J.C., Dyer, B.M., Leshoro, T.M., Upfold, L., 2006. The influence of food availability on breeding success of African Penguins *Spheniscus demersus* at Robben Island, South Africa. Biol. Cons. 132(1), 119-125.
- Crawford, R.J.M., Altwegg, R., Barham, B.J., Barham, P.J., Durant, J.M., *Dyer, B.M, Geldenhuys, D., Makhado, A.B., Pichegru, L., Ryan, P.G., Underhill, L.G., Upfold, L., Visagie, J., Waller, L.J., Whittington, P.A.,* 2011. Collapse of South Africa's penguins in the early 21st century. Afr. J. Mar. Sci. 33, 139-156.
- Crawford, R.J.M., Kemper J., *Underhill, L.G., 2013. African Penguin, Spheniscus demersus, in:* Garcia Borboroglu, P., Boersma, P. D (Eds.) Penguins: natural history and conservation, Seattle, WA: University of Washington Press*, pp. 211-231.*
- Davey, G., 2007. Visitors' Effects on the Welfare of Animals in the Zoo: A Review. J. Appl. Anim. Welfare Sci. 10(2), 169-183.
- Dehnhard, M., Clauss, M., Lechner-Doll, M., Meyer, H.H.D., Palme, R., 2001. Noninvasive monitoring of adrenocortical activity in Roe Deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites. Gen. Comp. Endocrinol. 123, 111-120.
- 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.
- Edwards, D.B., Chin, E.H., Burness, G., Gilchrist, H.G., Schulte-Hostedde, A.I., 2013. Linking sex difference in corticosterone with individual reproductive behaviour and

hatch success in two species of uniparental shorebirds. Comp. Biochem. Physiol., A: Comp. Physiol. 166, 169-176.

- Franceschini, M.D., Rubenstein, D.I., Low, B., Romaro, L.M., 2008. Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra. Anim. Conserv. 11, 263-269.
- Frost, P.G.H., Siegfried, W. R., Burger, A.E., 1976. Behavioral adaptations of the Jackass penguin *Sheniscus demersus* to a hot, arid environment. J. Zool. 179, 165-187.
- Furini, C., Miskiw, J., Izquierdo I., 2014. The learning of fear extinction. Neurosci. Biobehav. R. 47, 670-683.
- Garamszegi, L.Z., Eens, M., Török, J., 2008. Birds reveal their personality when singing. PLoS ONE 3, doi:10.1371/journal.pone.0002647.
- García, J.T., 2003. Is female investment in defence correlated with breeding success in Hen Harriers *Circus cyaneus*? Bird Study 50, 142-145.
- Goymann, W., Möstl, E., Van't Hof, T., East, M.L., Hofer, H., 1999. Noninvasive fecal monitoring of glucorticoids in spotted hyenas, *Crocuta crocuta*. Gen. Comp. Endocrinol. 144, 340-348.
- Goymann W., Möstl, E., Gwinner E., 2002. Corticosterone Metabolites can be Measured Noninvasively in Excreta of European Stonechats (*Saxicola torquata rubicola*). Auk 119(4), 1167-1173.
- Goymann, W., 2005. Noninvasive monitoring of hormones in bird droppings. Physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. Ann. N. Y. Acad. Sci. 1046, 35-53.
- Goymann, W., 2012. On the use of non-invasive hormone research in uncontrolled,

natural environments: the problem with sex, diet, metabolic rate and the individual. Methods Ecol. Evol. 3, 757–765.

- Harvey, P.H., Greenwood, P.J., 1978. Antipredator defence by Stonechats (*Saxicola torquata*). Anim. Behav. 28, 604-619.
- Hogan, L.A., Lisle, A.T., Johnston, S.D., Robertson, H., 2012. Non-invasive assessment of stress in captive numbats, *Myrmecobius fasciatus* (Mammalia: *Marsupialia*), using a faecal cortisol measurement. Gen. Comp. Endocrinol. 179, 376-383.
- Hulsman, A., Dalerum, F., Ganswindt, A., Muenscher, S., Bertschinger, H.J., Paris, M., 2011. Non-invasive monitoring of glucocorticoid metabolites in Brown Hyaena (*Hyaena brunnea*) feces. Zoo Biol. 30, 451-458.
- Keay, J.M., Singht 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(3), 234-244.
- Kotrschal, K., Hirschenhauser, K., Möstl, E., 1998. The relationship between social stress and dominance is seasonal in greylag geese. Anim. Behav. 55, 171-176.
- LeDoux, J., 1996. The emotional brain. Simon & Schuster, New York.
- Liptrap, R.M., 1993. Stress and reproduction in domestic animals. Ann. N. Y. Acad. Sci. 697, 275-284.
- Ludders, J.W., Langenberg, J.A., Czekala, N.M., Hollis, N.E., 2001. Fecal Corticosterone Reflects Serum Corticosterone in Florida Sandhill Cranes. J. Wildl. Dis. 37(3), 646-652.
- 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.

- Morgan, K.N., Tromborg, C.T., 2007. Sources of stress in captivity. Appl. Anim. Behav. Sci. 102, 262-302.
- Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrin. 23, 67-74.
- 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.
- Munck, A., Guyre, P.M., Holbrook N.J., 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr. Rev. 5(1), 25-44.
- Munck, A., Naráy-Fejes-Tóth, A., 1994. Glucocorticoids and stress: permissive and suppressive actions. Ann. N. Y. Acad. Sci. 746, 115-130.
- Nakagawa, S., Möstl, E., Waas, J.R., 2003. Validation of an enzyme immunoassay to measure faecal glucorticoid metabolites from Adélie penguins (*Pygoscelis adeliae*): a non-invasive tool for estimating stress? Polar Biol. 26, 491-493.
- Narayan, E.J., Webster, K., Nicolson, V., Mucci, A., Hero, J.M., 2013. Non-invasive evaluation of physiological stress in an iconic Australian marsupial: The Koala (*Phascolarctos cinereus*). Gen. Comp. Endocrinol. 187, 39-47.
- Ninnes, C.E., Waas, J.R., Ling, N., Nakagawa, S., Banks, J.C., Bell, D.G., Bright, A., Carey, P.W., Chandler, J., Hudson, Q.J., Ingram, J.R., Lyall, K., Morgan, D.K.J., Stevens, M.I., Wallace, J., Möstl, E., 2010. Comparing plasma and faecal measures of steroid hormones in Adelie penguins *Pygoscelis adeliae*. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 180, 83-94.

- Owen, M.A., Swaisgood, R.R., Czekala, N.M., Steinman, D.G., Lindburg, D.G., 2004 Monitoring stress in captive giant pandas (*Ailuropoda melanoleuca*): behavioral and hormonal responses to ambient noise. Zoo Biol. 23, 147-164.
- Palme, R., Robia, C., Baumgartner, W., Möstl, E., 2002. Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. Vet. Rec. 146, 108-109.
- 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, non invasive measurement in fecal samples. Ann. N. Y. Acad. Sci. 1040, 162-171.
- Palme, R., 2012. Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. Anim. Welf. 21, 331-337.
- Pearce, W., 2011. "*Spheniscus demersus*" (On-line), Animal Diversity Web. Accessed March 04, 2015 at http://animaldiversity.org/accounts/Spheniscus_demersus/
- Pribbenow, S., Jewgenow, K., Vargas, A., Serra, R., Naidenko, S., Dehnhard, M., 2013. Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in Eurasian (*Linx lynx*) and Iberian lynx (*Linx pardinus*). Gen. Comp. Endocrinol. 206, 166-177.
- Quinn, J., Cresswell, W., 2005. Personality, anti-predation behaviour and behavioural plasticity in the chaffinch *Fringilla coelebs*. Behaviour 142(9), 1377–1402.
- Rettenbacher, S., Palme, R., 2009. Biological validation of a non-invasive method for stress assessment in chickens. Berl. Munch. Tierarztl. Wochenschr. 122, 8-12.

- Romero, L.M., Wingfield, J.C., 1998. Seasonal changes in adrenal sensitivity alter corticosterone levels in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*). Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 11, 31–36.
- R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.
- Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869-887.
- Skutch, A.F., 1949. Do tropical birds rear as many young as they can nourish? Ibis 91, 430-455.
- Silverin, B., 1998. Stress responses in birds. Avian Poult. Biol. Rev. 9, 153-168.
- Terio, K.A., Citino, S.B., Brown, J.L., 1999. Fecal cortisol metabolite analysis for noninvasive monitoring of adrenocortical function in the cheetah (*Acinonyx jubatus*). J. Zoo Wildl. Med. 30(4), 484-491.
- Thiel, D., Jenni-Eiermann, S., Palme, R., 2005. Measuring corticosterone metabolites in droppings of Capercailliies (*Tetrao urogallus*). Ann. N. Y. Acad. Sci. 1046, 96-108.
- Touma, C., Palme, R., 2005. Measuring fecal glucocorticoids metabolites in mammals and birds: the importance of validation. Ann. N. Y. Acad. Sci. 1046, 54-74.
- Warham, J., 1990. The petrels: the behaviour, population biology and physiology. Academic Press, London.
- Washburn, B.E., Millspaugh, J.J., Shulz, J.H., Jones, S.B., Mong, T., 2003. Using fecal glucocorticoids for stress assessment in Mourning Doves. Condor 105, 696-706.
- Wasser, S.K., Kennet B., King G., Hanson E., 1997. Noninvasive physiological measure

of disturbance in the Northern Spotted Owl. Conserv. Biol. 11(4), 1019-1022.

- 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 non-domestic mammalian and avian species. Gen. Comp. Endocrinol. 120, 260–275.
- Watson, R., Coralie, M., Edwards, K.L., Norton, V., Brown, J.L., 2013. Development of a versatile enzyme immunoassay for non-invasive assessment of glucocorticoid metabolites in a diversity of taxonomic species. Gen. Comp. Endocrinol. 186, 16-24.
- Wells, K.M.S., Washburn, B.E., 2003. Effects of radio-transmitters on fecal glucocorticoid levels in captive Dickcissels. Condor 105, 805-810.
- 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 glucocorticoids analyses. Gen. Comp. Endocrinol. 137, 148-165.